BIOAVAILABILITY AND BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

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1 De bewering van Keuth & Rehm dat de exponentiële groeisnelheid van bacteriën toeneemt bij substraatconcentraties welke boven de maximale oplosbaarheid liggen, is onjuist.

Keuth S. and H.J. Rehm (1991) Biodegradation of phenanthrene by *Arthrobacter polychromogenes* isolated from a contaminated soil. Appl. Microbiol. Biotechnol. 34:804-808.

2 Het kunstmatig verontreinigen van grond door de verontreiniging in een kleine hoeveelheid vluchtig oplosmiddel aan de grond te voegen en het oplosmiddel vervolgens snel af te dampen levert een produkt op dat slechts in beperkte mate kan worden vergeleken met vervuilde grond zoals deze in de praktijk voorkomt.

Dit proefschrift

3 Het idee dat de verhoging van de snelheid van biologische afbraak van organische verontreinigingen in de bodem die bereikt wordt door het toepassen van surfactant-oplossingen, kan worden gekoppeld aan de mate van solubilisatie van de verontreiniging in de surfactant-oplossing, berust op een misvatting.

Dit proefschrift

4 Het in de bodemwetgeving gebruiken van verontreinigingsconcentraties die zijn bepaald via chemische extractie gaat goeddeels voorbij aan de biologische beschikbaarheid van de verontreinigingen, waardoor de ecotoxicologische onderbouwing van deze wetgeving discutabel is.

Dit proefschrift

5 Het bezuinigen op (experimenteel) wetenschappelijk onderzoek binnen het Rijksinstituut voor Volksgezondheid en Milieuhygiëne getuigt van een beperkte toekomstvisie, die niet past bij een instituut dat zich wil profileren als toonaangevend op het gebied van milieu- en volksgezondheids-toekomstverkenningen.

6 Literatuurbesprekingen geven vaak een nieuwe kijk op de waarde van wetenschappelijke publikaties en zijn bijzonder nuttig bij het zelf schrijven van manuscripten. Het is daarom voor (universitaire) onderzoeksgroepen aan te bevelen regelmatig dergelijke besprekingen te houden.

7 Een uitgebreide lunch is een goede voedingsbodem voor wetenschappelijk onderzoek.

8 In een tijd waarin het krijgen van kinderen is veranderd in het nemen van kinderen is, gezien de hoge bevolkingsdichtheid in dit land, het huidige Nederlandse systeem voor kinderbijslag een anachronisme.

ISBN 90-5485-575-4

VOORWOORD

Dit proefschrift is het resultaat van een project dat in het kader van het Speerpuntprogramma Bodemonderzoek is uitgevoerd bij de Afdeling Biotechnologisch Onderzoek (BTO) van het Laboratorium voor Afvalstoffen en Emissies (LAE) van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) in de periode 1989 - 1994. In eerste instantie betrof het een driejarig project, maar door een verlenging van anderhalf jaar, waarbij de vakgroep Milieutechnologie van de Landbouwuniversiteit Wageningen bij het project werd betrokken, kon het onderzoek uiteindelijk tot dit proefschrift leiden. Dat de samenwerking tussen de afdeling BTO en de vakgroep Milieutechnologie goed beviel blijkt ondermeer uit het feit er inmiddels een vervolg op dit onderzoek wordt uitgevoerd, waarbij een promovendus van de LUW bij het RIVM is gedetacheerd.

Achteraf gezien valt mijn periode bij de afdeling BTO in de bloeiperiode van de afdeling en mijn dank gaat dan ook in de eerste plaats uit naar Johan van Andel die het, mede door zijn mensenkennis, voor elkaar heeft gekregen een universitair aandoende onderzoeksgroep bij het RIVM op te zetten. Als afdelingshoofd was hij de prettigste "baas" die je je kunt wensen.

De dagelijkse begeleiding van het onderzoek was in handen van Ton Breure. Naast co-promotor, kamergenoot en onverbeterlijke sloddervos was hij toch vooral de enthousiaste, stimulerende en kritische wetenschapper die me over de moeilijke punten heen hielp. Zonder hem was dit proefschrift hoogstwaarschijnlijk niet tot stand gekomen.

Verder wil ik alle medewerkers en studenten van de afdeling BTO bedanken voor de prima sfeer, met name de "lunchclub". Ik mis de geweldige en gezellige lunches met inspirerende discussies over zowel onderzoek als meer wereldse zaken nog iedere dag. Klaas Doesburg, Wilfred van der Sterren en Rieke van der Wiel hebben in het kader van een stage of afstudeervak belangrijke bijdragen aan het onderzoek geleverd.

Wim Rulkens, als promotor pas de laatste jaren bij het project betrokken, heeft het vaak niet makkelijk gehad met "die microbiologen", maar wist door zijn grondige fysisch-chemische kennis het onderzoek zeker meer diepgang te geven.

De correcties van het Engels in dit proefschrift door Ruth de Wijs van het RIVM komen mij ook nu nog goed van pas bij het schrijven van Engelse teksten. Marian Vermuë wil ik graag bedanken voor het gebruik van haar computer bij Proceskunde. Ik hoop dat mijn gezwoeg in de avonduren en weekenden niet voor al te veel overlast heeft gezorgd.

Tenslotte gaat mijn dank natuurlijk nog uit naar Imke; haar heb ik zeker veel overlast bezorgd, maar binnenkort krijg ik de kans haar net zo goed te steunen als zij mij gedaan heeft!

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic pollutants abundantly present in the environment. This fact, along with their toxicity and mutagenicity, makes them priority pollutants. Experiments with PAHs under laboratory conditions have shown that these compounds can be degraded by various microorganisms. Under field conditions however, PAHs are known as persistent pollutants, forming an important problem for the biological remediation of PAH-polluted soil. This chapter will outline the physicochemical characteristics of PAHs, the environmental problems caused by the pollution of soil with PAHs, and the different aspects that play a role in the biological clean-up of these compounds.

Soil pollution

The contamination of soil with polluting compounds is one of the major environmental problems in both Europe and the United States. On the basis of how the pollution is present in the soil, two categories can be distinguished. The first is diffuse pollution, where the polluting compound is present at low concentrations in large areas. Atmospheric deposition is the major source of diffuse pollution. The second is spot pollution. The contaminated area is relatively small and very high concentrations of the pollutant may occur. The source of the contamination is usually a specific industrial activity which has been or is still being carried out at the spot. Since the 1970s, many polluted areas, causing vast social and economic problems, have been discovered. The number of known polluted sites in the Netherlands is approximately 120,000 and the costs for the clean-up of these sites has been estimated at more than 50 billion U.S. dollars (Stoop & Hesselink 1993).

Another distinction is based on the type of pollution: pollution with heavy metallic compounds and with organic compounds. Pollution with heavy metals is beyond the scope of this thesis and will not be dealt with.

Governments of several countries have set up rules concerning soil pollution. The EPA list (USA) and the Dutch standards (The Netherlands) are examples of guidelines giving regulations on pollutants in soil and groundwater.

Polycyclic aromatic hydrocarbons

Structures and properties of polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are organic molecules that consist of two or more fused benzene rings in linear, angular or cluster arrangement. The chemical structure of the most frequently occuring PAHs, all EPA priority pollutants, is presented in Figure 1. The simplest PAH is the two-ring compound naphthalene; a well-known example of a high molecular weight PAH is the five-ring compound benz[a]pyrene.

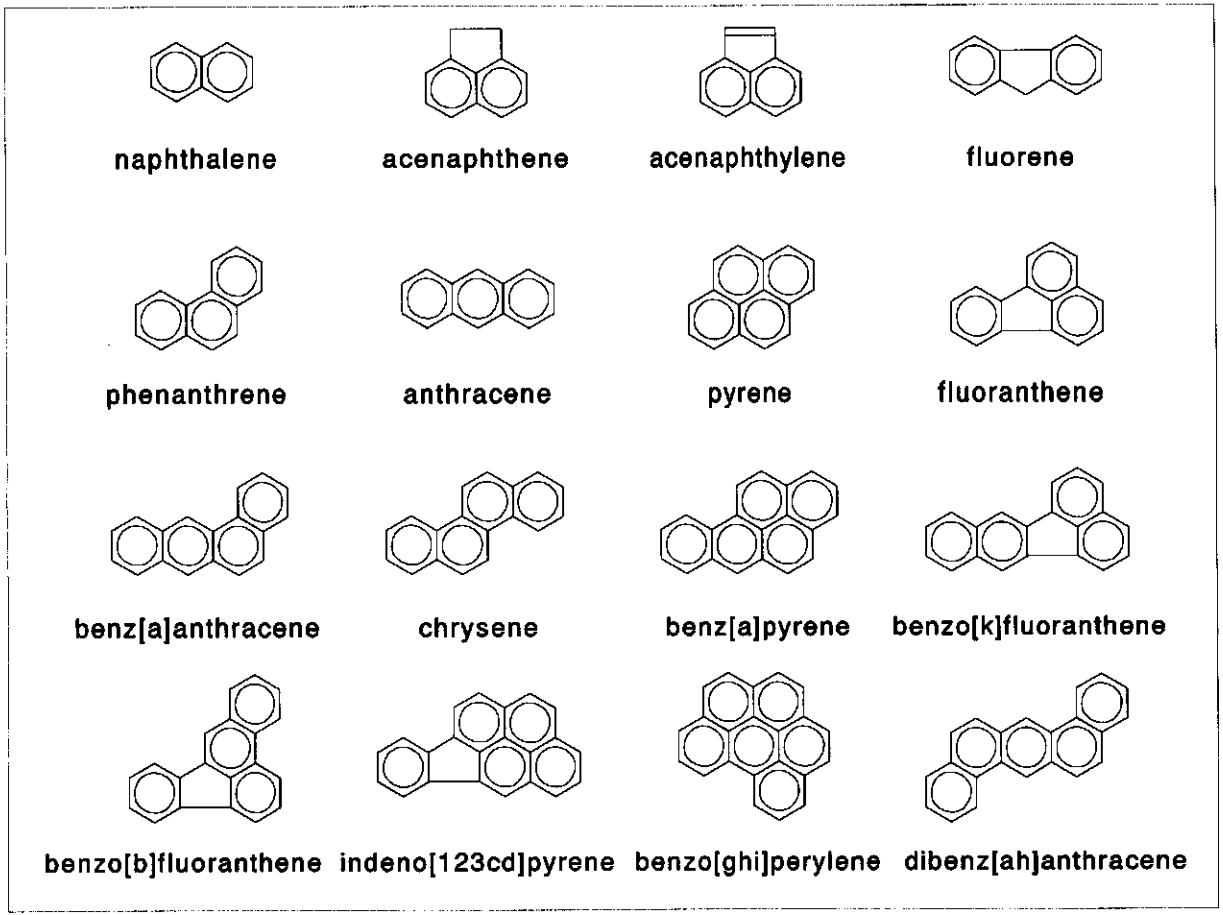

Figure 1: *Chemical structures of the most frequently occurring polycyclic aromatic hydrocarbons.*

Table 1 gives an overview of some physical and chemical properties of the most frequently occurring PAHs. Generally, the hydrophobicity is said to increase, and the aqueous solubility to decrease with an increasing number of aromatic rings.

Table 1: *Physical and chemical properties of the most frequently occurring PAHs (Sims & Overcash 1983).*

PAH	Molecular weight	Aqueous solubility at 30°C [mg·L^{-1}]	Vapour pressure [N·m^{-2} at 20°C]	Log octanol/ water partition coefficient
naphthalene*	128	31.7	6.56	3.37
acenaphthylene	152	3.93	3.87	4.07
acenaphthene	154	3.47	2.67	4.33
fluorene	166	1.98	1.73	4.18
phenanthrene*	178	1.29	$9.07 \cdot 10^{-2}$	4.46
anthracene*	178	$7.3 \cdot 10^{-2}$	$2.61 \cdot 10^{-2}$	4.45
pyrene	202	$1.35 \cdot 10^{-1}$	$8.00 \cdot 10^{-4}$	5.32
fluoranthene*	202	$2.60 \cdot 10^{-1}$	$9.11 \cdot 10^{-5}$	5.33
benz[a]anthracene*	228	$4.0 \cdot 10^{-2}$	$6.67 \cdot 10^{-7}$	5.61
chrysene*	228	$2.0 \cdot 10^{-3}$	$8.40 \cdot 10^{-5}$	5.61
benz[a]pyrene*	252	$4.0 \cdot 10^{-3}$	$6.67 \cdot 10^{-5}$	6.04
benzo[k]fluoranthene*	252	$1.2 \cdot 10^{3}$	$6.67 \cdot 10^{-5}$	6.57
benzo[b]fluoranthene	252	$5.5 \cdot 10^{-4}$	$6.67 \cdot 10^{-5}$	6.84
indeno[123cd]pyrene*	276	$6.2 \cdot 10^{-2}$	$1.33 \cdot 10^{-8}$	7.66
benzo[ghi]perylene*	276	$2.6 \cdot 10^{-4}$	$1.33 \cdot 10^{-8}$	7.23
dibenz[ah]anthracene	278	$5.0 \cdot 10^{-4}$	$1.33 \cdot 10^{-8}$	5.97

*: reference PAHs in Dutch list

Sources and environmental concern

PAHs occur naturally in the environment and as a result of human activities. Natural sources include the products from forest and bush fires, thermal geochemical processes (e.g. vulcanic activity) and synthesis by microorganisms and plants (Blumer 1976). Background concentrations of PAHs in pristine samples can range from 50 to 1100 µg·kg^{-1} in soil (Fritz & Engst 1975), 0.001 to 0.1 µg·L^{-1} in groundwater (Suess 1976), and 0.01 to 88 µg·kg^{-1} in plants (Sims & Overcash 1983).

The anthropogenic sources of PAH emissions can be divided into three main categories:

1 - the incomplete combustion of organic compounds (e.g. fossil fuels), resulting in the atmospheric deposition of PAHs
2 - the deposition of liquid/solid PAH-containing industrial products and waste, such as oil spillage and deposit of coal-tar on gas production sites
3 - the use of PAH-containing products, such as asphalt, coatings for ships, the wood-preserving creosote and anthracene oil, etc.

Concentrations in soil of PAHs originating from atmospheric deposition are variable, but measurements in the Netherlands have shown an average deposition of $2.5 \cdot 10^5$ kg per year (Sloof *et al.* 1989). PAH concentrations at heavily polluted sites may be as high as 12 $g \cdot kg^{-1}$ (Fritz & Engst 1975). An overview of PAH concentrations at different polluted industrial sites is given in Table 2. One of the best examples of the problems with PAH-contaminated sites is the pollution at sites of former gas production plants. The pollution consists mainly of high concentrations of PAHs, cyanides, and heavy metals. Of these contaminated sites 234 are known in the Netherlands, the costs of cleaning them up have been established at 400 million U.S. dollars (RIVM 1991).

The abundance of PAHs in the environment gives rise to concern because of the risk these compounds pose, both from an ecotoxicological and a human-health point of view. PAHs may accumulate in fatty tissues of mammals and specific PAHs have proved to be mutagenic, carcinogenic, and teratogenic (LaVoie *et al.* 1979). Because of this, the United States Environmental Protection Agency has placed 16 PAHs on their priority pollutant list (Keith & Telliard 1979). The Dutch standards for PAHs in soil and sludge are based on ecotoxicological research. When the concentration is below the reference value, the soil is considered clean and multifunctional. At concentrations higher than the intervention value, the soil is too polluted for further use and has to be treated. At intermediate pollution levels other factors, such as the function of the polluted site, are considered in deciding whether the soil is to be cleaned. The reference and intervention values for PAHs are based on the concentration of the ten PAHs on the Dutch list (see Table 1). The values are dependent on the amount of organic material present in the soil and can be calculated according to:

- reference value of total PAHs = 0.1 x %humic material [mg PAHs·kg^{-1} dry weight]
- intervention value of total PAHs = 4.0 x % humic material [mg PAHs·kg^{-1} dry weight]

For a soil with 10% organic matter the reference value is 1 $mg.kg^{-1}$ and the

intervention value 40 $mg.kg^{-1}$. Comparing these values with the concentrations in Table 2 , it can be seen that all of these soils are to be treated and that a reduction of the PAH concentration by a factor of 100-10,000 is necessary to obtain soil that can be used without restrictions.

Table 2: *Concentrations of PAHs in $mg{\cdot}kg^{-1}$ dry weight at selected contaminated sites (Wilson & Jones 1993).*

	Wood preserving		Creosote production		Wood Treatment	Coking plant	Coking plant	Gas works	
PAH	topsoil	subsoil	mean	range				mean	range
naphthalene*	1	3925	1313	<1-5769	92	56	59		
1-methyl naphthalene	1	1452	901	<1-1617			87		
2-methyl naphthalene	1	623	482	6-2926			112		
2,6-dimethyl naphthalene	2	296							
2,3-dimethyl naphthalene	1	168							
acenaphthylene	5	49	33	6-77			187		
acenaphthene	7	1368					29	2	0-11
fluorene	3	1792	650	49-1294	620	7	245	225	113-233
phenanthrene*	11	4434	1595	76-3402	1440	27	277	379	150-716
anthracene*	10	3037	334	15-693	766	6	130	156	57-295
2-methylanthracene	14	516							
fluoranthene*	35	1629	682	21-1464	1350	34		2174	614-3664
pyrene	49	1303	642	19-1303	983	28	285	491	170-833
chrysene*	38	481	614	8-1586	321	11	135	345	183-597
benz[a]pyrene*	28	82			94	14		92	45-159
benz[a]anthracene*	12	171			356	16	200	317	155-397
benzo[b]fluoranthene	}38	}140						260	108-552
benzo[k]fluoranthene*								238	152-446
dibenz[ah]anthracene					10	2		2451	950-3836
indeno[123cd]pyrene*	10	23						207	121-316
total PAHs	256	21466	7246		6022	199	1746	4679	
total reference PAHs	145	13782	4538		4419	164	801	3908	

**: reference PAHs in Dutch list*

Biological soil remediation

Introduction

There are several ways of dealing with heavily polluted soils and sediments. At present the techniques most used in the Netherlands for soils containing organic pollutants are incineration, extraction/classification, disposal at landfill sites, and, to a lesser extent, biological soil remediation. Incineration and extraction/classification are relatively expensive and destroy the soil as a living system. Landfilling is usually cheap, but only transfers the problem from one place to another. Moreover, it may cause unwanted emissions through volatization or leaching of pollutant. Other physicochemical techniques such as solvent extraction, critical CO_2 extraction, extraction with surfactant solutions, and glassification are still mostly in the development stage. However, they are cost-intensive and therefore only applicable to special waste streams. An overview of the different physicochemical techniques is given by Rulkens *et al.* (1993).

Biological soil remediation involves the use of microorganisms to degrade hazardous organic pollutants. It has several advantages over the other techniques in terms of costs and soil functionality. However, not every contaminated soil can be treated biologically. Therefore a characterization is necessary which includes:

- *type of pollutant.* Bioremediation is not applicable to non-biodegradable organic pollutants;
- *type of soil.* Soils with a high clay content are hard to treat using most biological methods;
- *other pollutants.* The presence of toxic compounds in high concentrations may prevent biodegradation, and bioremediation may be of little use when non-degradable pollutants (e.g. heavy metals) are present.

For successful biological clean-up of soil it is necessary to optimize the conditions for biodegradation of the pollutants. The most important parameters are (Thomas & Lester 1993):

- *presence of microorganisms.* Usually microorganisms capable of degrading the pollutants are present at the contaminated site. Many treatments involve the addition of specific bacteria, although the actual use is often questionable;
- *oxygen concentration.* The degradation of most hydrocarbons is slower or even inhibited under anaerobic conditions. An exception is formed by a number of highly halogenated compounds, which are dehalogenated under anaerobic

conditions only;

- *pH.* Hydrocarbon-degrading bacteria have a typical pH optimum of 6-8, fungi have a lower optimum, ranging from 4-5. Since the pollutant degradation may alter the pH, monitoring and controlling this parameter is important;
- *nutrient availability.* Microorganisms require a range of both macro- and micro-nutrients to maintain growth. The two most important nutrients for biological soil treatment are phosphorus and nitrogen;
- *temperature.* Temperature is one of the most important parameters in determining the rate of biodegradation. Optimal temperatures for biodegradation vary from 20-30°C;
- *moisture content.* Biodegradation is a water-based process and the presence of water is therefore essential. Excess water, however, may limit oxygen transfer.

The importance and the extent to which these parameters can be manipulated depends on the type of remediation technique applied. The three principle techniques that can be used for soil bioremediation are described below (Blackburn & Hafker 1993):

- With *in situ* treatment, the polluted soil is left in place and remains essentially undisturbed. The site can be isolated and is usually flushed with air (bioventing or biosparging), or with water. The first method is used mainly for volatile pollutants and the degradation in the soil is stimulated by enhanced oxygen supply. The off-gas may be cleaned in a biofilter. Washing with aqueous solution is used for non-volatile pollutants. Compounds with a high water solubility will be partly washed out and treated in a water treatment system. Water-insoluble compounds will have to be degraded in the soil. *In situ* treatment is usually cheap, but has the disadvantage that it is difficult to control the processes in the soil, although the washing water can be supplemented with e.g. nutrients and hydrogen peroxide (for oxygen supply).
- With landfarming, the polluted soil is excavated and transferred to an isolated site where it is spread in a layer with a defined height. Intensive landfarming usually involves mixing and addition of nutrients. Moreover, the process parameters such as pH, temperature, oxygen supply, and moisture content may be controlled. The advantage of this technique is that the soil can be treated under controlled conditions at relatively low costs. Landfarming, the best developed bioremediation technique, has been used on a large scale for oil-contaminated soils (Morgan & Watkinson 1989).

- Treatment in a bioreactor is the most intensive biological treatment and the input of energy in this technique is usually high. It is best suited for the treatment of soils with a high clay content or for sediments with a high water content. The conditions can be optimized and high degradation rates obtained. Bioreactors have not yet been used extensively for the treatment of polluted soil or sediment.

Role of bioavailability in soil bioremediation

In the Netherlands, about 10% of the polluted soil is biologically treated, most of it via landfarming. The main reasons for not using bioremediation more often are that the treatment times are usually too long and the residual pollutant concentrations often too high to permit unrestricted use of the treated soil. This is illustrated in Figure 2, which compares a typical timecourse of the pollutant concentration in a biological soil treatment process to that of the same pollutant under laboratory conditions. In the first stage, the pollutant is degraded rapidly and the concentration decreases rapidly. As the process continues, the degradation rate drops and in the final stage the decrease is very slow. This timecourse is similar to that of radioactive decay and therefore the degradability of organic pollutants is often expressed as the half-life ($t_{1/2}$) of the pollutant, although it is only an empirical approach.

Even the biodegradation rates of organic compounds that can easily be degraded by bacteria (e.g. phenol) are much lower in soil than under laboratory conditions. It is generally accepted that this slow biodegradation is caused by the pollutant not being present in the aqueous phase (Mihelcic *et al.* 1993). This is especially true for hydrophobic organic compounds such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and weathered mineral oils.

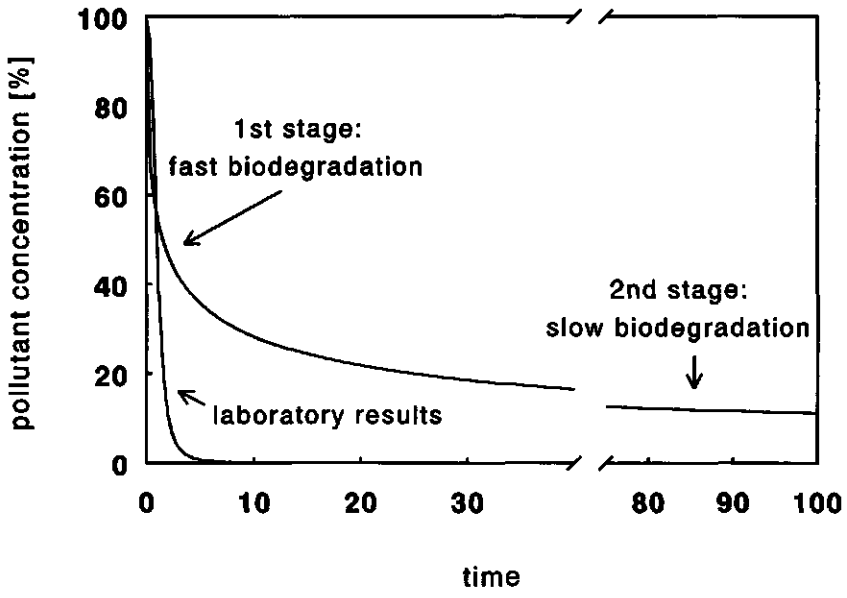

Figure 2: *A typical timecourse of the degradation of a hydrophobic organic pollutant in soil bioremediation.*

Studies have revealed that most organic compounds can only be taken up by bacteria when they are present in an aqueous phase and therefore the mass transfer of the pollutant to the bulk of the aqueous phase is a prerequisite for biodegradation. When the mass transfer limits the biodegradation process, this is termed limited bioavailability. The term bioavailability is also used with a somewhat different meaning by toxicologists and, to avoid confusion, several researchers have tried to give a definition for limited bioavailability. The definition to be used in this thesis is:

A pollutant has a limited bioavailability when its uptake rate by organisms is limited by a physicochemical barrier between the pollutant and the organism.

For a better insight into the processes that play a role in bioavailability, it is essential to understand the interactions between the soil matrix, the pollutant, the aqueous phase, and the microorganisms. These interactions are dependent on (i) the type and physicochemical state of the pollutant, (ii) the type and physicochemical state of the soil, (iii) the type and state of the microorganisms, and (iv) on external factors, such as temperature and presence of oxygen.

The first two factors determine the form in which the contaminants occur in soil.

The different physical forms possible for organic contaminants are illustrated in Figure 3. They can be dissolved in pore water, adsorbed onto soil particles, absorbed into soil particles, or be present as a separate phase, which can be a liquid or a solid phase. As bioavailability is based on mass transport rates, transport of the pollutant to the aqueous bulk phase is the key process governing the bioavailability of the contaminant.

Microorganisms may actively increase the bioavailability of hydrophobic compounds by excretion of enzymes or surface-active compounds, or by direct uptake of non-aqueous phase pollutant (Mihelcic *et al.* 1993). The extent of this increase is dependent on the other factors determining the bioavailability, and is difficult to predict.

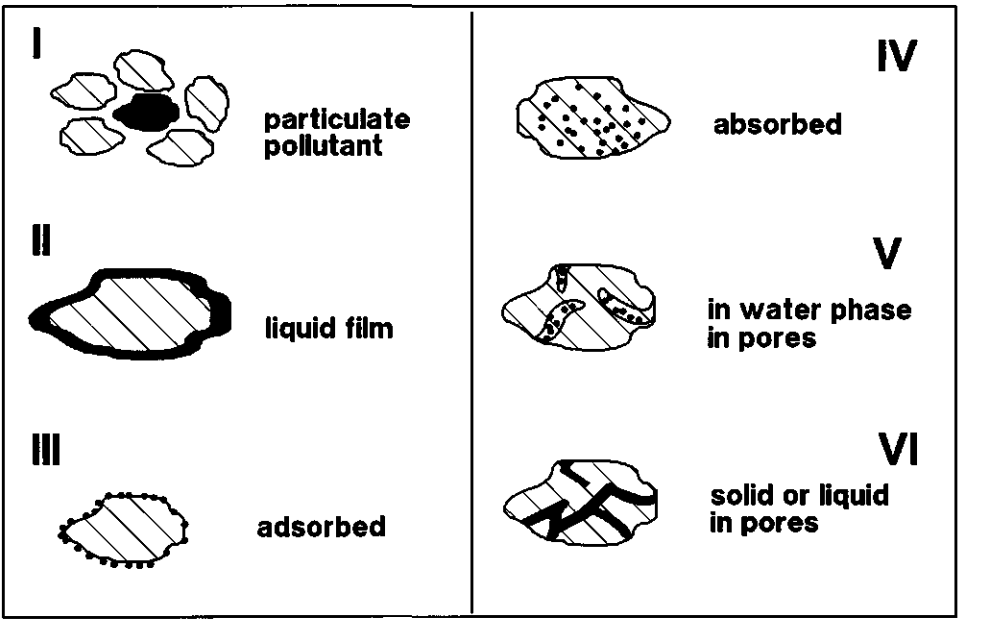

Figure 3: *Different physical forms of organic pollutants in soil: I: solid particles; II: liquid film; III: adsorbed onto soil; IV: absorbed into soil; V: in the waterphase of soil pores; VI as a separate phase in soil pores (adapted from Rulkens 1992).*

Biodegradation and bioavailability of polycyclic aromatic hydrocarbons

PAH-degrading microorganisms

As PAHs are naturally occurring compounds, it is not surprising that many different PAH-degrading microorganisms can be found in pristine environments (Sims & Overcash 1983). Most of the microorganisms that have been isolated, however, originate from PAH-contaminated sites. Many different species of bacteria (both gram-negative and gram-positive), fungi, and algae are known to degrade PAHs. An overview of the organisms capable of degrading the different PAHs is given by Cerniglia (1992).

Studies on the microbial ecology of PAH-contaminated soils have shown that the numbers of PAH-degrading microorganisms, as well as the degrading capacity, are much higher in PAH-contaminated soils than in pristine environments (Herbes & Schwall 1978). Thus, the indigenous microbial population changes towards PAH-tolerant and PAH-degrading microorganisms due to the presence of the pollution. It was also shown that bacteria are the most important group of soil microorganisms involved in the biodegradation of PAHs (Kastner *et al.* 1994, McGillivray & Shiaris 1994).

Microbial PAH metabolism

The microbial metabolism of PAHs has been studied extensively. Bacteria, fungi, yeasts, cyanobacteria, and algae are known to have the enzymatic capacity to oxidize PAHs, ranging in size from naphthalene to benz[a]pyrene. The aerobic hydroxylation of PAHs always involves the incorporation of molecular oxygen; there are, however, differences in the hydroxylation mechanisms of eukaryotic and prokaryotic organisms. At present, not much is known of the PAH metabolism of cyanobacteria and algae. Fungi with the cytochrome P-450 monooxygenase oxidize PAHs to form arene oxides, which can isomerize to phenols or undergo enzymatic hydration to yield *trans*-dihydrodiols (Figure 4A). White-rot fungi, such as *Phaenerochaete chrysosporium*, produce a-specific extracellular lignin-degrading enzymes. These enzymes seem to be responsible for the oxidation of high molecular weight PAHs, and pure enzymes catalyze the formation of quinones. Some of the fungal metabolites are more toxic than the parent compounds (Cerniglia 1984).

Since the bacterial PAH metabolism is of more importance for this thesis, it will

be dealt with in more detail. The initial attack of the aromatic ring is usually performed by a dioxygenase, forming a *cis*-dihydrodiol. This is then converted by a hydrogenase into a dihydroxylated derivative (Figure 4B).

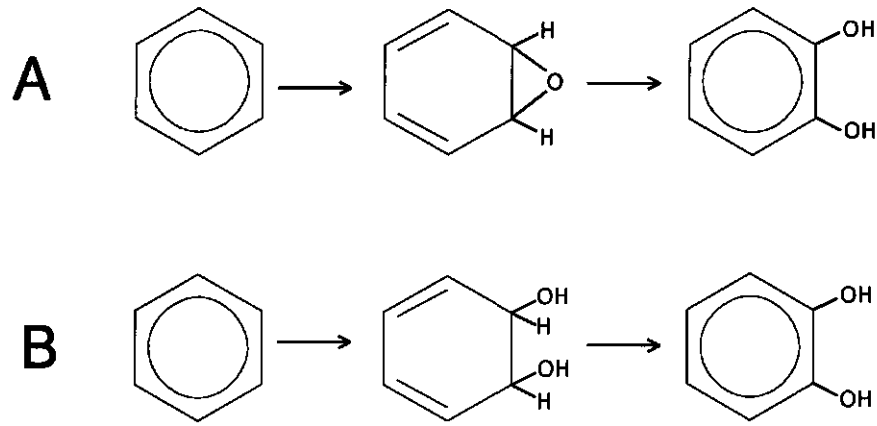

Figure 4: *Initial steps in the microbial catabolism of aromatic compounds A: fungi; B: bacteria.*

The next step in the degradation is ring fission, which proceeds analogue to the ring fission of catechol. It can take place via the *ortho* pathway, in which ring fission takes place between the two hydroxylated carbon atoms, or via the *meta* pathway, which involves cleavage of the bond adjacent to the hydroxyl groups. After this ring fission a number of reactions can occur. For the lower molecular weight PAHs, the most common route involves the fission into a C_3 compound and a hydroxy aromatic acid compound. The aromatic ring can thereafter either undergo direct fission or can be subjected to a decarboxylation, leading to the formation of a dihydroxylated compound. This compound can then be dissimilated as described above. When degraded via these pathways, the low molecular weight PAHs can be completely mineralized to CO_2 and H_2O. An overview of the mineralization pathways of naphthalene, phenanthrene, and anthracene, showing the similarities, is presented in Figure 5. The complete degradation of other low molecular weight PAHs, such as acenaphthene and acenaphthylene, has also been described (e.g. Komatsu *et al.* 1993). Partial degradation of low molecular weight PAHs can also occur, the products usually being hydroxy aromatic acids or hydroxy aromatics (Cerniglia 1992).

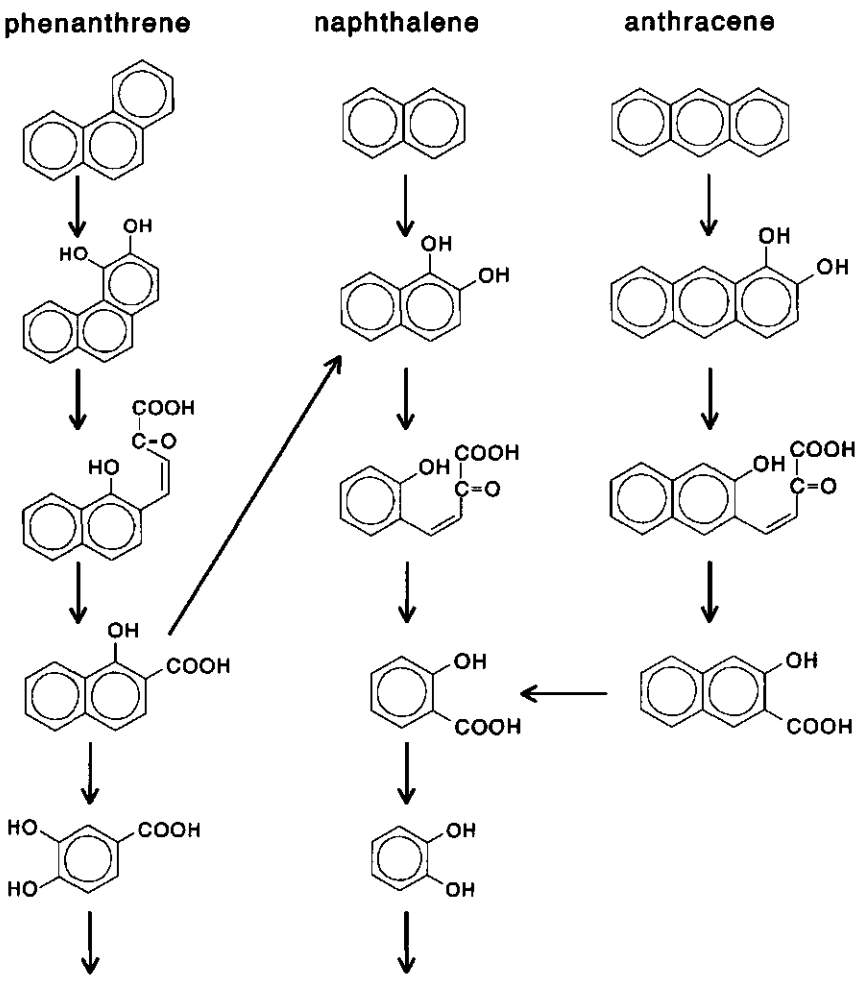

Figure 5: *Bacterial mineralization of naphthalene, phenanthrene, and anthracene.*

Detailed information on the biochemical pathways for bacterial catabolism of the high molecular weight PAHs is scarce and the use of these compounds as sole source of carbon and energy has only been reported for fluoranthene (Kelley *et al.* 1993, Mueller *et al.* 1990, Weissenfels *et al.* 1991), pyrene (Boldrin *et al.* 1993, Walter *et al.* 1991), and chrysene (Walter *et al.* 1991). Cometabolic, partial degradation has been shown for more PAHs, and often hydroxy polyaromatic acids are formed as end products. (Gibson *et al.* 1975, Heitkamp & Cerniglia 1988, Kelley

et al. 1993, Mahaffey *et al.* 1988). In biodegradation studies with the other PAHs it was found that all of the PAHs disappeared, although it could not be proven that this was caused by biodegradation.

Anoxic biodegradation by bacteria has only been found for the low molecular-weight PAHs acenapthene and naphthalene under denitrifying conditions (Mihelcic & Luthy 1987, 1988). No other evidence for the biodegradation of PAHs under anaerobic conditions could be found.

The regulation of the bacterial PAH metabolism is not yet very well established. The genetic information encoding for the enzymes involved in the initial steps of the degradation of naphthalene, phenanthrene, and anthracene has been shown to be located on plasmids. No information is available on the genetics of the degradation of high molecular weight PAHs (Menn *et al.* 1993, Sanseverino *et al.* 1993).

Biodegradation of PAHs in soil

Several studies have shown that biodegradation is the most important process responsible for the removal of PAHs in soil systems (e.g. Lee *et al.* 1978, Sims & Overcash 1983). For the more volatile two-ring PAHs, volatization may also play a role. In Table 2 it can be seen that polluted sites usually contain mixtures of several PAHs.

As it has been shown that the formation of aromatic metabolites is a common phenomenon in the bacterial PAH metabolism, the biodegradation of PAHs in soil can be expected to result in the accumulation of these metabolites. In most of the studies on the degradation of PAHs in natural systems, however, the results are expressed as the removal efficiency, determined by measurement of the residual PAH concentrations. Only few studies have considered the fate of PAHs in natural systems. Herbes & Schwall (1978) found that after incubation of ^{14}C-labelled PAHs in an acclimated soil, 20-60% of the amount of PAHs that had disappeared could be detected as soluble or bound ^{14}C compounds. Although the toxicity of the metabolites is often lower than the toxicity of the parent compound, their bioavailability may be higher because of their higher aqueous solubilities. Measuring the change in toxicity of the soil or groundwater before and after treatment forms a means of evaluating the results of bioremediation of contaminated soil that provides information on the problem of metabolite formation. Wang *et al.* (1990) and Baudgrasset *et al.* (1993) have used this method for PAHs. In both cases it was found that the toxicity of the contaminated soil decreased along with the PAH biodegradation. Thus, although the formation of metabolites is likely to occur, this was not reflected in the toxicity. A

possible explanation for this phenomenon is that the intermediates, which are more reactive than saturated PAHs, undergo a chemical reaction with the soil organic matter.

Bioavailability of polycyclic aromatic hydrocarbons

Although the biodegradation pathways of the different PAHs are very similar, as discussed above, their biodegradation rates in soil differ considerably. Generally, first-order degradation kinetics are observed and the degradation rate is found to decrease with an increasing number of rings. An overview of degradation rates of PAHs in contaminated soils is given by Wilson & Jones (1993). As an example, laboratory studies with creosote-polluted soil showed that two-ring PAHs exhibited half-lives < 10 days, three-ring PAHs showed half-lives <100 days, and four- and five-ring PAHs were found to have half-lives of >100 days (McGinnis *et al.* 1988). In another study (Herbes & Schwall 1978) the same differences in degradation rates of the different PAHs were observed, although slightly higher degradation rates were found.

Two factors are thought to be responsible for these different degradation rates. Firstly, the bacterial uptake rates of the compounds with higher molecular weights have shown to be lower than the uptake rates of low molecular weight PAHs (see Chapter 2). The second and most important factor, however, is the low bioavailability of PAHs. Because of their low aqueous solubilities, PAHs will occur in soil mainly in association with soil matter or as a separate phase. The last column of Table 1 shows the value of the octanol-water partition coefficient to generally increase with an increasing number of aromatic rings. Several studies have shown a good correlation between the octanol-water partition coefficient, the aqueous solubility, and the soil-water partition coefficient of a pollutant (e.g. Dzombak & Luthy 1984, Karickhoff *et al.* 1979). The fact that PAHs with the high molecular weights have the highest octanol-water partition coefficients explains the difference in the observed halflifes of PAHs in soil. When PAHs are present as a separate phase, such as tar particles, their bioavailability is even lower than that of sorbed PAHs (Weissenfels *et al.* 1992)

Besides the low degradation rates, the low bioavailability is also an important cause of another problem in the bioremediation of PAH-contaminated soil: the high residual PAH concentrations after clean-up. Erickson *et al.* (1993) investigated bioremediation of a PAH-contaminated manufactured-gas plant (MGP) site and showed that the limited bioavailability of the residual PAHs prevented further

biodegradation. Similar results were found by Bouwer *et al.* (1994) for naphthalene degradation at an MGP site and by Weissenfels *et al.* (1992) for PAHs occurring at a wood impregnation site. Generally, the residual PAH concentrations in soils after biological clean-up are found to be too high to allow unrestricted use of the soils according to most governmental guidelines (Wilson & Jones 1993).

Nevertheless, it can be said that biological remediation of PAH-polluted soil can in many situations be a good alternative for other remediation techniques. However to make biological clean-up of soil a grown-up technique, a solution for the problem of the limited bioavailability will have to be found.

Outline of this thesis

The aim of the study described in this thesis was to obtain insight into the microbiological, chemical, and physical processes involved in the bioavailability and biodegradation of polycyclic aromatic hydrocarbons. This insight can be used for modelling these processes and for optimization and prediction of biodegradation rates in the biological clean-up of soil. Moreover, it can serve as the basis for finding solutions to the problem of limited bioavailability.

Polycyclic aromatic hydrocarbons have been selected as model compounds in this study because they are frequently occurring priority pollutants and well-known representatives of a group of hydrophobic organic compounds which are biodegradable in laboratory experiments, but may persist in soil.

The first part of the research deals with the isolation and characterization of bacteria, growing on the PAHs naphthalene, phenanthrene, and anthracene. The organisms were cultivated in batch and continuous cultures and the most important microbiological kinetic parameters determined; this is described in Chapter 2.

