Biological remediation of soil contaminated with organic compounds can be limited by mass transfer rates. In these situations, the contaminants have a low bioavailability. The bioavailability of contaminants can be increased by biosurfactants, which are surfactants produced by microorganisms such as bacteria and yeasts. To optimize the use of biosurfactants for soil remediation, more insight is required in the effect of biosurfactants on contaminant bioavailability. It is necessary to determine which processes are stimulated by biosurfactants and why biodegradation by certain bacteria is stimulated whereas biodegradation by others is negatively affected.

INTRODUCTION

In the Netherlands, the number of sites contaminated with compounds of industrial origin is approximately 175,000 (Sociaal-economische raad, 1998). Many contaminants may adversely impact health and environmental quality, making remediation necessary. One of the available technologies is bioremediation, which uses the natural degradative potential of plants or microorganisms, usually bacteria and fungi, to convert contaminants into less toxic compounds, ideally CO₂ and water. This technology suffers from several bottlenecks, one of which is the low availability of hydrophobic organic contaminants (Fig. 1.1) to the organisms. This poor bioavailability is caused by low mass transfer rates of the contaminants to these microorganisms from sites where they are inaccessible. Several bacteria produce biosurfactants that may increase the bioavailability of hydrophobic compounds. Therefore, these biosurfactants may be used to enhance biodegradation rates of hydrophobic organic contaminants during soil remediation.

BIOAVAILABILITY

Bioavailability of contaminants in soil. The US Food and Drug Administration and the US Environmental Protection Agency define bioavailability as ‘the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action’. In the perspective of soil bioremediation, the term bioavailability is used to describe the extent to which mass transfer limitations determine a reaction (e.g. biodegradation rate or uptake rate by a specified organism) or an effect (e.g. toxicity to a specified organism or biodegradability by an organism). The bioavailability of soil contaminants to microorganisms that degrade these compounds therefore is determined by the processes that are involved in the transfer of the contaminants from soil to the enzymes which catalyze the first conversion step.

The biodegradation process consists of several steps (Fig. 1.2). Consider a substrate that is initially present in soil or a porous matrix where it is inaccessible to microorganisms. The substrate may be (ads)orbed to the matrix or may be present in the liquid or solid phase. First, this substrate has to be transferred to sites where it can come in direct contact with microorganisms. This can occur by desorption, dissolution, or mobilization of the contaminant from the soil 'phase' to the aqueous phase, and eventually by transport, i.e. convection and dispersion (Fig. 1.2). Subsequently, the substrate has to be taken up by the cells. After the substrate is taken up, it is finally converted. These steps will be described in some more detail below.

Desorption of contaminants from soil. Hydrophobic organic contaminants sorb strongly to soil. The extent and rate of
Chapter 1

Cl Cl Cl Cl Cl Cl

polycyclic aromatic hydrocarbons (3.4-7)

aliphatics (3 -9 and higher)

O

O

O

phthalate esters (1.5-6)

O

O

O

alkylated benzenes (2.7-5)

Cl Cl Cl Cl CCl3

chlorinated benzenes (3-5.5)

Cl Cl O Cl

dioxins (6.6 for cpd shown)

PCBs (4-8)

Cl Cl Cl Cl CI CI

DDT (6.4)

Figure 1.1. Representative compounds of several classes of hydrophobic soil contaminants. The number given in brackets is the range in log $K_{ow}$ values for members of that class of contaminants. The $K_{ow}$ (1-octanol water partitioning coefficient) value is a measure of the hydrophobicity of a compound. Many properties (solubility, sorption, desorption rates, etc) are correlated to the log $K_{ow}$ of the contaminants. The $K_{ow}$ values were taken from Schwarzenbach et al. (1993).

Sorption correlates to the organic matter content of the soil and the hydrophobicity (e.g. log $K_{ow}$) of the contaminant, indicating that organic matter plays a dominant role in determining sorption and sorption kinetics (Brusseau and Rao, 1989; Brusseau, 1993b). The importance of soil organic matter suggests that sorption is a partitioning process of the contaminant between an aqueous phase and soil organic matter. However, since isotherms often are nonlinear, sorption is not a true partitioning process (Weber et al., 1992). Since the distinction between adsorption and partitioning often cannot be made, the all-encompassing term 'sorption to soil' is widely preferred above the term 'absorption by soil organic matter' or 'adsorption to soil'.