The second part deals with the central issue of this thesis: the role of mass transport phenomena possibly occurring in soil on the biodegradation rate of PAHs. Chapter 3 describes bacterial the growth on crystalline PAH, one of the simplest systems in which the effect of the limited availability of substrate affects the bacterial growth kinetics. Chapter 4 deals with the biodegradation of PAH, sorbed onto a matrix, which can be either an artificial matrix or soil.

The final part of this thesis outlines a possible solution to the problem of limited availability of PAHs: the application of surface-active compounds. Chapter 5 reviews the use of these compounds in biological soil remediation. The following two

chapters describe the effect of surfactants on the bioavailability and biodegradation of crystalline PAHs (Chapter 6) and sorbed PAHs (Chapter 7). Finally, some general conclusions will be drawn in Chapter 8.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF PAH-DEGRADING BACTERIA

Abstract

Described here are the isolation and initial characterization of polycyclic aromatic hydrocarbon (PAH)-degrading microorganisms. Mixed cultures and pure bacterial strains were grown in batch on several PAHs and in chemostats on naphthalene and phenanthrene. The results of these experiments were used to determine the most important microbiological parameters for the biodegradation of PAHs.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that consist of two or more fused benzene rings. They occur in the environment at high concentrations, mainly due to human activities, and give rise to concern because of their toxicity and mutagenicity.

The biodegradation of PAHs by bacteria has been the subject of much research and many bacteria of different species have been isolated on many different PAHs. For the lower molecular weight PAHs the catabolic pathways have been revealed and it has been shown that the genetic information encoding for the first steps in the biodegradation is located on plasmids (Sanseverino *et al.* 1993). An overview of the biochemistry of PAH degradation is given by Cerniglia (1984).

The physiology of PAH-degradation has been investigated in less detail. For some isolated organisms the maximal growth rate (μ_{max}), temperature and pH optimum, formation of intermediates, or the substrate specificity have been determined (Boldrin *et al.* 1993, Guerin & Jones 1988, Keuth & Rehm 1991, Walter *et al.* 1991).

This study has been performed to obtain pure cultures growing on different PAHs and to study the growth kinetics of these organisms on the different PAHs. Insight into these growth kinetics is essential for studies in which these organisms are used to investigate the effect of substrate availability on biodegradation kinetics.

Materials and methods

Media and chemicals

All chemicals used were of an analytical grade, with the exception of Triton X-100. Organisms were grown at 30°C in mineral medium, essentially composed as described by Evans *et al.* (1970), with 1 mM EDTA as a chelating agent and the concentrations of other medium components being half those described. The mineral medium consisted of 50 mM NH_4Cl, 5 mM NaH_2PO_4, 5 mM KCl, 1 mM Na_2SO_4, 0.625 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.05 µM Na_2MoO_4 and 2.5 ml·L^{-1} of a spore solution containing 0.12 mM HCl, 5 mM ZnO, 20 mM $FeCl_3$, 10 mM $MnCl_2$, 1mM $CuCl_2$, 2 mM $CoCl_2$ and 0.8 mM H_3BO_3. When used for batch cultures the medium was buffered at pH 7.0 with 50 mM sodium phosphate.

Adaptation of activated sludge to PAH

Activated sludge samples from a domestic wastewater treatment plant (Gevudo, Dordrecht, The Netherlands) were suspended in buffered mineral medium described above. The enrichment cultures were aerated with naphthalene-, phenanthrene-, or anthracene-

saturated air at 60°C until growth occurred. From these cutures isolates were obtained by plating out on agar slants containing 1.5% agar in mineral medium. Phenanthrene and anthracene were added to the slants in crystalline form (0.1% w/v); naphthalene was added in the same way or via the gaseous phase by incubating slants at room temperature in an exsiccator in which naphthalene crystals were present.

Cell storage

Mixed cultures were maintained in sequential batch cultures (see iii below). Pure cultures were maintained:

(i) at 30°C on agar slants (storage at 4°C) identical to those described above, transferring the bacteria to new slants every 2-3 weeks;
(ii) at -70°C in mineral medium containing 15% glycerol;
(iii) as sequential batch cultures; 1 ml of these cultures was transferred to sterile Erlenmeyer flasks containing 100 ml fresh medium every 2-3 weeks and incubated at 30°C on a rotary shaker (200 rpm). Every four months a new batch culture was started from cells that had been stored at -70°C.

Batch growth experiments

Batch growth experiments were usually performed in 250-ml conical flasks or in 250-ml serum flasks containing 100 ml mineral medium. PAHs and hydroxynaphthoic acids were added in crystalline form after sterilization for 20 min. at 120°C; other substrates were dissolved in the mineral medium. The experiments were started by inoculating with 1 ml of the appropriate mixed or pure culture. The flasks were incubated on a rotary shaker (200 rpm) at 30°C.

Continuous growth experiments

Three different methods for PAH addition to continuous cultures were used: via the gas phase, dissolved in acetone, or dissolved in surfactant micelles. The first method was used for naphthalene only, the other two for both naphthalene and phenanthrene. All experiments were performed under carbon limitation at 30°C and the medium described above was used. Unless stated otherwise, the pH was maintained at 7.0 by titration with 0.5 M NaOH.

The continuous culture to which naphthalene was added via the gas phase was used for the production of naphthalene-grown strain 8909N cells and for determination of residual substrate concentrations only. A reactor (Applicon B.V., Schiedam,The Netherlands) with a working volume of 1.2 L was used. The air was passed through a 20-cm column (30°C) filled with naphthalene crystals to saturate it with naphthalene before being led through the reactor at a flow of 180 $L \cdot h^{-1}$. The dilution rate was varied from 0.05 to 0.35 h^{-1}. The non-linear fitting program Enzfitter (Biosoft, N.J., USA) was used to fit the data with the Monod equation.

Continuous experiments with acetone-dissolved PAH were performed to study the

kinetics of naphthalene and phenanthrene degradation. A reactor (L&H, UK) with a working volume of 800 ml was used. The PAH was added separately to the reactor as a solution of 30 g·L^{-1} in acetone. The flow of the acetone solution was dependant on the dilution rate and was set to achieve an overall ingoing naphthalene concentration of 62 mg·L^{-1}.The airflow was 1 vesselvolume per minute. Samples were taken to determine the dry weight, the optical density at 540 nm (OD540), the protein content, the residual PAH concentration, and the total organic carbon (TOC) content. Growth of strain 8909N on acetone was checked every two weeks by plating out on agar slants with 0.1% acetone.

When the PAH was added in surfactant micelles (little aggregates of surfactant molecules with a hydrophobic interior), a reactor setup was used in which oxygen supply was achieved by leading pure oxygen through the headspace of the reactor. For experiments with phenanthrene a 1-L Erlenmeyer flask with a working volume of 200 ml was used as a reactor. Mineral medium containing 2 g·L^{-1} Triton X-100 and approximately 70 mg·L^{-1} phenanthrene was pumped into the reactor at different flow rates. This setup did not allow the pH to be controlled and therefore the medium was buffered with 50 mM sodium phosphate buffer, pH 7. Continuous experiments with naphthalene dissolved in Triton X-100 micelles were performed in an L&H fermentor (see above) with a working volume of 300 ml. Growth of strains 8803F and 8909N on Triton X-100 was regularly checked by plating out on agar slants containing 0.1% (w/v) of Triton X-100. To check for oxygen limitation the strains were also cultured on succinate (2.0 g·L^{-1} in buffered mineral medium) in the same setup.

Analytical procedures

Biomass was essayed routinely by measuring the OD 540. Dry weights were determined according to Herbert *et al.* (1971); the carbon content of biomass was assumed to be 45% (Tempest & Strange 1966). Protein was assayed according to Peterson (1977). TOC was measured using a TOC analyzer (Model 700 TOC Analyzer O.I. Corporation, USA). The EDTA in the medium was measured as TOC.

CO_2 in the headspace gas of serum flasks was determined using a gas chromatograph (Hewlett Packard type 5890) equipped with a thermal conductivity detector and a Hayesep Q packed stainless steel column (diameter 1/8 in, length 2 m, Chrompack, Middelburg, The Netherlands). Helium was used as carrier gas with a flow rate of 30 ml·min.$^{-1}$. The injector temperature was 150°C, the oven temperature 80°C, and the detector temperature 200°C. The injection volume was 250 µl with splitless injection.

For analysis of aqueous PAH concentrations, samples were filtered with a 0.2 µm rotrand filter (Schleicher & Schuell, Germany), diluted 1:1 with acetonitrile, and injected on an HPLC using a ChromSpher 5 C18 (PAH) column (Chrompack). Eluens was a 80/20 mixture of acetonitrile/water. Peaks were detected by use of a fluorescence detector (Shimadzu, Kyoto, Japan). Wavelengths of excitation and emission were 278 nm and 324 nm for naphthalene, 253 and 333 nm for phenanthrene, and 253 and 373 nm for anthracene.

Results and discussion

Growth of mixed cultures and isolation of pure cultures

The enrichment procedures resulted in mixed bacterial cultures growing on naphthalene, phenanthrene, and anthracene. The maximum growth rates of these cultures are presented in Table 1.

Table 1: *Maximum growth rates of mixed cultures grown batchwise in mineral salts medium (pH = 7.0, T = 30°C).*

Substrate	Maximum solubility [mg·L^{-1}]	Growth rate obtained [h^{-1}]
naphthalene	31.7	0.3
phenanthrene	1.29	0.03
anthracene	0.073	0.003

It was found that all three culture types could grow on naphthalene, phenanthrene, and anthracene as sole source of carbon and energy. Several bacterial strains were isolated from the enrichment cultures growing on naphthalene, phenanthrene, and anthracene. They were all gram-negative motile rods and strains 8803F and 8909N were tentatively identified as *Pseudomonas* species. Because of the observed similarities, the other strains are likely to be *Pseudomonas* species as well. An overview of the isolated strains and their ability to grow on several substrates is shown in Table 2.

Batch growth of pure cultures

All strains growing on naphthalene were also capable of growth on salicylic acid, indicating that they degraded naphthalene via the pathway presented in Chapter 1, as proposed by Davies & Evans (1964). Growth of the cultures on naphthalene did not result in the formation of any measurable intermediates. Strain 8909N was selected for further studies. Its maximum growth rate in batch cultures was approximately 0.40 h^{-1} on naphthalene and 0.70 h^{-1} on succinate. Often when batch cultures with naphthalene were inoculated with cells of the strains 8806N or 8909N, a lag phase of 1-4 days was observed. No lag phase occurred when the inoculum had been growing in a medium with a high naphthalene concentration.

Table 2: *Growth of the isolated strains on different substrates.*

Code	Isolated on	Growth on						
		naph	phen	ant	flu	sal	12HNA	23HNA
8805N	naphthalene	+	+/-	+/-	-	+		
8806N	naphthalene	+	+/-	+/-	-	+	+	-
8909N	naphthalene	+	+/-	+/-	-	+	+	-
8801F	phenanthrene	-	+	+/-	-	+		
8803F	phenanthrene	+	+	+/-	-	+	+	+
8804F	phenanthrene	+	+	+/-	-	+		
8808F	phenanthrene	-	+	+/-	-	-	-	+
8809F	phenanthrene	+	+	+/-	-	+		
8911F	phenanthrene	+	+	+/-	-	+/-		
8901A	anthracene	+	+	+	-	+	+	+
8902A	anthracene	+	+	+	-	+	-	+

naph = naphthalene; phen = phenanthrene; ant = anthracene; flu = fluoranthene, sal = salicylate, 12HNA = 1-hydroxy,2-naphthoic acid, 23HNA = 2-hydroxy,3-naphthoic acid;
+: growth within 2 weeks; +/-: growth between 2 and 4 weeks; -: no growth within 4 weeks

High concentrations of naphthalene were concluded to be toxic to the bacteria, but bacteria can adapt to the high concentrations; a phenomenon that has been observed previously for growth on aromatic hydrocarbons (Weber *et al.* 1993).

Dense bacterial cultures growing on phenanthrene turned brown at the end of batch growth. The UV spectra of the supernatant of strain 8809F at different pH values showed good agreement with the spectra found by Evans *et al.* (1965) for the intermediate *cis*-4-(1-hydroxynaphth-2-yl)-2-oxo-3-butanoic acid (see Chapter 1, Figure 2). This, together with the fact that most strains isolated on phenanthrene could also grow on 1,2-hydroxynaphthoic acid, indicates that phenanthrene was degraded via the pathway proposed by Evans *et al.* (1965). The maximum growth rates of strains 8803F and 8808F in batch on phenanthrene were 0.11 and 0.10 h^{-1},

respectively. No phenanthrene toxicity was observed, probably due to its low aqueous solubility.

Growth on anthracene proceeded very slowly. Strain 8902A was the fastest growing strain, with a maximum growth rate on anthracene of 0.012 h^{-1}. No intermediates were found during growth on anthracene and no toxicity was observed. Table 3 compares the maximum specific growth rates found in this study with values from the literature and relates these to the solubilities of the substrates. Except for the fluorene, the maximal growth rate decreased remarkably parallel to a decrease in solubility of the substrates. Several explanations can be given for this phenomenon. Firstly, it is possible that even during the exponential phase low substrate solubilities determine the kinetics of microbial growth. Secondly, the transport of the molecules over the cell envelope may be more difficult for the larger PAHs. However, due to experimental problems (low biomass concentrations and extremely low growth rates), it is difficult to quantify this phenomenon in terms of the maximum growth rate μ_{max} and the Monod bacterial affinity constant K_s.

***Table 3**: Experimentally determined maximum growth rates and maximum growth rates reported in literature for several PAHs.*

Substrate	Aqueous solubility [mg·L^{-1}]	maximum growth rate [h^{-1}]
naphthalene	31.7	0.45
fluorene	1.98	0.03
phenanthrene	1.29	0.12; 0.11[a]; 0.069[c]
fluoranthene	0.26	0.040[c];
pyrene	0.14	0.023[b]; 0.056[c]
anthracene	0.073	0.012

[a]: Keuth & Rehm 1991; [b]: Walter *et al.* 1991; [c]: Boldrin *et al.* 1993;

The overall bacterial growth yields during batch growth were determined by measuring the residual PAH concentrations, the CO_2 concentration in headspace gases, the dry weights, and the protein content of cultures at the end of growth experiments. The results of these measurements are given in Table 4. No significant difference could be found between the yield of strain 8803F on phenanthrene and that of strain 8909N on naphthalene. Moreover, the yield remained constant with different amounts of PAHs.

Table 4: *Mass balance over batch growth of strain 8909N on naphthalene and strain 8803F on phenanthrene; data are means of data from three separate experiments.*

Initial substrate [C-mol]	Residual substrate [µM]	CO_2 produced [C-mol]	Dry weight [mg.L^{-1}]	Protein content [mg.L^{-1}]	CO_2 yield [C-mol.C-mol^{-1}]	Biomass yield[a] [C-mol.C-mol^{-1}]	Recovery[a] [%]
naphthalene							
0.78	< 0.1	0.54	1.02	55.9	0.69	0.25	94
1.56	< 0.1	1.12	N.D.	94.9	0.72	0.21	93
1.95	< 0.1	1.31	2.62	137	0.69	0.24	93
2.73	< 0.1	1.72	N.D.	185	0.63	0.24	87
3.90	< 0.1	2.53	4.82	258	0.65	0.23	88
phenanthrene							
0.79	< 0.25	0.55	1.02	56.9	0.70	0.26	96
1.57	< 0.25	0.99	N.D.	113	0.63	0.26	89
1.97	< 0.25	1.34	2.55	124	0.68	0.23	91
2.75	< 0.25	1.79	N.D.	194	0.65	0.25	90
3.93	< 0.25	2.59	4.93	264	0.66	0.24	90

a: calculated using the protein content and assuming that the protein content of the cells is 60% and that 45% of the cell mass is carbon

N.D.: not determined

Substrate specificity

Most of the strains isolated were capable of growth on several different PAHs (see Table 2). To investigate the substrate specificity of the bacteria, experiments have been performed with concentrated cell suspensions of the strains 8909N and 8803F. Strain 8909N did not show growth on phenanthrene. When this strain was precultured on naphthalene, addition of phenanthrene resulted in an intense yellow colouring of the medium. The UV spectrum of the supernatant showed good agreement with the spectrum of 3,4-dihydroxyphenanthrene. Incubation of strain 8909N cells, precultured on succinate, using phenanthrene as substrate did not result in detectable formation of intermediates. In contrast with strain 8909N, which could not grow on phenanthrene, strain 8803F was capable of growth on both naphthalene and phenanthrene. However, when strain 8803F cells, precultured on phenanthrene, were incubated with naphthalene, a lag phase of 2-4 days occurred; initially the production of 1,2 dihydroxy-naphthalene could be observed, indicating that the conversion of both PAHs to dihydroxylated compounds is mediated by the same enzymes and that different enzymes are involved in the ring fission, the next step in the degradation pathway.

Effect of co-occurring substrates

To assess the effect of the presence of different types of cosubstrates on the biodegradation of naphthalene, phenanthrene, and anthracene, the CO_2 production during growth on crystalline PAH was followed in the presence and absence of cosubstrates. An example is given in Figure 1, which shows the CO_2 production by strain 8803F growing on naphthalene, phenanthrene, and on a mixture of the two substrates. Adding up the CO_2 concentrations of the degradation curves of the two separate PAHs gives a curve that is almost identical to that of the CO_2 production in the presence of both substrates. Thus, it was concluded that both PAHs are degraded simultaneously. This type of experiment has been performed for mixtures of several substrates. The results of these experiments are summarized in Table 5.

Succinate can be concluded to be degraded preferentially over the other substrates. When two aromatic substrates are present, they are usually degraded simultaneously. The results of these experiments are consistent with the observations of Bauer & Capone (1988) and of Tiehm & Fritsche (1995), who found that the simultaneous presence of the PAHs naphthalene, phenanthrene, and anthracene did not alter the degradation patterns.

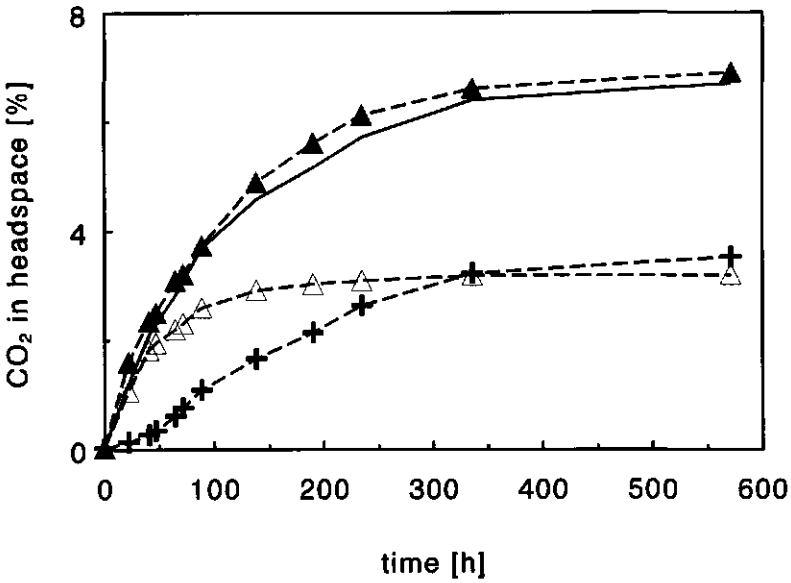

Figure 1: *CO_2 production of strain 8803F, on naphthalene (△), phenanthrene (+), and on a mixture of both PAHs (▲), the solid line represents the values obtained by addition of the CO_2 of the single substrates.*

Table 5: *growth of strain 8803F on different mixtures of two substrates; the substrate that was degraded first is given in italics.*

substrate	naphthalene	phenanthrene	12HNA	23HNA	salicylate
succinate	*succinate*	*succinate*	*succinate*	*succinate*	*succinate*
salicylate	*salicylate*	*s*	-	-	
23HNA	*s*	*s*	-		
12HNA	*12HNA*	*s*			
phenanthrene	*s*				

s: simultaneous growth
-: not determined

Growth of pure cultures in continuous cultures

The main problem in performing continuous growth experiments with PAHs is their low aqueous solubility. This makes it impossible to supply enough substrate via the aqueous phase to support detectable amounts of biomass. To solve this problem, three different methods of adding substrate have been used: via the gas phase (naphthalene only), dissolved in acetone, or dissolved in surfactant micelles (phenanthrene only). Chemostat experiments with naphthalene were performed with strain 8909N. The maximal growth rate and the overall growth yield of this strain were determined in a chemostat to which naphthalene was supplied as a solution in acetone. No growth on acetone occurred and the presence of acetone had no negative effects on the growth of the organisms on naphthalene. The maximum growth rate, determined by measuring the washout at a dilution rate of 0.5 h^{-1}, was found to be 0.45 h^{-1}. The results of the mass balance over this chemostat at different growth rates are presented in Table 6. Due to the presence of acetone, the TOC of the supernatant could not be used as a measure for excreted intermediates or other bacterial products. The residual naphthalene concentration was in all cases lower than 50 $\mu g \cdot L^{-1}$. The recovery was calculated as the percentage of the ingoing carbon that was measured as biomass or CO_2.

Table 6: *Yield and mass balance of strain 8909N growing on naphthalene in a chemostat under carbon limitation; naphthalene supplied in acetone*

growth rate [h^{-1}]	CO_2 yield [C-mol/C-mol]	biomass yield [C-mol/C-mol]	recovery [%][a]
0.1	0.53	0.34	87
0.21	0.33	0.61	94
0.3	0.37	0.53	90
0.41	0.35	0.45	80
0.44	0.33	0.48	81

[a]: calculated as the percentage of carbon measured as biomass or CO_2

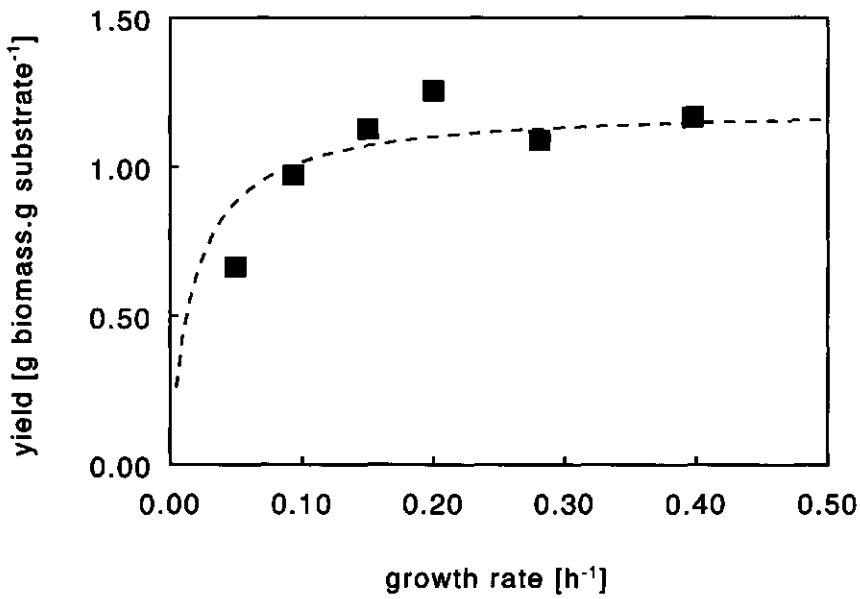

Figure 2: *Bacterial growth yield of strain 8909N on naphthalene in a chemostat under carbon limitation; naphthalene supplied in acetone.*

Figure 2 shows the overall growth yield as a function of the growth rate. This overall growth yield can be expressed as (Van 't Riet & Tramper 1991):

$$Y_{ov} = \frac{Y_{xs}}{1 + \frac{m_s \cdot Y_{xs}}{\mu}} \qquad (1)$$

where Y_{ov} is the overall growth yield [kg biomass·kg substrate^{-1}], Y_{xs} the maximal growth yield [kg biomass·kg substrate^{-1}], m_s the maintenance coefficient [kg substrate·kg biomass^{-1}·h^{-1}], and μ the growth rate [h^{-1}]. The values that have been obtained by fitting the result of Figure 2 with this equation are Y_{xs} = 1.2 and m_s = 0.015. The maximum theoretical growth yield is a factor 2 higher than the overall yield found in batch growth experiments. This can be explained by the fact that the growth rates in the batch experiments were low due to the slow dissolution of the PAH crystals.

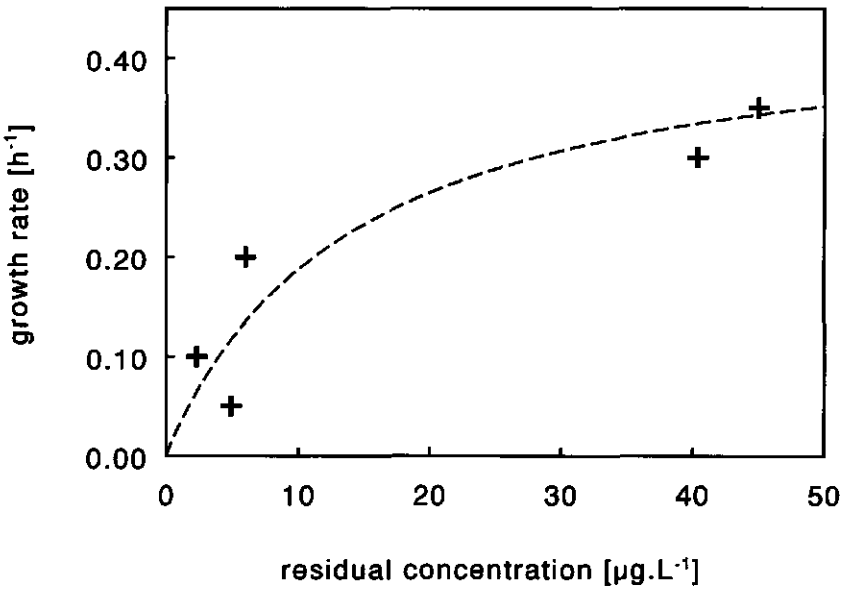

Figure 3: *Growth rate of strain 8909N growing on naphthalene in a chemostat under carbon limitation as a function of the residual naphthalene concentration; naphthalene supplied via the gaseous phase. Line represents fitting with Monod kinetics.*

To eliminate the possible effects of acetone, the continuous experiments in which the residual naphthalene concentration was measured were performed in a chemostat to which naphthalene was supplied via the gaseous phase. The results are shown in Figure 3. Although the measurements show some scattering, it is possible to estimate the K_s using Monod kinetics at a value of 14 ± 5 µg·L^{-1}.

The strains 8803F and 8809F were used for chemostat studies with phenanthrene. The first chemostat experiments were performed with strain 8809F in a reactor to which phenanthrene was supplied as a solution in acetone. The maximum growth rate obtained in this type of reactor was 0.04, but no steady states were found and crystallization of phenanthrene in the reactor was often observed. Attempts to supply phenanthrene via the gaseous phase did not result in detectable amounts of biomass in the reactor. Therefore a system was designed in which the medium was supplemented with the surfactant Triton X-100. At concentrations higher than 0.10 g·L^{-1}, Triton X-100 molecules cluster together, forming little

aggregates (micelles) which increase the apparent solubility of hydrophobic compounds. Using mineral medium kept at 30°C containing 2 g·L^{-1} Triton X-100, it was possible to dissolve 70-80 mg·L^{-1} of phenanthrene. When the surfactant was used, leading air through the liquid phase resulted in the formation of foam. Since the antifoaming agent that was used (Silicone antifoaming agent, BDH Chemicals Ltd, Poole, UK) was found to support growth of the strains used, a reactor setup was created in which the oxygen supply was achieved by leading pure oxygen through the headspace of the reactor. In this setup it was possible to grow strain 8803F at an OD540 ranging from 0.05 to 0.15 and a protein content ranging from 0.02 to 0.06 mg·L^{-1}. Using succinate as substrate for strain 8803F in the same experimental setup, it was possible to obtain an OD540 of 1.2 at a dilution rate of 0.2 h^{-1}. It was therefore concluded that no oxygen limitation occurred when the strain was growing on phenanthrene. No formation of coloured intermediates was observed during growth on phenanthrene, in contrast to all other experiments with phenanthrene. The results that have been found at the different steady states are shown in Table 7.

Table 7: *Yield and mass balance of strain 8803F growing on phenanthrene in a chemostat under carbon limitation; phenanthrene supplied in micelles.*

Growth rate [h^{-1}]	Protein content [mg·L^{-1}]	Biomass yield [g biomass/g substrate]	Biomass yield [C-mol/C-mol]	Recovery[a] [%]
0.015	24.00	0.53	0.28	N.D.
0.070	43.79	0.88	0.47	N.D.
0.095	49.71	0.99	0.55	94
0.11	44.22	0.94	0.50	N.D.

[a]: calculated as the percentage of carbon in the influent that could be measured as biomass or CO_2,assuming that 45% of the biomass is carbon.
N.D.: not determined

The high growth yield obtained indicates that most of the phenanthrene was converted into biomass, CO_2, and H_2O. It was not possible to achieve a complete mass balance over the reactor as the high concentration of surfactant in the medium interfered with the TOC analysis. The maximum growth rate of strain 8803F on phenanthrene, measured by washout at a dilution rate of 0.2 h^{-1}, was found to be 0.135 h^{-1}. In control experiments, no growth on Triton X-100 was observed and no effects of the surfactant were found on the growth of strain 8803F on succinate.

Measurement of the residual phenanthrene concentration in the chemostat experiments showed variable results, as can be seen in the fourth column of Table 8.

Table 8: *Residual substrate concentrations in chemostat with strain 8803F growing on phenanthrene under carbon limitation; phenanthrene supplied in micelles.*

Growth rate [h^{-1}]	Uptake rate [$mg \cdot L^{-1} \cdot s^{-1}$]	Maximal exit rate[a] [$mg \cdot L^{-1} \cdot s^{-1}$]	Overall residual concentration [$mg \cdot L^{-1}$]	Aqueous residual concentration [$mg \cdot L^{-1}$]
0.015	$1.0 \cdot 10^{-4}$	$0.7 \cdot 10^{3}$	0.07	$1.0 \cdot 10^{-3}$
0.07	$4.4 \cdot 10^{-4}$	$3.51 \cdot 10^{4}$	3.51	$5.21 \cdot 10^{-2}$
0.095	$6.4 \cdot 10^{-4}$	$8.5 \cdot 10^{3}$	0.85	$1.27 \cdot 10^{-2}$
0.11	$6.6 \cdot 10^{-4}$	$1.56 \cdot 10^{4}$	1.56	$2.33 \cdot 10^{-2}$

[a]: calculated according to Almgren *et al.* (1979) using a zero-order rate constant of 10^4 s^{-1} and assuming that the aqueous phenanthrene concentration is zero.

It should be noted that the major part of this residual substrate is present in the micellar phase and is therefore not directly available for uptake by the bacteria (Volkering *et al.* 1995). Since surfactant micelles have a very small diameter, usually 2-10 nm, the contact surface between the micellar phase and the aqueous phase is very large. Moreover, the mass transfer coefficient will have a very high value because of the small micelle diameter (Perry *et al.* 1963). This implies that the mass transfer of phenanthrene to the aqueous phase can proceed very quickly. Almgren *et al.* (1979) showed that diffusion rates of low molecular-weight PAHs from ionic micelles into an aqueous phase follow first-order kinetics. The first order rate constants that were found varied from > $5 \cdot 10^4$ s^{-1} for naphthalene to 10^3 s^{-1} for anthracene. Using a value of 10^4 for phenanthrene, it is possible to give a global estimation for the maximal exit rates at different residual concentrations. These rates are much higher than the bacterial uptake rates, as can be seen in Table 7. It can therefore be assumed that the concentration of phenanthrene in the micelles is in equilibrium with the aqueous phenanthrene concentration, making it possible to calculate the aqueous concentration according to Equation 2 (Volkering *et al.* 1995):

$$C_{aq} = \frac{C_{tot} \cdot V}{V + m \cdot V_{mic}} \qquad (2)$$

where C_{aq} is the aqueous concentration [$mg \cdot L^{-1}$], C_{tot} is the total concentration [$mg \cdot L^{-1}$], V the volume of the aqueous phase [L], m the micelle-water partition coefficient, previously determined to be $3.3 \cdot 10^4$ for phenanthrene (Volkering *et al.* 1995), and V_{mic} the micellar volume [L]. Although this equation has been validated

for high PAH concentrations only, deviations at low PAH concentrations will be small for non-ionic surfactants (Dougherty & Berg 1974). The calculated residual aqueous concentrations at the different steady states are shown in the last column of Table 8. Although these values are not very accurate, it is possible to estimate the K_s of strain 8803F on phenanthrene at 3-12 $\mu g \cdot L^{-1}$, a value of the same order of magnitude as the value for K_s for the growth of strain 8909N on naphthalene.

NOMENCLATURE

C_{aq}	aqueous concentration [$mg \cdot L^{-1}$]
C_{tot}	total concentration [$mg \cdot L^{-1}$]
K_s	bacterial affinity constant [$kg \cdot m^{-3}$]
m	micelle-water partition coefficient [-]
m_s	maintenance coefficient [kg substrate·kg biomass^{-1}·h^{-1}]
V	volume of aqueous phase [L^{-1}]
V_{mic}	micellar volume [L^{-1}]
Y_{ov}	overall growth yield [kg biomass·kg substrate^{-1}]
Y_{xs}	maximal growth yield [kg biomass·kg substrate^{-1}]
μ	growth rate [h^{-1}]
μ_{max}	maximum growth rate [h^{-1}]

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CHAPTER 3

BIODEGRADATION OF CRYSTALLINE POLYCYCLIC AROMATIC HYDROCARBONS

Abstract

Bacterial growth on crystalline naphthalene and phenanthrene has been demonstrated to be related to the dissolution rate of these compounds. No evidence for enhancement of substrate availability due to bacterial influence could be detected. Using a model based on dissolution kinetics for substrate availability and Monod kinetics for bacterial growth, it was possible to simulate bacterial growth on crystalline naphthalene. This model is widely applicable to growth of microorganisms on poorly water-soluble substrates and can be easily adapted to a more complex system such as microbial growth on substrates adsorbed to matrices.

This chapter is a revised form of two publications:
Volkering F., A.M. Breure, A. Sterkenburg, and J.G. van Andel (1992) Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. 36:548-552.
Volkering F., A.M. Breure, and J.G. van Andel (1993) Effect of micro-organisms on the bioavailability and biodegradation of crystalline naphthalene. Appl. Microbiol. Biotechnol. 40:535-540.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hazardous compounds originating from oil, tar, woodpreserving creosote, or from incomplete combustion of fossil fuels. The contamination of air (Daisey *et al.* 1979), soil (Bossert & Bartha 1984) and water (Andelman & Snodgrass 1974) by PAHs has been reported and several PAHs have shown to be mutagenic and/or carcinogenic (LaVoie *et al.* 1979). Since it has been shown that PAHs are biodegradable compounds (e.g. Davies & Evans 1964; Evans *et al*, 1965), biotechnological techniques might be applicable for remediation of PAH-polluted soil. However, application of biological soil remediation has demonstrated that PAHs (especially the ones with the higher molecular weights) are degraded very slowly, and that the residual PAH concentration is often too high to permit unrestricted application of the treated soil according to the Netherlands governmental guidelines (Ministry of Housing, Physical Planning and Environment 1987; Soczó & Staps 1988; Staps 1990).

Because of their hydrophobicity PAHs present in soil will occur mainly in non-aqueous phases. They can be absorbed onto soil particles, present in tar particles or in an oily phase, or occur as crystals. It has been shown that some microorganisms can take up non-aqueous hydrocarbons directly (Reddy *et al.*1982). For PAHs, only one study reports the colonization of phenanthrene crystals by a *Mycobacterium* sp., indicating direct substrate uptake (Guerin & Jones 1988). In all other documentation it has been found that pure cultures of bacteria can use PAHs in the dissolved state only (e.g. Thomas *et al.* 1986, Wodzinski & Bertolini 1972, Wodzinski & Coyle 1974). Therefore the dissolution of solid PAHs is a prerequisite for growth to occur. This implies that even in systems without intra-particle mass transfer limitation, like pure insoluble substrates in shaking cultures, mass transfer from the solid phase to the aqueous phase might be rate limiting. This can explain the often observed linear growth of bacteria and yeasts on slightly soluble substrates (e.g. McLee & Davies 1972, Prokop *et al.* 1971, Stucki & Alexander 1987; Thomas *et al.* 1986).

This study was performed to investigate the kinetics of PAH degradation in relation to the solubilization rate of PAHs. Insight into these kinetics could lead to a better understanding of the factors involved in the incomplete removal of PAHs in biological soil remediation processes.

Theory

The rates of biomass formation and substrate uptake can be described by following equation (Tempest, 1970):

$$\frac{dX_t}{dt} = -Y_{ov,t} \cdot \frac{dS_t}{dt} \qquad (1)$$

where X_t is the biomass concentration [kg.m^{-3}], S_t the total amount of substrate per unit of volume [kg·m^{-3}], $Y_{ov,t}$ the overall bacterial growth yield [kg biomass formed·kg substrate used^{-1}], and t the time [h]. The overall bacterial growth yield can be expressed as (Van 't Riet & Tramper 1991):

$$Y_{ov,t} = \frac{Y_{xs}}{1 + \frac{m_s \cdot Y_{xs}}{\mu_t}} \qquad (2)$$

with Y_{xs} being the maximal yield [kg biomass formed·kg substrate used^{-1}], m_s the maintenance coefficient [kg substrate·h^{-1}·kg biomass^{-1}], and μ_t the specific growth rate [h^{-1}]. When the yield changes during the experiment, the microbial cell mass is not a good measure for the number of cells per unit of volume N_t [m^{-3}], and is therefore not suited for calculating the growth rate. Because of this, the actual weight of one cell W_t [kg] has to be incorporated in the model:

$$W_t = W_0 \cdot \frac{Y_{xs}}{Y_{ov,t}} \qquad (3)$$

with W_0 being the initial weight of one cell [kg]. The change in the biomass concentration can then be expressed as:

$$\frac{dX_t}{dt} = W_t \cdot \frac{dN_t}{dt} \qquad (4)$$

The relation between the growth rate μ_t and the actual substrate concentration C_t can be expressed using the Monod equation:

$$\mu_t = \frac{1}{N_t} \cdot \frac{dN_t}{dt} = \mu_{max} \cdot \frac{C_t}{C_t + K_s} \tag{5}$$

where μ_{max} is the maximal specific growth rate [h^{-1}] and K_s the saturation constant [$kg \cdot m^{-3}$].

When the substrate is present in a solid and a liquid phase, the substrate concentration cannot be written as S_t, but has to be divided in the aqueous substrate concentration C_t [$kg \cdot m^{-3}$] and the amount of solid substrate Q_t [kg]. Equation (1) can then be written as:

$$\frac{dX_t}{dt} = -Y_{ov,t} \cdot \left(\frac{1}{V} \cdot \frac{dQ_t}{dt} + \frac{dC_t}{dt}\right) \tag{6}$$

where V is the aqueous volume [m^3]. The rate of mass transfer from the solid phase to the aqueous phase (dissolution) of spherical particles can be described as (Perry *et al.* 1963):

$$-\frac{dQ_t}{dt} = k_l \cdot A_t \cdot (C_{max} - C_t) \tag{7}$$

where k_l is the mass transfer coefficent [$m \cdot h^{-1}$], C_{max} the maximal solubility of the substrate [$kg.m^{-3}$], and A_t the actual contact surface area [m^2]. The mass transfer coefficient can be expressed semi-empirically as (Perry *et al.* 1963):

$$k_l = \frac{D}{d} \cdot \left(2 + 0.6 \cdot \left(\frac{\rho_l \cdot v_d \cdot d}{\eta}\right)^{1/2} \cdot \left(\frac{\eta}{\rho_l \cdot D}\right)^{1/3}\right) \tag{8}$$

in which D is the effective diffusion coefficient [$m^2 \cdot h^{-1}$]; d the particle diameter [m] ; ρ_l the liquid density [$kg \cdot m^{-3}$]; v_d the relative velocity of the particles [$m \cdot h^{-1}$], and η the liquid viscosity [$kg \cdot h^{-1} \cdot m^{-3}$]. This equation cannot be used for calculating k_l in shaking cultures since the relative velocity of the particles is unknown and probably different for particles with different diameters. However, it shows that k_l is dependent on the particle diameter and the liquid-mixing properties. With the assumption that the particles are spherical, the contact surface area can be expressed as:

$$A_t = 4 \cdot n_p \cdot \pi \cdot \left(\frac{3 \cdot Q_t}{4 \cdot n_p \cdot \rho_s \cdot \pi}\right)^{3/2} \qquad (9)$$

where n_p is the number of particles [-] and ρ_s the specific density of the solid substrate [kg·m^{-3}]. In most of the experiments presented here the change in diameter, and therefore the changes in k_l and in A_t, are small. By expressing dX_t/dt and dQ_t/dt as a function of C_t, as was done in the Equations 4, 5, and 7, Equation 6 becomes a linear differential equation that can be solved numerically.

In batch experiments at lower cell densities (when $C_t >> 0$) mass transfer is non-limiting and exponential growth can occur. At high cell densities in growing cultures, however, one may assume that C_t is negligible compared to C_{max} and constant ($dC_t/dt = 0$; $C_t = 0$) and, therefore, that the dissolution approaches its' maximal rate J_{max} (kg·m^{-3}·h^{-1}):

$$J_{max} = -\frac{1}{V} \cdot \left(\frac{dQ_t}{dt}\right)_{max} = \frac{k_l \cdot A_t \cdot C_{max}}{V} \qquad (10)$$

In this situation, the rate of substrate uptake by the biomass cannot exceed the dissolution rate and, by consequence, the biomass formation rate is limited to the mass transfer rate according to:

$$\frac{dX_t}{dt} = -\frac{Y_{ov,t}}{V} \cdot \left(\frac{dQ_t}{dt}\right)_{max} = \frac{Y_{ov,t} \cdot k_l \cdot A_t \cdot C_{max}}{V} \qquad (11)$$

Equation 11 describes the linear increase of the biomass concentration in time when the particle diameter and the yield are considered constant.

The computer program PSI/c (BOZA automatisering, Pijnacker, The Netherlands) was used to simulate microbial growth on crystalline naphthalene. The values for Y_{xs} and m_s, determined in chemostat experiments (Chapter 2), were 1.2 kg biomass·kg substrate^{-1} and 0.015 kg substrate·h^{-1}·kg biomass^{-1} respectively. The specific density ρ_s of naphthalene is 1200 kg·m^{-3}.

An example of a graphical representation of the model is given in Figure 1; in this simulaton it is assumed that the decrease in particle diameter is negligible, allowing calculation with a constant value for k_l.

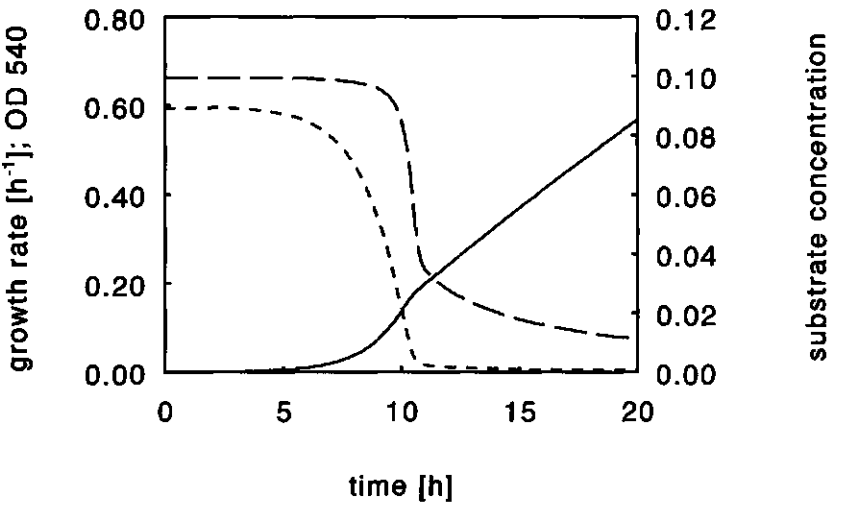

Figure 1: *Graphical representation of the model for batch growth on solid substrate: — biomass concentration X_t; - - - substrate concentration C_t; — — specific growth rate μ_t, calculated with the model described above.*

Materials and methods

Bacterial cultures

Mixed bacterial populations capable of growth on naphthalene, phenanthrene, or anthracene as the single source of carbon and energy were isolated by selective enrichment from a domestic waste-water treatment plant in Dordrecht (The Netherlands). They were all Gram-negative motile rods and could be identified as *Pseudomonas* spec. Strains 8909N, 8803F, and 8902A were used for growth experiments on naphthalene, phenanthrene, and anthracene, respectively. The strains 8805N, 8806N, and 8909N were used to investigate the influence of bacterial excretion products on the dissolution of naphthalene; strains 8803F and 8804F were used for the same type of experiments with phenanthrene.

Growth conditions

Bacteria were grown at 30°C in mineral medium, essentially composed as described by Evans *et al.* (1970), with 1 mM EDTA as a chelating agent and the concentrations of the other medium components being half those described. The medium consisted of 50 mM NH_4Cl, 5 mM NaH_2PO_4, 5 mM KCl, 1 mM Na_2SO_4, 0.625 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.05 µM Na_2MoO_4, and 2.5 ml·L^{-1} of a spore solution containing 0.12 mM HCl, 5 mM ZnO, 20 mM $FeCl_3$, 10 mM $MnCl_2$, 1mM $CuCl_2$, 2 mM $CoCl_2$, and 0.8 mM H_3BO_3. When used for batch

cultures the medium was buffered at pH 7.0 with 50 mM sodium phosphate. Pure cultures were maintained on agar slants containing 0.1% (w/v) of the PAH required (storage at 4°C). Mixed cultures degrading singly dosed PAH were maintained in sequential batch cultures in 300-ml conical flasks supplied with 100 ml medium. Crystalline PAHs were added as sole sources of carbon and energy. Strain 8909N was grown in a naphthalene-limited continuous culture using a stirred (1000 rpm) fermentor (Applicon, Schiedam,The Netherlands) with a working volume of 1.2 L at a dilution rate of 0.2 h^{-1}. The mineral medium used was the same as described above. The pH was maintained at 7.0 ± 0.1 with 0.5 N NaOH, the temperature set at 30°C. Aeration and substrate supply were accomplished by leading naphthalene-saturated air at 180 $L \cdot h^{-1}$, = $4.84 \cdot 10^{-4}$ mol naphthalene$\cdot h^{-1}$ through the culture.

Dissolution experiments

Dissolution experiments were performed at 30°C in a rotary shaker (200 rpm).The same type of flasks and aqueous volume were used as in the batch-growth experiments: 500-ml flasks with 150 ml sterile mineral medium for naphthalene and 250-ml conical flasks with 100 ml sterile mineral medium for phenanthrene and anthracene. The appropriate amount of PAH crystals was added to the flasks and samples were taken to determine the aqueous PAH concentration. To test the possible influence of bacterial excretion products on the dissolution of the substrate, experiments were performed in which supernatant of bacterial cultures, obtained by centrifugation (15 min, 12,000 g), was used instead of mineral medium. Samples were taken to determine the PAH concentration in solution. The non-linear fitting program Enzfitter (Biosoft, New Jersey, USA) was used to calculate C_{max} and the factor $k_f A_t$ from the dissolution curves using Equation 12:

$$C_t = C_{max} \cdot (1 - e^{-(\frac{k_f \cdot A_t \cdot t}{V})}) \qquad (12)$$

This equation is an integrated form of Equation 7, which is valid assuming the surface area of the crystals A_t is constant.

Biodegradation experiments

Batch-growth experiments with mixed cultures on naphthalene and phenanthrene were carried out at 30°C in 300-ml conical flasks containing 100 ml mineral medium. Substrate was added in crystalline form. Samples were taken for measurement of the optical density at 540 nm (OD540).

Batch-growth experiments with strain 8909N on naphthalene were carried out at 30°C in 500-ml conical flasks on a rotary shaker (150 or 200 rpm). The flasks contained 100 or 200 ml mineral medium supplied with the appropriate amount of naphthalene, sieved over a meshed wire sieve (Metaalgaas NV Twente, Hengelo, The Netherlands). The experiments

were started by inoculation with 2 or 5 ml of chemostat-grown cells of strain 8909N. The culture content was pumped continuously through a flow-through cuvette placed in a spectrophotometer (Perkin Elmer lambda 15) and the optical density at 540 nm was measured every 10 minutes. Linear regression was used to determine the linear increase in biomass concentration; dissolution rates were calculated from this increase using the overall bacterial growth yield, $Y_{ov,t}$, belonging to the average growth rate during this period (see Theory section above).

Batch growth experiments with strain 8803F on phenanthrene and strain 8902N on anthracene were performed in 250-ml serum flasks, supplied with 100 ml mineral medium and the appropriate amount of PAH crystals. The experiments were started by inoculating with 2 ml of active, PAH-grown cells. Bacterial growth was followed by measuring the CO_2 concentration in the headspace of the flasks during incubation (30°C, 200 rpm).

Analytical procedures

Bacterial growth was determined by measurement of the OD540, with the samples being diluted to an optical density < 1.0.

For determination of dissolved naphthalene in the culture fluid, 200 µl aliquots of supernatant samples were diluted with 800 µl acetonitrile and analyzed subsequently by HPLC using a ChromSpher C_{18} (PAH) column (Chrompack, Middelburg, The Netherlands). Eluens was a 80/20 mixture of acetonitrile/water. Peaks were detected using a fluorescence detector (Shimadzu, Kyoto, Japan). Wavelengths of excitation and emission were 278 nm and 324 nm, respectively, for naphthalene, and 253 and 333 nm for phenanthrene and for anthracene.

CO_2 in the headspace of serum flasks was determined using a gas chromatograph (Packard type 5890) fitted with a thermal conductivity detector and a Hayesep Q packed stainless steel column with a diameter of 1/8 in and a length of 2 m (Chrompack, Middelburg, The Netherlands). Helium was used as carrier gas with a flow rate of 30 ml·min.$^{-1}$. Injector temperature was 150°C, oven temperature 80°C and detector temperature 200°C. Injection volume was 250 µl with splitless injection.

Results

Growth experiments

During the enrichment procedures by which mixed bacterial cultures growing on naphthalene, anthracene or phenanthrene as the sole sources of carbon and energy were obtained, it was observed that growth occurred with a constant increase of biomass with time. Figure 2 shows the increase in OD540 observed with growth of these mixed cultures on crystalline naphthalene or phenanthrene. The linearity in this figure demonstrates that under these conditions of high cell density, batchwise

growth did not proceed according to the exponential kinetics by which growth occurred at low cell density.

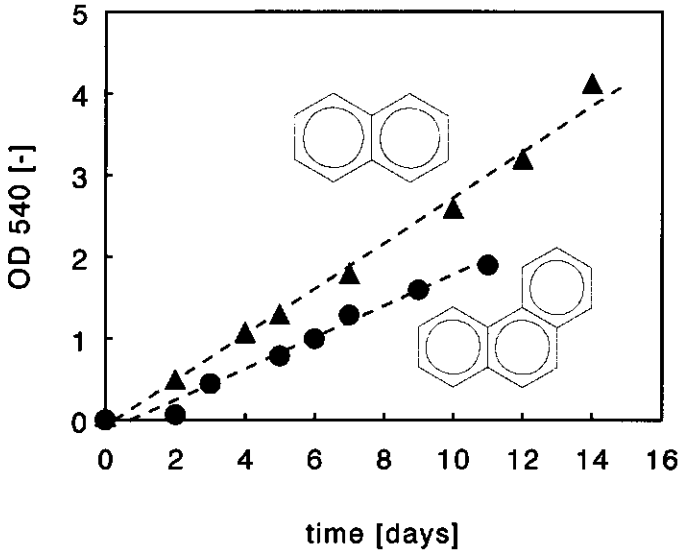

Figure 2: *Batch growth of a mixed bacterial culture on naphthalene (▲) and on phenanthrene (●) added in crystalline form.*

A typical curve for growth of a pure culture on naphthalene is given in Figure 3 for strain 8909N. In the initial stage of the experiment, at a low biomass concentration, a constant growth rate was found. After the naphthalene concentration in solution (C_t) had fallen to a value below 250 $\mu g \cdot L^{-1}$, the growth rate decreased rapidly. As solid naphthalene was still visible in the conical flask in which the batch experiment was performed, it was inferred that this decrease in naphthalene concentration was caused by the naphthalene uptake rate exceeding the maximal solubilization rate J_{max}. At the end of the growth curve the naphthalene concentration measured was 7 $\mu g.L^{-1}$ and the obserbved K_s, estimated from the course of μ_t and C_t, was 40 $\mu g \cdot L^{-1}$. The results of similar experiments with strain 8803F growing on phenanthrene and strain 8902A on anthracene are shown in Figure 4. The small phenanthrene and anthracene crystals interfered with the automated measurement of the optical density; therefore the CO_2 concentration in the headspace of the batch was used as a measure of bacterial growth. The observed maximum exponential growth rates were 0.09 and 0.012 h^{-1} on phenanthrene and anthracene, respectively. The

measurements of the aqueous PAH concentrations did not give enough information for estimating K_s.

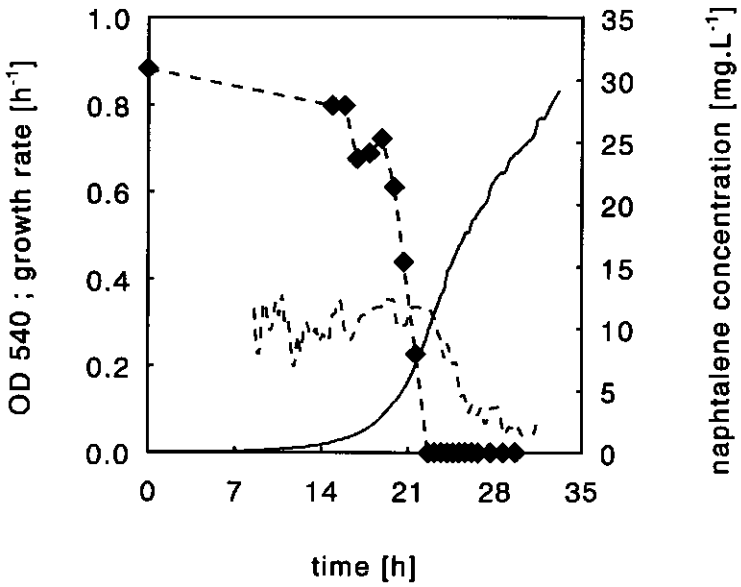

Figure 3: *Batch growth of strain 8909N on naphthalene: — OD at 540 nm; -◆- dissolved naphthalene concentration; - - - specific growth rate.*

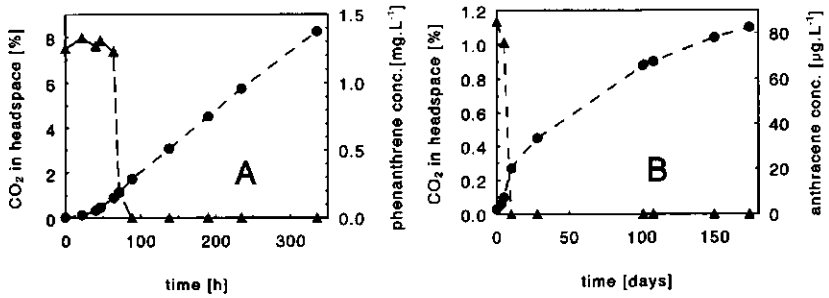

Figure 4: *CO_2 production during batch growth of strain 8803F on phenanthrene (A) and strain 8902A on anthracene (B): ●: CO_2 in headspace; ▲: aqueous PAH concentration.*

The maximal dissolution rate of a component, J_{max}, depends on the total interfacial area, A_t. When the increase in biomass is proportional to J_{max} it is, by consequence, also proportional to A_t. This was tested by inoculation of strain 8909N in batch cultures with equal amounts of naphthalene having different particle diameters, after which the biomass concentration was followed with time. Figure 5 shows that the slope in the dissolution-limited part of the growth curves thus obtained increased with decreasing diameter of the substrate particles, and hence depended on the available surface area of the crystals. For phenanthrene crystals different sieve fractions were not available. Therefore, the crystal area was varied by varying the amount of crystals. The results of growth experiments with different amounts of phenanthrene are shown in Figure 6.

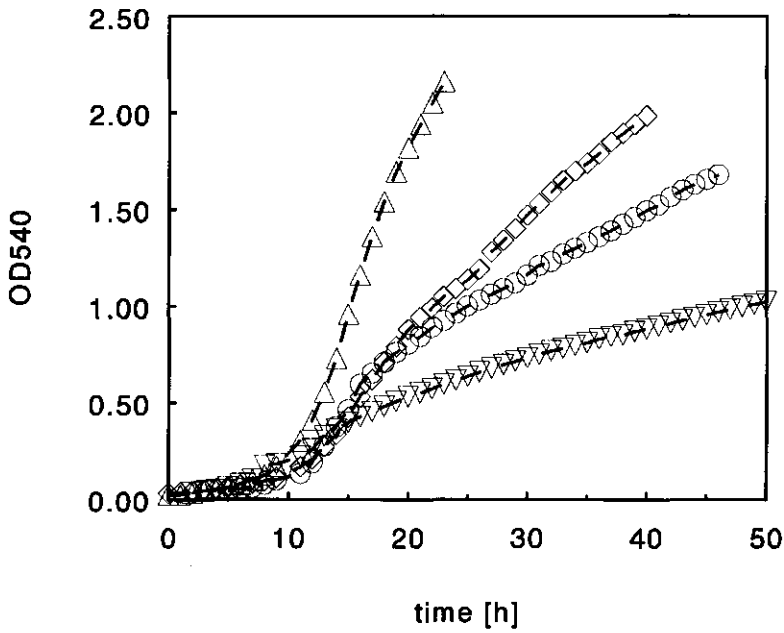

Figure 5: *Batch growth of strain 8909N on sieved fractions of crystalline naphthalene with different diameters. Fractions with a diameter of: ▵, 600-1000 µm; ◇, 1000-2400 µm; ○, 2400-3350 µm; ▽, > 3350 µm.*

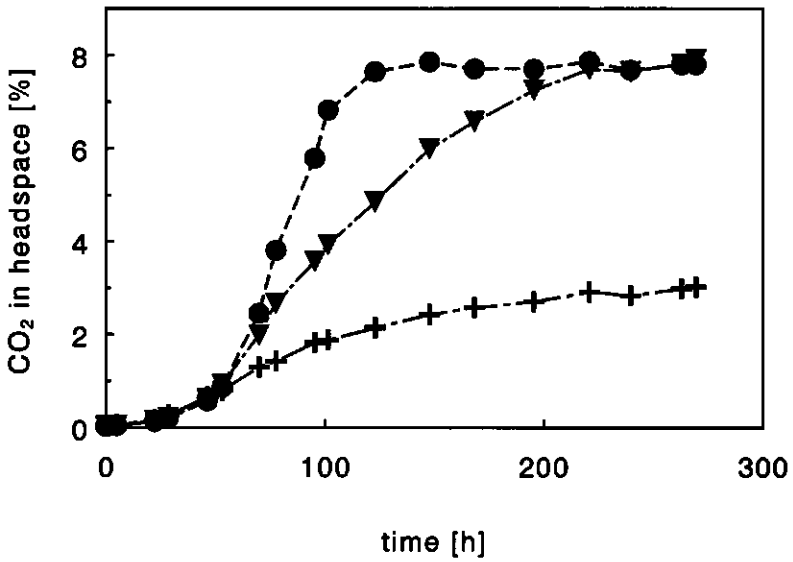

Figure 6: *CO_2 production during batch growth of strain 8803F on different amounts of crystalline phenanthrene: +: 10 mg; ▼: 25 mg; ●: 50 mg.*

Dissolution experiments

By measuring the dissolution of the crystalline substrate in abiotic systems, it is possible to estimate the factor $k_f A_t$, and with it the maximal dissolution rate, J_{max}. An example of the results of such dissolution experiments with naphthalene is shown in Figure 7. As can be expected, the dissolution rate is dependant on the surface area which changes by altering the size of the crystals. Assuming that the crystals are spherical, the surface area of the different sieve fractions can be estimated by measuring the number of crystals per g PAH. The values for k_f that have been found for the different sieve fractions of naphthalene and for phenanthrene are given in Table 1 and shown graphically in Figure 8. It can be seen that k_f decreases with an increasing particle diameter. Using Equation 9, it is possible to calculate the relative particle velocity, v_p. The results of these calculations are also shown in Figure 8. The velocity is higher for crystals with a larger diameter. This can be explained by visual observations, which revealed that large crystals remained in a rather constant position, whereas small crystals moved along with the fluid.

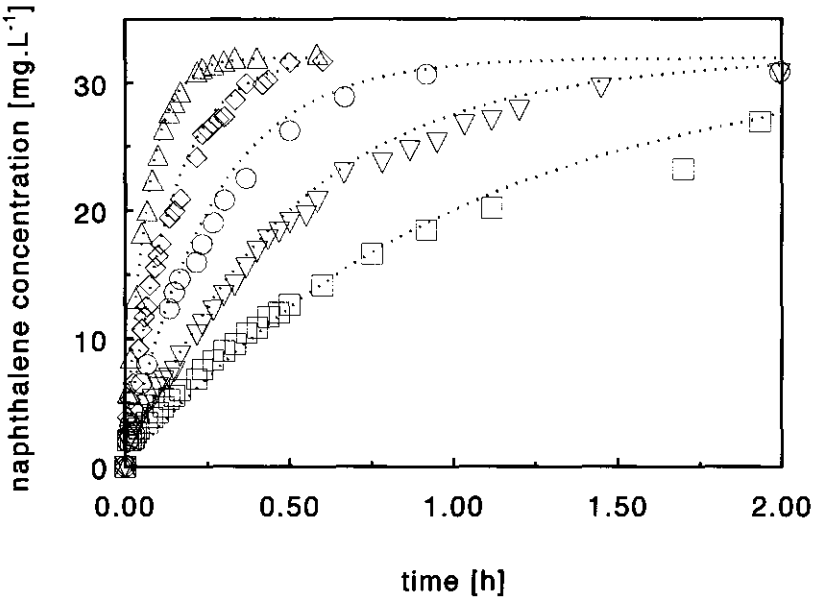

Figure 7: *Dissolution kinetics of 1 g of different sieve fractions of naphthalene in 200 ml mineral medium shaking (200 rpm) at 30°C in 500-ml conical flasks; particle diameters (µm): - Δ - <600, - ◊ - 600-1000, - o - 1000-2400, - ▽ - 2400-3350, - □ - >3350.*

Table 1: *Data on the dissolution kinetics of sieved fraction of naphthalene and phenanthrene, incubated shaking (200 rpm) at 30°C.*

PAH	sieve fraction	$k_l \cdot A_t / V$ [h^{-1}]	k_l [$m \cdot h^{-1}$]	J_{max} [$kg \cdot m^{-3} \cdot h^{-1}$]	dX_t/dt [$kg \cdot m^{-3} \cdot h^{-1}$]
naphthalene	< 600 µm	15.5	0.245	0.495	N.D.[a]
	600-1000 µm	7.46	0.238	0.239	0.306
	1000-2400 µm	3.93	0.267	0.126	0.135
	2400-3350 µm	1.98	0.278	0.064	0.079
	> 3350 µm	1.52	0.243	0.049	0.034
phenanthrene	-	6.60	-[b]	$6.9 \cdot 10^{-3}$	N.D.

[a]: The substrate limitation could not be measured due to oxygen limitation;
[b]: The specific surface area of the phenanthrene crystals is unknown;
N.D.: not determined.

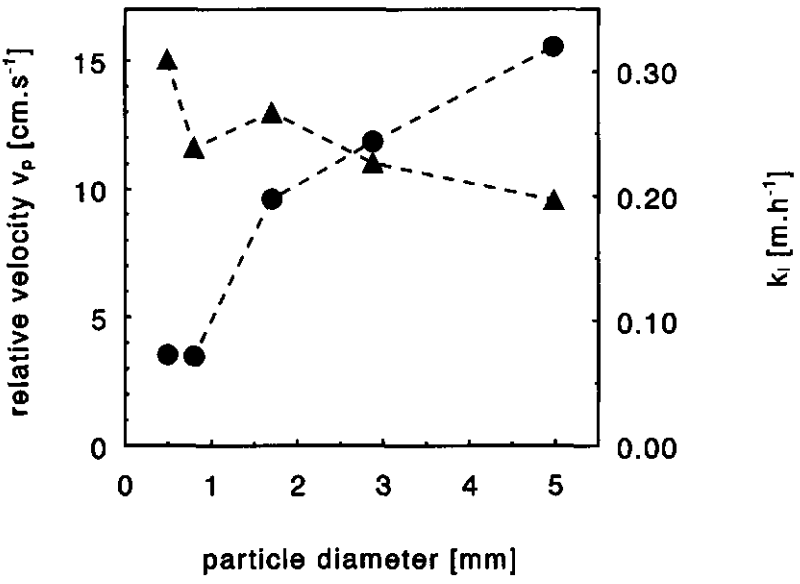

Figure 8: *The parameters k_l (▲) and v_p (●), calculated from the result of dissolution experiments, as a function of the particle diameter.*

Effect of bacteria on substrate availability

By comparing the maximal dissolution rates (from results of the dissolution experiments) with the slopes of linear biomass increase found in the batch-growth experiments described above, it is possible to determine whether the bacterial growth is limited by the availability of the substrate. The maximal dissolution rates ($J_{max} = k_l A_t C_{max} / V$) for the different sieve fractions of naphthalene and for phenanthrene are given in Table 1.

In Figure 9 the measured maximal dissolution rates of naphthalene are shown as a function of the particle diameter and compared with the dissolution rates calculated from the linear growth curves. If the bacteria have no influence on the substrate availability, there should be no difference between the measured and the calculated rates. The difference that can be observed could suggest a small influence of the bacteria on the bioavailability. For phenanthrene the maximal dissolution rate calculated from the CO_2 production rate of the experiment shown in Figure 4A was 7.8 kg·m^{-3}.h^{-1}, whereas the dissolution rate calculated from the dissolution experiments was 6.9 kg·m^{-3}.h^{-1}.

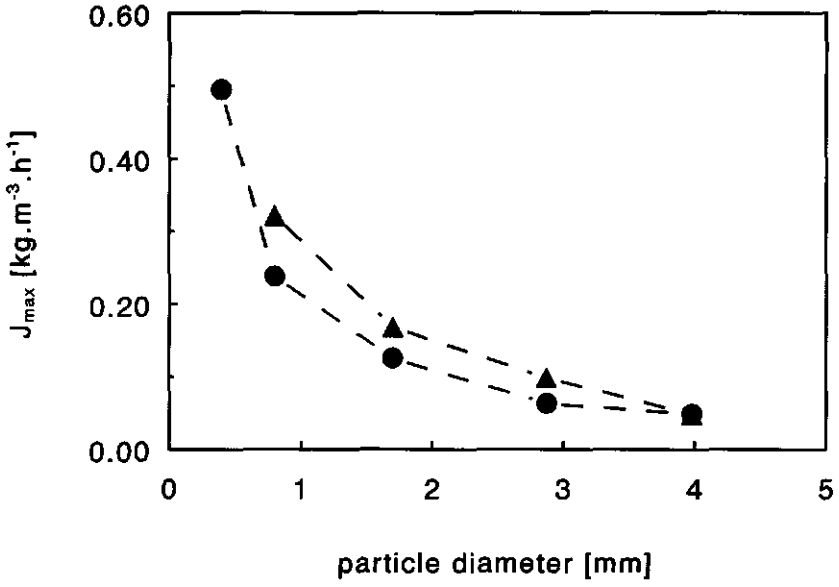

Figure 9: *The maximal dissolution rate, J_{max} calculated from growth experiments (▲) determined in dissolution experiments (●) as a function of the particle diameter.*

To test if this influence on the bioavailability is caused by solubility-enhancing compounds produced by the bacteria, dissolution experiments with naphthalene and phenanthrene have been performed in supernatants of different bacterial strains growing in batch under conditions in which mass transfer was limiting. From the results of these experiments shown in Figure 10A and B it can be concluded that there is no enhancement of the solubility and solubilization rates of naphthalene and phenanthrene in the supernatant of the *Pseudomonas* strains used and therefore the effect of the bacteria on the substrate availability cannot be attributed to the excretion of solubility-enhancing compounds. Since microscopical examination revealed that no growth of bacteria on the surface of the crystals occurred, excluding direct uptake of PAH, the small enhancement of substrate availability is likely to be due to deviations in the calculation factors used.

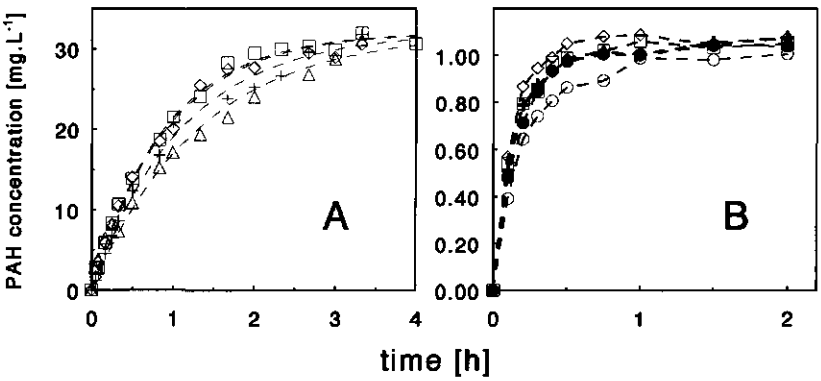

Figure 10: *Dissolution of naphthalene (A) and phenanthrene (B) in mineral medium or supernatants of different bacterial strains growing on the PAH involved. A: □ mineral medium, + sup. 8805N, ◊ sup. 8806N, - Δ sup. 8909N. B: □ mineral medium, + sup. 8803F, ◊ sup. 8804F, - ● - sup 8808F, o dead cells 8803F.*

Modelling of microbial growth

In the experiments described above, PAH crystals were present in abundance Hence, the surface area and the diameter of the naphthalene crystals could be considered constant, resulting in constant dissolution rates. Under natural conditions, however, substrate limitations are usually caused by desorption processes. Desorption rates are generally not constant for prolonged times, making it impossible to find a linear section in the growth curve. Estimation of the substrate availability during batch growth experiments can then be done by simulation of the process. As an example of a system without a constant mass transfer rate, dissolution experiments and growth experiments with a small amount of naphthalene have been performed and simulated with the model presented in the Theory section. Due to the small amount of substrate the change in the surface area during bacterial growth is considerable and it is hard to find a linear growth rate and thus to estimate J_{max}. However, by simulation it is possible to estimate the important process parameters. The results of two growth experiments and their simulations are shown in Figure 11. The results of the dissolution experiments and the values of the parameters used for the simulations are shown in Table 2. The model describes the growth well and the values for k_l found in the growth experiments and in the dissolution experiments are in the same range.

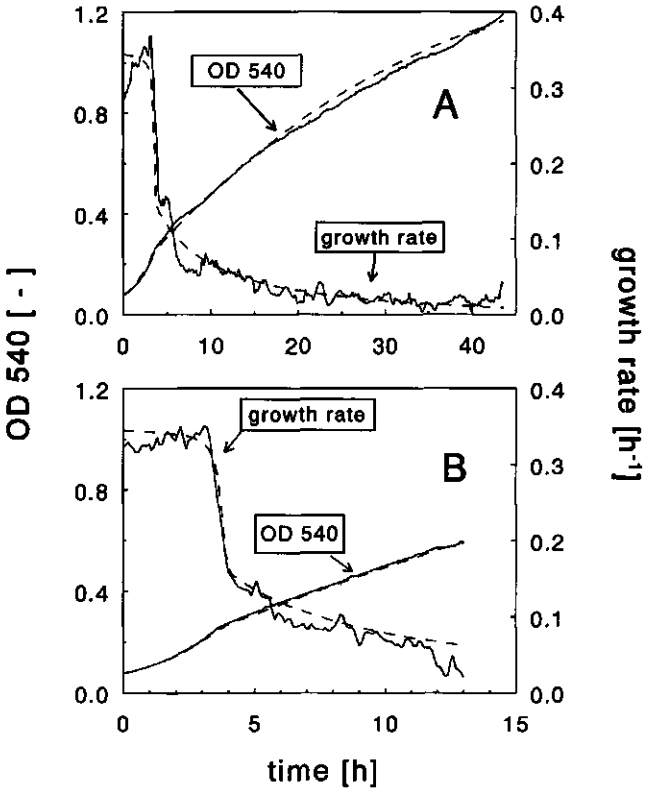

Figure 11: *Growth kinetics of strain 8909N on naphthalene (A: 0.12 g and B: 0.25 g) in 100 ml mineral medium shaking (150 rpm) at 30°C in 500-ml conical flasks: —— measured values; - - - - calculated values. The parameters used for simulation are given in Table 2.*

Table 2: *Parameters used for simulating the growth of strain 8909N on naphthalene presented in Figure 11A and B.*

Parameter	μ_{max} [h^{-1}]	K_S [$kg \cdot m^{-3}$]	N_0 [L^{-1}]	k_f [$m \cdot h^{-1}$]	n_p [-]	Q_0 [g]	C_{max} [$kg \cdot m^{-3}$]	V [L]
Dissolution 0.25 gr	-	-	-	0.21	18	0.250	-	0.15
Growth 10A	0.33	$1 \cdot 10^{-5}$	$1.25 \cdot 10^{11}$	0.18	8	0.117	$3.2 \cdot 10^{-2}$	0.1
Growth 10B	0.33	$1 \cdot 10^{-5}$	$1.25 \cdot 10^{11}$	0.19	17	0.245	$3.2 \cdot 10^{-2}$	0.1

Discussion

In the experiments described in Figures 1-4, the hypothesis was confirmed that the rate of substrate dissolution could restrict bacterial growth on polycyclic aromatic hydrocarbons. By comparing the maximal dissolution rate (measured in dissolution experiments) with the growth rate in the dissolution-limited phase, it was shown that the presence of bacteria and bacterial excretion products had no significant effect on the maximal dissolution rate of naphthalene and phenanthrene. The estimation of specific bacterial growth rates on these poorly soluble substrates in batch is often not very reliable. The observation of Keuth & Rehm (1991), that the specific growth rate increases with increasing amounts of (solid) substrate, can be explained by the mechanism proposed in this paper.

The model presented here, in which dissolution kinetics and Monod kinetics are coupled, can be concluded to be suitable for describing bacterial growth in a system where the substrate supply is mass transfer limited. Thus it can form a basis for a more complex model describing bacterial growth on substrates adsorbed to heterogeneous surfaces, such as soil. Furthermore, it allows insight into the effect of the physical and biological parameters in a system in which the biodegradation is limited by mass transfer. A way of enhancing the microbial clean-up of polluted soils is augmentation with bacteria that have the capability of quickly degrading the polluting compound and have a high affinity for it. Since the important microbial parameters μ_{max} and K_s and the initial number of bacteria N_0 are incorporated into the model presented above, it is possible to show the effect of these parameters on the course of the biomass and substrate curves. Figure 12 represents simulations in which μ_{max}, K_s, and N_0 are varied over a wide range. It can be seen that the growth rate and the affinity constant of the bacteria have little influence on the amount of remaining substrate after 150 hours. Augmentation with extra bacteria biodegradation is of no use unless the initial concentration of active biomass is very low. Concluding, it can be said that application of this kind of specialized bacteria in the biotechnological treatment of polluted soils is not effective when the mass transfer of the polluting compound is the rate-limiting factor.

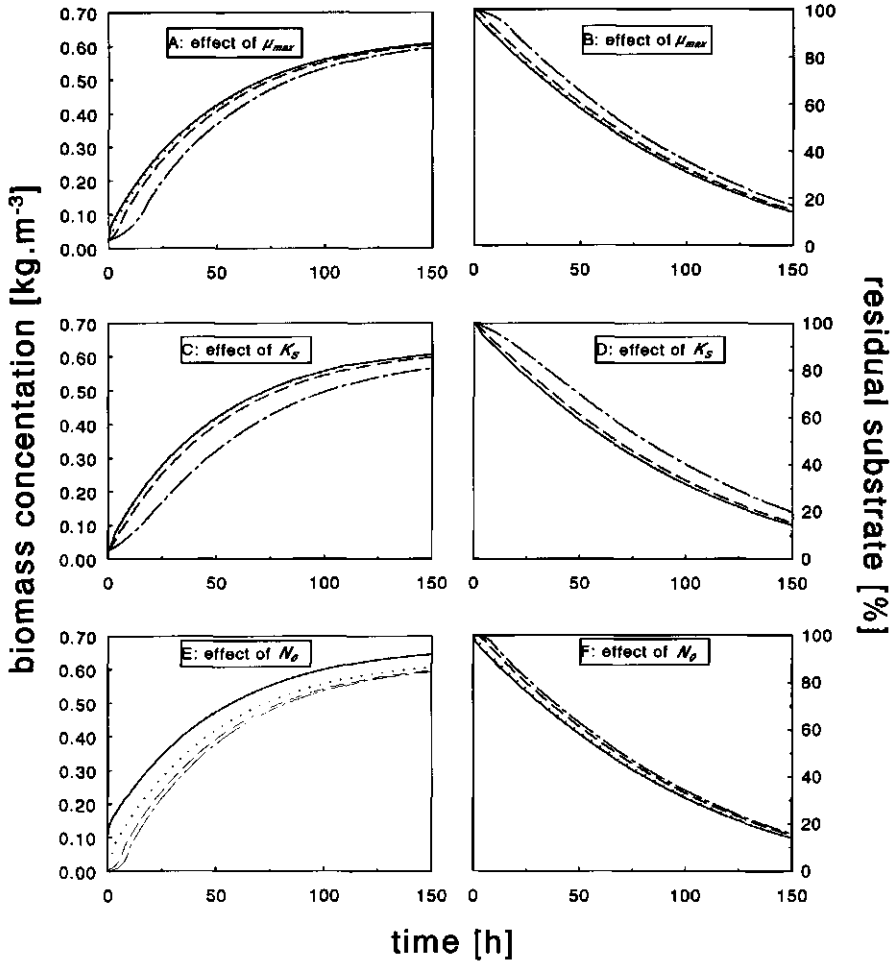

Figure 12: *Effect of varying μ_{max} (A,B), K_s (C,D) and inoculation size (E,F) on biomass and residual substrate concentration during growth on solid substrate, as calculated using the model presented; other parameters the same as in Figure 10A .*

A,B: μ_{max} = 1.0; ·····μ_{max} = 0.4; ----μ_{max} = 0.2; · μ_{max} = 0.1 (h^{-1})
C,D: $K_s = 1 \cdot 10^{-5}$; ·····$K_s = 1 \cdot 10^{-4}$; ----$K_s = 1 \cdot 10^{-3}$; · $K_s = 1 \cdot 10^{-2}$ ($kg \cdot m^{-3}$)
E,F: $N_0 = 5 \cdot 10^{11}$; ·····$N_0 = 1.25 \cdot 10^{11}$; ----$N_0 = 2.5 \cdot 10^{10}$; · $N_0 = 1 \cdot 10^{10}$ (L^{-1})

When the mass transfer rate is limiting, the degradation rate can only be increased by increasing this mass transfer rate, as can be seen in Figure 13. The lines in this figure represent model simulation as described above, with different initial values for k_l. As expected, the initial stage in the figure is very similar to Figure 5, in which the factor $k_l A_t$ was varied by changing the contact area. Enhancing the mass transfer can be done by improving mixing characteristics, by enlarging the contact surface area, or by increasing the aqueous solubility.

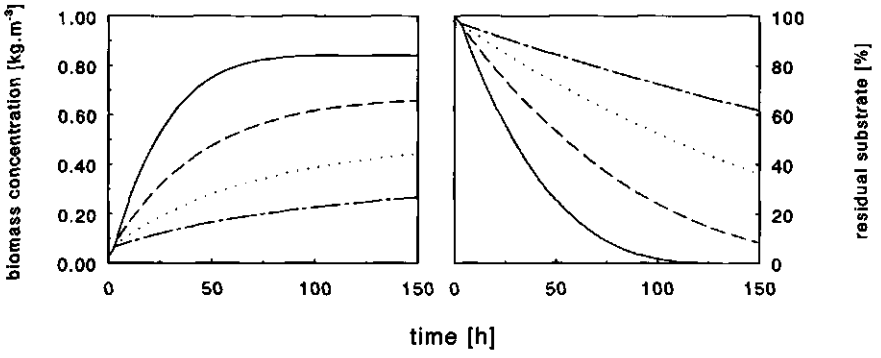

Figure 13: *Effect of varying the initial value of K_l on the growth on solid substrate calculated with to the model presented; other parameters the same as in Figure 4A : k_l = 0.4; - - - k_l = 0.2; ···· k_l = 0.1; · k_l = 0.05.*

A way of increasing the aqueous solubility that is widely under investigation at present is the addition of surface-active compounds. However, introduction of synthetic surfactants can be introduction of another pollutant and is therefore debatable. As an alternative, application of bacteria that produce surface-active compounds could enhance biodegradation rates (e.g. Bury & Miller 1992, Reddy *et al.* 1982). In the experiments described here no enhancement of dissolution and biodegradation was found. This is not surprising since the solubilization effect of surfactants is attributed mainly to the formation of micelles (Laha & Luthy 1991), which for most of the biosurfactants takes place at high concentrations (~20 mg·L^{-1}) for surfactant produced by *Pseudomonas aeruginosa* UG1, Jain *et al.* 1992). Moreover, when bacteria are present to keep the aqueous substrate concentration low, the dissolution rate is at its maximum. The presence of micelles under these

conditions will probably have very little or no influence on the aqueous substrate concentration and will therefore not have a significant effect on the dissolution rate of crystalline substrates.

In the case of substrate adsorbed to soil, however, surface-active compounds may have a more pronounced effect on the substrate availability. This can be a positive effect, like mobilizing substrate in ganglia (Vigon & Rubin 1989), as well as a negative effect, like clogging of pores by dispersion of clay particles (Abdul *et al.* 1990). Literature on the effect of surfactants on biodegradation also shows varying results, ranging from enhancement (e.g. Aronstein *et al.* 1991, Oberbremer *et al.* 1990) to inhibition (e.g. Laha & Luthy 1992) of biodegradation. We are now investigating microbial growth on sorbed PAHs and the possible influence of (bio)surfactants to gain a better insight in this process.

In the experiments described here, conversion of substrates was coupled directly to bacterial growth, as the substrate to be converted was the only carbon and energy source. Therefore, the conversion rate of the substrate is directly coupled to the microbial growth rate. However, if the pollutant is not the only carbon and energy source, and is converted by cometabolic processes, the conversion rate can still be limited by the mass transfer rate and the model can be easily adapted to this situation.

Nomenclature

A_t	surface area [m^2]
C_{max}	maximal aqueous concentration [$kg \cdot m^{-3}$]
C_t	actual aqueous substrate concentration [$kg \cdot m^{-3}$]
J_{max}	maximal dissolution rate [$kg \cdot m^{-3} \cdot h^{-1}$]
k_l	mass transfer coefficient [$m \cdot h^{-1}$]
K_s	bacterial affinity constant [$kg \cdot m^{-3}$]
m_s	bacterial maintenance coefficient [kg substrate$\cdot h^{-1} \cdot$kg biomass^{-1}]
n_p	number of PAH crystals [-]
N_0	initial number of cells per m^3 [m^{-3}]
N_t	number of cells per m^3 [m^{-3}]
OD540	optical density at 540 nm [-]
Q_t	amount of solid substrate [kg]
v_p	realtive particle velocity [$m.h^{-1}$]

V	volume of the aqueous phase [m^3]
W_0	initial weight of one cell [kg]
W_t	weight of one cell [kg]
X_t	biomass concentration [$kg \cdot m^{-3}$]
$Y_{ov,t}$	overall bacterial growth yield [kg biomass·kg substrate^{-1}]
Y_{xs}	maximal bacterial growth yield [kg biomass·kg substrate^{-1}]
η	viscosity [$kg \cdot h^{-1} \cdot m^{-3}$]
μ, μ_t	bacterial growth rate [h^{-1}]
μ_{max}	maximal bacterial growth rate [h^{-1}]
ρ_s	specific density of the substrate [$kg \cdot m^{-3}$]
ρ_l	specific density of the liquid phase [$kg \cdot m^{-3}$]

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CHAPTER 4

BIODEGRADATION OF SORBED POLYCYCLIC AROMATIC HYDROCARBONS

Abstract

This study describes the effect of sorption on the bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Desorption and biodegradation experiments have been performed with activated carbon, synthetic carrier materials, and different soils, loaded with naphthalene or phenanthrene. With all of these matrices sorption is shown to reduce the degradation rates, and therefore the bioavailability of the PAHs. The desorption of naphthalene and phenanthrene from the synthetic matrices Amberlite XAD-4 and XAD-7 and from the soils could be described with a two-compartment model, but not with a radial diffusion model. For soils the physical interpretation of the parameters obtained was limited. When comparing desorption and biodegradation of sorbed PAHs, it was found that for the porous synthetic matrices the biodegradation proceeded faster that could be expected from desorption alone. In contrast, the biodegradation of soil-sorbed naphthalene and phenanthrene could be explained by degradation of aqueous phase PAH only.