Establishment of sorption kinetics allows estimation of cleanup times and of environmental risks. Furthermore, the study of sorption kinetics can aid to obtain mechanistic insight into sorption process. The rate of the actual desorption step, as determined by the Gibbs energy of activation of this step, will not often be limiting the rate of release of contaminants from soil (Pignatello and Xing, 1996). Instead, ad- and desorption rates are thought to be determined by mass transfer processes such as intra-organic matter diffusion and retarded pore diffusion. The rate of intra-organic matter diffusion is determined by properties of the organic matter of the soil, whereas the rate of retarded pore diffusion is determined by the pore size, tortuosity, and the extent of adsorption of the contaminant to the pore walls. Pore diffusion may include liquid and surface diffusion. Although both intra-organic matter diffusion and pore diffusion may occur in soil, the former will often be the slowest and dominant step.

Desorption of hydrophobic contaminants to soil is often characterised by the existence fast and slow stages, indicating the occurrence of several processes on different time scales. An increase in the contact time between a hydrophobic organic compound and soil results in an increase in the fraction of the compound for which desorption would be slow (Steinberg et al., 1987; Pignatello et al., 1993). This indicates that soil consists of several sorptive 'domains' with different kinetic properties. These domains might consist of (glassy) and expanded (rubbery) organic material (Pignatello and Xing, 1996; Huang and Weber, 1997a; Leboeuf and Weber, 1997; Luthy et al., 1997).

Dissolution of contaminants. Contaminants can be present in soil as non-aqueous phase liquids (NAPLs, Fig. 1.2). NAPLs occur as pools lying on top of the water table, as sink layers on top of a soil layer with low permeability, as a film coated
to soil particles, or as globules present in soil pores. Removal of these NAPLS can be very slow due to low dissolution rates and the high amount of mass that can be present. In these cases, treatment times tend to be very long because large NAPL pools function as long-term sources of contamination.

The NAPL dissolution rate $J$ (g L$^{-1}$ h$^{-1}$) is determined by the specific NAPL-water interfacial area $a_{nw}$ (cm$^2$cm$^{-3}$) and a mass transfer coefficient $k_i$ (cm h$^{-1}$) as

$$J = k_i a_{nw} (C_{aq} - C_{sat})$$  \hspace{1cm} (1.1)

where $C_{aq}$ (g/L) is the dissolved concentration of a contaminant and $C_{sat}$ (g/L) is its solubility (Powers et al., 1994). Equation 1.1 indicates that the dissolution rate is higher for a compound with a higher solubility and for a NAPL with a higher specific NAPL-water interfacial area. During NAPL dissolution, $a_{nw}$ declines, which in turn reduces the dissolution rate and slows down the remediation process. Therefore, the dependence of the specific NAPL-water interfacial area $a_{nw}$ on the amount of NAPL present must be incorporated in NAPL dissolution models (Powers et al., 1994). Dissolution of solid-phase soil contaminants (Fig. 1.2) can be described in a similar way (Grimberg et al., 1995; Volkering et al., 1995; Mulder et al., 1998b).

**Mobilization of contaminants.** Under certain conditions a NAPL can be displaced in nondissolved state and behave as a second mobile phase. This mobilization of NAPL occurs when the viscous and buoyancy forces that act to displace a NAPL globule overcome the capillary (and shear) forces that retain the globule in the porous medium (Pennell et al., 1996). The effect of buoyancy forces on NAPL mobilization is dependent on the direction of the flow. An analysis of the viscous, buoyancy, and capillary forces yields an expression that describes the critical condition where a NAPL becomes mobile.

**Figure 1.2.** Processes involved in the biodegradation of contaminants that are initially present in soil. Processes involved in the transfer of compounds between the soil phase and the bulk aqueous phase: 1: desorption; 2: dissolution; 3: detachment; 4: mobilization. Processes involved in the uptake of contaminants by cells: a: uptake of dissolved substrate; b: uptake of 'pseudo-solubilized' substrate; c: uptake of substrate by direct attachment of the organism to substrate droplets.
This critical condition is determined, amongst others, by the difference in density of NAPL and water and by the interfacial tension between NAPL and aqueous phase (Pennell et al., 1996). The interfacial tension can be reduced by using compounds such as surfactants (Pennell et al., 1996; Saripalli et al., 1997a). Therefore, surfactants may enhance mobilization. The difference in density between aqueous and nonaqueous phases can be modified, for instance by using brine to increase the density of the aqueous phase or by using alcohols that partition into (dense) NAPLs to decrease the density of these NAPLs (Imhoff et al., 1995). The reduction in the difference in density between aqueous and nonaqueous phases may decrease the vertical mobilization of NAPLs.

It is evident that NAPL removal by mobilization is more efficient than by solubilization. However, an unwanted side effect of the increase in NAPL mobilization is the displacement of NAPLs that are more dense than water to deeper layers.