Introduction

The slow desorption of hydrophobic organic compounds (HOCs) from soil has been identified as one of the most important reasons for the slow removal of these compounds from contaminated sites in biological soil remediation processes (Mihelcic *et al.* 1993). Sorption processes are governed by the type and physical state of the pollutant, by the type and structure of the soil, and by other factors such as temperature, pH, presence of oxygen, etc. Especially the heterogeneity of soils makes prediction of sorption processes in situ very difficult. The processes that play a role in biological soil remediation are even more complicated because of the interactions of microorganisms with the soil and pollutants. Some evidence has been found that direct uptake of sorbed substrate by microorganisms is possible (Guerin & Boyd 1992, Hermannson & Marshall 1985). Most studies, however, showed that degradation is limited by desorption of the substrate and that therefore only aqueous-phase substrate is directly available for uptake. As yet, no definite answer to this question has been found. However, even when sorbed substrate could directly be degraded by microorganisms, it was found to be less bioavailable than free solute substrate (Gordon & Millero 1985). Desorption therefore is a crucial process in the biodegradation of sorbed substrates.

In this study the relation between desorption and biodegradation will be investigated using three different types of sorbent: activated carbon, synthetic carrier materials, and soils. The activated carbon is used as an example of a system with sorbed substrate. The synthetic materials have been used as model systems in which desorption and biodegradation can be measured accurately. The soils used were are a sandy soil, an anthropogenic sandy soil and a peat soil.

Theory and modeling

Models that couple biodegradation and desorption kinetics can be a powerful tool in predicting biodegradation rates. These models generally consist of one part describing desorption kinetics and the other part describing degradation kinetics. Due to the complexity of soil, models describing the desorption kinetics will always be a simplification.

An important parameter in all of these models is the equilibrium partitioning of the HOC over the soil and the aqueous phase. Depending on the type and

concentration of HOC and on the type of soil, different equations can be used to describe this partitioning (Weber 1972). At low pollutant concentrations the sorption isotherm is often found to be proportional, in which case the partitioning can be characterized by a constant soil-water partition coefficient (Equation 1):

$$Q_{eq} = k_p \cdot C_{eq} \quad (1)$$

where Q_{eq} is the equilibrium sorbed pollutant concentration [mg·g^{-1}], k_p the soil-water partition coefficient [L·g^{-1}], and C_{eq} the equilibrium pollutant concentration in solution [mg·L^{-1}]. In attempts to predict the partition coefficient, it is often described as a function of parameters such as the organic carbon content, pH, salinity, etc. (e.g. Dzombak & Luthy 1984, Hegeman *et al.* 1995, Karickhoff *et al.* 1979, McCarthy *et al.* 1989, Means *et al.* 1980). At higher concentrations isotherms are usually not linear. The two most used non-linear models are the Langmuir model and the Freundlich model (Voice & Weber 1983). The Langmuir model (Equation 2) is a model for adsorption, in which it is assumed that the maximum sorption capacity is reached when the total adsorbing surface is covered with a monomolecular layer of the adsorbing compound:

$$Q_{eq} = Q_{max} \cdot \frac{k_a \cdot C_{eq}}{1 + k_a \cdot C_{eq}} \quad (2)$$

where Q_{max} is the maximum sorbed polltuant concentration [mg·g^{-1}] and k_a the Langmuir constant [L·g^{-1}].

The Freundlich model is a model that can be used for describing both absorption and adsorption and can be described as in Equation 3:

$$Q_{eq} = K_f \cdot (C_{eq})^{1/n} \quad (3)$$

where K_f is the Freundlich sorption capacity constant [mg$^{1-1/n}$·L^{1-n}·g^{-1}] and n (>1) the Freundlich sorption energy constant [-]. The Freundlich model is largely empirical, although attempts have been made to give a thermodynamical basis for it (Weber 1972).

To describe the desorption kinetics of pollutants from soil, different models have been developed. The two most used types are multi-compartment models (e.g. Brusseau *et al.* 1991, Karickhoff 1980, Marca Schrap 1990, Peel & Benedek 1980) and radial diffusion models (e.g. Carroll *et al.* 1994, Rijnaarts *et al.* 1990, Scow & Alexander 1992, Scow & Hutson 1992, Wu & Gschwend 1986). In multi-compartment models the soil is divided into separate compartments (usually two)

with different sorption characteristics. Although this type of model is largely empirical, it usually fits the desorption data well (Carroll *et al.* 1994). In radial diffusion models the soil is considered homogeneous and the desorption is limited by the diffusion of the pollutant through the matrix. In this study a simple two-compartment model and a radial diffusion model are compared. The two-compartment model used is based on the following assumptions:

- the solid phase is a porous material with two different types of pores; shallow and deep pores, each having equal adsorption properties;
- the pores are in direct contact with the aqueous bulk phase, not interconnected, and straight;
- the pollutant can occur in the aqueous bulk phase, adsorbed onto the pore wall or in the pore liquid;
- the amount of pollutant in the pore liquid is negligible compared to the amount of adsorbed pollutant;
- transport of the pollutant in the pores occurs via pore walls (surface diffusion);
- transport of the pollutant from the pores to the aqueous bulk phase occurs via the contact length between the pores and the aqueous bulk phase;
- mass transfer limitation outside the solid phase can be neglected.

A schematic illustration of the model is given in Figure 1.

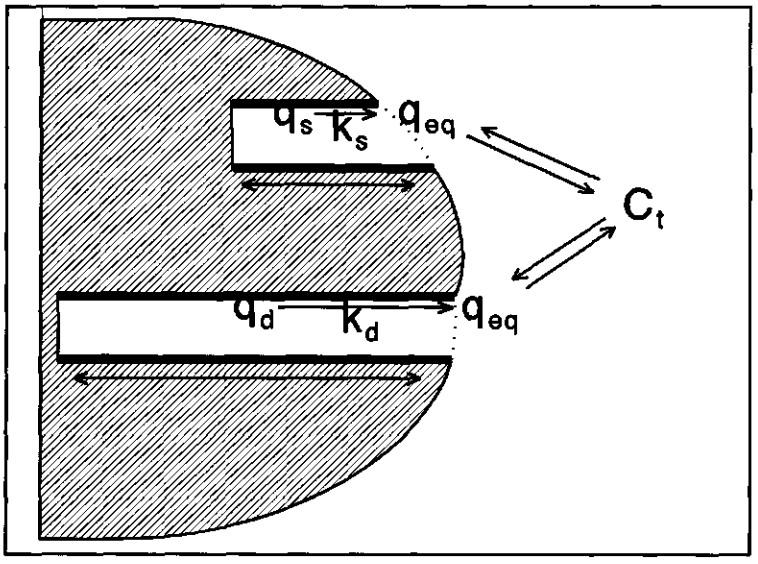

Figure 1: *Schematical illustration of the two-compartment model; for explanation of the symbols see text.*

The mass balance over the different phases is given in Equation 4 below:

$$V_x \cdot S_x \cdot (f \cdot \bar{q}_{s,0} + (1-f) \cdot \bar{q}_{d,0}) + V \cdot C_0 = V_x \cdot S_x \cdot (f \cdot \bar{q}_{s,t} + (1-f) \cdot \bar{q}_{d,t}) + V \cdot C_t \quad (4)$$

where V_x is the amount of solid phase [g], S_x the specific surface area of the porous particles [$m^2 \cdot g^{-1}$], f the fraction of the surface area that consists of shallow pores [-], $\bar{q}_s$ the average loading of the shallow pores [$mg \cdot m^{-2}$], $\bar{q}_d$ the average loading of the deep pores [$mg \cdot m^{-2}$], V the aqueous volume [L], and C the concentration in the aqueous bulk phase [$mg \cdot L^{-1}$]. The subscripts *0* an *t* refer to inital and actual values, respectively.

The transport from a deep pore to the aqueous phase is dependent on the length of the pore l_d [m], the effective surface diffusion coefficient $D_{s,eff}$ [$m^2 \cdot h^{-1}$], the length of the contact between the pore wall and the aqueous phase ($\pi \cdot d_d$, d_d = pore diameter [m]) and the driving force, as shown in Equation 5:

$$-\frac{d(l_d \cdot \pi \cdot d_d \cdot \bar{q}_{d,t})}{dt} = \frac{D_{s,eff}}{0.5 \cdot l_d} \cdot \pi \cdot d_d \cdot (\bar{q}_{d,t} - q_{eq,t}) \quad (5)$$

The equilibrium adsorbed concentration $q_{eq,t}$ [$mg \cdot m^{-2}$] can be calculated from the aqueous bulk concentration using the appropriate isotherm model. Rearranging this equation yields:

$$-\frac{d\bar{q}_{d,t}}{dt} = k_d \cdot (\bar{q}_{d,t} - q_{eq,t}) \quad (6)$$

in which $k_d = D_{s,eff}/0.5 \cdot l_d^2$ [h^{-1}]. Likewise, the transport from a shallow pore to the aqueous bulk phase can be described as:

$$-\frac{d\bar{q}_{s,t}}{dt} = k_s \cdot (\bar{q}_{s,t} - q_{eq,t}) \quad (7)$$

with $k_s = D_{s,eff}/0.5 \cdot l_s^2$ [h^{-1}], where l_s is the shallow pore length [m].

By differentiating Equation 4 and combining it with Equations 6 and 7, the change in the aqueous bulk concentration can be expressed as:

$$V \cdot \frac{dC_t}{dt} = -(1-f) \cdot V_x \cdot S_x \cdot \frac{d\bar{q}_{d,t}}{dt} - f \cdot V_x \cdot S_x \cdot \frac{d\bar{q}_{s,t}}{dt} \quad (8)$$

A typical example of modeling batch desorption with the two-compartment model is shown in Figure 2. The desorption rate in the first phase is mainly dependent on the value of k_s, the rate in the second phase is governed by the value of k_d. The fraction of shallow pores, *f*, determines the position where the curve bends. This is consistant with the setup of the model.

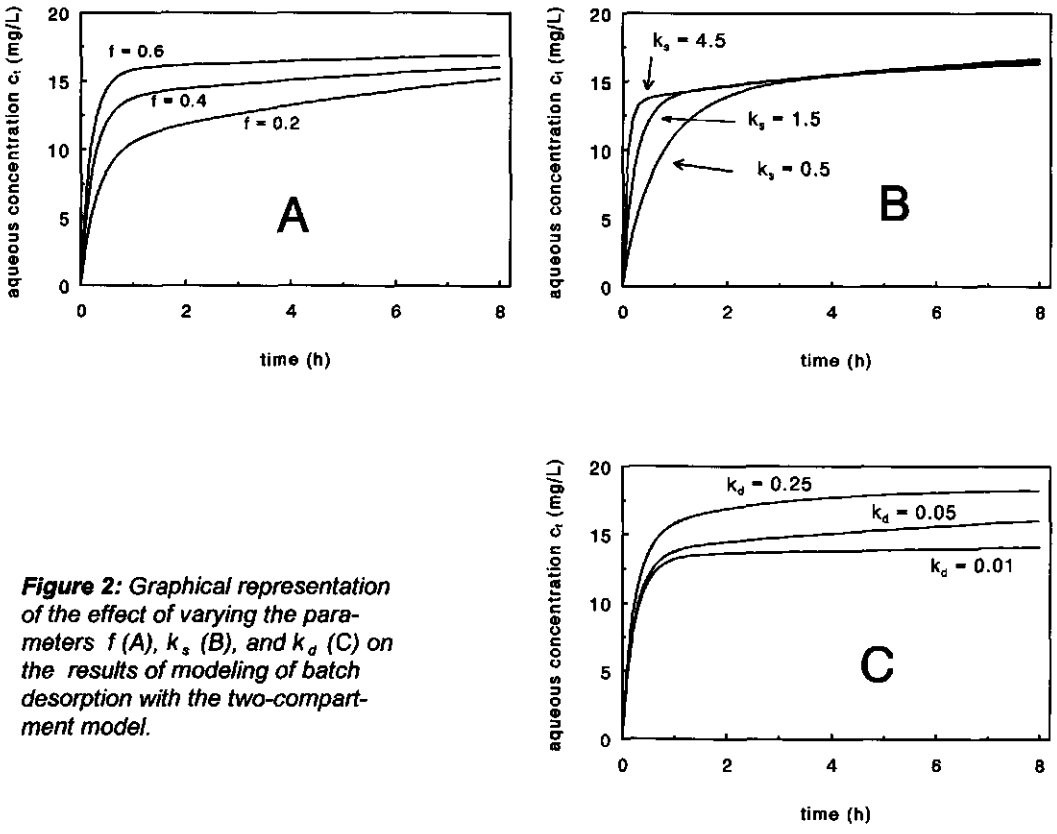

Figure 2: *Graphical representation of the effect of varying the parameters f (A), k_s (B), and k_d (C) on the results of modeling of batch desorption with the two-compartment model.*

In the radial diffusion model for the desorption used, diffusion of the pollutant through homogeneous particles is assumed to be the rate-limiting process. The diffusion from homogeneously loaded spherical particles into a well-stirred solution of limited volume that is initially free of pollutant was modeled according to Crank (1975). The mass balance over the two phases is given in Equation 9 below:

$$V_x \cdot Q_0 = V_x \cdot Q_t + V.C_t \tag{9}$$

where Q_0 is the initial loading [mg·g^{-1}], and Q_t the actual average concentration in the particles [mg·g^{-1}]. The change in the concentration in the particles is given by:

$$\frac{\partial Q_t}{\partial t} = D \cdot \left(\frac{\partial^2 Q_t}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial Q_t}{\partial r}\right) \tag{10}$$

in which D is the apparent diffusion coefficient [m^2·h^{-1}] which is considered constant, and r the radial distance [m]. Under the assumption that the isotherm is proportional, and that the particle density is equal to the density of water, this equation can be solved for batch desorption according to Crank (1975):

$$\frac{Q_0 - Q_t}{Q_0 - Q_{eq}} = 1 - \sum_{n=1}^{\infty} \frac{6 \cdot \alpha \cdot (1+\alpha) \cdot e^{(-D \cdot q_n^2 \cdot t/a^2)}}{9 + 9 \cdot \alpha + q_n^2 \cdot \alpha^2} \tag{11}$$

where Q_{eq} is the equilibrium sorbed pollutant concentration [mg·g^{-1}], a the particle radius [m], α, a parameter that describes the final fractional amount desorbed, can be expressed as in Equation 12, and in which q_n are the non-zero roots of Equation 13.

$$\frac{1}{1 + 1/\alpha} = \frac{Q_0 - Q_{eq}}{Q_0} \tag{12}$$

$$\tan(q_n) = \frac{3 \cdot q_n}{3 + \alpha \cdot q_n^2} \tag{13}$$

For modelling the results of the biodegradation experiments, the two-compartment model described above was coupled to Monod kinetics in a similar way as described for crystalline substrates in Chapter 3. It was assumed that only PAHs present in the aqueous bulk phase are directly available for bacterial uptake.

Computer simulations with the different models were performed using the computerprogram PSI/C (Boza automatisering, The Netherlands). Results were fitted by using the least square root area method.

Materials and Methods

Microorganisms and media

Mixed cultures growing on mixtures of PAHs adsorbed to activated carbon (see below) were frozen in 15% glycerol at -196°C and maintained at -30°C. The isolation and maintenance of the strains 8909N and 8803F growing on naphthalene and phenanthrene, respectively, as well as the mineral medium (pH 7) used, are described in Chapter 2. This mineral medium was also used in the desorption experiments.

Sorbents

Three different types of sorbent have been used to study the desorption and biodegradation of PAHs:

- activated carbon (ROW 0.8 type, Norit NV, Amersfoort, The Netherlands);
- inert synthetic matrices: Amberlite XAD-4 and XAD-7 (Supelco, Bellefonte, USA), hydrophobic spherical porous resins designed for adsorption of HOCs from aqueous solutions, and a non-porous polymer with a coating of hydroxyethylmethacrylate;
- three different types of soil: a sandy soil with 2.9% organic carbon, an anthropogenic sandy soil named Eerdsoil with 6.6 % carbon, and a peat soil named Koopveen with 28.9% organic carbon. The soils were sieved over a 1-mm meshed wire sieve to remove large soil particles.

Loading of the materials with PAHs

Activated carbon. PAHs originating from extraction sludge, the main stream of waste resulting from the extraction/classification procedure of soil remediation, were concentrated 10-fold onto activated carbon. To achieve this, 200 g of sludge was added to 500 ml of acetone, and the suspension was mixed thoroughly for 10 min. Thereafter 1 L of hexane was added. After thorough mixing for another 10 min the suspension was centrifuged for 20 min at 5000 *g*. The supernatant organic phase was washed three times with demineralized water. After this, 20 g of pelleted activated carbon was added to the hexane and the PAHs were allowed to adsorb by mixing for 10 min under lowered pressure (2 kPa) and followed by removal of the organic solvent by distillation in a rotavapory apparatus (2 kPa, 40°C). Via this procedure a loading amounting to 71.0 µg total PAH per mg dry weight of activated carbon was achieved.

Synthetic matrices. Before loading, the matrices were washed thoroughly with de-ionized water to remove contaminations. The washing was ended when the UV spectrum of liquid could no longer be distinguished from the spectrum of clean water. After this, the material was stored at 4°C in a closed glass jar to prevent dehydration. To load the matrices, 20-30 g of the wet material was transferred into 500-ml serum bottles containing 250 ml of de-ionized water. After pasteurization of the material at 80°C for three hours, closed tubular

dialysis membranes (cutoff 1000 Dalton) filled with 15 g of naphthalene crystals and 25 ml of water were added. The bottles were closed with a screwcap with a teflon lining and incubated horizontally in a rotary shaker (90 rpm) at 30°C. Once a week samples were taken to determine the naphthalene concentration in the aqueous phase. After 1 month the aqueous phase concentration was equal to the maximum solubility and the bottles were opened; the membranes with the remaining naphthalene crystals were removed and the water was decanted. The materials were dewatered for 5 min on filtration paper and transferred into a glass jar. Nitrogen gas was led through the jars to remove oxygen, after which the jars were closed and stored at 4°C. A small amount of the freshly loaded materials was dewatered at 80°C to constant weight to determine the dry weight; another small amount was extracted to determine the initial naphthalene concentrations (see below). After a period of three months the loading of the materials was determined again to control the stability of the loaded materials.

Soils. Soils were loaded with naphthalene and phenanthrene using two different methods. With the first method (the acetone method), 25 g of dry soil was added to serum flasks containing 50 ml of a 10 $g \cdot L^{-1}$ solution of the PAH in acetone. The bottles were closed and incubated for either 2 h or 5 days at 30°C. Thereafter most of the acetone was decanted and the residual acetone was removed by flushing with nitrogen gas. The second method for loading soils (the water method) was very similar to the method used for loading of the synthetic matrices. In this method, 1-L serum bottles were filled with 100 g of soil, 400 ml of deionized water, and 15 g of PAH crystals in a sealed dialysis membrane. The bottles were incubated horizontally on a rotary shaker (90 rpm) at 60°C for a period of one month for naphthalene and three months for phenanthrene. After this period the temperature was lowered by 5°C per day to a final temperature of 30°C. The bottles were then opened, the membranes with the PAH crystals removed and the water removed by leading the soil slurries over a glass-fibre filter. The remaining soils were dried further in an exsiccator containing silica gel and PAH crystals. After drying, the soils were ground in a mortar and sieved over a meshed wire sieve to remove particles with a diameter > 1 mm. The initial loading of the soils was determined by extraction (see below).

Batch desorption experiments

The batch desorption experiments were performed in 250-ml serum flasks on a rotary shaker (200 rpm) at 30°C. The experiments were started by adding 0.025 to 0.1 g of loaded synthetic matrix or soil to the flasks containing 100 or 150 ml of sterile buffered mineral medium. At regular intervals filtered samples of 0.75 ml were removed to determine the PAH concentration in the liquid phase. In the experiments with the synthetic matrices the liquid was decanted after approximately one week and the equilibrium naphthalene concentration of both liquid and solid phases was determined. To test for the influence of bacterial excretion products on the desorption of naphthalene and phenanthrene from the synthetic

matrices, similar experiments have been performed in which filtered (0.2 µm rotrand filter, Schleicher & Schuell, Germany) supernatants of batch cultures growing on naphthalene (strain 8909N) or phenanthrene (strain 8803F) were used instead of mineral medium.

Continuous desorption experiments

The continuous desorption experiments or leaching experiments with naphthalene-loaded XAD-4 and XAD-7 were performed in a mixed system. Mineral medium was pumped into 35-ml serum bottles containing 12 to 15 ml of liquid and 0.1 g of naphthalene-loaded resin. Sintered glass filters were fixed into the outlet of the bottles to protect the resins from washing out. The serum bottles were placed in a rotary shaker (230 rpm) at 30°C. The naphthalene concentration in the leachates was determined by measuring the absorbance at 275 nm (A_{275}) every 12 min with a spectrophotometer using a flow-through cuvette. At regular intervals samples were taken to determine the effluent naphthalene concentrations with HPLC. The pump flow, set at ± 30 $ml.h^{-1}$, was measured at the beginning and at the end of the experiment. The leachates were collected in 5-L jars which contained 25 ml of a 5 M NaOH solution to prevent biodegradation of the desorbed naphthalene. At the end of the experiment, the volumes of the leachate, the naphthalene concentration in the jars and the residual loadings of the matrices were determined.

Biodegradation experiments

Biodegradation experiments were performed in 250-ml serum flasks on a rotary shaker (150 or 200 rpm) at 30°C. The flasks contained 100 ml of mineral medium and were supplemented with 0.1 to 0.5 g of loaded material. In the experiments with XAD-4 and XAD-7 the headspace of the flasks was filled with oxygen gas to eliminate the possibility of oxygen limitation. The experiments were started by inoculation with 1 ml of active batch-grown cells of strain 8909N or strain 8803F for experiments with naphthalene or phenanthrene, respectively. In the experiments with soils and synthetic matrices the biodegradation of naphthalene and phenanthrene was followed by measurement of the percentage of CO_2 in the headspace gas of the bottles. At the end of the experiments 1 ml of a 12 M HCl solution was added to the flasks to remove the dissolved CO_2, and the CO_2 concentration in the headspace gas was measured once more. The residual naphthalene concentrations of the liquid and the solid phases were determined.

In the experiments with activated carbon the biodegradation of PAHs was followed by measurement of the protein content of the liquid phase.

Analytical procedures

Protein was assayed after alkali treatment of cells with the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. CO_2 in the headspace gas of serum flasks was determined using a gas chromatograph (Hewlett Packard type 5890) equipped with a

thermal conductivity detector and a Hayesep Q packed stainless steel column (diameter 1/8 inch, length 2 m, Chrompack, The Netherlands). Helium was used as carrier gas with a flow rate of 30 ml·min^{-1}. The injector temperature was 150°C, the oven temperature 80°C, and the detector temperature 200°C. The injection volume was 250 µl with splitless injection. Aqueous naphthalene concentrations were determined by injection of filtered (0.2 µm), with acetonitrile diluted (1:1) samples on a HPLC with an Chromspher C_{18} (PAH) column (Chrompack, The Netherlands). Eluent was an 85/15 mixture of acetonitrile/water. Peaks were detected with a UV detector by measurement of the A_{275} for naphthalene and of the A_{254} for phenanthrene. Extraction samples were measured likewise, but were diluted to a naphthalene concentration lower than 100 mg·L^{-1}. Extraction of naphthalene from the solid phases was performed by adding 50 or 100 ml of acetonitrile to the matrix and incubating for one week, after which the naphthalene concentration in the acetonitrile was measured. When the extraction was performed with highly loaded material (determination of initial loadings), the acetonitrile was replaced by the same volume of clean acetonitrile and the procedure was repeated. For the synthetic matrices, the dry weight of the matrix was determined by drying a known amount of wet material at 80°C to constant weight.

Results and discussion

Experiments with activated carbon

Biodegradation experiments with a mixture of PAHs originating from polluted soil have been performed with activated carbon as the sorbate. Batch cultures with 250 and 500 mg of loaded activated carbon in 100 ml mineral medium were inoculated with a mixed PAH-degrading culture and the increase in biomass was followed by determination of cellular protein. Figure 3 shows that with PAHs adsorbed onto activated carbon a linear increase in biomass was found, similar to the linear growth on crystalline PAHs (Chapter 3). Moreover it can be seen that for adsorbed substrate growth was proportional to the amount of PAH on activated carbon added, *i.e.*, the total desorbing surface. This shows that the biodegradation of PAHs sorbed onto activated carbon was limited by the desorption process.

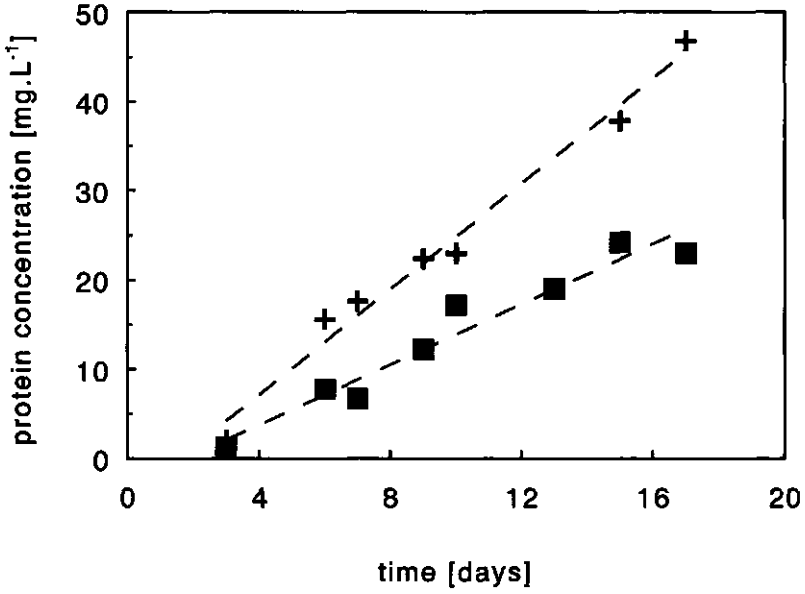

Figure 3: *Batch growth of a mixed bacterial culture on a mixture of polycyclic aromatic hydrocarbons, adsorbed onto 250 (■) or 500 (+) mg loaded active carbon.*

Experiments with synthetic matrices

Loading of the matrices. With the method used it was possible to obtain loaded carrier material that remained stable for longer periods. The maximal loading of the XAD-4 resin was 140-150 $mg \cdot g^{-1}$, which is equivalent to 310-330 $mg \cdot g^{-1}$ dry weight. Since this resin has a specific surface area of 727 $m^2 \cdot g^{-1}$ and the surface area of 1 molecule of naphthalene is $4.29 \cdot 10^{-19}$ m^2 (Radt 1948), it can be calculated that about 87% of the resin surface area was covered with naphthalene. This indicates that the naphthalene was evenly distributed over the resin. For XAD-7 the maximal loading was 68 $mg \cdot g^{-1}$, which amounts to a coverage of 89%. The surface area of the coated polymer was unknown; the maximal loading obtained was 6.1 $mg \cdot g^{-1}$.

Batch desorption. Batch desorption experiments were performed by adding different amounts of matrix to a known volume of mineral medium and following the aqueous

naphthalene concentration. The equilibrium concentrations that were found at the end of the desorption experiments were used to construct the desorption isotherms, which for the three materials could be described using the Freundlich equation (Equation 1). The results of the measurements and the fitting are shown in Figure 4; the Freundlich constants that were found are given in Table 1.

Table 1: *Freundlich constants for desorption of naphthalene from the synthetic matrices.*

material	K_f [$mg^{1-1/n} \cdot L^{1-n} \cdot g^{-1}$]	n [-]
polymer	0.44	1.59
XAD-7	5.11	1.47
XAD-4	39.5	2.50

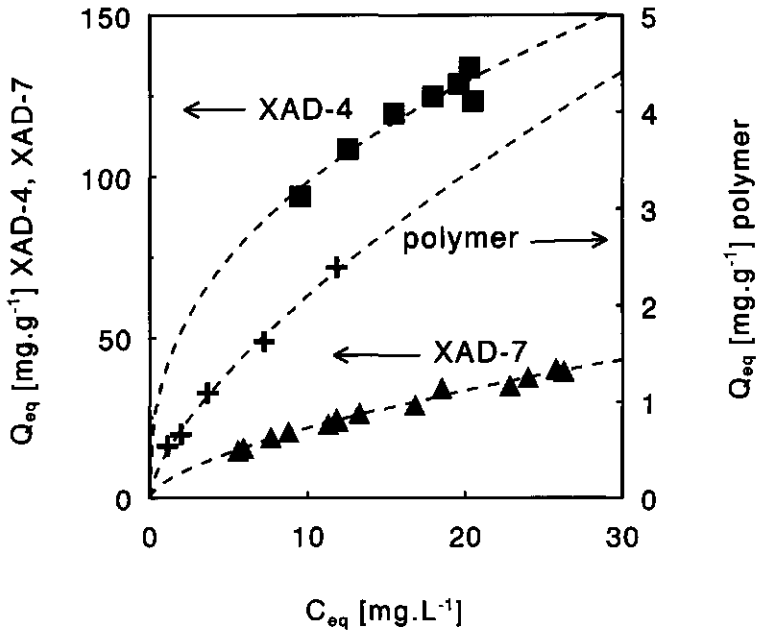

Figure 4: *Desorption isotherm of naphthalene loaded onto XAD-4 (■), XAD-7 (▲), or the coated polymer (✚).*

In the desorption experiments with the coated polymer, equilibrium was reached within 1 h. This provides too little data to follow the desorption kinetics. The results of the desorption experiments with XAD-4 and XAD-7 were fitted with both the two-compartment and the radial diffusion models. The non-proportinality of the isotherms forms a problem for using the radial diffusion model. The analytical solution presented by Crank (1975) is only valid when partitioning is proportional. For non-linear isotherms no analytical solutions were available and therefore the isotherms of XAD-4 and XAD-7 were linearized. This resulted in partition coefficients of 6.96 and 1.74 $L \cdot g^{-1}$ for XAD-4 and XAD-7, respectively. The best results of fitting the desorption of 0.1 g of naphthalene-loaded XAD-4 in 100 ml of mineral medium with the radial diffusion model are shown in Figure 5 as an example (solid line). As can be seen, it was not possible to produce a curve that gave a good description of the experimental data. Although this may be partly caused by the linearization of the isotherm, the radial diffusion model cannot explain the biphasic character of the desorption curve observed. It is likely that better results can be obtained with a more complicated model, combining adsorption and diffusion process. However, the construction of such a model was not within the scope of this study.

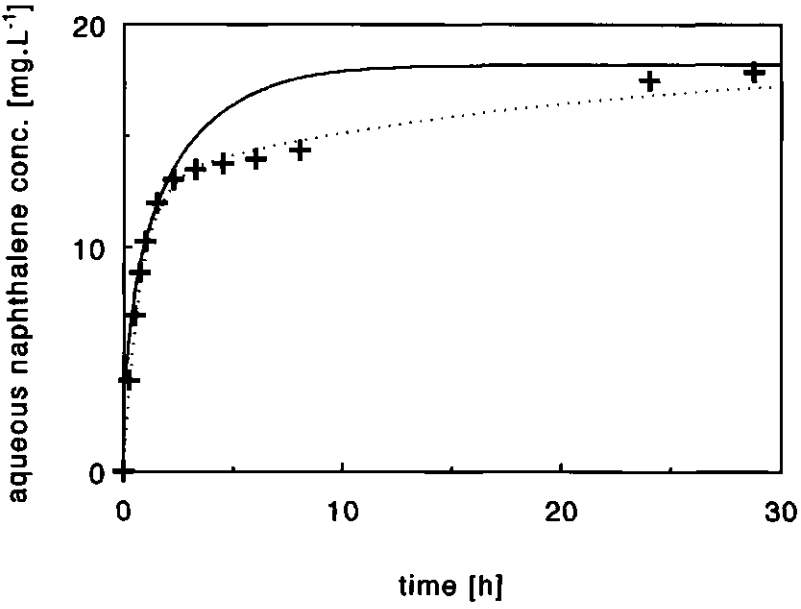

Figure 5: *Batch desorption kinetics of naphthalene loaded onto XAD-4 (+). Lines represent fitting with the two-compartment (dotted lines) and radial diffusion model (solid lines).*

Using the two-compartment model it was possible to fit the experimental results well, as can be seen in Figure 5 too (dotted line). However, since the curves could be fitted with different sets of values for the desorption variables f, k_d, and k_s, the accuracy by which these variables could be determined was low (Table 2). Therefore, the results of the batch desorption experiments could not be used to make a reliable estimation of the three parameters.

Table 2: *Desorption parameters found by fitting batch and continuous desorption of naphthalene from XAD-4 and XAD-7 with the two-compartment model.*

resin	batch desorption			continuous desorption		
	f [-]	k_d [h^{-1}]	k_s [h^{-1}]	f [-]	k_d [h^{-1}]	k_s [h^{-1}]
XAD-4	0.20 - 0.45	0.02-0.06	0.5-1.5	0.26-0.29	0.048-0.052	0.69-0.75
XAD-7	0.50 - 0.80	0.01-0.05	0.35-0.55	0.63-0.67	0.024-0.028	0.39-0.43

Continuous desorption. Continuous desorption experiments were performed in a mixed system. This has the advantage over column experiments in that a better comparison with the biodegradation experiments is possible. The results of the measurements of the naphthalene concentration in the leachates are shown in for XAD-4 and XAD-7 in Figure 6A. In Figure 6B these data have been converted to the desorbed fractions of the napthalene initially present desorbed. It can be seen that the desorption from XAD-7 proceeds much faster than from XAD-4. The dashed lines in the figures show curves which were fitted using the two-compartment model, assuming ideal mixing in the bulk phase. The values for f, k_d, and k_s that have been obtained by fitting the measured data are presented in Table 2. The fact that these values could also be used to describe the results of the batch desorption experiments shows that the two-compartment model can be used to describe the desorption of naphthalene from the XAD resins.

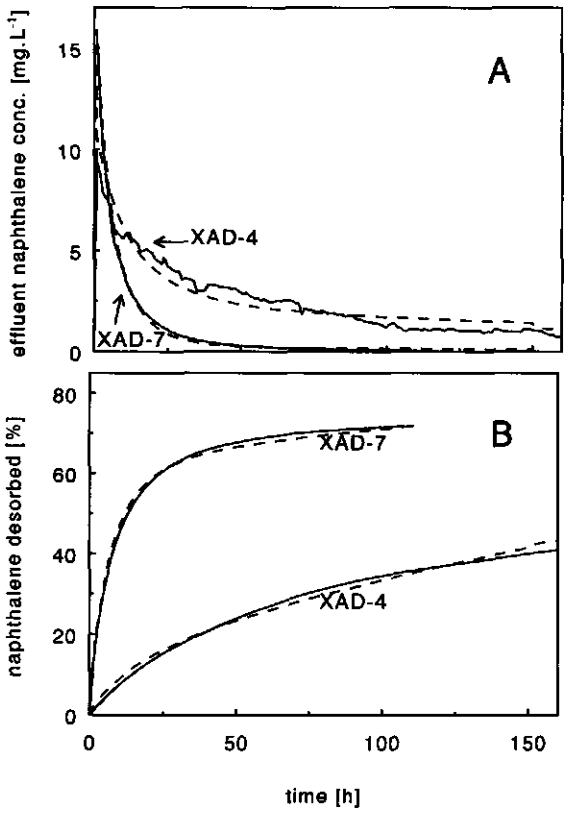

Figure 6: *Continuous desorption of naphthalene from XAD-4 and XAD-7: A: measured effluent concentration, B: % initial naphthalene that has been desorbed. Solid lines represent measured data; dashed lines give curves fitted with the two-compartment model.*

Biodegradation. In Figure 7 an example of the results of a biodegradation experiment with naphthalene-loaded synthetic materials is shown. The data are corrected for CO_2 concentrations measured in blank experiments containing unloaded resins. Using the yield factor as obtained previously (Chapter 2) and carbonate equilibria (Lindsay 1979) it is possible to relate the CO_2 concentration in the headspace gas to the total amount of CO_2 produced and with it to the amount of naphthalene degraded. In these experiments 53% of the initially present carbon was recovered as

CO_2, a slightly lower figure than that found for the yield in the batch-growth experiments with crystalline naphthalene (Chapter 2). Due to the attachment of the bacteria to the matrix it was not possible to obtain a carbon balance. The average growth rate in the experiment presented in Figure 7 was approximately 0.035 h^{-1} and, using Equation 1 of Chapter 2 and assuming that 91% of the degraded naphthalene is converted to CO_2 and biomass (Chapter 2, Table 4), it can be calculated that 55% of the naphthalene is converted to CO_2 in the chemostat experiments at a growth rate of 0.035 h^{-1}. This shows that the recovery of 53% of the carbon found for the growth on sorbed substrate in the experiments described here is a realistic value. In none of the biodegradation experiments performed with the coated polymer it was possible to find a desorption-limited growth phase. In the biodegradation experiments with napthalene sorbed onto XAD-4 and XAD-7 exponential growth could only be observed in the first stage of the experiments. Measurement of naphthalene concentrations in the non-exponential growth stages showed that the aqueous-phase naphthalene concentrations were virtually zero (data not shown). Therefore it was concluded that in these stages the biodegradation process was limited by the desorption of naphthalene from the resins.

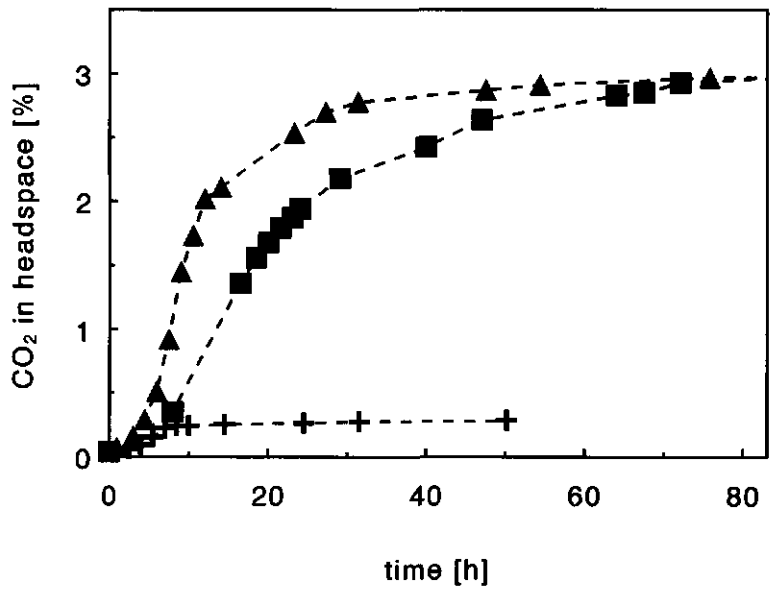

Figure 7: *CO_2 production of strain 8909N on naphthalene sorbed onto XAD-4 (■), XAD-7 (▲), or the coated polymer (✚).*

The amount of degraded naphthalene that was calculated using the yield factor discussed above is shown for for XAD-4 and XAD-7 in Figure 8. As both the physical conditions (temperature, mixing) and the time span of the biodegradation experiments and the continuous desorption experiments are comparable, the values for the desorption parameters found in these experiments (Table 2) were used for modelling the biodegradation experiments. Microbial growth was modeled using Monod kinetics as presented in Chapter 3. The lines without markers in Figure 8 represent simulations in which it was assumed that the only effect of the presence of bacteria is the degradation of naphthalene present in the aqueous bulk phase.

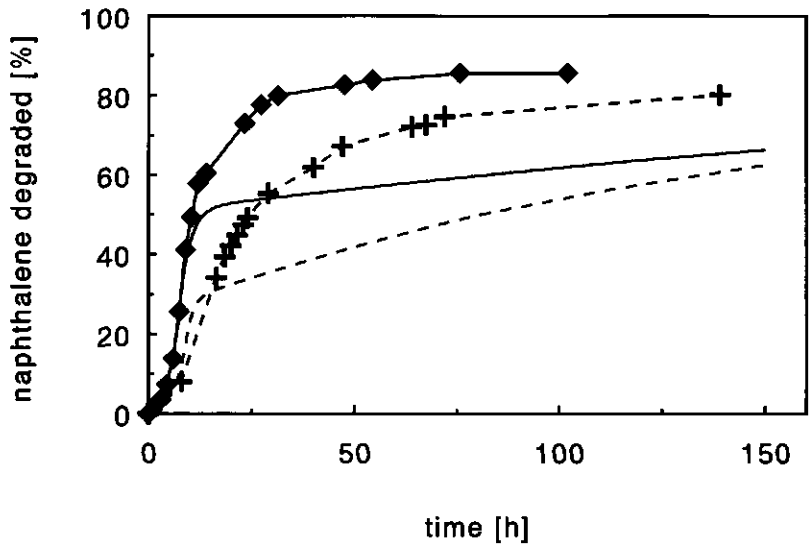

Figure 8: *Biodegradation of naphthalene during batch growth of strain 8909N on naphthalene sorbed onto XAD-4 (✚) or XAD-7 (◆). Lines represent simulated data for XAD-4 (dashed line) and XAD-7 (solid line),obtained with the two-compartment model using the parameters from Table 2.*

As can be seen in the figure, the simulations clearly underestimate the measured data, indicating that biodegradation proceeds faster than can be explained by desorption alone. This phenomenon, found previously by other researchers (Griffith & Fletcher 1991, Guerin & Boyd 1992) has several explanations, such as direct uptake of sorbed substrate (grazing), growth of bacteria in pores, and bacterial excretion of bioavailability-enhancing compounds. To test this last hypothesis, batch

desorption of naphthalene from XAD-4 and XAD-7 in supernatant of strain 8909N, cultured on naphthalene under dissolution-limiting conditions, was investigated. No difference between the results of the experiments with supernatant and blank experiments with mineral medium could be observed (data not shown). This excludes excretion of bacterial products as the mechanism for the enhanced bioavailability. In the growth experiments it was observed that attachment of the cells onto the matrix occurred during growth. Therefore both direct uptake of sorbed substrate and growth in the pores of the matrix may explain the difference that was found between the biodegradation rate and the desorption rate.

Experiments with soil

Soil loading. Table 3 gives an overview of the initial loadings of the different soil types with naphthalene and phenanthrene. It can be seen that more PAH could be sorbed onto the soil using the acetone method than using the water method. It should be noticed that crystalline PAHs are likey to be present in soils loaded via the acetone method, especially for the soils with a loading time of 2 h. The amount of PAH finally sorbed onto the soils shows a positive correlation with the fraction organic matter of the soil.

Table 3: *Initial loading of different soils with naphthalene and phenanthrene*

soil	organic matter [%]	initial loading [mg·g^{-1}]					
		water method		acetone method			
		naphthalene	phenanthrene	naphthalene		phenanthrene	
				2 h[a]	5 days[a]	2 h[a]	5 days[a]
Sandy soil	2.9	1.6	1.1	2.2	7.3	2.4	7.4
Eerdsoil	6.6	2.7	1.6	2.7	10.7	2.4	9.4
Koopveen	28.9	7.4	3.2	3.3	16.1	4.2	11.1

[a]: incubation time before acetone decanting

Batch desorption. The difference between the two methods of loading is also expressed in the results of batch desorption experiments, as can be seen in Figure 9 for phenanthrene. The desorption of naphthalene and phenanthrene from the soil loaded via the acetone methods was faster than from the soil that was loaded via the

water method. Moreover, it was observed that for soils loaded via the acetone-method (loading time 2h) the final aqueous concentration (measured after four days) was lower than the maximal measured concentration, which was reached after 2-3 h. This may be explained by adsorption of the PAH onto the soil after it has been dissolved. The soils that were loaded via the acetone-method with a loading time of five days were used for further experiments.

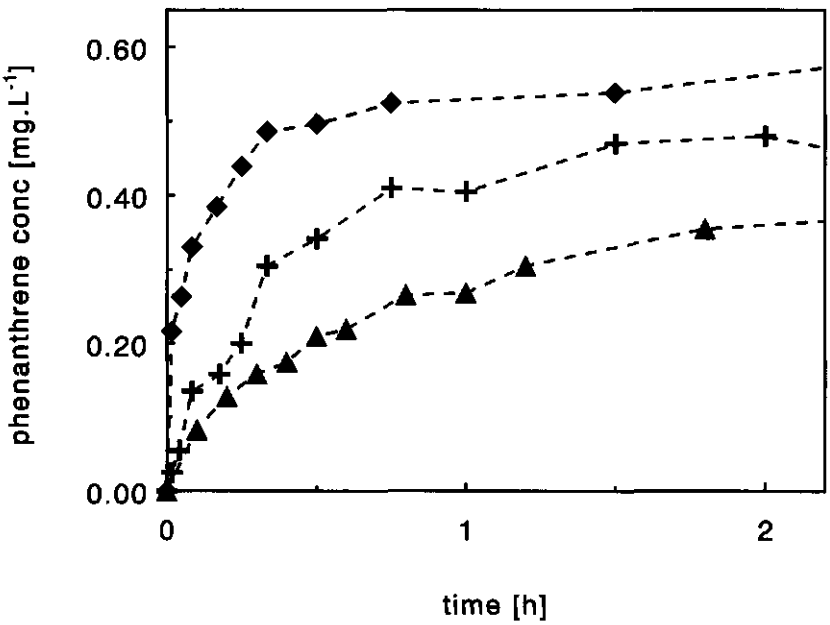

Figure 9: *Batch desorption of phenanthrene from Eerdsoil, which was loaded via three different methods. Symbols: ▲: loaded via the water method; ✚: loaded via the acetone method, loading time 5 days; ◆: loaded via the acetone method, loading time 2 h.*

The equilibrium concentrations and loadings that were found at the end of the batch desorption experiments were used to construct the desorption isotherms, which could be described with the Freundlich equation (Equation 3). The results are shown in Figure 10 for naphthalene (A) and phenanthrene (B), the Freundlich constants found for the naphthalene-loaded and phenanthrene-loaded soils are given in Table 4.

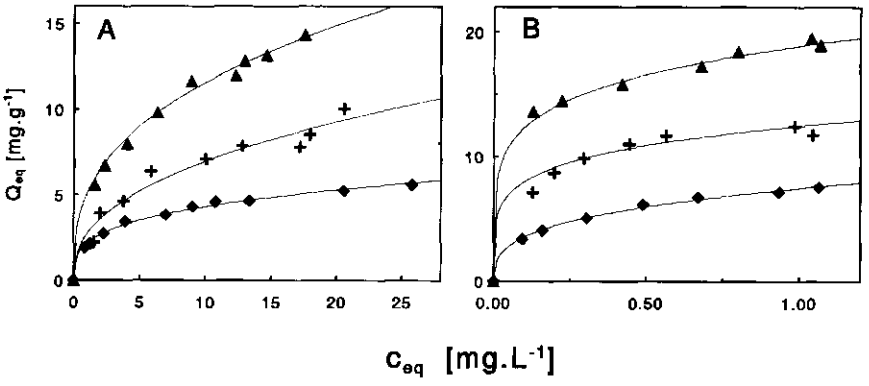

Figure 10: *Isotherms for desorption of naphthalene (A) and phenanthrene (B) from sandy soil (◆), Eerdsoil (✚), and Peat soil (▲).*

Table 4: *Freundlich constants for the desorption of naphthalene and phenanthrene from different soils loaded via acetone.*

soil	naphthalene		phenanthrene	
	K_f [mg$^{1-1/n}$·L^{1-n}·g^{-1}]	n [-]	K_f [mg$^{1-1/n}$·L^{1-n}·g^{-1}]	n [-]
Sandy soil	2.15	3.33	7.5	3.07
Eerdsoil	2.66	2.40	12.5	5.15
Koopveen	5.01	2.75	18.9	5.39

Batch desorption experiments were also used for studying the desorption kinetics. The typical pattern with initially fast desorption, followed by a phase with slow desorption, could not be described with the radial diffusion model. Using the two-compartment model, it was possible to describe the measured data well, as can be seen for different amounts of naphthalene-loaded Eerdsoil in Figure 11.

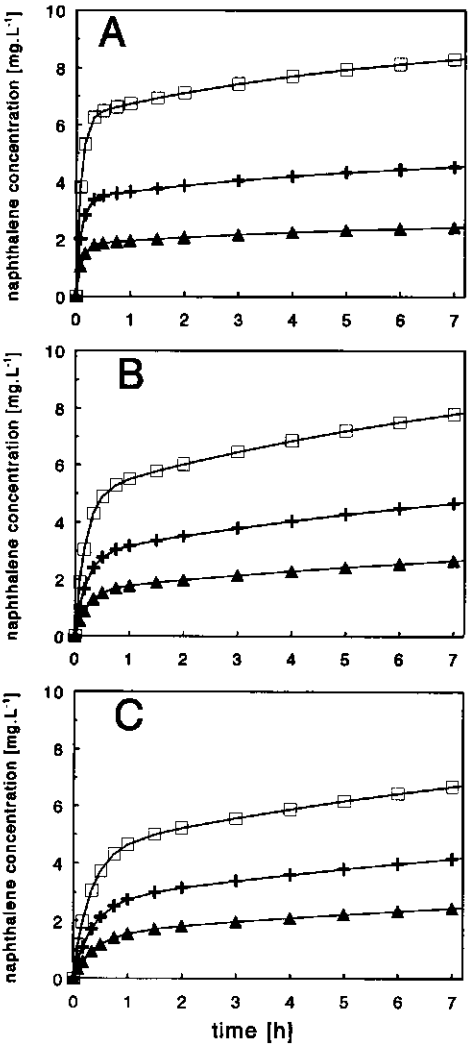

Figure 11: *Desorption of naphthalene from different amounts of Sandy soil (A), Eerdsoil (B), and Koopveen (C) in 100 ml mineral medium. Symbols: ▲: 0.05 g soil, ✚: 0.1 g soil, □: 0.2 g soil. Solid lines represent fitted data using the two-compartment model.*

Similar results have been found for the other types of soil and for phenanthrene. In contrast to the batch desorption experiments with the synthetic matrices, the data from the experiments with soil could be used to get a good estimation for the parameters f, k_d, and k_s. The values of the parameters found by fitting the desorption of naphthalene and phenanthrene from the different soils with the two-compartment model are presented in Table 5. Comparing the results with Table 3, it can be seen that the deep pore fraction (1 - f) is correlated with the fraction organic matter in the soil. No correlation between the type of soil and the mass transfer coefficients could be found. This supports the viewpoint that the model does not provide a good physical description of the acual desorption process in the soils. It can, however, be used to describe the experimental data.

Table 5: *Parameters obtained by fitting batch desorption of naphthalene and phenanthrene from different types of soil with the two-compartment model.*

soil	naphthalene			phenanthrene		
	f [-]	k_d [h^{-1}]	k_s [h^{-1}]	f [-]	k_d [h^{-1}]	k_s [h^{-1}]
Sandy soil	0.63-0.65	0.15-0.17	8.1-10.1	0.41-0.44	0.035-0.039	0.85-0.95
Eerd soil	0.33-0.37	0.081-0.091	3.4-4.3	0.27-0.29	0.045-0.051	0.51-0.0.55
Koopveen	0.21-0.23	0.054-0.058	1.94-1.99	0.16-0.18	0.10-0.13	0.62-0.64

Biodegradation. Biodegradation of soil-sorbed naphthalene and phenanthrene was monitored by measurement of the CO_2 production in batch experiments with strain 8909N and 8803F respectively. Typical examples of the CO_2 production on the different naphthalene- and phenanthrene-loaded soils are shown in Figure 12A-F, the values were corrected for CO_2 production with unloaded soils. After 10-12 days, when the CO_2 concentration in the headspaces remained almost constant, the experiments were stopped by adding 1 ml of 12 M HCl. After this, the final CO_2 concentration in the headspace gases, the aqueous naphthalene concentrations, and the residual loadings of the soils were determined. In none of the experiments naphthalene or phenanthrene was detected in the aqueous phase. The average mass balances over the experiments presented in Figure 12 are given in Table 6. In all of the experiments approximately 52% of the initial carbon present as PAH was converted to CO_2. This is very similar to the values found with crystalline PAHs (Chapter 2) and PAHs sorbed onto inert matrices (see above). The solid lines in the

Figures 12A-F represent the data that were found by modelling, assuming that only aqueous phase substrate is available for biodegradation. The desorption was modeled with the two-compartment model, using the parameters that were obtained with the desorption experiments (Table 5). Microbial growth was modeled using Monod kinetics as presented in the previous chapter. The results that were obtained with the Sandy soil and the Eerdsoil show good agreement with the modeled data. For the Koopveen soil, biodegradation in the desorption-limited phase seems to proceed slightly faster than could be explained by desorption alone. However, this difference is not significant due to the limited applicability of the model. Therefore biodegradation was concluded to be limited by desorption of PAHs from the soil.

Table 6: *Mass balance over growth experiments of strain 8909N on naphthalene and strain 8803F on phenanthrene sorbed onto different types of soil; data represent means of two experiments.*

soil type	soil amount [g]	naphthalene		phenanthrene	
		residual loading [%]	CO_2 yield [C-mol/C-mol]	residual loading [%]	CO_2 yield [C-mol/C-mol]
Sandy soil	0.5	4.6	0.53	12.6	0.51
	1.0	5.4	0.53	14.8	0.52
	2.0	5.1	0.51	13.8	0.52
Eerdsoil	0.5	3.6	0.52	27.7	0.51
	1.0	4.7	0.53	27.6	0.52
	2.0	3.4	0.50	26.9	0.51
Koopveen	0.5	8.3	0.54	42.6	0.54
	1.0	8.6	0.50	41.4	0.52
	2.0	8.6	0.51	42.9	0.55

Conclusions

In biodegradation experiments with activated carbon, synthetic matrices, and soil, it was shown that the sorption of the PAHs naphthalene and phenanthrene onto these materials resulted in a decrease in the bioavailability of these PAHs.

The desorption of naphthalene from the synthetic porous resins XAD-4 and XAD-7 could be described using a two-compartment model as was shown in batch

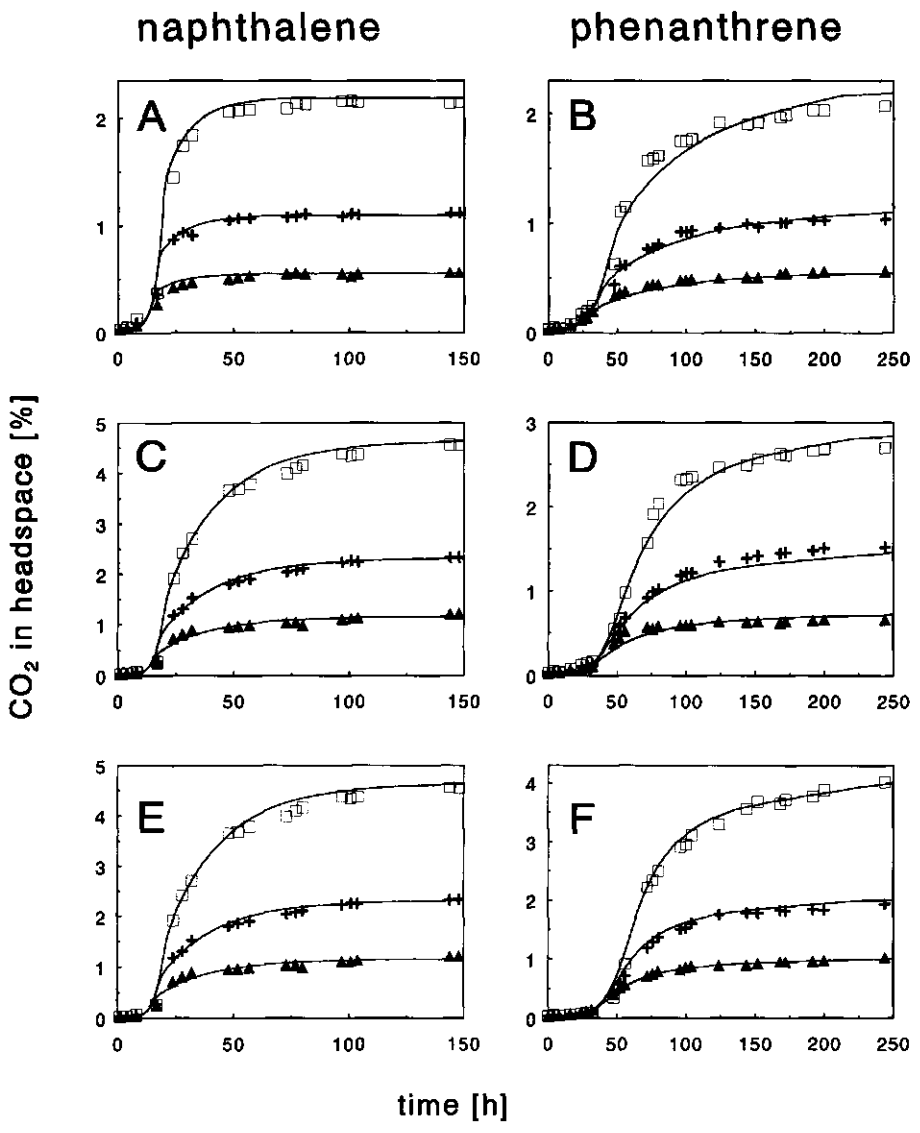

Figure 12: *CO_2 production of strain 8909N on different amounts of naphthalene-loaded soils and of strain 8803F on phenanthrene-loaded soils. Soil types: A,B: sandy soil; C,D: Eerdsoil; E,F: Koopveen. Symbols: ▲: 0.5 g soil, ✚: 1.0 g soil, □ : 2.0 g soil. Solid lines represent data modeled with the two-compartment model using the parameters from Table 5.*

and continuous experiments. In biodegradation experiments with these resins it was found that the biodegradation of sorbed naphthalene proceeded faster than the desorption of naphthalene from the matrix, indicating that the microorganisms have some means of gaining access to sorbed substrate.

In similar experiments with different types of soil, the biodegradation rates were found to match the desorption rates. Two explanation, based on the difference between the resins and the soils, can be put forward for this difference. The first difference is the porosity of the material. One can imagine that bacteria are able to grow in the largest pores of the resins. This reduces the length of the diffusion distance and therewith the time necessary for desorption. Secondly it is possible that the sorption of PAHs onto the synthetic materials will occur mainly as adsorption, whereas sorption onto the soils, and especially onto the soil organic matter, may occur as absorption. When bacteria are capable of directly taking up sorbed substrates, it is likely that adsorbed substrates are more available for the bacteria. As a general conclusion from the experiments presented here it can be said that the bioavailability of hydrophobic pollutants such as PAHs is greatly reduced when the pollutant occurs in a sorbed state, but that the extent of the reduction can be different under different conditions.

Nomenclature

a	particle radius [m]
α	final fractional amount that has been desorbed [-]
C	concentration in the aqueous bulk phase [mg·L^{-1}]
C_0	initial concentration in the aqueous bulk phase [mg·L^{-1}]
C_{eq}	equilibrium pollutant concentration in solution [mg·L^{-1}]
d_d	pore diameter [m]
D	apparent diffusion coefficient [m^2·h^{-1}]
$D_{s,eff}$	effective surface diffusion coefficient [m^2 ·h^{-1}]
f	fraction of the surface area present in shallow pores [-]
k_a	Langmuir constant [L·g^{-1}]
k_d	deep pore desorption constant [h^{-1}]
k_p	soil-water partition coefficient [L·g^{-1}]
k_s	shallow pore desorption constant [h^{-1}]
K_f	Freundlich sorption capacity constant [mg$^{1-1/n}$·L^{1-n}·g^{-1}]

l_d	length of the deep pore [m]
l_s	length of the shallow pore [m]
n	Freundlich sorption energy constant [-]
$\overline{q}_d$	average loading of the deep pores [mg·m^{-2}]
q_{eq}	equilibrium adsorbed concentration [mg·m^{-2}]
$\overline{q}_s$	average loading of the shallow pores [mg·m^{-2}]
Q_{eq}	equilibrium sorbed pollutant concentration [mg·g^{-1}]
Q_{max}	maximum sorbed pollutant concentration [mg·g^{-1}]
Q_0	initial loading [mg·g^{-1}]
Q_t	average concentration in the particles [mg·g^{-1}]
r	radial distance [m]
S_x	specific surface area [m^2·g^{-1}]
t	time [h]
V	aqueous volume [L]
V_x	amount of solid phase [g]

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CHAPTER 5

MICROBIOLOGICAL ASPECTS OF THE USE OF SURFACTANTS IN BIOLOGICAL SOIL REMEDIATION

Abstract

The biodegradation of hydrophobic organic compounds in polluted soil is a process that involves interactions among soil, pollutants, and microorganisms. Surfactants or surface-active agents are compounds that may affect these interactions. Therefore the use of surfactants may be a means of overcoming the problem of limited bioavailability of hydrophobic organic pollutants in biological soil remediation. The effects of surfactants on the physiology of microorganisms may range from inhibition of growth due to surfactant toxicity to stimulation of growth caused by the use of surfactants as cosubstrate. The most important effect of surfactants on the interactions among soil and pollutant is stimulating mass transport of the pollutant from the soil to the aqueous phase. This may be caused by three different mechanisms: emulsification of liquid pollutant, micellar solubilization, and facilitated transport. The importance of these mechanisms for the effect of surfactants on the bioavailability of organic pollutants present in different physical states is discussed.

The complexity of the effect of surfactants on pollutant bioavailability is reflected by the results in the literature, which range from stimulation to inhibition of both desorption and biodegradation of the polluting compounds. Moreover, no general trends can be found in the results. Therefore more research is necessary before the application of surfactants can become a standard tool in biological soil remediation.

Introduction

Many studies on the biodegradation of hydrophobic organic compounds (HOCs) in soil have shown that the limited bioavailability of these compounds, i.e. the slow release of these compounds to the aqueous phase, is often the rate-limiting step in the process. A review on this subject was published recently by Mihelcic *et al.* (1993).

Of the possible technological solutions to limited pollutant bioavailability, a very promising one is the use of surfactants to mobilize the pollutant (Morris & Pritchard 1993). Other solutions are (i) still in the developing phase, such as the application of acoustic techniques; the use of organic solvents, chemical oxidants, and fungi or fungal enzymes, or (ii) generally too expensive, such as increasing the soil temperature. A realistic solution is formed by the mechanical reduction of the size of soil aggregates, but this technique cannot be applied with *in situ* soil remediation.

The first research on the application of surfactants in soil was carried out in the petroleum industry. Using the dispersive and surface-active action of surfactants, the objective was to improve the recovery of oil from oil wells by flushing with surfactant solutions. It is therefore not surprising that the first study on the use of surfactant for clean-up of contaminated soils was performed by the American Petroleum Institute (1979). In this article and in many other articles on the use of surfactants for soil remediation, the washing of polluted soil with surfactant solutions is studied. They deal mainly with the effect of soil-surfactant and soil-pollutant interactions on the solubilization of HOCs, and on the transport of HOCs through soil. Less has been published on the use of surfactants in biological soil remediation. This study presents an overview of the use of surfactants in soil bioremediation, focussing on the microbiologically important aspects such as surfactant-microorganism interactions and the bioavailability of the pollutants.

Physicochemical properties of surfactants

Surface-active agents or surfactants are organic molecules that usually consist of a hydrophobic and a hydrophilic part. The hydrophilic part makes surfactants soluble in water, the hydrophobic part makes them tend to concentrate at interfaces. The presence of surfactant molecules at air- water interfaces results in a reduction of the surface tension of the solution, and leads to the stabilization of foams. Many

surfactants can reduce the surface tension of aqueous solutions from 72 mN/m (the value for water) to 25 ± 5 mN/m, depending on type and concentration of the surfactant. In the presence of a non-aqueous phase liquid (NAPL) surfactants concentrate at the liquid-liquid interface, reducing the interfacial tension. This may cause dispersion of NAPL droplets and stabilization of emulsions. Concentration of surfactant molecules at solid surfaces also leads to a reduction of the interfacial tension, which may improve the contact between the aqueous and the solid phase.

Another characteristic of surfactants is the formation of micelles, little aggregates of surfactant molecules. At low concentrations in aqueous solutions, single molecules are present. Beyond a certain concentration, called the critical micelle concentration (CMC), the surfactant molecules cluster together, forming aggregates of 20-200 molecules. The CMC is different for every surfactant and typically ranges from 0.1 to 10 mM. A schematic representation of a micelle is given in Figure 1. Simplified, these micelles can be seen as little (2-4 nm) drops with a hydrophobic core and a hydrophilic shell. The presence of micelles leads to an increase in the apparent solubility of HOCs; this is also referred to as solubilization. At higher surfactant concentrations other types of surfactant aggregates may be formed. These high concentrations are not interesting for soil remediation processes and will not be discussed any further. In the presence of a solid surface, micelle-like structures called admicelles may be formed at the solid-liquid interface. The surfactant concentration at which this phenomenon occurs is called the "critical admicelle concentration" or CAC; this concentration is usually lower than the CMC (West & Harwell 1992, Nayyar *et al.*1994).

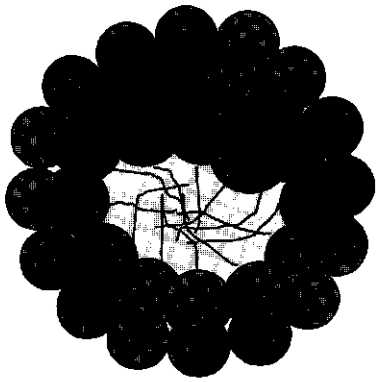

Figure 1: *Schematic representation of a surfactant micelle.*

Depending on the nature of the hydrophilic group, four types of surfactants can be distinguished: anionic, cationic, zwitterionic, and nonionic surfactants. Surfactants can be produced chemically (synthetic surfactants) and biologically (biosurfactants). The most common hydrophobic parts of synthetic surfactants are paraffins, olefins,

alkylbenzenes, alkylphenols and alcohols; the hydrophilic group is usually sulphate, sulphonate, carboxylate (anionic), quaternary ammonium (cationic), polyoxyethylene, sucrose, or polypeptide (nonionic). The major classes of biosurfactants include glycolipids, phospholipids, fatty acids, lipopeptides/lipoproteins, and biopolymeric surfactants. Surfactants are produced on large scale and have numerous applications, e.g. as additives in cleaning agents, food, and cosmetics, and in mining and road construction. An extensive overview of the composition and physicochemical properties of surfactants is given by Rosen (1989).

Surfactant-microorganism interactions

Surfactant toxicity

Introduction of a surfactant in the environment will always lead to contamination with this surfactant and is therefore of little use when the compound itself gives rise to environmental concern. Consequently, the toxicity of the surfactant and its potential degradation products is one of the most important criteria for the selection of surfactant in soil clean-up. Because of the use of surfactants on large scale in detergents, the toxicity of these compounds has been tested relatively well. An overview of the toxicity of the different surfactant types towards numerous species is given by De Oude (1992). The toxic effect of surfactants on bacteria can be explained by two main factors (Helenius & Simons 1975): (i) disruption of cellular membranes by interaction with lipid components and (ii) reactions of surfactant molecules with proteins essential to the functioning of the cell. At a pH of 7 and higher, cationic surfactants are the most toxic ones, at lower pH anionic surfactants display the most toxic behavior. Nonionic surfactants are in general less active against bacteria than ionic surfactants. Biologically produced surfactants occur naturally in soil and the use of these surfactants in bioremediation processes will be more acceptable from a social point of view. In comparison with synthetic surfactants, a lower toxicity can be expected from most biosurfactants, although some biosurfactants can be as toxic as synthetic surfactants (Lang & Wagner 1993).

For microorganisms degrading hydrophobic hydrocarbons the presence of surfactants, especially in concentrations above the CMC, has been observed to have an inhibiting effect (Laha & Luthy 1991, 1992, Roch & Alexander 1995, Tsomides *et al.* 1995). As yet, the reason for this effect of the presence of micelles is not understood.

Surfactant biodegradation

The biodegradation of the surfactant is a factor that can have both negative and positive effects in the use of surfactants for bioremediation. Negative effects can be caused by (i) depletion of minerals or oxygen, (ii) toxicity of surfactant intermediates, which are often more toxic than the parent compounds (Holt *et al.* 1992), or (iii) preferential degradation of the surfactant, slowing the pollutant degradation. Moreover the degradation of the surfactant will reduce the bioavailability-enhancing effects (Holt *et al.* 1992, Oberbremer *et al.* 1990). The most obvious positive effect of surfactant degradation is the removal of the surfactant from the polluted site. Secondly, the presence of a degradable surfactant may enhance the uptake rate of hydrocarbons. This was found, for example, by Miller & Bartha (1989), who proved that the degradation of long-chain *n*-alkanes was limited by transport of the substrate over the bacterial membrane. They showed that solubilization of the alkanes in liposomes, micelles constructed of phosholipids, could overcome this limitation. The authors inferred that this was caused by fusion of the liposomes with the cell membrane. More evidence for this mechanism is given by Bury & Miller (1993) who found that uptake of micellar *n*-decane and *n*-tetradecane was stimulated by a biodegradable surfactant, resulting in higher growth rates. This was probably caused by direct uptake of the hydrocarbons along with the micelles. Another positive effect is that a degradable surfactant might be used as a cosubstrate when the pollutant is degraded cometabolically, although to our knowledge no study has been published on this subject. Extensive information on the biodegradability of numerous surfactants has been collected by Swisher (1987).

Surfactant absorption

Biological membranes are mainly built up of phospholipid molecules, which are very similar to surfactants. Hence these membranes are very effective in absorbing surface-active compounds (Sikkema *et al.* 1995). Apart from the toxic effect and the possibility of fusion with micelles, which have been discussed above, two more consequences of this absorption have been described. Firstly, the aqueous concentration of the surfactant will be lowered, a process similar to the adsorption of surfactant to soil (Swisher 1987). Secondly, it may have an effect on the permeability of the membrane for HOCs, (Mihelcic *et al.* 1993, Van der Werf *et al.* 1995), thus enhancing the uptake of hydrophobic substrates.

Bacterial mobility

Some researchers have reported that surfactants may affect the mobility of bacteria in soil. Marchesi *et al.* (1991) have found that anionic sulphate surfactants can cause attachment of bacteria degrading these surfactants to sediment. The addition of pyruvate or of non-biodegradable surfactants did not induce bacterial attachment. The authors implied that the adherence was related to the bacterial uptake of the sorbed surfactant. Jackson *et al.* (1994) observed the opposite effect, namely a decrease in the attachment of *Pseudomonas pseudoalcaligenes* to clay when an anionic sulphate surfactant was present. Three possible explanations for the effect were given. Firstly, the surfactant could cause a decrease in the reversible adsorption of the bacteria due to a change in charge density. Secondly, the surfactant could prevent flocculation, thus increasing the transport of bacteria. The last explanation involved the dissolution of extracellular polymers, which are responsible for the irreversible adsorption. Since attached microorganisms often show activities different from free cells (Van Loosdrecht *et al.* 1990), this effect of surfactants can be of importance for soil remediation. This is especially true for *in situ* bioremediation, in which transport of bacteria can be an essential process.

Surfactants and hydrocarbon bioavailability

Role of surfactants in bacterial uptake of hydrophobic compounds

The enhancement of the bioavailability of hydrophobic organic pollutants by surface-active compounds can be caused by three main mechanisms:

1. *Emulsification of liquid-phase pollutant.* As mentioned above, surfactants can decrease the interfacial tension between an aqueous and a nonaqueous phase. This may lead to the formation of micro-emulsions or, with energy input, to the formation of emulsions. This results in an increase in the contact area, enabling improved mass transport of the pollutant to the aqueous phase and in mobilization of sorbed liquid-phase pollutant;
2. *Enhancement of apparent solubility of the pollutant.* This so called "solubilization" is caused by the presence of micelles. Hydrophobic organic compounds will solubilize mainly in the core of the micelles, whereas more hydrophilic molecules such as mono-aromatic compounds may be present in both the core and the shell of the micelles. The transport of micellar hydrocarbon to the aqueous phase can be very rapid due to the small size of the micelles (Almgren *et al.* 1979), but it is

not clear whether solubilized hydrocarbons are directly available to the degrading microorganisms (Grimberg *et al.* 1995, Roch & Alexander 1995);

3. *"Facilitated transport" of the pollutant.* This term covers several different processes such as the interaction of pollutant with single surfactant molecules, the interaction of surfactants with solid or sorbed hydrocarbons (both as single surfactant molecules and as micelle-like aggregates), and mobilization of pollutant trapped in soil ganglia by lowering the surface tension of the pore water in soil particles.

These three mechanisms may all cause enhanced mass transport and are therefore often hard to distinguish. Apart from positive effects, surfactants can also have negative effects on the bioavailability of HOCs. For instance, surfactants can inhibit bacterial attachment, disperse soil colloids, causing clogging of pores, or interfere with the natural interactions of microorganisms with the pollutant. In the following sections the positive and negative effects mentioned above will be discussed for the case of non-aqueous phase liquid phase pollutant, solid pollutant, and sorbed pollutant.

Non-aqueous phase liquid pollutant

In the literature three mechanisms for the microbial uptake of liquid hydrocarbons have been proposed: (i) uptake of hydrocarbon dissolved in the aqueous phase; (ii) direct uptake of hydrocarbons from the liquid–liquid interface; (iii) uptake of "pseudosolubilized" hydrocarbon (Hommel 1990, Bury & Miller 1993). The first mechanism is usually found for compounds with a relatively high aqueous solubility (Goswami *et al.* 1983), although Yoshida *et al.* (1971) found that the uptake of C_{16} to C_{18} alkanes by the yeast *Candida tropicalis* proceeded via the aqueous phase. Direct uptake of NAPLs, involving bacterial attachment to the liquid-liquid interface, has often been described for poorly soluble substrates (e.g. Efroymson & Alexander 1994, Prokop *et al.* 1971, Wodzinski & Larocca 1977). Zhang & Miller (1994) showed that cell hydrophobicity plays an important role in the attachment. The presence of bacterial emulsifiers and of the bacteria themselves at the interface lowers the interfacial tension, thus causing enhanced dispersion of the NAPL. This results in an increase in surface area and with it an increase in biodegradation rates. Pseudosolubilization of NAPLs by microbial excretion products has been studied extensively by the group of Singh and Baruah (Goswami *et al.* 1983, Reddy *et al.* 1982). Depending on the organism and the hydrocarbon, uptake may take place through either one or a combination of the above mechanisms.

In a system with two liquid phases, the addition of extra surfactant can have two consequences. Firstly, it may improve dispersion and pseudosolubilization of the NAPLs, as was shown by Breuil & Kushner (1980) who showed that C_{16} and C_{18} fatty acids, lipids, and the synthetic surfactants Triton X-100, FL-70 and Brij 35 stimulated growth of *Acinetobacter lwoffi* and of *Pseudomonas aeruginosa* on hexadecane. Secondly, the presence of surfactants at the liquid-liquid interface may inhibit the bacterial attachment to this interface, thereby reducing the degradation rate. This was first shown by Aiba *et al.* (1969) for the degradation of dodecane and tetradecane in the presence of Tween 20 and for hexadecane by Mimura *et al.* (1971). In a recent study, Efroymson & Alexander (1994) reported that the presence of Triton X-100 in a shaking culture completely inhibited the growth of an *Arthrobacter* sp. on hexadecane, solubilized in the inert NAPL heptamethylnonane. They showed that in the presence of the surfactant the number of bacteria in the aqueous phase was higher and concluded that the surfactant prevented the attachment to the liquid-liquid interface and thereby inhibited the hydrocarbon degradation. In contrast, Triton X-100 stimulated growth of the same organism on naphthalene. In conclusion it can be said that the best results of the application of surfactants can be expected for NAPL pollutants that are not degraded via direct uptake of hydrocarbons from the liquid-liquid interface.

Solid pollutant

Direct uptake via attachment of bacteria to solid hydrocarbon surfaces is possible, as described by Goswami *et al.* (1983) for growth on sterols, by Zilber *et al.* (1980) for the growth of a marine pseudomonad on *n*-tetracosane, and by Guerin & Jones (1988) for the growth of a *Mycobacterium* sp. on phenanthrene. More often, however, no bacterial attachment on the surface of solid substrate is found (Cameotra *et al.* 1983, Chakravarty *et al.* 1972, Stucki & Alexander 1987, Thomas *et al.* 1986, Volkering *et al.* 1992, Wodzinski & Bertolini 1972, Wodzinski & Coyle 1974). If the bacteria are not attached to the solid surface, the substrate has to be dissolved before it can be taken up. The effects of dissolution of solid substrate on bacterial growth kinetics have been described by Chakravarty *et al.* (1972) and Volkering *et al.* (1992, 1993). The first authors presented a mathematical model in which the biodegradation of *n*-eicosane by a *Pseudomonas* sp. was limited by the dissolution rate of the substrate. In this model, the authors assumed that growing cells produce a metabolite which helps the solubilization of the substrate. Unfortunately, a synthetic emulsifier was used in the experiments performed to

validate the model, making the interpretation of the data very difficult. In a later study of the same group (Cameotra *et al.* 1983) evidence is presented that the uptake of *n*-docosane by the yeast *Endomycopsis lipolytica* YM and the uptake of *n*-tetradocosane by *Pseudomonas* PG-1 occurred primarily through pseudosolubilization by microbial excretion products. In our studies (Volkering *et al.* 1992,1993) we found that the degradation of naphthalene by a *Pseudomonas* sp. was limited by the substrate dissolution rate and that no solubility-enhancing excretion products were involved.

The effect of surfactant addition on the biodegradation of solid hydrocarbons has been described in a limited number of articles. In contrast to liquid phase pollutants, no dispersion can occur, excluding the first mechanism of bioavailability enhancement. When bacterial attachment occurs, the effect of surfactant can be the same as described above for liquid hydrocarbon, i.e. inhibition of biodegradation by inhibition of attachment. This has been reported by Goswami *et al.* (1983), who found that the synthetic alkylphenol polyglycol ether HYOXYD AAO prevented attachment of *Arthrobacter* 317 to β-sitosterol and strongly inhibited the degradation of the sterol. In contrast, other synthetic surfactants such as Tween 40, Tween 80, and Unisperse-P had a slightly increasing effect on the growth on β-sitosterol.

When the substrate is used in the dissolved state only, surfactant can enhance the bioavailability in two ways. Firstly, the dissolution rate can be increased by partitioning of the hydrocarbon into micelles. We inferred earlier (Volkering *et al.* 1993, 1995) that this effect is of little importance in the dissolution-limited growth phase (bioavailability-limited phase), as the aqueous hydrocarbon concentration will be very low in this phase. This is confirmed by experiments with octadecane, performed by Zhang & Miller (1992), in which it was found that the addition of 300 $mg.L^{-1}$ of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* ATCC 9027 increased the solubility of the alkane by a factor 10^4, whereas the octadecane mineralization rate by the same organism was only increased by a factor 4. Secondly, the surfactant can affect the dissolution process by interaction with the surface of the substrate, as suggested by Gerson (1993). A possible mechanism for this interaction could be the formation of micelle-like structures at the solid surface (Nayyar *et al.* 1994). This will affect the maximal dissolution rate and may increase microbial growth in the dissolution-limited phase.

It was found by Tiehm (1994) that different synthetic nonionic surfactants stimulated the growth of mixed cultures on several polycyclic aromatic hydrocarbons, but from the data presented it cannot be concluded whether this stimulation was

caused by growth on micellar substrate, pseudosolubilized during the first period of the experiment, or by an increased maximal dissolution rate. Similar results were found by Hunt *et al.* (1993) for the biodegradation of phenanthrene in the presence of a rhamnolipid biosurfactant.

Soil-sorbed pollutant

Most studies on the biodegradation of sorbed compounds have been performed with artificially contaminated soils. The majority of these studies revealed that microorganisms can only take up dissolved substrate. There are, however, a few publications in which it was found that sorbed compounds are to some extent available for microorganisms without prior desorption (Gordon & Millero 1985, Guerin & Boyd 1992, Hermannson & Marshall 1985). However, even when sorbed substrates were found to be directly degradable, they were less bioavailable than aqueous-phase substrates (Gordon & Millero 1985). Therefore desorption plays an essential role in the microbial growth on soil-sorbed substrate.

The use of surfactants in soil bioremediation leads to complex interactions between surfactant, soil, pollutant, and microorganisms. A simplified schematic overview of these interactions is shown in Figure 2. In reality the interactions will be more complex due to the heterogeneity of the soil.

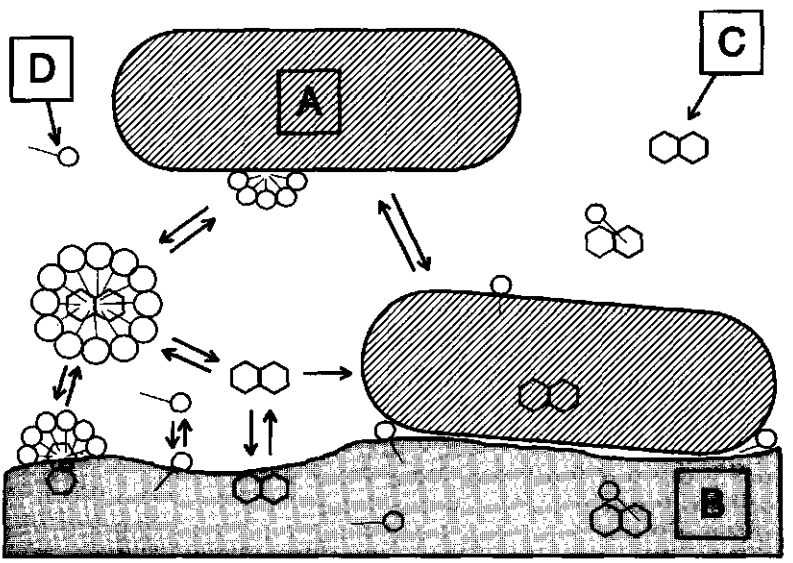

Figure 2: *Schematic overview of the interactions between microorganisms (A), soil (B), pollutant (C), and surfactants (D).*

The adsorption of surfactant molecules onto soil is dependent on the properties of both soil and surfactant (Edwards *et al.* 1991, 1992, 1994). Although surfactant adsorption is an unfavourable process for micellar solubilization (West & Harwell 1992), it may play an essential role in the facilitated transport of sorbed substrate.

The effect of surfactants on the equilibrium desorption or partitioning of HOCs between the soil and the aqueous phase, important in soil washing, has been studied by many researchers for both synthetic surfactants (e.g. Edwards *et al.* 1991, 1992, 1994, 1994a, Liu *et al.* 1992, 1992a, Park & Jaffé 1993, Sun & Boyd 1993, Vigon & Rubin 1989) and biosurfactants (Harvey *et al.* 1990, Scheibenbogen *et al.* 1994, Van Dyke *et al.* 1993, 1993a). Generally it can be said that sorbed surfactant may increase the sorbing capacity of the soil, whereas solute surfactant increases the aqueous solubility of HOCs. The net effect from these two mechanisms is dependant on the surfactant concentration. At concentrations well above the CMC, a decrease in the soil-water partition coefficient of the pollutant can be expected, especially for very hydrophobic pollutants. At surfactant concentrations below or near the CMC, the effect on the partitioning is dependant on soil and surfactant properties. It should be noted that the CMC of the surfactant is based on the surfactant concentration in the aqueous phase. This implies that the presence of a sorbing agent, such as soil, will lead to an increase in the apparent CMC (Edwards *et al.* 1994a). In contrast, cationic surfactants can be used to promote sorption of HOCs to more hydrophilic surfaces, even at high concentrations (Wagner *et al.* 1994).