**Detachment of microdroplets.** A third mechanism of NAPL removal from soil is the release of NAPL microdroplets or emulsions from the immobile NAPL phase and is called either detachment, dispersion or, when surfactants are present, emulsification (Okuda et al., 1996; Bai et al., 1997; Fortin et al., 1997; Chapter 5). It is distinguished from solubilization by the fact that the amount of contaminant in the aqueous phase can exceed the solubility limit of the contaminant. It is distinguished from mobilization by the fact that the NAPL does not behave as a true second mobile phase and that no emulsion forms during mobilization (Bai et al., 1997). However, the distinction between detachment and mobilization may be gradual.

**Transport.** When a spatial separation exists between the contaminant in soil and the bacteria that degrade the contaminant, the contaminant must be transported to the cells (Harms, 1997; Herman et al., 1997a; Angelova and Schmauder, 1999). The transport velocity of a compound is determined by convection, diffusion, dispersion, and sorption of the contaminant.

Transport of contaminants through soil is also of importance when contaminants are to be removed by pump and treat technology (Pennell et al., 1993). Furthermore, by measuring transport of contaminants through soil columns, (de)sorption and dissolution of contaminants can be studied.

**Uptake of contaminants by bacteria.** Three modes of substrate uptake are usually discerned (Nakahara et al., 1977; Singer and Finnerty, 1984; Hommel, 1990; Goswami and Singh, 1991; Miller, 1995; Bouchez-Naitali et al., 1999) (Fig. 1.2): uptake of dissolved substrate, uptake of substrate by direct attachment of the organism to (liquid) substrate droplets that are much larger than the cells, and uptake of 'pseudo-solubilized' substrate. The term 'pseudo-solubilized' substrate is used to describe substrate that is solubilized, emulsified, or otherwise surfactant-bound.

For very hydrophobic substrates, the aqueous solubilities are too low to account for the observed growth rates when only dissolved substrate is taken up (Miller, 1995). This indicates that these substrates are taken up by either of the two other modes of uptake (Miller, 1995). Solid compounds can not be taken up by attachment of the bacteria to the solid surface, indicating that the substrate has to dissolve prior to uptake (Volkering et al., 1998). Sorbed contaminants also have to desorb before they can be taken up (Volkering et al., 1998). However, several studies suggest that sorbed compounds can be directly taken up (Shimp and Young, 1988; Guerin and Boyd, 1992; Calvillo and Alexander, 1996; Tang et al., 1998). However, it is more likely that in these cases the bacteria take up substrate from the aqueous phase directly at the solid-water interface, thereby creating a steep concentration gradient that drives desorption of substrate that is not surface-located (Guerin and Boyd, 1992).
Of the two membranes in Gram negative bacteria, the outer membrane, especially the LPS layer, poses the greatest barrier to uptake of hydrophobic compounds (Hancock and Bell, 1988; De Bont, 1998). Several mechanisms have been proposed to describe the actual passage of compounds over the outer membranes of these bacteria. Most low molecular weight compounds pass the outer membrane by diffusion through large (0.6-2.3 nm) water-filled aspecific channels formed by oligomeric porin proteins (Hancock and Bell, 1988). Cationic compounds may displace divalent cations that bridge LPS aggregates, thereby enhancing the entry of these compounds (Hancock and Bell, 1988). Hydrophobic compounds pass the LPS layer and the membrane without using porins (Hancock and Bell, 1988). Uptake of hydrophobic compounds by certain Gram negative bacteria, including Pseudomonads, is notoriously slow (Hancock and Bell, 1988; De Bont, 1998). For instance, these microorganisms are highly resistant to hydrophobic antibiotics due to the slow uptake of these compounds (Hancock and Bell, 1988). Summarising, bacteria have different uptake modes of hydrophobic contaminants and the mode of uptake can directly determine the bioavailability of substrate.

Metabolism. After the substrate has entered the cell, it is converted. Either it is used as growth substrate or it is converted cometabolically. An enormous amount of research is devoted to the metabolism of organic contaminants, to the organisms involved, and to the pathways by which the compounds are degraded. This research was reviewed recently by Van Agteren et al. (1998). The pathways for the aerobic degradation of phenanthrene and hexadecane, two model contaminants that were used in this study, are briefly summarized below.

Biodegradation of phenanthrene commences by attack of a dioxygenase on the aromatic ring, yielding a cis-dihydrodiol. After hydrogenation, ring fission, and further oxidation, the compound is completely converted to CO₂ and water (Van Agteren et al., 1998). Biodegradation of hexadecane and other long chain alkanes is less well understood on a molecular level than biotransformation of short chain alkanes. The degradation of short chain n-alkanes (C₅ - C₁₀) starts with conversion of the terminal methyl group of the alkane to an alcohol by a monoxygenase, followed by further oxidation in two steps to a carboxylic acid. The fatty acids are further metabolized in the β-oxidation pathway. Subterminal oxidation may also occur in some bacteria, yielding a secondary alcohol that is further converted to a ketone. The pathway for long chain alkanes might be similar. Rhodococcus sp. strain Q15 oxidizes alkanes (C₁₀-C₂₁) by the terminal and subterminal oxidation pathways (Whyte et al., 1998) and an Acinetobacter calcoaceticus S30 converted octacosane (C₂₈) to octacosanol and octacosanoic acid (Lal and Khanna, 1996). However, a different pathway is proposed for Acinetobacter sp. M-1. This strain apparently uses a dioxygenase to convert long chain n-alkanes (C₁₃-C₄₄) to the n-alkyl hydroperoxides (Sakai et al., 1996).

Relation between mass transfer rates and biodegradation rates. Vast amount of data show that desorption and dissolution rates can limit biodegradation rates (Manilal and Alexander, 1991; Volkering et al., 1992, 1998; Weissenfels et al., 1992; Mulder et al., 1998a). However, in the literature it has also been claimed repeatedly that the rate of biodegradation exceeded the rate of one of the upstream processes. For instance, the biodegradation rates of poorly available substrates have been observed to exceed the rates predicted from desorption rates (Guerin and Boyd, 1992), or dissolution rates (Thomas et al., 1986; Efroymson and Alexander, 1991, 1994). A likely explanation of the observed data is that the bacteria used in those studies reside directly at the interface and create a larger concentration gradient than in the abiotic experiments used to determine desorption or dissolution rates.
It must be concluded that the mass transfer process that limits the biodegradation rate may differ between different bacteria.

Relevance of the various processes for soil remediation. The relevance of desorption, dissolution, transport, uptake, and biodegradation for determining the biodegradation rate can be assessed from an analysis of the time constants for these processes (Fig. 1.3A). Remediation of soil can take months to years (Fig. 1.3A). It is evident that this time scale is much larger than the time scale for biodegradation (e.g., enzyme turnover), indicating that limitations exist. These limitations may be caused by transport of contaminants and nutrients over length scales of $10^{-2}$-10$^{-1}$ m (Fig. 1.3B). Uptake of substrate by cells, protein synthesis, and cell multiplication are relatively fast processes compared to desorption and dissolution. This makes clear that desorption and dissolution will often be rate-limiting. In these cases, addition of cells or stimulation of substrate uptake will not have a stimulatory effect on the rate of soil remediation. Adaptation of the indigenous microorganisms to changing conditions or to the contamination can be limiting. Soil is heterogeneous on all length scales including the sub-particle scale ($<10^{-3}$ m) and the macroscopic scale ($>10^{-1}$ m) (Fig. 1.3B). This is of special importance when translating experimental results to situations of a higher (length) scale, since the limiting mass transfer process may be dependent on the scale of the experimental setup. This analysis shows that in situations where soil bioremediation is limited by the bioavailability the remediation rate will often be determined by the rate of desorption or dissolution of the contaminants.

Increasing contaminant bioavailability in soil. The bioavailability of soil contaminants can be increased by stimulating the process that is limiting the rate of biodegradation (Chung et al., 1993). Stimulation of desorption and dissolution rates can in principle be accomplished by all kinds of physical and chemical means. For instance, the temperature can be raised, soil might be pulverized to increase access and decrease diffusional distances, soil may be agitated, acoustic techniques may increase bioavailability, or soil organic matter may be oxidized using chemical agents (Bollag and Bollag, 1995). However, the most promising way to increase a contaminant's bioavailability is thought to be the addition of agents that stimulate mass transfer rates. Agents used to enhance biodegradation include cyclodextrin (Schwartz and Bar, 1995; Bizzigotti et al., 1997; Wang et al., 1998), surfactants, and biosurfactants (vide infra).