For assessing the effect of surfactants on the mobilization of sorbed substrate, desorption kinetics are more important than equilibrium desorption data. Batch experiments can be used for studying desorption kinetics, but continuous desorption experiments ("leaching experiments") provide more useful data. Most of the studies published on this subject demonstrate that the desorption and mobilization of hydrophobic pollutants from soil columns is enhanced by the addition of surfactants (e.g. Abdul & Gibson 1991, Abriola *et al.* 1993, Dohse & Lion 1994, Pennell *et al.* 1993, 1994, Roy *et al.* 1994).

The effect of surfactants on the biodegradation of soil-sorbed HOCs has been the subject of several studies, of which a summary of the results is given in Table 1. Due to the complexity of the interactions described above, many different effects of surfactant in this system can be expected.

Laha & Luthy (1991, 1992) found for various surfactants that the presence of these compounds in concentrations above the CMC inhibited the mineralization of

phenanthrene by a mixed culture in a soil-water system. At concentrations below the CMC, no inhibition was observed and dilution of the solutions to a surfactant concentration below the CMC removed the inhibitory effect. The mineralization of glucose in mineral medium and in a soil-water system was not significantly affected by surfactant, and the use of glucose as a cosubstrate inhibited the degradation of phenanthrene only temporarily. The authors concluded that the inhibition was not caused by toxicity or by preferential degradation of the surfactant, but could not give an explanation for the inhibition. For polychlorinated biphenyls Viney & Bewley (1990) found that the nonionic surfactants Triton X-100 and Tensoxid S50 stimulated the equilibrium desorption from soil, but inhibited the degradation of all PCB congeners by several isolates. In two research articles published by the group of Alexander (Aronstein *et al.* 1991, Aronstein & Alexander 1992), the effect of the non-ionic surfactants, Alfonic 810-60 and Novel II 1422-56, in concentrations below the CMC on the desorption and mineralization of phenanthrene and biphenyl in batch systems was documented. Although the surfactants at these low concentrations did not result in an appreciable change in the partition of the hydrocarbons over the soil and the aqueous phase, the mineralization by both indigenous and introduced bacteria was markedly enhanced. The authors noted rightfully that the extent of desorption is of less importance than the desorption rate, but presented no experiments that could explain the observed effect. Moreover, the procedure used to introduce the substrate into the soil will lead to the formation of hydrocarbon crystals. Thus, not only desorption, but also dissolution will occur. Another study by the same group (Aronstein & Alexander 1993) concerned the biodegradation of phenanthrene and biphenyl in soil columns through which solutions with low concentrations of the surfactant Novel II 1412-56 were pumped. Mineralization of both hydrocarbons was stimulated by the presence of the surfactant, but since no desorption experiments with the soil columns under sterile conditions were performed, it is not possible to draw conclusions on the mechanism of the stimulation. A promising aspect of the study was the fact that the presence of surfactant did not cause an increase in the effluent concentrations of the two hydrocarbons and their degradation products.

Oily pollutants in soil will often partially occur as NAPLs. Because of their emulsifying properties, surfactants are thought to be especially effective in enhancing the biodegradation of this type of pollution. Although Rittmann & Johnson (1989) did not perform desorption experiments, they found the dispersant Corexit 7664 to have no effect on the biodegradation of lubricating oil in soil in microcosm and reactor experiments. Testing synthetic nonionic and anionic surfactants in microcosms,

Rasiah & Voroney (1993) found that 2 out of 6 surfactants (1 nonionic and 1 anionic) were significantly effective in enhancing biodegradation of oil from a polluted soil. The best results were obtained with the only biodegradable surfactant tested. In column experiments with sandy soil Ducreux *et al.* (1993) used a nonionic surfactant to enhance oil biodegradation. It was found that a 0.05% (w/v) surfactant solution increased the removal of oil by 50% when compared to experiments without surfactant.

The effect of biologically produced surfactants on the degradation of sorbed hydrocarbons has also been investigated. In bioreactor experiments, performed by Oberbremer *et al.* (1990), with a 10% soil slurry amended with 1.35% of a hydrocarbon mixture, microbiologically produced glycolipids promoted the onset and increased the extent of biodegradation. Using a surface active compound produced by *Pseudomonas aeruginosa* UG2, Jain *et al.* (1992) showed that, at the highest concentration used (100 $\mu g \cdot g^{-1}$ soil), the surfactant significantly enhanced the disappearance of tetradecane, pristane, and hexadecane, but not of 2-methylnaphthalene. Addition of the surfactant-producing bacteria at densities up to $10^8 \times g^{-1}$ soil had no effect on the hydrocarbon disappearance. Since the hydrocarbons used were not radioactively labelled, and since no measure for bacterial growth was presented, it is not certain whether the disappearance of the hydrocarbons was caused by biodegradation or by some undesired effect of the surfactant on the extraction procedure. Dohse & Lion (1994) studied the desorption and mineralization of phenanthrene sorbed onto a low-carbon sand in the presence of several different microbial polymers. Of the 28 polymers tested, 24 decreased the soil-water partition coefficient of phenanthrene. One polymer was used for column experiments in which the breakthrough of phenanthrene was investigated and it was found that the mobility of phenanthrene was increased by the polymer. In mineralization experiments, however, no significant effect of the polymer on the biodegradation of sorbed phenanthrene was found.

Table 1: *Literature overview on the effect of surfactants on biodegradation of hydrophobic organic compounds added to soil.*

Authors	Rittman & Johnson 1989	Oberbremer *et al.* 1990	Viney & Bewley 1990	Aronstein *et al.* 1991, Aronstein *et al.* 1992, Aronstein & Alexander 1993	Laha & Luthy 1991 Laha & Luthy 1992
Soil type	silty clay loam	unknown	sand	sand (0.4% o.c.), silt loam (7.6% o.c.), muck (32.9% o.c.)	silt loam (1.5% o. c.)
Contamination	lubricating oil	mixture of alkanes and aromatics	polychlorinated biphenyls	phenanthrene, biphenyl	phenanthrene
Microorganisms	mixed culture	mixed culture	several isolates and pure strains	mixed culture	mixed culture
Setup	microcosm and slurry reactor	slurry reactor	microcosm	shaking soil slurries and column experiments	shaking soil slurries
Surfactant type	dispersant Corexit 7664	several biosurfactants	alkylphenol ethoxylates	alcohol ethoxylates	several nonionic
Surfactant concentration	5% of added nutrient solution (microcosm); 0.11 and 0.22% (reactors)	200 $mg.L^{-1}$	5 $g.L^{-1}$	sub-CMC	varying
Effect on desorption	N. D.	N.D.	stimulation	no effect	stimulation of partitioning
Effect on biodegradation	no effect	stimulation	inhibition	stimulation	inhibition at high surfactant conc.
Explanation	insufficient mixing	-	surfactant toxicity	-	- (no toxicity)

N.D.: not determined; o.c: organic carbon

***Table 1** (continued).*

Authors	Jain *et al.* 1992	Ducreux *et al.* *1993*	Rasiah & Voroney 1993	Dohse & Lion 1994	Providenti *et al.* 1995
Soil type	silt loam (2.1% o.c.)	silica sand	clay loam (1.2% o.c.)	sand (0.05% o.c.)	sandy loam
Contamination	oily mixture of alkanes and methylnaphthalene	diesel oil	oil refinery waste	phenanthrene	phenanthrene, creosote
Microorganisms	*Pseudomonas aeruginosa*	mixed culture	indigeneous population	mixed culture	*Pseudomonas aeruginosa*
Setup	microcosms	columns	microcosms	shaking soil slurries	shaking soil slurries
Surfactant type	biosurfactant	nonionic	several nonionic and anionic	microbial polymers	rhamnolipid biosurfactants
Surfactant concentration	25 or 100 $\mu g.g^{-1}$ soil	0.05% w/w	0.35 $\mu l.g^{-1}$ soil	30 or 100 mg $TOC.L^{-1}$	100-400 $mg.L^{-1}$
Effect on desorption	N.D.	stimulation	N.D.	stimulation	N.D.
Effect on biodegradation	stimulation for alkanes, no effect for methylnaphthalene	stimulation	stimulation (best result with degradable surfactant)	no effect	mixed
Explanation	-	-	oil emulsification, surfactant used as cosubstrate	-	-

N.D.: not determined; o.c.: organic carbon

Studies with polluted soil

Little detailed information is available on the use of surfactants in field studies; an overview is presented in Table 2. In a full scale *in situ* biological treatment of a former gas-work site, Bewley *et al.* (1989) used surfactants to enhance the bioavailability of the pollutants. Although the performance of the process was satisfactory, no blank experiments without surfactants were performed. The type of surfactant was not reported. In a study by Ellis *et al.* (1990) it was found that the degradation of heavy petroleum oil in a soil from an oil refinery in a microcosm was increased from 79% to 95% by the addition of the biodegradable surface-active agent Cyanamer P70. It was also reported that *in situ* biodegradation of petroleum hydrocarbons was stimulated using the same surfactant (Balba *et al.* 1993). Marks *et al.* (1992) used the nonionic surfactants Triton N-100 and Triton X-100 as additives in continuous reactor experiments with PAH-contaminated sludges. The oleophilic fertilizer Inipol EAP 22, a combination of surfactant and fertilizer, was used on a large scale to stimulate biological clean-up of shorelines contaminated with oil originating from the Exxon Valdez oil spill (Glaser 1991). Compared to that of other fertilizers, the addition of Inipol EAP 22 was more successful in stimulating the oil degradation. It was not investigated whether this stimulation was caused by a more efficient nutrient addition or by enhanced bioavailability of the oil due to the surface-active properties of Inipol EAP 22. Recent work by Churchill *et al.* (1995), however, showed that Inipol EAP 22 also stimulated hydrocarbon degradation under conditions without N or P limitation and the authors suggested that the positive effect of the fertilizer on the biodegradation of the oil spilled by the Exxon Valdez may have been significantly caused by the surface-active properties. In a recent study by Belkin *et al.* (1994) it was reported that the use of an unknown surfactant changed the course of the toxicity of the leachate from columns filled with PAH-polluted soil in which biodegradation occurred. At the beginning of the treatment a toxicity increase was observed, but at the end of the experiment the toxicity of the surfactant-amended soil was lower than the control soil without surfactant.

Table 2: *Literature overview on the effect of surfactants on the remediation of contaminated soil and sludge.*

Authors	Bewley *et al.* 1989	Ellis *et al.* 1990, Balba *et al.* 1993	Ellis *et al.* 1991	Marks *et al.* 1992
Soil type	complex	unknown	sandy clay, clay loam	refining sludge and petrochemical sludge
Contamination	coal tar (PAHs)	refinery oil	creosote	PAHs
Microorganisms	mixed culture	strains isolated from indigenous population	*Pseudomonas sp.*	mixed culture
Setup	*in situ* treatment and landfarming	microcosms, *in situ* treatment and landfarming	microcosms, columns	continuous slurry reactors
Surfactant type	unknown	Cyanamer P70	Ethylan CD 916	Triton N-100, Triton X-100
Surfactant concentration	unknown	unknown	0.5% w/v (desorption); 200 $\mu g.g^{-1}$ (biodegradation)	1 $g.L^{-1}$
Effect on desorption	N.D.	stimulation	stimulation of solubilization and leaching	possible stimulation of partitioning
Effect on biodegradation	not clear, possible stimulation	stimulation in microcosms, possible stimulation in full scale treatment	possible stimulation	not clear, possible stimulation
Explanation	no blanks, several parameters changed	no blanks, several parameters changed	two parameters changed	no blanks, several parameters changed

N.D.: not determined

Selection of surfactants

For successful application of surface-active compounds in bioremediation, the choice of the surfactant is of crucial importance. Of all the aspects mentioned earlier in this review, the surfactant toxicity is the only one that is always unfavorable. The other aspects can have both positive and negative effects on the clean-up of soil, depending on the external conditions. These conditions can be different for every case and include the following factors:

- type and physicochemical state of the pollutant (NAPL, solid, sorbed);
- type and physicochemical state of the soil (pH, presence of oxygen, moisture content, presence of nutrients, permeability, etc.);
- type and state of the degrading microorganisms.

It is important to have at least a global impression of these factors before deciding to apply surfactants in a biological soil treatment process. There are several parameters to characterize the surfactants, the most important of which are listed below:

- *HLB number or hydrophile lipophile balance.* An empirical parameter that describes the relative contribution of the hydrophilic group. HLB numbers are supplied by most of the surfactant manufacturers. Surfactants with an HLB number of 3-6 are lipophilic and can be used to form water in oil emulsions, whereas surfactants with an HLB number in the range of 10 to 18 are more hydrophilic and can be used to form oil in water emulsions. The surfactants that are most successful in the washing of oil-contaminated soils usually have an HLB higher than 10. The relation between HLB and solubilizing properties of alkyl ethoxylates has been studied by Diallo *et al.* (1994).
- *reduction of the surface tension.* Surface tension reduction has been identified as the most important parameter for the effectiveness of surfactants in oil recovery (Hill *et al.* 1973).
- *reduction of the interfacial tension.* When a pollutant is present as a NAPL, its mobilization is largely determined by the interfacial tension. The interfacial tension reduction can be evaluated by making a Winsor diagram, which is based on the emulsification of the pollutant (West & Harwell 1992)
- *solubilization efficiency.* This can be expressed as the volume of oil solubilized per unit volume of surfactant (Barakat and Gracia 1983). When all other parameters are equal, the surfactant with the highest solubilization efficiency will give the best results in soil washing (Vigon & Rubin 1989).

Although these parameters have been used in several publications, there is no general rule on which parameter is the best selection criterium. As it was inferred that facilitated transport in soil is the most important effect of surfactants in bioremediation, the surface or interfacial tension reduction are probably the best parameters for selecting surfactants for biological soil remediation, especially when the pollutant is present as or in an NAPL. Besides this it is advisable to perform simple standardized laboratory experiments in which the effect of the surfactant on the desorption and biodegradation of the pollutant is determined.

Future prospects

Reviewing the results of the studies involving the effect of surfactants on hydrocarbon degradation in soil, it can be stated that many different surfactants have been tested with many different types of soil and pollutants. However, although surfactants have been found to stimulate the degradation of hydrophobic pollutant, no general trends can be found using these results. Therefore more laboratory research using simple model systems is necessary. It is likely that the insights to be obtained from such research can be used to understand the more complicated processes in field situations.

Since the application of surfactants is one of the few realistic technological solutions for the problem of limited pollutant bioavailability, the use of surfactants as a tool in the biological clean-up of soil has promising possibilities. This is especially true for *in situ* techniques. Although the effect of surfactants in landfarming may be limited by the lack of mixing, application in intensive landfarming could be used to enhance the clean-up process. The application of surfactants in aerobic bioreactors may cause unwanted foaming and seems therefore unfavorable (Oberbremer & Müller-Hurtig 1989).

Both from an economic and scientific point of view the best perspectives are to be expected when surfactants are used at low concentrations, with the possible exception of pollutants present as a liquid phase. Dispersion of this type of pollutants is an effective way of increasing its bioavailability, but may require high surfactant concentrations.

Biosurfactants have the advantage over those chemically produced in that they are socially more acceptable and can be expected to be less toxic. As it has been shown that biologically produced surfactants may be specifically effective for the

growth substrate of the surfactant-producing microorganism (Falatko & Novak 1992), the stimulation of the biosurfactant production by the pollutant-degrading organisms is a promising option. This is likely to be cost-effective and excludes possible interference with the natural interactions among the degrading population and the pollutant.

In conclusion, much more knowledge can be said to be required before the application of surfactants can be used as a standard technique in soil remediation. Criteria will have to be developed for making decisions to use surfactants. These criteria may include simple desorption and/or biodegradation experiments. To make all of this possible more insight is needed. Therefore laboratory studies, field studies, and especially *in situ* studies on the use of surfactants with proper blank experiments are essential.

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CHAPTER 6

INFLUENCE OF NONIONIC SURFACTANTS ON BIOAVAILABILITY AND BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

Abstract

The presence of the synthetic nonionic surfactants Triton X-100, Tergitol NPX, Brij 35, and Igepal CA-720 resulted not only in increased apparent solubilities, but also in increased maximal dissolution rates of crystalline naphthalene and phenanthrene. A model, based on the assumption that surfactant micelles are formed, which act as a separate phase, underestimated the dissolution rates; this led to the conclusion that surfactants present at concentrations higher than the critical micelle concentration affect the dissolution process. This conclusion was confirmed by the results of batch growth experiments, which showed that the rates of biodegradation of naphthalene and phenanthrene in the dissolution-limited growth phase were increased by the addition of surfactant, indicating that the dissolution rates were higher than in the absence of surfactant. In activity and growth experiments, no toxic effects of the surfactants at concentrations up to 10 $g.L^{-1}$ were observed. Substrate present in the micellar phase was shown not to be readily available for degradation by the microorganisms. This finding has important consequences for the application of (bio)surfactants in biological soil remediation.

This chapter has been published as:
Volkering F., A.M. Breure, J.G. van Andel, and W.H. Rulkens (1995) Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 61:1699-1705.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are poorly soluble, hydrophobic organic compounds (HOCs) which have been released into the environment on a large scale. This fact, along with their toxicity and mutagenicity, makes these compounds priority pollutants. Although laboratory studies have revealed that virtually all PAHs are biodegradable (Cerniglia 1992), the rates of PAH degradation at contaminated sites are often much lower than the rates expected on the basis of the results of laboratory studies. It is generally accepted that a low level of bioavailability (i.e. slow release of the pollutant from solid phases to the aqueous phase) is one of the most important factors involved in the slow biodegradation of HOCs in soil (Mihelcic *et al.* 1993).

A possible way of enhancing the bioavailability of HOCs is the application of (bio)surfactants, molecules which consist of a hydrophilic part and a hydrophobic part. Because of this property these molecules tend to concentrate at surfaces and interfaces, and to decrease levels of surface tension and interfacial tension. Another important characteristic of surfactants is the fact that above a certain concentration (the critical micelle concentration or CMC) aggregates of 10 to 200 molecules, which are called micelles, are formed. The effect of a surfactant on the bioavailability of organic compounds can be explained by three main mechanisms: (i) dispersion of nonaqueous-phase liquid hydrocarbons, leading to an increase in contact area, which is caused by a reduction in the interfacial tension between the aqueous and the nonaqueous phase; (ii) increased solubility of the pollutant, caused by the presence of micelles which may contain high concentrations of HOCs, a mechanism which has been studied extensively previously (Edwards *et al.* 1991, 1992, Liu *et al.* 1992); and (iii) "facilitated transport" of the pollutant from the solid phase to the aqueous phase, which can be caused by a number of phenomena, such as lowering of the surface tension of the pore water in soil particles, interaction of the surfactant with solid interfaces, and interaction of the pollutant with single surfactant molecules.

The first mechanism is involved only when liquid-phase hydrocarbon is present and is not discussed further in this paper. Both of the other mechanisms can cause an increase in the rate of mass transfer to the aqueous phase, and therefore the relative contributions of these two mechanisms to the enhancement of bioavailability are often unknown. When biodegradation is limited by the bioavailability of the substrate, the substrate concentration in the bulk liquid is much lower than the

saturation concentration. Because of this, we inferred previously (Volkering *et al.* 1993) that for bioremediation processes the contribution of solubility enhancement is probably small. This means that facilitated transport is the most important mechanism for enhancement of biodegradation in these processes.

Many studies on the use of (bio)surfactants for soil remediation have dealt either with the effect of surfactants on desorption and biodegradation of organic contaminants sorbed onto soil (Aronstein *et al.* 1991, 1992, 1993, Laha & Luthy 1991, 1992) or with the practical problems of application of surfactants to soil (Abdul *et al.* 1990, Van Dyke *et al.* 1993, Vigon & Rubin 1989). The results of these studies are often difficult to interpret because of the complexity of the interactions among soil, pollutant, surfactant, and microorganisms.

In this study, we investigated the effects of four nonionic surfactants on the bioavailability and the rates of biodegradation of crystalline naphthalene and phenanthrene. Crystalline PAHs can occur in contaminated soil, but the main reason for choosing this system is its relative simplicity. Previous research has given us a good insight in the biodegradation kinetics of crystalline PAHs (Volkering *et al.* 1992, 1993), and therefore the effects of the presence of surfactants on the bioavailability and biodegradation can be interpreted unambiguously. Insight into these effects can be used to gain a better understanding of the more complicated processes involved in the biodegradation of sorbed substrates in the presence of surfactants.

Materials and methods

Bacterial cultures

The isolation of strain 8909N growing on naphthalene has been described previously (Volkering *et al.* 1992). Strain 8803F was isolated in cultures containing phenanthrene in the same way. Both of these strains are gram-negative *Pseudomonas* strains.

Media and culture conditions

Organisms were grown at 30°C in a mineral medium that was essentially the medium described by Evans *et al.* (1970) except that the concentrations of the medium components were one-tenth of those described by Evans *et al.* This medium contained 10 mM NH_4Cl, 1 mM NaH_2PO_4, 1 mM KCl, 0.2 mM Na_2SO_4, 0.125 mM $MgCl_2$, 2 µM $CaCl_2$, 0.01 µM Na_2MoO_4, and 0.5 ml of a spore solution per liter; the spore solution contained 0.12 mM HCl, 5 mM ZnO, 20 mM $FeCl_3$, 10 mM $MnCl_2$, 1mM $CuCl_2$, 2 mM $CoCl_2$, and 0.8 mM H_3BO_3. No chelating agent was used. The medium was buffered at pH 7.0 with 50 mM sodium phosphate. Pure cultures were maintained on mineral medium agar slants containing 1.5%

agar and the PAH required at a concentration of 0.1% (w/v) and were stored at 4°C. To obtain washed cells, the PAH crystals were removed from an exponentially growing batch culture by decanting, after which the broth was centrifuged for 15 min. at 12,000 *g* and washed twice with 50 mM potassium phosphate buffer (pH 7).

Dissolution experiments

The dissolution experiments were performed in 250-ml serum flasks on a rotary shaker (200 rpm) at 30°C. The experiments were started by adding 0.50 g of naphthalene (particle diameter 1000-2400 µm) or 0.15 g of phenanthrene (particle diameter 600-1000 µm) to flasks containing 150 ml of sterile buffered mineral medium and the appropriate amount of surfactant. At regular intervals samples (0.75 ml) were filtered with a regenerated cellulose filter (pore size 0.2 µm, Schleicher & Schuell, Dassel, Germany) and diluted 1:1 with acetonitrile to determine the PAH concentration in the liquid phase.

Surfactant toxicity and biodegradability

The effects of surfactants on the maximal growth rate of strain 8909N on succinate and naphthalene were determined in batch growth experiments by measuring the CO_2 concentration in the headspace gas. For experiments with succinate, 100 ml of buffered mineral medium supplemented with 2 $g \cdot L^{-1}$ sodium succinate and the appropriate amount of surfactant was added to a 250-ml serum flask, which was then inoculated with 1 ml of a succinate-grown culture of strain 8909N. For experiments with naphthalene, 100 ml of buffered mineral medium supplemented with the appropriate surfactant concentration was saturated overnight with crystalline naphthalene. The crystals were removed by decanting before the preparation was inoculated with 1 ml of naphthalene-grown strain 8909N cells. The maximal growth rate was determined as the slope of the linear part of the growth curve plotted on a log scale. Experiments with strain 8803F with succinate and phenanthrene as the substrates were performed at a surfactant concentration of 1 $g \cdot L^{-1}$ only. To test for growth on surfactant, CO_2 production in batches containing surfactant at a concentration of 1 $g \cdot L^{-1}$ was monitored after inoculation with strain 8909N or 8803F cells.

Acute surfactant toxicity was determined in oxygen uptake experiments. Oxygen uptake experiments performed with succinate and succinate-grown cells were started by adding 0.1 ml of a 0.1 M sodium succinate solution to 4.9 ml of a 50 mM potassium phosphate buffer solution containing the appropriate surfactant concentration and 0.1 ml of cell suspension. Oxygen uptake experiments with naphthalene were started by adding 0.1 ml of a suspension of naphthalene-grown strain 8909N cells to 4.9 ml of naphthalene-saturated mineral medium containing the appropriate surfactant concentration. Oxygen uptake was monitored, and the activity of the cells was measured as the slope of the initial oxygen uptake rate.

Biodegradation of crystalline PAHs

Biodegradation experiments with naphthalene were performed in 500-ml serum flasks on a rotary shaker (150 or 200 rpm). Each flask contained 100 or 150 ml of mineral medium supplemented with 0.3 g of naphthalene crystals. The headspace gas of each flask was replaced with oxygen to eliminate the possibility of oxygen limitation. The experiments were started by inoculating each flask with 2 or 5 ml of active naphthalene-grown strain 8909N cells. The culture contents were pumped continuously through a flowthrough cuvette placed in a Perkin-Elmer lambda 15 spectrophotometer and the optical density at 540 nm was determined every 6 min. The appropriate amount of surfactant was added when the dissolution-limited phase was reached. In blank experiments, which were designed to test for growth on the surfactant, the PAH crystals were removed from the cultures before the surfactant was added. The effects of surfactants on the biodegradation of phenanthrene by strain 8803F were determined by measuring the CO_2 production in the headspace gas of 250-ml serum flasks containing 100 ml of mineral medium and 0.06 g of phenanthrene crystals after inoculation with 5 ml of a suspension of active phenanthrene-grown cells. Surfactant was added when the dissolution-limited growth phase was reached. At different times after the surfactant was added liquid samples were removed to determine the overall PAH concentration.

Bioavailability of micellar substrate

Oxygen uptake experiments with naphthalene were performed with freshly harvested active naphthalene-grown cells. The experiments were started by adding 0.1 ml of the cell suspension to 5.0 ml of an air-saturated 50 mM potassium phosphate buffer solution (pH 7.0) containing no surfactant or 5.0 g surfactant per liter and different naphthalene concentrations, and the oxygen uptake was monitored. The initial uptake rate was calculated from the slope of the decrease in oxygen concentration that occured after the cells were added and was corrected for endogeneous cell respiration. The initial oxygen uptake rates were fitted with Michaelis-Menten kinetics by using the nonlinear fitting program Enzfitter (Biosoft, Cambridge, United Kingdom).

Analytical procedures

Bacterial growth was determined by measuring the optical density at 540 nm (OD540). Levels of dissolved PAHs were determined by injecting samples into a HPLC (series HP 1050; Hewlett Packard GmbH, Waldbronn, FRG) equipped with a ChromSpher C_{18} (PAH) column (Chrompack, Middelburg, The Netherlands). The eluens used was a mixture of acetonitrile and water (85:15). Peaks were detected with a UV detector by measuring the A_{275} (absorbance at 275 nm) for naphthalene and the A_{254} for phenanthrene. The CO_2 contents of the headspace gases in serum flasks were determined with a Hewlett Packard type 5890 gas chromatograph equipped with a thermal conductivity detector and a Hayesep

Q packed stainless steel column (diameter, 0.125 in. [3.175 mm]; length, 2 m; Chrompack). Helium was used as the carrier gas and the flow rate was 30 ml·min.$^{-1}$. The injector temperature was 150°C, the oven temperature was 80°C, and the detector temperature was 200°C. The injection volume was 250 µl with splitless injection. The dissolved oxygen contents were determined with a biological oxygen monitor as described by Dubinsky *et al.* (1987). The viscosity of a solution was determined with a Ubelohde viscosimeter by measuring the time needed for the fluid to pass through a capillary tube at 30°C.

Chemicals

The following surfactants were used without any purification: the octylphenol ethoxylate ethers Triton X-100 (density at 20°C, 1,065 g·L^{-1}) and Igepal CA-720 (density at 20°C, 1,104 g·L^{-1}), the nonylphenol ethoxylate ether Tergitol NPX (density at 20°C, 1,060 g·L^{-1}), and the dodecanol ethoxylate ether Brij 35 (solid at 20°C). All other chemicals were of analytical grade.

Modeling

Dissolution kinetics in the presence of surfactants were described with a model based on the model for dissolution that has been described previously (Volkering *et al.* 1993). The model used to describe the effects of surfactants was formulated on the basis of the following assumptions:

1. at surfactant concentrations above the CMC, the surfactants form micelles;
2. nonmicellar surfactant does not affect the solubility of PAHs;
3. at equilibrium, the concentration of PAHs in the micelles is a linear function of the PAH concentration in the water phase;
4. the limitation of mass transfer from the water phase to the micellar phase occurs in the water phase; and
5. the micelles act as a separate phase and only increase the apparent solubility of the PAHs.

Assumptions 1 through 3 are generally accepted (Edwards *et al.* 1991), and the fourth assumption is based on the small diameter of the micelles. Assumption 5 is the central issue of the model. This assumption needs to be verified by comparing calculated values with experimental data. A model based on these assumptions was constructed (Equations 1 to 5). Equation 1 gives the mass balance of the PAH over the three phases:

$$-\frac{dQ_t}{dt} = \frac{dC_t}{dt} \cdot V + \frac{dC_{mic,t}}{dt} \cdot V_{mic} \qquad (1)$$

where Q_t is the amount of solid PAH [kg], C_t the aqueous PAH concentration [kg.m^{-3}], V the

aqueous volume [m^3], $C_{mic,t}$ the micellar PAH concentration [$kg \cdot m^{-3}$], and V_{mic} the micellar volume [m^3].
Equation 2 describes the mass transfer from the crystals to the aqueous phase (dissolution):

$$-\frac{dQ_t}{dt} = k_{l,s} \cdot A_t \cdot (C_{max} - C_t) \quad (2)$$

where $k_{l,s}$ is the liquid-solid mass transfer coefficient [$m \cdot h^{-1}$], A_t the surface area of crystals [m^2], and C_{max} the maximal aqueous PAH concentration [$kg \cdot m^{-3}$].
As the mass transfer limitation for transport from the aqueous phase to the micellar phase occurs in the aqueous phase (assumption 4), the mass transfer from the aqueous phase to the micelles can be described with Equation 3:

$$\frac{dC_{mic,t}}{dt} = \frac{k_{l,mic} \cdot A_{mic}}{V_{mic}} \cdot (C_t - \frac{C_{mic,t}}{m}) \quad (3)$$

Where $k_{l,mic}$ is the liquid-micelle mass transfer coefficient [$m \cdot h^{-1}$],A_{mic} the surface area of the micelles [m^2], and m the water-micelle partition coefficient, which is defined as the ratio of the concentration in the micellar phase and the concentration in the aqueous phase when the system is in equilibrium [-]. Combining these equations yields Equation 4:

$$\frac{dC_t}{dt} = \frac{k_{l,c} \cdot A_t}{V} \cdot (C_{max} - C_t) - \frac{k_{l,mic} \cdot A_{mic}}{V} \cdot (C_t - \frac{C_{mic,t}}{m}) \quad (4)$$

The total PAH concentration (C_{tot} [$kg \cdot m^{-3}$])in the solution can be calculated with Equation 5:

$$C_{tot} = \frac{C_t \cdot V + C_{mic,t} \cdot V_{mic}}{V + V_{mic}} \quad (5)$$

The micelle diameter was assumed to be $3 \cdot 10^{-9}$ m (Bury & Miller 1993). Because of the small micelle diameter, the water-micelle interfacial area was much larger than the water-crystal interfacial area; for example, at a surfactant concentration of 1 $g \cdot L^{-1}$, the water-micelle area was 218 m^2, whereas in the dissolution experiments the water-crystal area was approximately $1.38 \cdot 10^{-3}$ m^2 for naphthalene and $9.84 \cdot 10^{-4}$ m^2 for phenanthrene. This means that the mass transport from the aqueous phase to the micellar phase was much faster than the mass transport from the crystals to the water phase. Thus, the partitioning of the PAH molecules over the aqueous phase and the micellar phase occurs almost instantaneously compared with the dissolution process. The computer program PSI/c (BOZA automatisering, Pijnacker, The Netherlands) was used for the modeling study.

Results

Dissolution experiments

The dissolution kinetics of naphthalene crystals in the presence of different concentrations of Tergitol NPX are shown in Fig. 1. As expected, the presence of surfactant at concentrations below the CMC had no effect on the dissolution of naphthalene, whereas the presence of micelles resulted in higher apparent levels of solubility. In Fig. 2 the maximal level of apparent naphthalene solubility is plotted as a function of the surfactant concentration. From the slope of the linear part of the graph it is possible to calculate the micelle-water partition coefficient *m*. This kind of experiment was performed with naphthalene and phenanthrene for the four surfactants. For comparison, partition was also expressed as the molar water partition coefficient ($K_{m,w}$), which was calculated as described by Edwards *et al.* (1991). The values for *m* and log $K_{m,w}$ resulting from the dissolution experiments are shown in Table 1.

Table 1: *Water-micelle partition coefficients m and log $K_{m,w}$ values of the surfactants for naphthalene and phenanthrene.*

Surfactant	Naphthalene		Phenanthrene	
	m	log $K_{m,w}$[a]	*m*	log $K_{m,w}$[a]
Triton X-100	$1.83 \cdot 10^3$	4.68	$3.43 \cdot 10^4$	6.02
Tergitol NPX	$2.19 \cdot 10^3$	4.79	$4.12 \cdot 10^4$	6.20
Igepal CA-720	$1.35 \cdot 10^3$	4.64	$2.86 \cdot 10^4$	5.97
Brij 35	$9.0 \cdot 10^2$	4.48	$2.02 \cdot 10^4$	5.83

[a]: $K_{m,w}$ was calculated as descibed by Edwards *et al.* (1991).

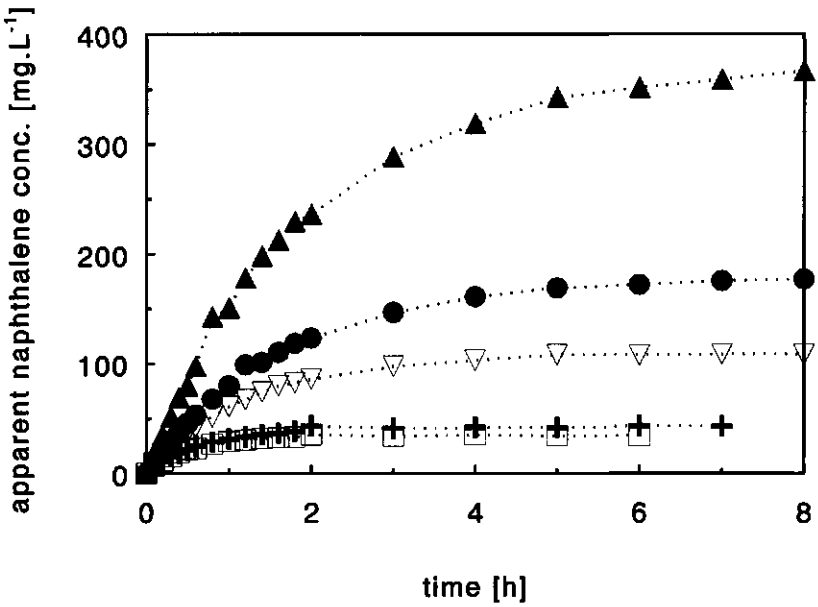

Figure 1: *Effects of different concentrations of Tergitol NPX on the dissolution kinetics of naphthalene. The preparations contained no surfactant (□) or surfactant at a concentration of 0.1 g·L⁻¹ (+), 1.0 g·L⁻¹ (▽), 2.0 g·L⁻¹ (●), or 5.0 g·L⁻¹ (▲).*

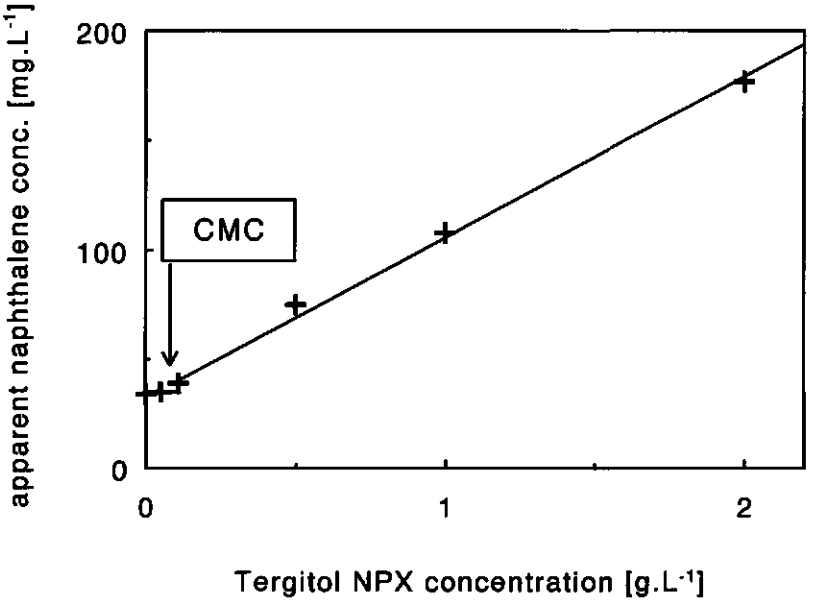

Figure 2: *Maximum solubility of naphthalene as a function of the Tergitol NPX concentration.*

Surfactant toxicity and biodegradability

Growth experiments performed with the four surfactants at concentrations of 1 $g \cdot L^{-1}$ revealed no inhibition of CO_2 production by strain 8909N when the organism was grown on succinate or naphthalene or by strain 8803F when the organism was grown on succinate or phenanthrene. No extra CO_2 production due to growth on surfactant was observed, and no growth occurred when 1 $g \cdot L^{-1}$ surfactant was used as the sole substrate. The possible toxic effects of Tergitol NPX and Triton X-100 on strain 8909N were investigated in more detail. Oxygen uptake and batch growth experiments with succinate and naphthalene were performed with surfactants present at concentrations up to 10 $g \cdot L^{-1}$. These experiments showed that the maximal growth rate and the oxygen uptake rate of resting cells on both substrates were not affected by the presence of surfactant. Moreover, no effect on the overall bacterial growth yield was observed.

Biodegradation of PAHs

Batchwise bacterial growth on crystalline PAH results in a typical growth curve: exponential growth, followed by a linear growth phase in which the dissolution rate of the PAH is the limiting factor (Volkering *et al.* 1992). To investigate the effects of surfactants on the biodegradation of PAHs, surfactants were added at different concentrations to batch cultures that were in the linear growth phase.

Figure 3 shows the results of the experiments in which naphthalene was used with the surfactants Triton X-100 and Tergitol NPX. In separate experiments in which naphthalene crystals were removed before any surfactant was added, no bacterial growth was observed after surfactant addition, and therefore the increase in the linear growth rate was not caused by the organisms using the surfactant as a source of carbon and energy.

Figure 4 shows the results of similar experiments performed with strain 8803F and phenanthrene as the substrate. The PAH concentration in solution in the dissolution-limited growth phase ranged from 20 to 50 $\mu g \cdot L^{-1}$ for both naphthalene and phenanthrene. These results are in good agreement with the low aqueous PAH concentrations measured previously in biodegradation experiments (Volkering *et al.* 1992). As some of the PAH is present in the micellar phase, the aqueous PAH concentration is even lower. At the end of the experiment, when crystals were no longer visible and the CO_2 concentration remained constant, no difference in the CO_2 levels in the cultures with and without surfactant was detected, indicating that no surfactant biodegradation had occurred.

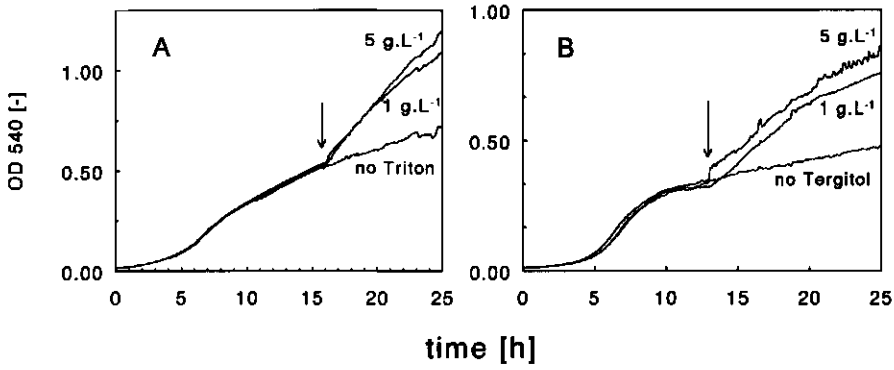

Figure 3: *Effects of adding Triton X-100 (A) and Tergitol NPX (B) on the growth of strain 8909N on crystalline naphthalene. The surfactant concentrations used are indicated in the figure; the arrows indicate the moment when surfactant was added.*

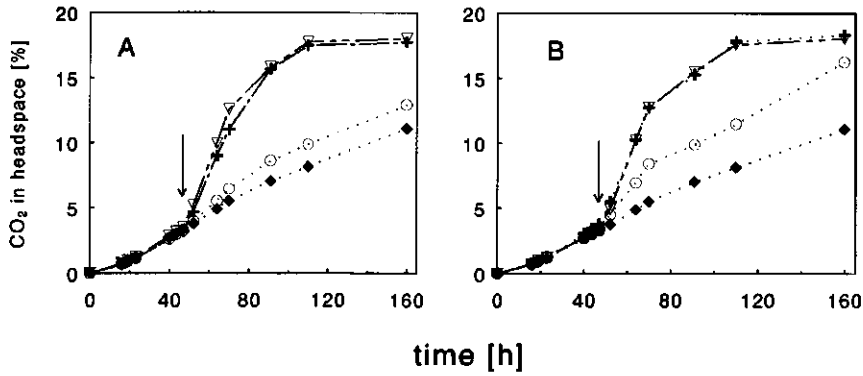

Figure 4: *Effects of adding Triton X-100 (A) and Tergitol NPX (B) on CO_2 production by strain 8803F growing on crystalline phenanthrene. Symbols: - -◆- -: no surfactant, - -O- - : 0.1 g·L^{-1},- -✚- -: 1 g·L^{-1}, - -▽- -: 5 g·L^{-1} surfactant. The arrows indicate the moment when surfactant was added.*

Bioavailability of micellar PAHs

The first step in the biodegradation of PAHs is an oxidation step; therefore, the initial oxygen uptake rate of washed cells can be used as a measure of bacterial activity on PAHs. Because of the short time needed for the measurements, the possibility that growth occurred can be eliminated, and Michaelis-Menten kinetics can

be used to describe the relationship between activity and substrate concentration (Wilkinson & Harrison 1973). Figure 5 shows the results of oxygen uptake experiments performed with strain 8909N and naphtalene with and without Triton X-100 and Tergitol NPX. The values for K_m, the Michaelis-Menten affinity constant, obtained by fitting the data with Michaelis-Menten kinetics are also shown. The K_m found in this way can be used as a measure of bacterial activity, but has no physiological background and therefore can not be used as a characteristic for microorganisms.