BIOSURFACTANTS

Many bacteria, yeasts, and fungi produce extracellular or membrane-associated surface active compounds, called biosurfactants (for reviews see Zajic and Seffens, 1984; Rosenberg, 1986; Hommel, 1990; Fiechter, 1992; Lang and Wagner, 1994; Banat, 1995; Lin, S.C., 1996; Desai and Banat, 1997). The term biosurfactant refers to any type of compound produced by microorganisms with
General Introduction

Isolation of biosurfactant-producing organisms. Biosurfactant-producing strains can be easily isolated by enrichment. Screening for biosurfactant production can be done by measuring hemolysis of red blood cells (Passeri et al., 1991), lysis of filamentous bacteria (Jain et al., 1991; Kitatsuji et al., 1996), reduction in surface tension (Van der Vegt et al., 1991; Busscher et al., 1994; Willumsen and Karlson, 1997), reduction in interfacial tension, the presence of emulsifying activity (Volkering and Noordman, 1996; Willumsen and Karlson, 1997), cell surface hydrophobicity (Neu and Poralla, 1990), and by measurement of contact angles or the wetting of water-repelling materials (Jain et al., 1991). The reduction of interfacial or surface tension by biosurfactants can also determined with non-specialized equipment and on a small scale that enables facile screening of a large number of cultures (Jain et al., 1991; Bodour and MillerMaier, 1998). The reduction of surface tension and emulsifying activity do not necessarily correlate (Willumsen and Karlson, 1997). Furthermore, the bacterial cells themselves can posses surface active properties (Rosenberg, 1986). Therefore, it is necessary to choose a screening assay based on the type of biosurfactants one wants to isolate or to use complementary assays (Volkering and Noordman, 1996).

Structure. Based on their chemical structure, biosurfactants can be classified as glycolipids, lipopeptides, fatty acids and phospholipids, high molecular-weight or polymeric biosurfactants, and 'particulate' biosurfactants (Rosenberg, 1986). Glycolipids are low molecular weight compounds containing a mono- or disaccharide unit linked to a fatty acid moiety. The structural diversity of the glycolipids is high (Haferburg et al., 1986) (Fig. 1.4). Still many new glycolipids are being discovered (Arino et al., 1998). The best known lipopeptides are the surface active antibiotics (bactericidal, fungicidal) produced by Bacillus. These compounds are often cyclic and are composed of 7-16 amino acids and a fatty acid side chain (Rosenberg, 1986; Fiechter, 1992). The polymeric biosurfactants include (hetero)polysaccharides (e.g. emulsan), polysaccharide-protein complexes, lipopolysaccharides, or proteins with emulsifying properties (Rosenberg, 1986). Extracellular vesicles that play a role in hydrocarbon uptake by cells, and microbial cells with surface active properties are referred to as 'particulate' biosurfactants (Rosenberg, 1986). Biosurfactants with modified structures may be produced by using genetic engineering (Fiechter, 1992).

Biosynthesis. Biosurfactants and parts thereof may either be synthesized de novo or may be assembled from substrates and
commonly available central intermediates. For instance, *Arthrobacter paraffineus* KY4303 produced fructose-lipid when grown on fructose, trehalose-lipid when grown on *n*-alkane, and sucrose lipid when grown on sucrose (Itoh and Suzuki, 1974). *Arthrobacter paraffineus* DSM2567 incorporated mannose, glucose, maltose, cellobiose, and maltotriose into glycolipid structures, dependent on which of the corresponding carbohydrates was used as substrate (Li, Z.-Y. et al., 1984). The fatty acid moiety of trehalose lipid produced by *Rhodococcus erythropolis* depends on the chain length of the alkane feed (Kretschmer and Wagner, 1983). Similarly, a correlation between the lipidic composition of the sophorolipid products and the nature of the fatty acids of the rape seed esters was observed for the yeast *Candida bombicola* CBS 6009 (Davila et al., 1992). These data show that for these strains either the hydrophobic or hydrophilic moiety of the biosurfactants was not synthesized *de novo*.

The biosynthesis of rhamnolipid by Pseudomonads involves the sequential addition of two molecules of deoxythymidine-diphospho-L-rhamnose to a β-hydroxy fatty acid. Each step is catalyzed by a separate rhamnosyltransferase. These rhamnosyltransferases can accept various β-hydroxy fatty acids, resulting in rhamnolipids containing C_{10}, C_8-C_{10}, C_{10}-C_{10}, C_{10}-C_{12}, C_{10}-C_{12:1} moieties. The structural genes that code for the first rhamnosyltransferase (*rhlAB*) and regulatory genes that are involved in the biosynthesis of rhamnolipid (*rhlRI*) have been cloned and sequenced and have been transferred to other organisms to allow rhamnolipid production in heterologous hosts (Ochsner et al., 1995). Genes coding for the second rhamnosyltransferase (*rhlC*) and genes involved in biosynthesis of deoxythymidine-diphospho-L-rhamnose (*rmlBCAD*) have recently been characterized (Olvera-Carranza et al., 1999; Rahim et al., 1999).