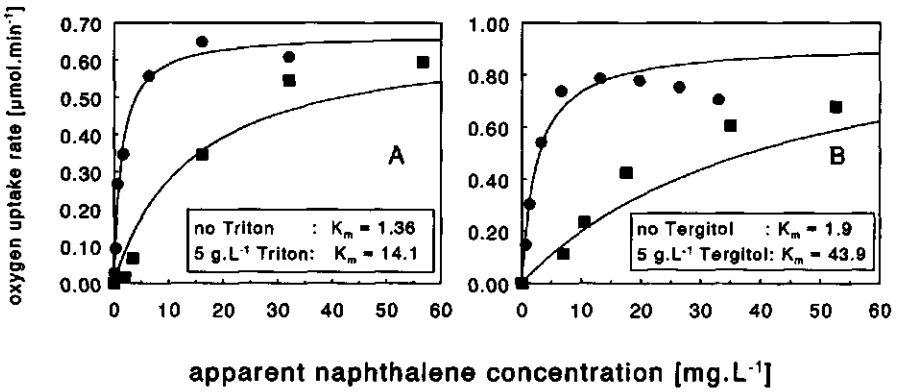

Figure 5: *Oxygen uptake rate of strain 8909N on naphthalene as a function of the apparent napthalene concentration with and without surfactants Triton X-100 (A) or Tergitol NPX (B). Symbols ●: no surfactant, ■: 5 g·L⁻¹ surfactant. The curves represent fitted Michaelis-Menten kinetics.*

Discussion

Effect of surfactants on dissolution kinetics

The increase in the level of apparent solubility due to the presence of surfactant micelles which we observed is consistent with the results of Edwards *et al.* (1991),

who obtained slightly lower values for log $K_{m,w}$, probably because of their use of 1% methanol in the aqueous phase. Although the water-micelle partition coefficient is a useful parameter, it provides no information about dissolution kinetics. Therefore, a dissolution model was constructed in which it was assumed that the increase in the apparent level of solubility was the only effect of the micelles. As an example, the results of modeling for dissolution of naphthalene when Tergitol NPX was the surfactant are shown in Fig. 6. The factor $k_{l,s} \cdot A_t$, a measure of the maximum dissolution rate, was calculated from the experiment performed without surfactant as described previously (Volkering *et al.* 1992); the same value was used for modeling the experiments with surfactant (Fig. 6 and Table 2).

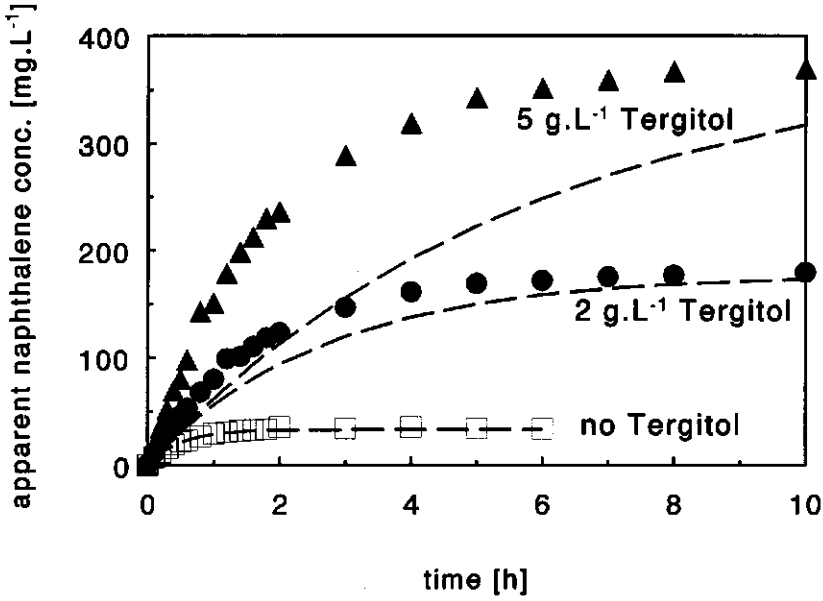

Figure 6: *Modeling of dissolution kinetics in the presence of different concentrations of Tergitol NPX. The symbols represent measured data, dashed lines represent model predictions.*

The model predictions for dissolution kinetics clearly underestimate the dissolution rates of PAHs at surfactant concentrations higher than the CMC. By increasing the factor $k_{l,s} \cdot A_t$, however, it was possible to obtain a good description of the dissolution kinetics. The maximal dissolution rates ($J_{max} = k_l \cdot A_t \cdot C_{max} / V$ [kg·m^{-3}·h^{-1}]), found by increasing the $k_{l,s} \cdot A_t$ values for the different surfactants, are given in Table 2.

Table 2: *Maximum dissolution rates (J_{max}) calculated from dissolution experimental data obtained with crystalline naphthalene and phenanthrene in the presence of different concentrations of nonionic surfactants.*

Surfactant concentration [g·L^{-1}]	J_{max} naphthalene [g·L^{-1}·h^{-1}]				J_{max} phenanthrene [g·L^{-1}·h^{-1}]			
	Triton X-100	Tergitol NPX	Igepal CA 720	Brij 35	Triton X-100	Tergitol NPX	Igepal CA 720	Brij 35
0	0.013	0.013	0.013	0.012	0.001	0.001	0.001	0.001
0.1	0.014	0.013	0.014	0.011	-	-	-	-
0.5	0.019	0.015	0.017	0.013	0.010	0.010	0.009	0.008
1	0.025	0.016	0.022	0.015	0.017	0.016	0.017	0.013
2	0.029	0.019	0.026	0.017	0.031	0.027	0.028	0.022
5	0.035	0.034	0.037	0.024	0.068	0.060	0.072	0.047

-: not determined

The effects of the surfactants were more pronounced for phenanthrene than for naphthalene; the reason for this difference is not clear. The high values for $k_{l,s}$·A_t cannot be explained by decreases in viscosity, as viscosity measurements revealed that the viscosity of a surfactant solution increased slightly as the surfactant concentration increased; this is consistent with the observations of Vigon & Rubin (1989). Therefore, the presence of micelles affects the dissolution rate, and the assumption that the micelles only act as a separate phase is not valid. Two possible mechanisms for the effect of surfactant micelles on the dissolution rate can be put forward. In the first mechanism the micelles are present not only in the aqueous bulk phase, but also in the stagnant layer surrounding the PAH crystals. This should decrease the PAH concentration in the layer and thus increase the $k_{l,s}$ value. More likely, however, is the possibility that a hemicellular layer of surfactant molecules is formed around the crystals. This allows a dynamic exchange of the micellar PAH to occur. The latter mechanism is very similar to the interactions of surfactant molecules with soil interfaces (Nayyar *et al.* 1994) and has also been proposed by Gerson (1993), although Gerson presented no evidence. On the basis of the results of the experiments described above, no distinction between the two mechanisms can be made.

Surfactant toxicity

It is well known that surfactants can be toxic to bacteria. Although nonionic surfactants are generally less toxic than ionic surfactants and although gram-negative bacteria are generally less sensitive than gram-positive bacteria (Swisher 1987), it seemed likely that the presence of surfactants at the high concentrations used in this study (up to 10 $g \cdot L^{-1}$) could have a negative effect on the bacterial strains used. However, in all of the growth and activity experiments that were performed, no toxic effect of the surfactants on the microorganisms was found.

Biodegradation experiments

Addition of surfactant to batch cultures growing on crystalline naphthalene or phenanthrene during the dissolution-limited phase results in an increase in the linear growth rate. The effect is more pronounced for phenanthrene than for naphthalene. This effect cannot be caused by an increase in the apparent PAH solubility since the aqueous PAH concentration in the dissolution-limited phase is virtually zero. This implies that the maximal dissolution rate increases when surfactant is present (facilitated transport). These results confirm the increase in the dissolution rate which we observed in the dissolution experiments. However, the increase in the growth rate was less than the increase in the maximal dissolution rate found in the dissolution experiments. This can be explained by the fact that there was less PAH present in the biodegradation experiments than in the dissolution experiments. An additional explanation involves sorption of the surfactant onto the bacteria, a common phenomenon (Swisher 1987) which results in lower aqueous surfactant concentrations.

When surfactant was added at the beginning of batch growth experiments with naphthalene, the exponential growth phase was longer, but the maximal growth rate on naphthalene did not increase. In contrast, Bury and Miller (1993) observed higher maximal growth rates on *n*-decane and *n*-tetradecane in the presence of surfactants than in the absence of surfactants. The surfactants which these authors used, however, were degraded along with the alkanes, and the increase could have been caused by direct bacterial uptake of micelles filled with alkanes.

Bioavailability of micellar PAHs

In several studies micellar solubilization has been used to enhance the availability of poorly soluble substrates (Bury & Miller 1993, Guerin & Jones 1988, Thiem 1994), and the role of solubilization by microbial excretion products in the growth on alkanes

has been studied extensively (Reddy *et al.* 1982). The high exit rates of micellar substrate allow exponential growth at high cell densities. It should be noted that this is no evidence that the substrate in the micelles is readily available to the microorganisms. The oxygen uptake rate of strain 8909N on naphthalene was affected by the presence of surfactants, as shown in Fig. 5 for Triton X-100 and Tergitol NPX. This effect cannot be attributed to toxicity of the surfactants. One explanation for this is the possibility that the PAHs were partitioned between the water phase and the micellar phase. Figures 5 and 7 show the results of the same experiments, but in Fig. 7 the *x*-axis shows the aqueous naphthalene concentration which was calculated from the total naphthalene concentration with the partition coefficients in Table 1. The K_m values obtained by fitting these results with Michaelis-Menten kinetics are also shown in Figures 5 and 7. From the corresponding lines and values for K_m, it is clear that the naphthalene concentration in the water phase controls the bacterial activity. More evidence for this was obtained from growth experiments performed with strain 8909N cells, which were not adapted to high concentrations of naphthalene. We observed that in batch growth experiments with naphthalene (data not shown) inoculation with active cells originating from batch cultures containing low naphthalene concentrations (linear growth phase) resulted in a lag phase of 10-24 h, whereas after inoculation with active cells originating from cultures containing high naphthalene concentrations (exponential growth phase) there was no lag phase. This indicates that high concentrations of naphthalene can be toxic to the bacteria and that the bacteria can adapt to these high concentrations. This is not an uncommon phenomenon for gram-negative bacteria (Weber *et al.* 1993). Growth experiments performed with naphthalene and unadapted strain 8809N cells showed that in cultures to which 1 to 5 $g \cdot L^{-1}$ Triton X-100 was added before inoculation no lag phase occurred. This illustrates that the toxicity of naphthalene is reduced when it is present in micelles. This is a good explanation for the fact that adaptation times are shorter in the presence of biosurfactants than in the absence of biosurfactants, as described by Oberbremer *et al.* (1990).

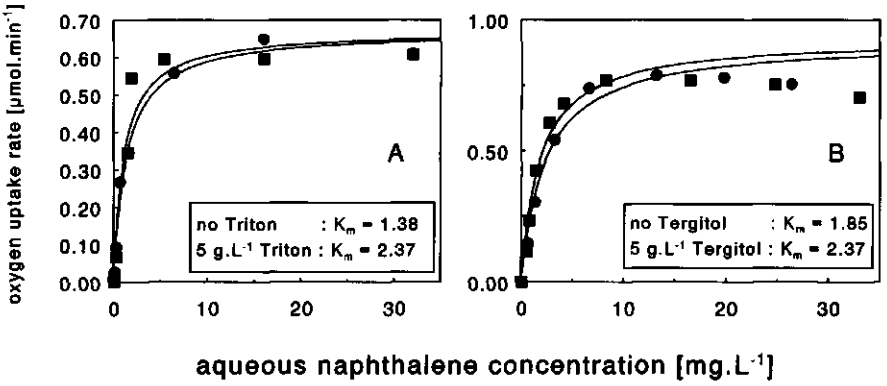

Figure 7: *Oxygen uptake rate of strain 8909N on naphthalene as a function of the aqueous napthalene concentration with and without the surfactants Triton X-100 (A) or Tergitol NPX (B) Symbols: ●: no surfactant, ■: 5 g·L⁻¹ surfactant. The curves represent fitted Michaelis-Menten kinetics.*

The most important conclusion that can be drawn from the data described above is that PAHs in the micellar phase are not readily available to microorganisms. Thus, micellar PAH is a protected reservoir that may replenish aqueous-phase PAH when it is depleted by biodegradation. This has been stated previously by Zhang and Miller (1992), but the data these authors presented do not justify their conclusion, as they compared equilibrium solubility with growth kinetics. The fact that micellar substrate is not readily bioavailable has important consequences for the application of surfactants in bioremediation. First, the presence of micelles may lower the concentration of contaminant in the water phase, thereby reducing the bacterial activity or growth. This was seen in the oxygen uptake experiments whose results are shown in Fig. 5. At the same total naphthalene concentration, the activity of cells is reduced by the presence of surfactant. This effect, combined with the toxicity of the surfactants, could explain the inhibition of phenanthrene mineralization by micellar surfactant, as described by Laha and Luthy (1991, 1992). Second, in *in situ* processes, the nonavailable substrate may be washed out and thus cause unwanted contamination of groundwater. For the reasons described above, careful study is needed before the use of surfactants in biological soil treatment can be recommended.

Nomenclature

A_{mic}	micellar surface area [m^2]
A_t	crystal surface area [m^2]
C_{max}	maximum aqueous PAH concentration [$kg \cdot m^{-3}$]
$C_{mic,t}$	micellar PAH concentration [$kg \cdot m^{-3}$]
C_t	aqueous PAH concentration [$kg \cdot m^{-3}$]
C_{tot}	overall PAH concentration in solution [$kg \cdot m^{-3}$]
J_{max}	maximum dissolution rate [$kg \cdot m^{-3} \cdot h^{-1}$]
$k_{l,mic}$	liquid-micelle mass transfer coefficient [$m \cdot h^{-1}$]
$k_{l,s}$	liquid-solid mass transfer coefficient [$m \cdot h^{-1}$]
K_m	Michaelis-Menten affinity constant [$mg \cdot L^{-1}$]
$K_{m,w}$	molar water partition coefficient [-]
m	water-micelle partition coefficient [-]
Q_t	amount of solid PAH [kg]
t	time [h]
V	aqueous volume [m^3]
V_{mic}	micellar volume [m^3]

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CHAPTER 7

DESORPTION AND BIODEGRADATION OF SORBED NAPHTHALENE IN THE PRESENCE OF NONIONIC SURFACTANTS

Abstract

The effect of the nonionic surfactants Triton X-100 and Brij 35 on the desorption and biodegradation of the polycyclic aromatic hydrocarbon naphthalene sorbed onto two well defined synthetic matrices was investigated. In batch desorption experiments it was found that the presence of surfactant lowered the solid-liquid partition coefficient, even at concentrations lower than the critical micelle concentration. The effect of surfactant on desorption kinetics was investigated in continuous desorption experiments. At concentrations below and above the critical micelle concentration, the presence of surfactant was found to increase the desorption rates. Therefore micellar solubilization was concluded to be not the only mechanism by which the surfactants stimulate desorption. Biodegradation of sorbed naphthalene was stimulated when surfactant was added in the desorption-limited phase, supporting the results found in the desorption experiments. When desorption and biodegradation were compared, it was found that the extent of the stimulation by surfactant was less for biodegradation than for desorption. Moreover it was observed that biodegradation proceeded faster than could be explained by desorption alone.

This chapter has been submitted for publication in Environmental Science & Technology as "Desorption and biodegradation of sorbed naphthalene in the presence of nonionic surfactants" by the authors F. Volkering, A.M. Breure, J.G. van Andel, and W.H. Rulkens.

Introduction

Hydrophobic organic pollutants in soils usually occur attached to the soil matrix. Some researchers have found that microorganisms can take up sorbed substrates directly (Guerin & Boyd 1992, Hermannson & Marshall 1985), but in most studies it has been demonstrated that only aqueous phase substrate is directly available for biodegradation (e.g. Mihelcic & Luthy 1991, Robinson *et al.* 1990, Scow & Alexander 1992). This implies that sorbed pollutant has to be desorbed before it can be degraded. The slow desorption of hydrophobic organic compounds (HOCs) is the main reason for the slow biodegradation of these compounds in the biological clean-up of soil (Mihelcic *et al.* 1993, Providenti *et al.* 1994). This phenomenon is called limited bioavailability.

The use of surfactants to enhance the mobility of the pollutant is one possible way of solving the problem of limited bioavailability. Surfactants are molecules that usually consist of a hydrophobic and a hydrophilic part. Because of this they tend to concentrate at surfaces and interfaces. The presence of surfactants lowers the surface tension of aqueous solutions and the interfacial tension at liquid-liquid and solid-liquid interfaces. At concentrations higher than the critical micelle concentration (CMC), surfactant molecules form small aggregates, micelles, which increase the apparent aqueous solubility of HOCs (solubilization). Micellar solubilization may enhance the desorption of sorbed pollutants as was found in most of the literature describing studies on the effect of surfactants on the desorption of HOCs from soil (e.g. Edwards *et al.* 1992, Liu *et al.* 1992, Van Dyke *et al.*1993). The presence of surfactants may also cause facilitated transport of the sorbed pollutant. This term covers several processes by which the desorption of pollutants is stimulated, such as mobilization of pollutants trapped in soil ganglia (Vigon & Rubin 1989), interaction of pollutants with single surfactants molecules (Edwards *et al.* 1992), and solubilization in admicelles, micelle-like structures that are formed at solid surfaces (Nayyar *et al.* 1994).

Surfactants may also decrease the bioavailability of HOCs by promoting sorption of the pollutant onto soil (Wagner *et al.* 1994), by interference with the natural interactions among microorganisms and pollutants (Efroymson & Alexander 1994), or by clogging of soil pores (Abdul *et al.* 1990). Other negative effects of applying surfactants in soil bioremediation may be caused by surfactant toxicity or by preferential degradation of the surfactant.

Studying the effect of surfactants on the biodegradation of HOCs in soil is complex due to the interactions among soil, bacteria, pollutant, and surfactant. This complexity is reflected in the different results found in the studies on the effect of surfactants on the biodegradation of HOCs sorbed onto soil. Some authors found that surfactants had no effect on the desorption, but that they stimulated bacterial mineralization (Aronstein *et al.* 1991, Aronstein & Alexander 1992). Others found that while the desorption was stimulated, the biodegradation was not affected (Dohse & Lion 1994) or even inhibited (Laha & Luthy 1991, 1992). In all these studies there were too many unknown variables to draw general conclusions.Therefore we have chosen to study the effect of surfactants on desorption and biodegradation in a system that is better defined than soil. The polycyclic aromatic hydrocarbon (PAH) naphthalene, used as model HOC, was sorbed onto inert, synthetic matrices. The two nonionic surfactants selected, Triton X-100 and Brij 35, were used in previous research concerning the interactions among naphthalene, surfactants, and the naphthalene-degrading bacterial strain (Volkering *et al.* 1995). This setup thus allows us to assess the action of the surfactants on the desorption of naphthalene, and the effect of this action on the biodegradation of naphthalene.

Materials and methods

Bacterial cultures

The isolation of strain 8909N, growing on naphthalene, has been described previously (Volkering *et al.* 1992). The organism, a gram-negative *Pseudomonas* species, has also been used in previous studies on the biodegradation of naphthalene (Volkering *et al.* 1992, 1993, 1995).

Media and culture conditions

Organisms were grown at 30°C in mineral medium, with essentially the same composition as described by Evans *et al.* (1970), with the medium components being one-tenth of those described. The medium consisted of 10 mM NH_4Cl, 1 mM NaH_2PO_4, 1 mM KCl, 0.2 mM Na_2SO_4, 0.125 mM $MgCl_2$, 2 µM $CaCl_2$, 0.01 µM Na_2MoO_4 and 0.5 ml·L^{-1} of a spore solution containing 0.12 mM HCl, 5 mM ZnO, 20 mM $FeCl_3$, 10 mM $MnCl_2$, 1mM $CuCl_2$, 2 mM $CoCl_2$ and 0.8 mM H_3BO_3. No chelating agent was added. The medium was buffered at pH 7.0 with 50 mM sodium phosphate. Pure cultures were maintained on mineral medium agar slants containing 1.5% agar and 0.1% (w/v) of naphthalene (storage at 4°C).

Loading of naphthalene onto the matrices

The matrices used in this study were the resins Amberlite XAD-4 and XAD-7 (Supleco, Bellefonte, USA). Some physical properties of XAD-4 and XAD-7 are shown in Table 1; these resins have been designed for the adsorption of organic compounds from aqueous solutions. Before loading, the resins were washed with de-ionized water to remove contaminations. This was repeated until the UV spectrum of the washing water, after being in contact with the resin for 24 h, showed no difference with the spectrum of clean de-ionized water. The wet resins were stored at 4°C in a closed glass jar to prevent dehydration. To load the matrices, 20-30 g of the material was transferred into a 500-ml serum bottle with 250 ml of de-ionized water. After pasteurization at 80°C for 3 h, a closed tubular dialysis membrane (cutoff 1000 Dalton), filled with 15 g of naphthalene crystals and 25 ml of water was added. Thereupon the bottle was closed with a screwcap with teflon lining and was incubated horizontally in a rotary shaker (90 rpm) at 30°C for one month. After this period the bottle was opened, the membrane with the naphthalene crystals was removed, and the bottle was closed and incubated for one more day. Next, a sample of the aqueous phase was taken for measurement of the naphthalene concentration, and the water was decanted. The material was dried for 5 min on filtration paper and transferrred into a glass jar. Nitrogen gas was led through the jar to remove oxygen, after which the jar was closed and stored at 4°C. A small amount of the freshly loaded material was dried to determine the dry weight; another small amount was extracted to determine the initial naphthalene loading. After a period of three months the loading of the resins was again determined.

Table 1: *Physical properties of the resins used.*

resin	chemical nature	porosity [vol. %]	density [$kg \cdot m^{-3}$]	surface area [$m^2 \cdot g^{-1}$]	Av. pore diameter [Å]
XAD-4	polyaromatic	45	1080	725	40
XAD-7	acrylic ester	55	1240	450	90

Adsorption of surfactant

To assess the adsorption of surfactant onto the matrices, 0.05 to 0.5 g of unloaded matrix was added to 250-ml serum flasks containing 100 ml of a solution of 0.1 to 5 $g \cdot L^{-1}$ Triton X-100 in mineral medium. The flasks were placed on a rotary shaker (200 rpm) at 30°C. After 3 to 5 days the aqueous surfactant concentration was determined by measuring the absorbance at 254 nm (A_{254}) with a spectrophotometer (Perkin Elmer Lambda 15, USA). In some experiments the adsorption kinetics were monitored by continuously pumping a small part of the liquid phase through the spectrophotometer.

Batch desorption of naphthalene

Batch desorption experiments, performed in 250-ml serum flasks on a rotary shaker (200 rpm, 30°C), were started up by adding 0.05 to 0.5 g of loaded material to the flasks containing 150 ml of sterile buffered mineral medium and the appropriate amount of surfactant. At regular intervals samples of 0.75 ml were taken to determine the naphthalene concentration in the liquid phase. After approximately one week the liquid phase was decanted and the naphthalene concentration of both the liquid and the solid phases determined by HPLC analysis.

Continuous desorption of naphthalene

The continuous desorption leaching experiments were performed in a mixed system. Sterile mineral medium with the appropriate surfactant concentration was pumped into 35-ml serum bottles with a working volume of 12 to 15 ml that contained 0.1 (XAD-4) or 0.2 g (XAD-7) of naphthalene-loaded resin. Sintered glass filters were fixed into the outlet of the bottles to protect the resins from washing out. The bottles were placed in a rotary shaker (230 rpm) at 30°C. The pump flow, set at ± 30 $ml \cdot h^{-1}$, was measured at the beginning and at the end of the experiment. The little change in flow that was observed was considered to be linear with time. The naphthalene concentration in the leachates were determined by measuring the A_{275} every 12 min with a spectrophotometer, using flow-through cuvettes. At regular intervals samples of the leachates were taken to determine the naphthalene concentration with HPLC. The leachates were collected in 5-L jars containing 25 ml of a 5 M NaOH solution to prevent biodegradation. To make mass balances, the final volume of the leachates, the final naphthalene concentration in the jars, and the residual naphthalene loading of the matrices were determined at the end of the experiment.

Biodegradation of naphthalene

Biodegradation experiments were performed at 30°C in closed 250-ml serum flasks on a rotary shaker (150 or 200 rpm). The flasks contained 100 ml of mineral medium with the appropriate amount of surfactant and were supplied with 0.25 g of loaded matrix. To eliminate the possibility of oxygen limitation, the headspace of the flasks was filled with oxygen. The experiments were started by inoculation with 1 ml of active batch-grown strain 8909N cells. The biodegradation of naphthalene was monitored by measuring the percentage of CO_2 in the headspace gas of the bottle. As the pH shift over the experiment was negligible, this percentage could be used as a measure for the total amount of CO_2 produced and thus as a measure of biodegradation. At the end of the experiment 1 ml of 12 M HCl was added to the flasks to remove the dissolved CO_2, and the CO_2 concentration in the headspace gas was measured. The residual naphthalene concentration of the liquid and the solid phase was determined.

Analytical procedures

Aqueous naphthalene concentrations were determined by injection of filtered (0.2 µm rotrand filter, Schleicher & Schuell, Germany), with acetonitrile diluted (1:1) samples on an HPLC with a ChromSpher C18 (PAH) column (Chrompack, Middelburg, The Netherlands). The eluent used was a mixture of acetonitrile and water (85:15). Peaks were detected with a UV detector by measuring the A_{275}. Extraction samples were similarly measured, but diluted to a naphthalene concentration lower than 100 mg·L^{-1}. Extraction of naphthalene from the matrix was performed by adding 50 or 100 ml of acetonitrile to the matrix, shaking for one week and measuring the naphthalene concentration in the acetonitrile. For extraction of highly loaded matrices (determination of starting concentrations), this procedure was repeated once more. The dry weight of the matrix was determined by drying a known amount of wet material at 80°C until the weight was constant. The CO_2 in the headspace gas of serum flasks was determined using a gas chromatograph (Hewlett Packard type 5890) equipped with a thermal conductivity detector and a Hayesep Q packed stainless steel column (diameter 1/8 inch, length 2 m, Chrompack). Helium was used as carrier gas with a flow rate of 30 ml·min^{-1}. The injector temperature was 150°C, the oven temperature 80°C, and the detector temperature 200°C. The injection volume was 250 µl with splitless injection. The specific area of Triton X-100 was determined by measuring the surface tension of aqueous solutions with different surfactant concentrations, as described by Lyklema (1991).

Results

Loading of the resins with naphthalene

The loading method used made it possible to obtain high loadings without using hydrophobic solvents, which would interfere in the desorption experiments. The aqueous naphthalene concentrations, measured after the membranes were removed, were equal to the maximum concentration. This indicates that equilibrium was reached, and therefore that the materials were homogeneously loaded. The final naphthalene loadings of the matrices were 68 mg·g^{-1} for XAD-7, and 145 mg·g^{-1} for XAD-4. Extractions revealed that under anoxic conditions at 4°C these loadings remained stable for at least three months.

Adsorption of surfactant

To asses the effect of surfactant on the desorption of naphthalene, it is necessary to obtain insight into the sorption of the surfactant onto the matrices. The adsorption isotherms of Triton X-100 onto XAD-4 and XAD-7 are shown in Figure 1.

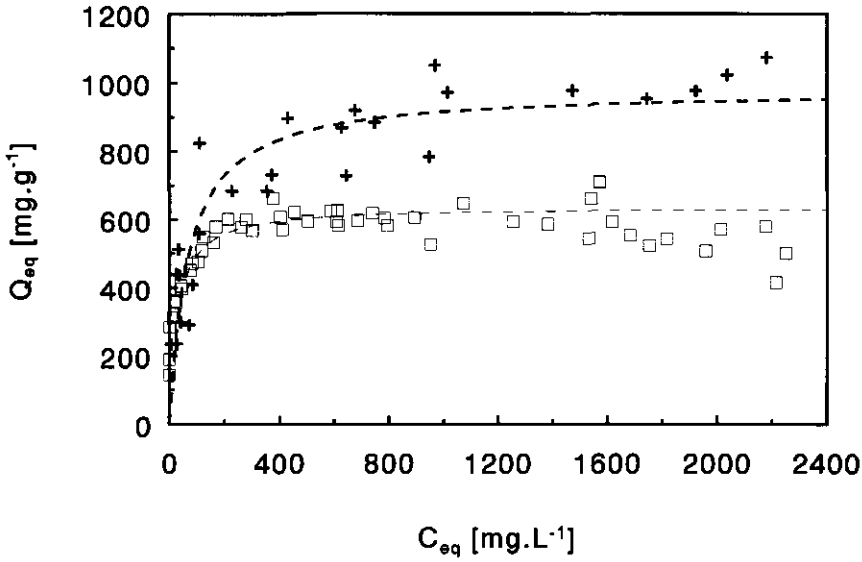

Figure 1: *Isotherms for adsorption of Triton X-100 onto XAD-7(□) and XAD-4 (✚); lines represent isotherms fitted using the Langmuir equation.*

The isotherms could be described according to the Langmuir equation as:

$$Q_{eq} = Q_{max} \cdot \frac{k_a \cdot C_{eq}}{1 + k_a \cdot C_{eq}} \qquad (1)$$

where Q_{eq} is the equilibrium sorbed surfactant concentration [mg·g^{-1}], Q_{max} is the maximal sorbed surfactant concentration [mg·g^{-1}], k_a is the Langmuir constant [L·mg^{-1}], and C_{eq} is the equilibrium surfactant concentration in solution [mg·L^{-1}]. The values for Q_{max} and k_a found for the two adsorbents are shown in Table 2.

Table 2: *Equilibrium data on the adsorption of Triton X-100 onto XAD-4 and XAD-7*

resin	Q_{max} [mg·g^{-1}]	k_a [L·mg^{-1}]	coverage [a] [%]
XAD-4	980	14.4	87
XAD-7	638	35.5	91

[a]: calculated using a specific area of $6.7 \cdot 10^{-19}$ m^2 for Triton X-100.

The adsorption of Triton X-100 onto XAD-7 was completely reversible, while adsorption onto XAD-4 showed adsorption-desorption hysteresis (data not shown).

Batch desorption of naphthalene

Batch desorption experiments may give information on both equilibrium concentrations and desorption kinetics. Figure 2 shows the desorption isotherms of naphthalene in the absence of surfactants for XAD-4 and XAD-7.

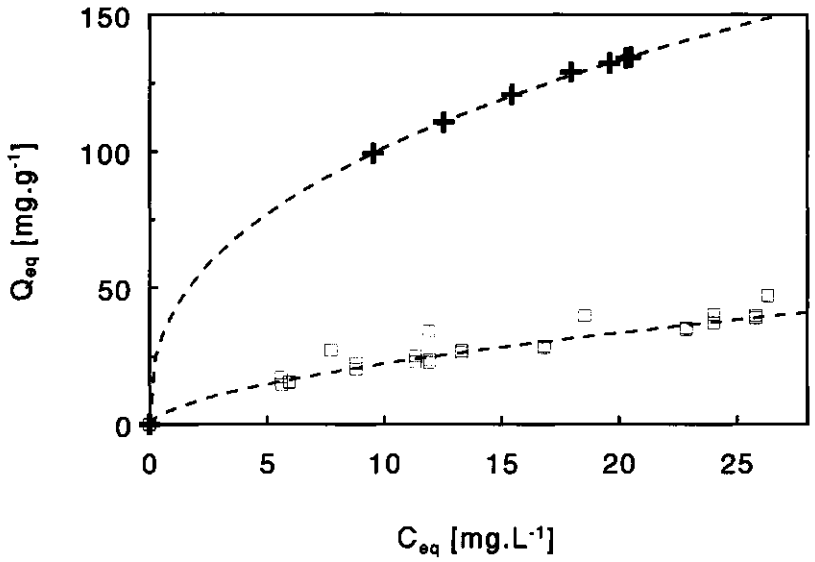

Figure 2: *Isotherms for desorption of naphthalene from XAD-7 (□) and XAD-4 (✚); lines represent fitted isotherms using the Freundlich equation.*

Since the loading of the resins did not approach maximum values at high aqueous naphthalene concentrations, these isotherms could not be described using the Langmuir equation. As can be seen in Figure 2 the data could be fitted using the Freundlich equation:

$$Q_{eq} = K_f \cdot (C_{eq})^{1/n} \qquad (2)$$

where K_f is the Freundlich sorption capacity constant [$mg^{1-1/n} \cdot L^{1/n} \cdot g^{-1}$] and n is the Freundlich sorption energy constant [-]. The values of K_f and n obtained by fitting the measured data are presented in Table 3.

Table 3: *Equilibrium data on the sorption of naphthalene onto the different resins.*

resin	K_f [$mg^{1-1/n} \cdot L^{1/n} \cdot g^{-1}$]	n [-]	Q_{max} [a] [$mg \cdot g^{-1}$]	coverage [b] [%]
XAD-4	39.5	0.40	145	87
XAD-7	5.11	0.63	68	89

[a]: *determined in loading experiments;*

[b]: *calculated using a specific area for naphthalene of* $4.29 \cdot 10^{-19}$ m^2 *(Radt, 1948).*

The effect of Triton X-100 on the desorption kinetics of naphthalene from XAD-4 is shown in Figure 3.

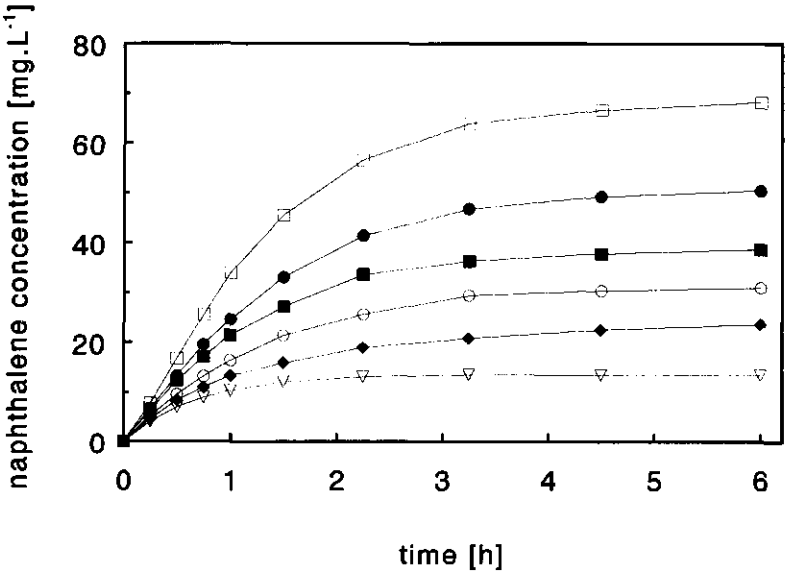

Figure 3: *Effect of Triton X-100 on the desorption of naphthalene from XAD-4; surfactant concentrations:* ▽: *0 g.L*$^{-1}$, ◆: *0.1 g.L*$^{-1}$, ○: *0.25 g.L*$^{-1}$, ■: *0.5 g.L*$^{-1}$, ●: *1.0 g.L*$^{-1}$, □: *2.0 g.L*$^{-1}$.

The presence of surfactant changes the partitioning of naphthalene over the solid and the liquid phase. Although the desorption proceeded faster at high surfactant concentrations, it was not possible to determine the effect of the surfactant on the initial desorption rate in these experiments. Using XAD-7, similar results were found, but equilibrium was reached quicker.

The effect of Triton X-100 on the partitioning of naphthalene over the liquid and the solid phase is shown in Table 4 for XAD-4 and XAD-7.

Table 4: *Effect of Triton X-100 on the partitioning of naphthalene over the solid and the liquid phase in batch desorption experiments with 0.1 g resin in 100 ml mineral medium.*

surfactant concentration [g·L^{-1}]	XAD-4			XAD-7		
	C_{eq} [mg·L^{-1}]	Q_{eq} [mg·g^{-1}]	Q_{eq}/C_{eq} [L·g^{-1}]	C_{eq} [mg·L^{-1}]	Q_{eq} [mg·g^{-1}]	Q_{eq}/C_{eq} [L·g^{-1}]
0	18.5	118	6.38	20.3	37.9	1.87
0.1	31	105	3.38	20.5	37.7	1.84
0.25	31.7	104	3.28	20.8	27.3	1.31
0.5	38.8	97	2.50	23.1	22.8	0.98
1.0	51.7	84	1.62	28.4	19.2	0.67
2.0	69	67	0.97	34.4	15.2	0.44

Continuous desorption of naphthalene

The batch desorption experiments did not provide enough distinct information on the kinetics of desorption to determine whether facilitated transport played a role in the effects the surfactant had on the desorption process. For this reason continuous desorption experiments in the presence of surfactant were performed. In the instrumental setup used for these experiments, the naphthalene concentration was determined by measuring the A_{275}. Since Triton X-100 also absorbs at this wavelength (due to the presence of an aromatic ring) the linear alkyl ethoxy ether Brij 35 was used as surfactant in the experiments in which the effluent concentration was continuously measured. Figure 4A represents the results of the naphthalene concentration measurements in the leachate for the experiments with XAD-7. These data can be used to calculate the percentage of desorbed naphthalene as shown in Figure 4B. The results show a relatively quick complete desorption and little effect of the use of the surfactant Brij 35, even at concentrations as high as 2 g·L^{-1}.

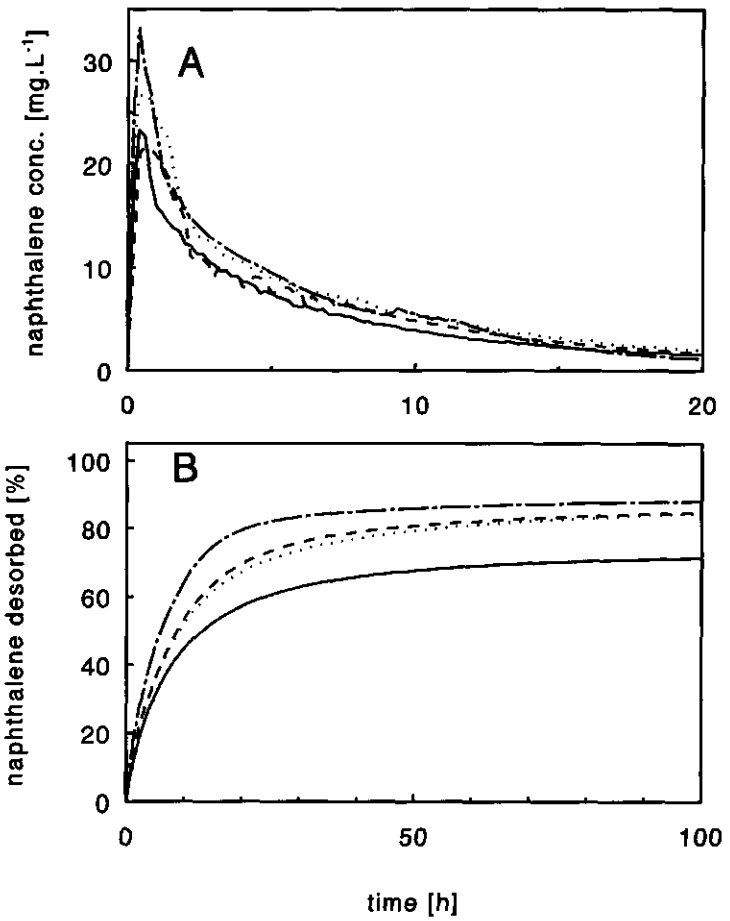

Figure 4: *Continuous desorption of naphthalene from XAD-7 in the presence of different concentrations of Brij 35. A: naphthalene concentration in the leachate, B: % naphthalene desorberd; surfactant concentrations: ——: 0 g·L^{-1}, - - -: 0.1 g·L^{-1}, ·····: 0.5 g·L^{-1}, - · -: 2.0 g·L^{-1}.*

A more pronounced effect was found for naphthalene sorbed onto XAD-4 as can be seen in the Figures 5A and 5B. Due to the formation of bubbles in the cuvette in the experiment with 0.1 g.L^{-1} Brij 35, the measurement was unreliable from 54 h onwards. It can be seen, however, that even at this sub-CMC concentration, the desorption of the sorbed naphthalene was enhanced markedly.

The above results are confirmed by the measurement of the residual loadings and the naphthalene concentrations in the leachate at the end of the experiments. These results and the mass balance (expressed as the % naphthalene recovered) over the continuous desorption experiments with XAD-4 and Brij 35 are presented in Table 5.

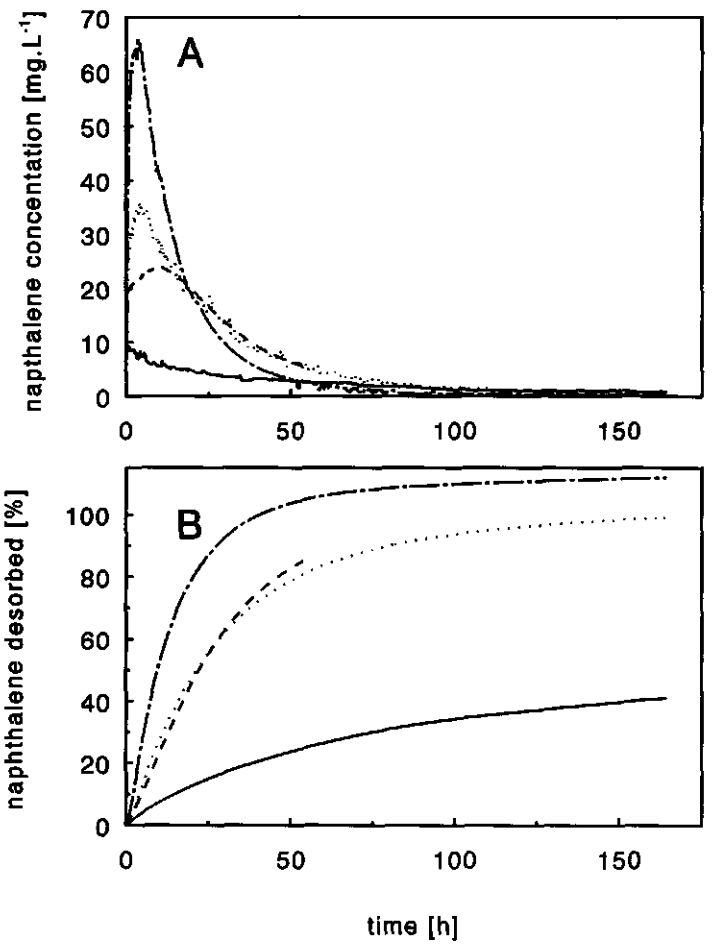

Figure 5: *Continuous desorption of naphthalene from XAD-4 in the presence of different concentrations of Brij 35. A: naphthalene concentration of the leachate, B: % naphthalene desorberd; surfactant concentrations: ——: 0 g·L^{-1},- - -: 0.1 g·L^{-1}, ·····: 0.5 g·L^{-1}, - · -·: 2.0 g·L^{-1}.*

This table also shows the results of the experiments with XAD-4 and Triton X-100. In these experiments the naphthalene concentration in the leachate could not be continuously monitored; therefore only overall data, very similar to the data obtained with Brij 35, are presented.