**Regulation.** Production of sophorolipids, trehalose lipids, and glycolipid-EM by *Pseudomonas aeruginosa* is induced by aliphatic substrates (Desai and Banat, 1997). Production of biosurfactants can also be regulated by repression, for instance by organic acids or glucose (Desai and Banat, 1997). Production of rhamnolipid is notably increased during limitation of nitrogen (Ochsner et al., 1995), phosphate (Mulligan et al., 1989), or multivalent cations (Guerrasantos et al., 1986; Ochsner et al., 1995).

**Physico-chemical properties.** The range in physico-chemical properties of biosurfactants reflects their structural diversity. For instance, biosurfactants are known that create water-in-oil emulsions as well as biosurfactants that create oil-in-water emulsions. Critical micelle concentrations range from 10^{-3} to 10 g/L (Haferburg et al., 1986). Many biosurfactants reduce the surface tension to values below 30 nN m^{-1} and interfacial tension to values below 1 mN m^{-1} (Haferburg et al., 1986).

**Function.** The production of biosurfactants seems to be a prerequisite for the ability of microorganisms to grow on poorly soluble hydrocarbons (Lang and Wagner, 1987; Sylldatk and Wagner, 1987; Hommel, 1990). Biosurfactant-production is foremost associated with strains growing on aliphatic compounds, but also biosurfactant-producing strains are known that degrade polycyclic aromatic hydrocarbons (Deziel et al., 1996; Dagher et al., 1997; Willumsen and Karlson, 1997) and other aromatic compounds (Gibbons and Alexander, 1988; Phale et al., 1995). Biosurfactants may be active either in cell-bound or in extracellular form (Hommel, 1990). For instance, emulsan is excreted into the medium, but is active only when cell bound (Pines and Gutnick, 1986). It has been proposed that the extracellular (anionic) biosurfactants function to promote uptake of pseudosolubilized substrate, whether the cell-associated (nonionic) biosurfactants function to promote uptake of substrate by facilitating attachment.
of the cells to the separate-phase substrate (Hommel, 1990; Volkering et al., 1998). While the anionic extracellular rhamnolipid or sophorolipids may indeed promote uptake of pseudosolubilized substrate and the cell-associated nonionic trehalose lipid may promote attachment of the cells to the substrate (Rapp et al., 1979), the cell-associated emulsan does not stimulate attachment of the cells to the substrate (Pines and Gutnick, 1986; Rosenberg, 1986). This indicates that the statement by Hommel (1990) that is cited above is not generally true. The stimulating action of a biosurfactant may be rather specific for the strain that produces the biosurfactant (Hommel, 1990; vide infra, Chapter 7).

The fact that many biosurfactants are produced even when the cells are not grown on hydrophobic substrates suggests that their function is not solely restricted to the stimulation of substrate availability (Koch et al., 1991; Bouchez-Naïtali et al., 1999). An other function might be to regulate cell adhesion to and cell detachment from surfaces, which controls mobility of the cells (Rosenberg, 1986; Neu and Poralla, 1990; Busscher et al., 1996). Biosurfactants may function as antibiotics. The biocidic action of rhamnolipid, mycolic acid-type biosurfactants from Actinomycetes, and of lipopeptides produced by Bacillus are well documented (Haferburg et al., 1986; Jenny et al., 1991).

**Applications.** Biosurfactants may be applied in foods and pharmaceutical products (Velikonja and Kosaric, 1994; Desai and Banat, 1997), enhanced oil recovery and cleaning of oil tanks (Fiechter, 1992; Desai and Banat, 1997), and for soil remediation (Müller-Hurtig et al., 1994; Banat, 1995). Biosurfactants may also be used as anti-viral and anti-bacterial agents (Stanghellini and Miller, 1997; Vollenbroich et al., 1997; Lang and Wullbrandt, 1999). Rhamnolipid may be used as a source of rhamnose (Linhardt et al., 1989; Lang and Wullbrandt, 1999) or R-3-hydroxydecanoic acid (Lang and Wullbrandt, 1999). Biosurfactants might be used for enhancing the biotransformation rate of lipophilic compounds such as sterols or steroids when uptake by cells is rate limiting (Angelova and Schmauder, 1999).

Advantages of biosurfactants over synthetic surfactants include the high structural diversity and concomitant diversity in properties, the possibility to produce these biosurfactants from renewable resources, and the fact that they are biodegradable. In addition, these compounds are natural, which facilitates the public acceptance of their application in the environment (Haferburg et al., 1986; Fiechter, 1992). Biosurfactants generally are more expensive that synthetic surfactants (Fiechter, 1992; Lang and Wullbrandt, 1999). Therefore, the use of purified biosurfactants is restricted to high-added value products unless methods for cheap production and purification are developed.

**HOW SURFACTANTS INCREASE BIOAVAILABILITY**

*Effects of surfactants on distribution of contaminants in soil.* It is well established that surfactants reduce sorption of contaminants to soil by solubilization when their aqueous concentration exceeds the critical micelle concentration (Edwards et al., 1991). On the other hand, adsorbed surfactants provide additional sorptive capacity to the soil, which can enhance sorption of hydrophobic compounds (Edwards et al., 1994; Brown and Burris, 1996; Sheng et al., 1996). This effect, known as admicellar sorption or adsolubilization, can negatively influence the amount of contaminants present in the (mobile) aqueous phase and potentially the availability of substrate to microorganisms. Not much is known in detail about the effect of surfactants on desorption kinetics. However, recent research has shown that surfactants can increase desorption rate constants. This probably occurs due to surfactant-induced swelling of the soil organic matter, which increases the diffusivity of the sorbate
through the expanded sorbent (Deitsch and Smith, 1995; Yeom et al., 1996a; Sahoo and Smith, 1997; Chapter 2). Surfactants enhance dissolution rates of liquid and solid contaminants (Grimberg et al., 1995, 1996a; Yeom et al., 1996b; Zhang et al., 1997; Mulder et al., 1998b). Surfactants may also enhance mobilization of NAPL by reducing the interfacial tension (Okuda et al., 1996; Pennell et al., 1996). Surfactants can emulsify compounds present as non-aqueous phase liquids (NAPLs) (Okuda et al., 1996). Emulsified substrate has a larger substrate-water interfacial area, enabling faster mass transfer and creating more opportunity for direct contact between cells and substrate. Specific surfactant-cell interactions might enable faster uptake of substrate in the presence of surfactants (Ratledge, 1988; Van Hoof and Jafvert, 1996). Recently, several studies were aimed at determining whether micelle-partitioned substrate can directly be taken up, i.e. without first passing the aqueous phase. Uptake of micellar-partitioned substrate was 0.0-0.8 times as efficient as uptake of aqueous substrate, depending on the type of surfactant and the surfactant concentration (Guha and Jaffé, 1995, 1996; Roch and Alexander, 1995; Volkering et al., 1995; Zhang et al., 1997; Guha et al., 1998a; Willumsen and Arvin, 1999). This indicates that uptake of surfactant-solubilized substrate was less efficient for these bacteria than was uptake of dissolved substrate. Concluding, surfactants stimulate many processes that can possibly limit the bioavailability of contaminants in soil.

Effects of surfactants on biodegradation of soil contaminants. In many cases, addition of surfactants to cultures enhances biodegradation of hydrophobic organic components (for reviews see Rouse et al., 1994; Miller, 1995; Volkering et al., 1998). However, the positive cases are counterbalanced by almost as many negative results. For instance, biodegradation of soil sorbed phenanthrene in several studies was enhanced by addition of nonionic alkylethoxylate surfactants (Aronstein et al., 1991; Aronstein and Alexander, 1992; Tsomides et al., 1995), while in another study similar alkylethoxylates had no effect or inhibited biodegradation of soil-sorbed phenanthrene (Laha and Luthy, 1991, 1992). Since the processes involved in the biodegradation of a contaminant are dependent on the physical state of the contaminant (i.e. dissolved, sorbed, solid, liquid, Fig. 1.2), it might be expected that the effect of surfactants depends on the physical state. However, positive and negative effects of surfactant addition have been observed irrespective of the initial state of the contaminant. For instance, the biodegradation of dissolved naphthalene was either be unaffected (Liu et al., 1995) or inhibited by Triton X-100 (Volkering et al., 1995), biodegradation of liquid aliphatic compounds by a Pseudomonas sp. was either enhanced (Breuil and Kushner, 1980) or inhibited (Lupton and Marshall, 1979) by surfactants, and the biodegradation of solid polycyclic aromatic hydrocarbons was either enhanced by surfactants (Mueller et al., 1990; Volkering et al., 1993, 1995; Tiehm, 1994; Mulder et al., 1998b) or was inhibited (Tiehm, 1994; Stelmack et al., 1999). Furthermore, no general distinction is observed between the effects of anionic or nonionic surfactants. The effect of surfactants is highly strain-dependent (Zhang and Miller, 1994; Churchill, P.F. and Churchill, 1997). It is often difficult to determine how the effects of surfactants on biodegradation come about, since often the effects of the surfactants on the separate processes (Fig. 1.2) have not been investigated (Oberbremer et al., 1990; Jain et al., 1992; Aronstein and Alexander, 1993; Miller, 1995; Providenti et al., 1995a; Volkering et al., 1998). In the following, some general conclusions are reviewed that can be derived from studies on surfactant-enhanced biodegradation.