Table 5: *Mass balance over the continuous desorption of naphthalene from XAD-4 in the presence of different concentrations of Brij 35 or Triton X-100.*

surfactant conc. [g·L^{-1}]	Brij 35					Triton X-100		
	naphthalene desorbed [mg·g^{-1}]		residual loading [mg·g^{-1}]	recovery [%]		naphthalene desorbed [mg·g^{-1}][a]	residual loading [mg·g^{-1}][a]	recovery [%][a]
	*[a]	*[b]		*[a]	*[b]			
0	59	43.6	55	83	63	71.1	40	76
0.1	> 115	107	5.1	> 88	82	109	2.3	76
0.5	143	120	4.5	108	91	139	1.8	97
1.0	151	129	3.7	112	97	148	1.6	103
2.0	161	133	1.3	119	98	N.D.	N.D.	N.D.

[a]: calculated from the on line measurement of the effluent concentration

[b]: determined by measurement of the overall concentration in the eluted solution

N.D.: not determined

Naphthalene biodegradation

Bacterial attachment onto the matrix was observed to occur during growth on XAD-sorbed naphthalene. Because of this the suspended biomass concentration was not a good measure for bacterial growth. Biodegradation was therefore monitored by measurement of the CO_2 concentration in the headspace gas of batch cultures. Typically, growth on sorbed naphthalene starts with an exponential growth phase, followed by a phase in which the bacterial growth is limited by the desorption of naphthalene from the matrix. The aqueous-phase naphthalene concentration in this stage will be virtually zero (Volkering *et al.* 1993). To assess the effect of surfactant on the biodegradation of sorbed naphthalene, the surfactant was added either at the beginning of the experiment or during the desorption-limited growth phase. In the experiments with XAD-7, a short desorption-limited growth phase was observed with only little effect of the surfactants on the biodegradation. In the non-exponentential growth stage of experiments with XAD-4, no naphthalene could be

detected in the aqueous phase (data not shown), and desorption of naphthalene from the matrix was concluded to be rate-limiting. Adding Triton X-100 or Brij 35 at the start of experiments with XAD-4 resulted in longer exponential growth phases than in experiments without surfactant (Figure 6). Due to this prolongued exponential phase it was not possible to compare the desorption-limited phases of the blank experiments with those observed in the experiments with surfactant. Therefore experiments were performed in which the surfactant was added in the limited growth phase. The CO_2 production rates increased after surfactant was added as can be seen in Figure 7. At the end of the experiments no residual naphthalene could be detected in the liquid phases. In Table 6 gives the residual naphthalene loadings of the XAD-4 and the mass balances over the biodegradation experiments. To confirm that the observed increase in the CO_2 production rate was caused only by the degradation of naphthalene, several blank experiments have been performed. No CO_2 production was found in inoculated cultures that contained only surfactant or unloaded matrixes, and surfactant did not affect the carbonate equilibria, as was tested by adding 1 ml of a 12 M HCl solution to the cultures. Moreover, the CO_2 yield found is in good agreement with the yield found for the batch growth of strain 8909N on crystalline naphthalene (Chapter 3).

Table 6: *Mass balance over batch experiments with strain 8909N growing on naphthalene sorbed onto XAD-4.*

surfactant concentration [g·L^{-1}]	residual loading [mg·g^{-1}]	CO_2 produced [mmol]	CO_2-yield [a] [C-mol·C-mol^{-1}]
0	20.6	0.051	0.52
0.1	4.7	0.052	0.54
0.5	0.5	0.052	0.54
1.0	1.1	0.053	0.55
2.0	0.3	0.051	0.53

[a]: calculated as C-mol CO_2 produced per C-mol naphthalene disappeared.

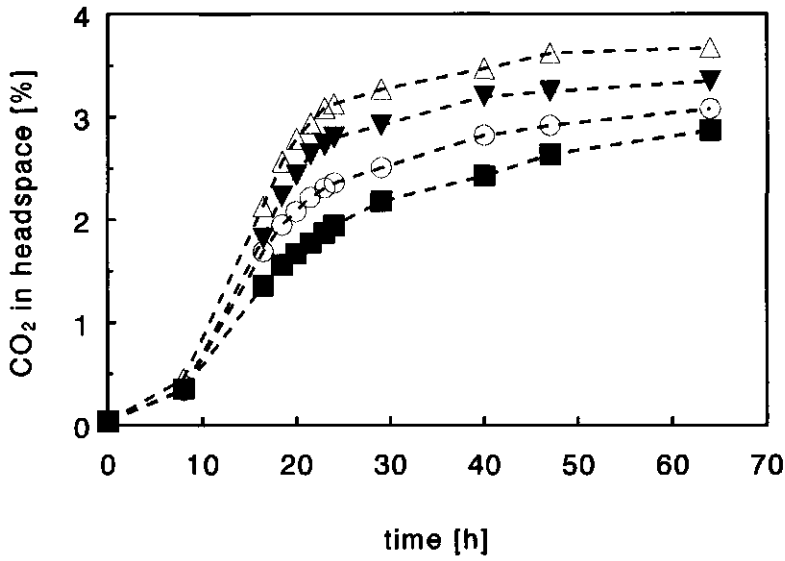

Figure 6: *CO_2 production of strain 8909N on naphthalene sorbed onto XAD-4, Triton X-100 added at t = 0; surfactant concentrations: -■-: 0 g·L^{-1}, -○-: 0.1 g·L^{-1}, -▼-: 0.5 g·L^{-1}, -Δ- 2.0 g·L^{-1}.*

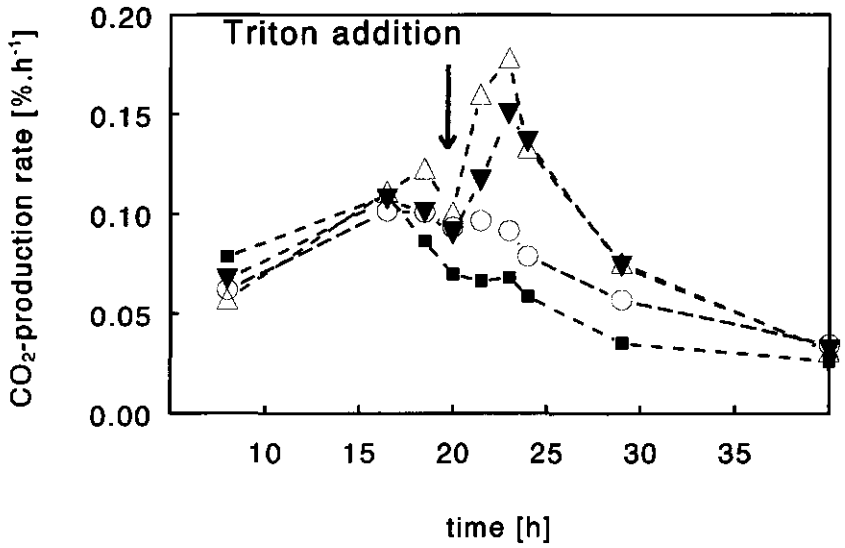

Figure 7: *Rate of CO_2 production of strain 8909N on naphthalene sorbed onto XAD-4, arrow indicates time of Triton X-100 addition, surfactant concentrations: -■-: 0 g·L^{-1}, -○-: 0.1 g·L^{-1}, -▼-: 0.5 g·L^{-1}, -Δ- 2.0 g·L^{-1}.*

DISCUSSION

Batch sorption of Triton X-100 and naphthalene

Adsorption of surfactants onto solid surfaces is a complicated process that may involve the formation of surfactant aggregates called admicelles or hemicelles, depending on whether the aggregates consist of 1 or 2 layers of surfactant molecules (West & Harwell 1992). For the adsorption of nonionic surfactants from an aqueous solution onto a hydrophobic surface such as the resins used here, a monomolecular layer can be expected. This appears to be confirmed in the adsorption experiments using Triton X-100, and XAD-4 and XAD-7. An estimation of the coverage of the materials can be made using the specific surface areas of the matrices and a surface area of $6.7 \cdot 10^{-19}$ m^2 per molecule of Triton X-100. These calculations revealed that about 90% of the resin surface area was covered with surfactant molecules (Table 2). Moreover, the adsorption isotherms could be described with the Langmuir model, which is based on the assumption that a monomolecular layer of molecules is formed on the adsorbing surface.

For the adsorption of naphthalene onto the matrices similar results were found (Table 3). With naphthalene (molecular surface area $4.29 \cdot 10^{-19}$ m^2, Radt 1948), a coverage of approximately 90% was found for both XAD-4 and XAD-7. In contrast to Triton X-100, the desorption isotherms of naphthalene could not be described with the Langmuir model. This difference may be caused by the low aqueous solubility of naphthalene.

From the batch desorption experiments with naphthalene that have been performed in this study, it can be concluded that the presence of surfactants at high concentrations results in a decrease in the solid-liquid partition coefficient due to solubilization of naphthalene in surfactant micelles. This is consistent with the results of most studies on sorption of HOCs in the presence of surfactants (e.g. Edwards *et al.* 1991, 1992, Liu *et al.* 1992, Park & Jaffé 1993, Sun & Boyd 1993, Vigon & Rubin 1989). More interesting is the decrease in the partition coefficient at low surfactant concentrations (0.1 and 0.25 $g \cdot L^{-1}$). Since these are the initial concentrations, the aqueous surfactant concentrations at equilibrium will be considerably lower than the CMC (approximately 0.1 $g \cdot L^{-1}$ for Triton X-100, Volkering *et al.* 1995), because of adsorption of surfactant molecules onto the matrix. Therefore no surfactant micelles will be present, eliminating solubilization as the reason for the increased aqueous naphthalene concentrations. Competition between naphthalene and Triton X-100 for the available sorbing surface is the most likely explanation for this phenomenon.

The results of the batchwise desorption experiments do not allow distinct conclusions about the effect of surfactant on the initial desorption rate of naphthalene (facilitated transport). The reason the desorption proceeds faster in the experiments with high surfactant concentrations may be the partitioning of naphthalene into the micelles, rather than facilitated transport. This partitioning will cause a slower decrease in the difference between the equilibrium concentration and the actual concentration, the driving force behind the desorption process, and will thus lead to a higher desorption rate.

Continuous desorption of naphthalene

The leaching experiments were performed at one surfactant concentration below the CMC and three concentrations above it. The mass balances over the experiments with XAD-4, and Brij 35 and Triton X-100 (Table 5) show that for the sub-CMC surfactant concentations (0 and 0.1 $g \cdot L^{-1}$) the recovery of naphthalene was rather poor. The most likely explanation for this is that, although as much stainless steel tubing as possible was used, liquid-phase naphthalene was lost due to absorption or volatization. This loss will be less at surfactant concentrations higher than the CMC, since then a large amount of the solubilized naphthalene will be present in the micellar phase. This means that for the experiments without surfactant and with 0.1 $g \cdot L^{-1}$ surfactant, the desorbed amounts of naphthalene are likely to be higher than the amount recovered in the leachates. This does not interfere with the conclusion that the presence of 0.1 $g.L^{-1}$ Brij 35 and Triton X-100 (sub-CMC) clearly stimulates the desorption of naphthalene. This can also be seen from the residual loadings of the matrices after leaching, which were a factor 10-20 lower when surfactant was present (Table 5). The solubility enhancement of naphthalene in aqueous solutions at this surfactant concentration is negligible (Volkering *et al.* 1995). Therefore it can be concluded that the surfactant stimulates the desorption of naphthalene through facilitated transport. However, since the desorption was even faster at surfactant concentrations higher than the CMC, solubilization may also play a role in these experiments. We inferred earlier (Volkering *et al* 1993, 1995) that for enhancing the biodegradation of soil-sorbed pollutant (but not for soil washing), facilitated transport of pollutant is likely to be the most important effect of surfactants. Thus, the relatively large effect found at the concentration below the CMC is promising, also from an economic point of view.

Biodegradation of sorbed naphthalene

Bacterial growth on sorbed hydrocarbons in batch cultures can be described analogue to the growth on solid hydrocarbons (Volkering 1992, 1993). Initially, the desorption rate is higher than the degradation rate, and exponential growth and CO_2 production occurs (exponential stage). The degradation rate will increase with increasing cell numbers and will eventually exceed the desorption rate, causing the aqueous hydrocarbon concentration to drop. This results in a stage in which the bacterial growth is limited by the desorption of the HOC (desorption-limited stage). The presence of surfactant in this system may stimulate the biodegradation of the hydrocarbon in two ways.

Firstly, at concentrations higher than the CMC, more hydrocarbon will be desorbed in the first stage due to micellar solubilization, resulting in a longer exponential growth stage. Secondly it may cause facilitated transport of sorbed hydrocarbon to the aqueous phase, which results in both a longer exponential stage and an increased growth rate in the desorption-limited stage. In the experiments with XAD-4 described here, the addition of surfactant at the start of the experiment resulted, as expected, in a prolongued exponential stage (Figure 6). Addition of the surfactant in the desorption-limited stage resulted also in a stimulation of the CO_2-production on the sorbed substrate, as can be seen in Figure 7. This was not caused by degradation of the surfactant or by a change in the bacterial yield on naphthalene as shown in Table 6. Therefore, these results confirm the conclusion from the continuous desorption experiments, i.e. that the presence of surfactants results in facilitated transport of sorbed naphthalene. This is also supported by the results of the measurements of the residual naphthalene loadings, which were performed after the experiment was stopped by addition of 1 ml 12 M HCl. The residual loadings in the bottles without surfactant were significantly higher than in the other experiments, showing that more naphthalene was degraded when surfactant was present. As to the mechanism of this mobilization, little can be said from the data presented here.

Comparison of desorption and biodegradation

Using a yield factor as described previously (Volkering *et al.* 1993) and carbonate equilibria (Lindsay 1979), it is possible to relate the CO_2 concentration in the headspace gas to total amount of CO_2 produced and thus to the amount of naphthalene that was degraded. This is shown in Figure 8 for the experiments with and without 2.0 $g \cdot L^{-1}$ surfactant (addition at t=0). To compare biodegradation and desorption, the amounts of naphthalene desorbed in the leaching experiments

without surfactant and with 2.0 g·L^{-1} surfactant are also given in the figure. Although the experimental setup was different in both experiments, several observations can be made from the data presented in Figure 8.

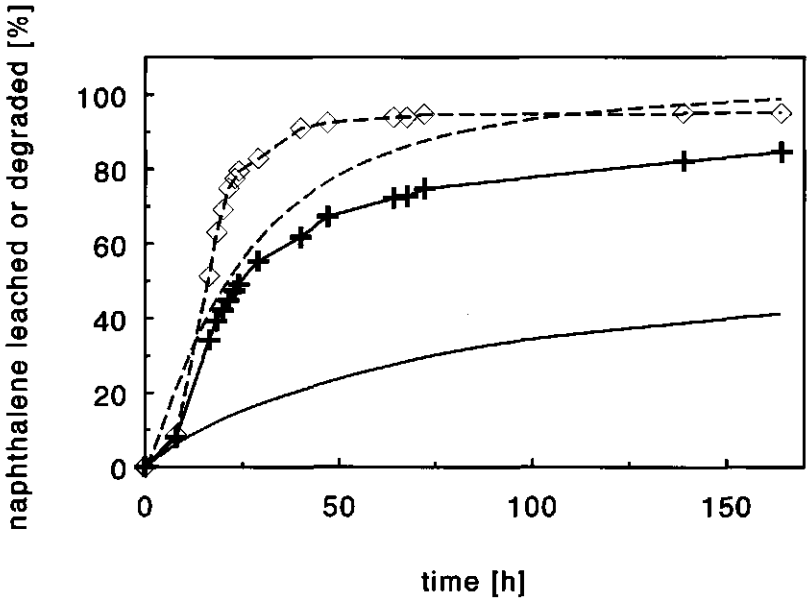

Figure 8: *Effect of surfactant on biodegradation (symbols+lines) and on continuous desorption (lines only) for naphthalene sorbed onto XAD-4; —: no surfactant, - - -: 2.0 g·L^{-1} surfactant*

Comparing the solid lines it appears that in the absence of surfactant the biodegradation proceeds faster than the desorption. This phenomenon has been discussed previously in Chapter 4.

When surfactant is present it is more difficult to compare batch growth experiments with continuous desorption experiments, because the presence of surfactant causes an important difference in the two types of experiments. In the batch experiments, a known amount of surfactant is present, which will partly adsorb onto the matrix. In the leaching experiments, however, fresh medium with the same surfactant concentration is continuously supplied. Hence, more surfactant will be absorbed onto the matrix with equal (initial) aqueous surfactant concentrations and the effect of the surfactant molecules on the desorption will be different in each case. However, in both the desorption experiments (Table 5) and the biodegradation

experiments (Table 6) little difference was found between the results of the experiments with the highest surfactant concentrations (1.0 and 2.0 $g \cdot L^{-1}$). Therefore it is possible to draw two more conclusions from Figure 8: (i) the effect of surfactants is less pronounced in the biodegradation experiments than in the desorption experiments, and (ii) the effect of the microorganisms is less pronounced when surfactant is present (dashed lines). It appears that both the presence of surfactant and of growing bacteria have a positive effect on the bioavailability, and that the effect of the one is decreased by the effect of the other. Nevertheless, it can be concluded that application of surfactants, under the right conditions, can stimulate the degradation of organic pollutants and can therefore be seen as an interesting tool for soil bioremediation.

NOMENCLATURE

C_{eq}	equilibrium surfactant concentration in solution [$mg \cdot L^{-1}$]
k_s	Langmuir constant [$L \cdot mg^{-1}$]
K_f	Freundlich sorption capacity constant [$mg^{1-1/n} \cdot L^{1/n} \cdot g^{-1}$]
n	Freundlich sorption energy constant [-]
Q_{eq}	equilibrium sorbed surfactant concentration [$mg \cdot g^{-1}$]
Q_{max}	maximum sorbed surfactant concentration [$mg \cdot g^{-1}$]

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CHAPTER 8

CONCLUDING REMARKS

Bioavailability and biodegradation of polycyclic aromatic hydrocarbons

This thesis has discussed the research on the biodegradation of polycyclic aromatic hydrocarbons. These compounds are representative of a group of hydrophobic organic pollutants which are biodegradable under laboratory conditions, but are persistent in soil. In 1990, when this project started, the realization that the occurence of mass transfer limitation in soil could be the main cause for this contradiction gradually became evident. The main goal of this research therefore was to reveal the relation between the bioavailability of the hydrophobic pollutants (PAHs) and their biodegradation rates.

The first step in the research was the isolation and characterization of PAH-degrading bacteria, which were then used for studying the effect of mass transfer limitations on the degradation of PAHs in different systems. For crystalline PAHs it was shown that the degradation rates could be directly coupled to the dissolution rates of the different PAHs. For PAHs sorbed onto a matrix, more complex results were found. Using synthetic porous materials as the sorbing matrix, it was found that the biodegradation rate of naphthalene was 2-3 times higher than the calculated maximal desorption rate. Therefore it must be concluded that with this type of matrices the bacteria must have some means of gaining better access to the sorbed naphthalene. In contrast with this, it was found in experiments with soil-sorbed naphthalene and phenanthrene that the same microorganisms degraded aqueous-phase PAHs only. These results show that the biodegradation of hydrophobic pollutants in soil is a complicated matter involving both physicochemical and biological processes. In all cases, however, it was found that the biodegradation rate of separate-phase PAHs was lower than that of free-phase PAHs, showing that the bioavailability of PAHs can be an important factor limiting their biodegradation.

Consequences of bioavailability limitations for biological soil remediation

In recent studies on biological soil remediation, limited bioavailability is generally considered to be one of the most important factors determining the result of bioremediation of poorly soluble pollutants such as PAHs, PCBs, and weathered oil (Blackburn & Hafker 1993, Mihelcic *et al.* 1993, Providenti *et al.* 1993, Schulz-Berendt 1994, Thomas & Lester 1993, Wilson & Jones 1993). This is also reflected by the fact that at the "Third In Situ and On Site Bioremediation Symposium" in San Diego (april 1995), two full sessions were dedicated to the phenomenon of bioavailability. This shows that bioavailability is a "hot topic" in the research concerning biological clean-up of soils. When compared to other factors that play a role in soil remediation, bioavailability is most notable for its vagueness. Its definitions, such as the one presented in the introduction, are usually generic. For a good understanding of bioavailability it is essential to realize that it is a time-based concept. Moreover, bioavailability can be different for different organisms and is dependent on a large number of physico-chemical parameters, as discussed in the previous chapters. It is therefore difficult to make predictions on the rates of biodegradation in bioremediation processes, and thus to predict the time necessary for soil clean-up. The poor predictability and reliability are the main reasons for the present limited application of biological soil remediation.

It is therefore essential that standard methods are developed by which the bioavailability of pollutants in soils and sediments can be characterized. These may be biological methods, in which the effect of the pollutant in the soil or sediment on standard organisms is measured, or physicochemical methods, in which the mass transfer of the pollutant to the aqueous phase is measured. Biological methods are generally more laborious and therefore more expensive than physicochemical methods. The problem with physicochemical methods is that the differences in bioavailability for different organisms are not taken into account. However, from the results presented in this thesis and from most other studies, it can be concluded that the bioavailability of sorbed pollutants is much lower than that of aqueous-phase pollutants. Thus, by choosing the right conditions and using a proper experimental setup, it should be possible to predict biodegradation rates using physicochemical methods. This is confirmed by the results with soil presented in Chapter 4, as was discussed above. The type of physicochemical experiments best suited to this are

probably column-leaching experiments. In addition to these, it may be necessary to perform simple biological experiments.

Application of surfactants as a solution to limited bioavailability

The possibility for solving the problem of limited bioavailability by technological means is dependent on the type of biological soil treatment that is used.

When the polluted soil is excavated, several different techniques are available: (i) mechanical reduction of the size of the soil aggregates to reduce the length of the desorption pathways and thus to accelerate the desorption; (ii) increasing the temperature to speed up desorption rates, (ii) separation of the highly polluted fraction of the soil, and (iv) addition of bioavailability-enhancing compounds. These techniques can be applied both before the biological process and simultanuous to it

With *in situ* treatment the number of technological solutions is limited. Improving bioavailability of the pollutants can be established by (i) increasing the temperature, which is in most cases too expensive, (ii) using acoustic techniques, a method that is still in the developing stage and only applicable to liquid-phase pollutants, or (iii) adding compounds that may enhance the bioavailability of th pollutants. These include co-solvents, fungal enzymes, chemical oxidants and surface-active compounds, the latter being the solution that has been applied the most.

The technological solution for the problem of limited availability of hydrophobic organic pollutants dealt with in most detail in this thesis is the use of surface-active agents. As discussed in Chapter 5, the three main mechanism by which surfactants can enhance the bioavailability of hydrophobic pollutants are (1) solubilization, (2) emulsification, and (3) facilitated transport. Solubilization can only occur when micelles, aggregates of 20-20 surfactant molecules, are present. The presence of micelles results in increased (apparent) solubilities of hydrophobic pollutants. In soil however, micelle formation occurs at rather high surfactant concentrations due to adsorption of surfactant molecules. The emulsifying action of surfactant is caused by the decrease in the surface tension between the aqueous phase and the pollutant pase and can only occur when the pollutant is present as a liquid-phase. Facilitated transport is a term that covers several processes, such as the release of pollutant in trapped soil pores, interaction of pollutant with single surfactant molecules, and direct interactions of micellar structures with sorbed pollutants (adsolubilization).

There are, however, also negative effects of the use of surfactants, such as toxic behavior or preferential degradation of the surfactants, clogging of soil pores, or

interference of the surfactants with the natural interactions among the micro-organisms and the pollutant.

Due to the complexity of these effects in soil, present knowledge is not enough to predict the effect of the application of surfactants in soil remediation. Nevertheless, surfactants are frequently used to prevent bioavailability problems in bioremediation processes, both with *ex situ* (e.g. Joshi & Lee 1995, Schmidt & Hahn 1995) and *in situ* processes (e.g. Ducreux *et al.* 1995, Guttman 1995, Rhodes *et al.* 1995, Ross *et al.* 1995, Van Vree *et al.* 1993).

As the amount of surfactant used may be one of the factors determining the costs of the treatment, optimizing the surfactant addition is an important prerequisite for its use. Several considerations can be taken to minimize the use of surfactant. Firstly, surfactant addition will only be useful when the bioavailability of the pollutant is limiting the biodegradation rate as was discussed in the Chapters 5-7. Therefore addition of surfactants at the start of the bioremediation process is likely to be of little use. Secondly, it seems ineffective to apply surfactant in high concentrations, as was also discussed in the Chapters 5-7. Finally, it might be useful to add surfactants on an intermittent basis.

Another promising option to reducing the costs of surfactants may be stimulation of the *in situ* production of biosurfactants by the natural population or by introduced micro-organisms. Although the use of biosurfactants in soil remediation has received considerable attention (see Chapter 5) and although some studies on the use of biosurfactant-producing bacteria have been published (Jain *et al.* 1992, Providenti *et al.* 1995), the understanding of processes involved in biosurfactant production during bioremediation still requires fundamental microbological research.

Consequences of limited bioavailability for soil-quality limits

Although technological solutions for bioavailability limitations are being studied as discussed above, another side of bioavailability is often forgotten. The term bioavailability originates from the field of toxicology, and it is easy to understand that a polluted soil in which the pollution has a low bioavailability will pose a lower ecotoxicological risk than a soil in which the same pollutant has a high bioavailability, even when the levels of the pollutant concentration are the same. However, this insight is not reflected in most of the regulations concerning soil pollution. All these regulations are based on pollutant concentrations measured by extraction of the soil with organic solvents, although in some guidelines the organic carbon content of the

soils is incorporated. By not taking the bioavailability of the pollutant into account, these regulations may lead to an overestimation of the ecotoxicological risks (LaGoy & Quirck 1994). An alternative to measurement of pollutant concentrations by solvent extraction is measurement of the leaching behavior of the pollutant, as explained in the previous section. At the moment the Dutch legislation on the quality of building materials is in revision. The new rules are based on the results of leaching experiments and standard methods are now under development (Ministry of Housing, Spatial Planning, and the Environment 1994). Rules based on this type of experiments have much better scientific background than the present rules for soil pollution. A similar approach has recently been proposed by Beck *et al.* (1995). The authors defined kinetically constrained soil-quality limits (KCSQLs). These are based on the timecourse of the decrease of the pollutant concentration. The most important agreement between the KCSQL-concept and the one presented here is that the risk of pollution does not mainly depend on its concentration in the soil, but rather on the rate at which it may come available for uptake by organisms.

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SUMMARY

One of the main problems in biological soil remediation is the slow or incomplete degradation of hydrophobic organic pollutants. The principal reason for this problem is the fact that these compounds bind strongly to the soil matrix or occur as a separate non-aqueous phase in the soil. As most microbiological processes take place in the water phase, transport of the polluting compound to this phase is essential for biodegradation to occur. When this transport is the limiting factor in the biodegradation process, this is termed limiting bioavailability.

This thesis deals with the effect of bioavailability on the biodegradation of polycyclic aromatic hydrocarbons (PAHs). PAHs are hydrophobic organic pollutants that are abundantly present in contaminated soils and give raise to environmental concern because of their toxicity and mutagenicity. Most PAHs are degradable by microorganisms and the important biochemical aspects of the PAH-degradation have been revealed. PAHs are nevertheless considered persistent pollutants in soil, a fact that is attributed to their limited bioavailability.

The first part of the research consisted of the isolation of bacteria capable of degrading the PAHs, naphthalene, phenanthrene, and anthracene. Subsequently a number of isolated bacterial strains were grown in batch and continuous cultures to determine the most important microbial growth parameters, such as the maximum growth rate, the Monod saturation constant, and the bacterial growth yield.

The effect of bioavailability on the biodegradation of PAHs was studied in two model systems: (i) crystalline PAHs and (ii) PAHs bound to a matrix.

For studying the bioavailability of crystalline PAHs the results of dissolution and biodegradation experiments were compared. In the degradation experiments it was found that two phases could be observed during batch growth: an exponential growth phase, followed by a linear growth phase, in which biomass formation was limited by the availability of the PAHs. By using a model in which Monod kinetics for bacterial growth were coupled to dissolution kinetics for substrate availability, it was shown that the observed degradation rates were matched by the rates of dissolution of the PAHs to the aqueous phase. Therefore it was concluded that in this system only aqueous phase PAHs were available for bacterial uptake and that the bioavailability of the PAHs was not directly stimulated by the presence of the microorganisms.

With matrix-bound PAHs desorption and biodegradation experiments were conducted. The first matrices studied were the synthetic porous resins XAD-4 and

XAD-7. The desorption of naphthalene from these materials was studied in batch and continuous desorption experiments. The results from these experiments could be described using a two-compartment model in which the matrix is divided in a fraction with shallow pores and one with deep pores. In biodegradation experiments with naphthalene-loaded resins the same type of batch-growth kinetics was observed as described above for crystalline substrates: exponential growth, followed by a phase in which substrate availability limits the degradation rate. By comparing the results of the desorption experiments and the biodegradation experiments it was shown that the biodegradation proceeded faster than could be explained by desorption alone. Therefore it was concluded that the bacteria had a positive effect on the bioavailability of naphthalene that was adsorbed onto the resins. This effect was not caused by the presence of bacterial excretion products.

In contrast to this it was found that the biodegradation of soil-bound naphthalene and phenanthrene could be explained by degradation of PAHs present in the aqueous bulk phase only. Thus, the bioavailability of sorbed PAHs depends on the type of matrix the PAHs are sorbed onto .

The second part of this thesis deals with the most widely applied solution for the problem of limited bioavailability: the application of surface-active agents or surfactants. Surfactants are molecules that usually consist of a hydrophillic and a hydrophobic part. Due to this they have a tendency to concentrate at surfaces and interfaces and to form new interfaces. There are several different ways by which surfactants may increase the bioavailability of hydrophobic compounds in soil:

- solubilization in the aqueous phase by the presence of micelles, aggregates of 20-200 surfactant molecules with a hydrophobic interior;
- emulsification of liquid hydrocarbons in the waterphase;
- facilitated transport, a term that covers several processes, such as mobilisation of pollutant present in soil pores or interaction pollutant with single surfactant molecules.

Surfactants may also have a negative effect on pollutant bioavailability, for instance by the toxic effect or preferential degradation of the surfactant, or by interference with the natural interactions among microorganisms and pollutant.

The effect of several nonionic surfactants on the bioavailability of PAHs was studied in the same model systems as described above: crystalline PAHs and PAHs sorbed onto a matrix.

Dissolution experiments with crystalline naphthalene and phenanthrene showed that the presence of surfactants caused an increase in the apparent solubility and in

the maximum dissolution rate of these PAHs. Both phenomena have an effect on the bioavailability of PAHs. Although it was found that micellar PAHs were not readily available for uptake by the bacteria, the transport of PAHs from the micelles is sufficiently fast to allow almost complete exponential growth on solubilized PAHs. The effect on the maximum dissolution rate is probably more important because this is the most relevant factor under bioavailability-limiting conditions. Addition of surfactant to cultures growing on PAH in the dissolution-limited phase resulted in an increase in the linear growth rate. This shows that for crystalline PAHs surfactants can be used to increase the bioavailabilty

For sorbed naphthalene similar results were found. In desorption experiments it was shown that in the presence of surfactant, the partitioning of naphthalene to the waterphase as well as the maximum desorption rate was increased. Addition of surfactants to cultures growing on sorbed naphthalene in the desorption-limited phase resulted in an increase in the degradation rate. This shows that surfactants can be used for enhancing the bioavailability of sorbed PAHs.

The first general conclusion from this thesis is that the bioavailability of hydrophobic pollutants in soil is a complex matter and therefore difficult to quantify. In model systems under laboratory conditions, however, it was possible to simulate the essential processes. This experimental work revealed the most important mechanisms that play a role in bioavailability limtations. Because of the large impact of bioavailability on both the performance of biological soil remediation and on the risks posed by soil contamination, it is essential that standard methods be developed which provide criteria for bioavailability. These criteria may be used to predict the results of biological soil remediation processes and may form a basis for soil quality limits in which the bioavailability of the pollutant is considered.

Secondly, the application of surfactants can be concluded to be a promising option for enhancing the bioavailability of hydrophobic pollutants. In two model sytems it was shown that addition of surfactants speeded up the biological degradation of PAHs markedly and some explanations for this phenomenon have been found. However, to allow the use of surfactants as a standard technique in biological soil remediation, more insight into the complex interactions involved in the introduction of surfactants into soil is necessary.

SAMENVATTING

Bij de biologische reiniging van met organische verbindingen verontreinigde grond wordt één van de grootste problemen gevormd door de langzame en slechts gedeeltelijke afbraak van hydrofobe verontreinigingen. De belangrijkste reden voor dit verschijnsel is het feit dat deze verbindingen in sterke mate hechten aan de bodemmatrix of als een aparte fase in de grond voorkomen. Omdat de biologische afbraak voor het grootste deel in de waterfase plaatsvindt, moeten de verontreinigingen eerst naar deze fase worden getransporteerd, wat in veel gevallen de beperkende factor in het reinigingsproces vormt. Dit verschijnsel wordt limiterende biobeschikbaarheid genoemd en staat momenteel sterk in de wetenschappelijke belangstelling.

In dit proefschrift wordt het effect van biobeschikbaarheid op de biologische afbraak van polycyclische aromatische koolwaterstoffen (PAKs) beschreven. PAKs zijn hydrofobe organische verbindingen welke op grote schaal in de bodem voorkomen en bekend staan om hun toxische en carcinogene eigenschappen. Vrijwel alle PAKs zijn microbiologisch afbreekbaar en de biochemische aspecten van deze afbraak zijn vrij goed bekend. Desondanks staan PAKs bekend als persistente bodemverontreinigingen. De voornaamste reden hiervoor is de slechte biobeschikbaarheid van deze verbindingen in de bodem.

De eerste stap in het onderzoek was het isoleren van bacteriën welke in staat waren om de PAKs naftaleen, fenanthreen en anthraceen af te breken. Een aantal van de geïsoleerde stammen is vervolgens in batch- en continue-cultures gekweekt om de belangrijkste parameters voor de groei van deze micro-organismen op PAKs vast te stellen, zoals de maximale groeisnelheid, de Monod affiniteitsconstante en de yield.

Vervolgens is de relatie tussen biobeschikbaarheid en biodegradatie van PAKs onderzocht in twee modelsystemen: (i) kristallijne PAKs en (ii) aan een matrix gebonden PAKs. Het eerste en meest eenvoudige systeem is de afbraak van kristallijne PAKs. Batchgroei op slecht oplosbare verbindingen als PAKs bestaat meestal uit twee fasen: een exponentiële groeifase en een fase waarin de groei wordt gelimiteerd door de beschikbaarheid (oplossnelheid) van het substraat. Voor de afbraak van kristallijn naftaleen en fenanthreen kon worden aangetoond dat de afbraak even snel verliep als de oplossnelheid van de kristallen. De bacteriën waren blijkbaar in staat om alleen opgelost substraat af te breken en hadden dus geen directe invloed op de biobeschikbaarheid van kristallijn naftaleen en fenanthreen.

Bij de afbraak van aan een matrix gebonden PAKs bleek dat verschillende effecten konden worden waargenomen. De afbraak van aan synthetische hars-achtige materialen gebonden naftaleen verliep sneller dan kon worden verklaard door desorptie. Daarom moeten de bacteriën op een of andere manier in staat zijn de biobeschikbaarheid van het gebonden naftaleen te beïnvloeden. In tegenstelling hiermee bleek dat de afbraak van aan verschillende standaardgronden gebonden naftaleen en fenanthreen wel kon worden verklaard door ervan uit te gaan dat alleen gedesorbeerde PAKs kunnen worden afgebroken. In alle gevallen werd gevonden dat sorptie aan een vaste matrix de biobeschikbaarheid van de PAKs reduceert, maar het was niet mogelijk de mate van deze reductie te voorspellen

Het tweede deel van dit proefschrft behandelt de meest toegepaste oplossing voor biobeschikbaarheidsproblemen: het gebruik van oppervlakte-actieve stoffen, ook wel surfactants genaamd. Surfactants zijn moleculen die meestal uit een hydrofoob en een hydrofiel deel bestaan en zich daardoor concentreren aan oppervlakken en grensvlakken. Deze verbindingen zijn via een aantal verschillende mechanismen in staat slecht in water oplosbare verontreinigingen in de bodem te mobiliseren:

- solubilisatie in de waterfase via micellen, kleine aggregaten van surfactant moleculen waarin hydrofobe verbinding kunnen oplossen;
- emulgering van vloeibare verontreinigingen in de waterfase;
- mobilisatie van verontreinigingen in de poriën van bodemdeeltjes door verlaging van de oppervlaktespanning.

Daarnaast kunnen ook negatieve effecten optreden, bijvoorbeeld door de toxische werking of preferente degradatie van de surfactant, of door verstoring van de natuurlijke interacties tussen de micro-organismen en de verontreiniging.

Het onderzoek naar het effect van surfactants op de biobeschikbaarheid en biodegradatie van PAKs is uitgevoerd met dezelfde twee typen modelsysteem als boven beschreven: kristallijne PAKs en aan een matrix gebonden PAKs. Bij experimenten met kristallijn naftaleen en fenanthreen bleek dat de aanwezigheid van verschillende niet-ionogene surfactants leidde tot een verhoging van zowel de schijnbare oplosbaarheid (door micelvorming) als de maximale oplossnelheid van deze PAKs. Beide verschijnselen hebben een effect op de biobeschikbaarheid van de PAKs. Omdat de biobeschikbaarheid van hydrofobe verontreinigingen in de grond wordt bepaald door transportsnelheden, is het effect van de surfactants op de oplossnelheid waarschijnlijk het meest belangrijk. Het toevoegen van surfactants aan batchcultures die oplos-gelimiteerd op naftaleen of fenanthreen groeiden resulteerde

in een hogere groeisnelheid. Het toepassen van surfactants verhoogde dus de biobeschikbaarheid van de kristallijne PAKs.

Soortgelijke effecten werden waargenomen in experimenten met aan inerte matrices geadsorbeerd naftaleen. In de aanwezigheid van surfactants werd bij desorptie-experimenten meer naftaleen van de matrices gedesorbeerd en was de maximale desorptie-snelheid hoger dan in de afwezigheid van surfactants. De groei op geadsorbeerd naftaleen tijdens de desorptie-gelimiteerde fase werd door het toevoegen van surfactants gestimuleerd. Het blijkt dus dat ook de biobeschikbaarheid van geadsorbeerde PAKs vergroot kan worden door het toepassen van surfactants.

De eerste algemene conclusie van dit proefschrift is dat de biobeschikbaarheid van hydrofobe organische verontreinigingen in de bodem een fenomeen is dat wordt bepaald door een compex geheel van factoren en daarom moeilijk kwantificeerbaar is. Het is echter wel mogelijk gebleken in modelsystemen de verschillende deelprocessen na te bootsen en grotendeels te verklaren, waardoor meer inzicht is verkregen in de achterliggende mechanismen. Door het grote belang van biobeschikbaarheid voor zowel de biologische reiniging van vervuilde grond als voor het risico dat door bodemverontreinigingen wordt gevormd is het sterk aan te raden dat standaardmethoden worden ontwikkeld waarmee criteria voor de biobeschikbaarheid van bodemverontreinigingen kan worden vastgesteld. Deze criteria kunnen gebruikt worden bij de voorspelling van de resultaten die met biologische bodemreiniging behaald kunnen worden en als basis dienen voor bodemverontreinigingsnormen waarin de biobeschikbaarheid van de verontreinigingen wordt meegewogen.

Ten tweede is gebleken dat het gebruik van surfactants een veelbelovende manier is om de biobeschikbaarheid van hydrofobe verontreinigingen te vergroten. In een tweetal modelsystemen is gevonden dat toepassing van surfactants de biologische afbraak van PAKs duidelijk kan bevorderen en zijn een aantal duidelijke aanwijzingen voor de verklaring voor dit fenomeen gevonden. Echter, voordat de toepassing van surfactants als standaardmethode bij biologische reiniging van verontreinigde bodem kan worden toegepast, is meer inzicht nodig in de complexe interacties die bij de introductie van surfactants in de bodem een rol spelen.

CURRICULUM VITAE

Frank Volkering werd op 26 november 1963 geboren te 's Gravenhage. In 1982 behaalde hij het VWO-diploma aan de Rijswijkse Openbare Scholengemeenschap.

In dat zelfde jaar begon hij met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. In 1988 studeerde hij af met als afstudeervakken Proceskunde en Technische Microbiologie. Hij heeft stage gedaan bij TNO-MT te Zeist.

In 1988 is hij werkzaam geweest als toegevoegd onderzoeker bij de secties Proceskunde en Industriële Microbiologie van de vakgroep Levensmiddelentechnologie van de LUW. Van oktober 1989 tot en met augustus 1994 was hij werkzaam als projectmedewerker bij de afdeling Biotechnologisch Onderzoek van het Laboratorium van Afvalstoffen en Emissies van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne te Bilthoven. De resultaten van het daar uitgevoerde onderzoek staan beschreven in dit proefschrift.

Sinds 15 mei 1995 is hij werkzaam als milieutechnoloog bij MTI Milieutechnologie C.V. te Nijmegen.

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