Negative effects of surfactants on biodegradation may result from surfactant toxicity (Helenius and Simons, 1975; Tiehm,
General Introduction

Surfactant toxicity in turn may result from the disruption and permeabilization of membranes by surfactants (De la Maza et al., 1992) or from specific interactions of surfactants with proteins (Helenius and Simons, 1975). Especially cationic surfactants are toxic, which is not surprising given their strong interactions with the negatively charged membranes (Lupton and Marshall, 1979).

The effect of surfactants on the biodegradation of contaminants may be influenced by the biodegradation of the surfactants themselves in several ways. First, surfactants can be used preferentially to the contaminants, inhibiting the degradation rate of the latter (Laha and Luthy, 1991; Deschenes et al., 1996). Second, biodegradation of surfactants can yield energy or increase biomass, which can result in increased biodegradation rates of the contaminants. This may be especially profitable when the surfactant is the primary substrate and the contaminant is degraded cometabolically (Volkering et al., 1998). Actually, this concept is exploited by the construction of a surfactant-degrading strain that cometabolically converts PCB (Lajoie et al., 1994). The surfactant functioned as a primary substrate and increased the bioavailability of the PCB that was degraded cometabolically (Lajoie et al., 1997). Third, the biodegradation of a surfactant results in surfactant loss which may result in loss of the stimulating effect (Oberbremer et al., 1990). Fourth, biodegradation of surfactants may result in a deficiency in oxygen (Tiehm et al., 1997) or other nutrients. These deficiencies may in turn limit biodegradation of the contaminants.

The stimulation of mass transfer rates by surfactants is more important for determining their effect on contaminant biodegradation than is their effect on partitioning equilibria (Reddy et al., 1983). Often no direct relation is found between degradation rates and the extent of solubilization or emulsification of the substrate. For instance, surfactants added at submicellar concentrations enhanced the mineralization rate of phenanthrene sorbed to soil without increasing the (extent of) desorption. This suggests that the enhanced biodegradation was caused by the effect of surfactants on desorption kinetics (Aronstein et al., 1991; Aronstein and Alexander, 1993). In an other study, rhamnolipid stimulated the dispersion of n-octadecane by a factor 10^4 while it stimulated the biodegradation rate of this substrate by P. aeruginosa only four-fold (Zhang and Miller, 1992). The same was found for the surfactant-enhanced biodegradation of 2-methylnaphthalene (Churchill, S.A. et al., 1995).

In several cases, the effects of surfactants both on mass transfer rates and on the biodegradation kinetics were determined independently (Grimberg et al., 1996b; Zhang et al., 1997; Mulder et al., 1998b; Willumsen and Arvin, 1999). In these cases, the effect of surfactants on biodegradation can be predicted from their effect on mass transfer processes. Mulder et al. (1998b) and Grimberg et al. (1996b) showed that the effect of nonionic surfactants on biodegradation of phenanthrene by these strains could be well explained by an effect on dissolution of phenanthrene. These studies, that were performed with strains that take up dissolved substrate, indicate that the effect of surfactants on biodegradation rates in these cases was exclusively caused by their effect on dissolution rates.

When substrate is not taken up from the dissolved aqueous phase but by direct attachment of the cells to the substrate, surfactants may negatively affect uptake and biodegradation (Churchill, P.F. and Churchill, 1997). Surfactants render these substrate droplets hydrophilic and inhibit bacterial attachment (Foght et al., 1989; Efroymson and Alexander, 1991; Stelmack et al., 1999).
substrate than for cells that take up dissolved or solubilized substrate. The quantitative analysis of degradation kinetics for strains using the former uptake mode deserves further attention.

Several studies have investigated the effect of surfactants on biodegradation of contaminants in field-contaminated soil, as reviewed by Volkering (1998). Volkering concludes that more positive results generally were found during experiments with field-contaminated soil than with soil contaminated in the laboratory (Volkering et al., 1998). This might indicate that surfactants generally perform better during situations with lower bioavailability of contaminants. Few field-scale applications of surfactants during bioremediation have been reported (Volkering et al., 1998). It is possible that more field-scale experiments have been performed, e.g. by engineering companies, but that these have not appeared in the open literature.

**Biosurfactants and bioavailability.**

Similar to what was found for synthetic surfactants, the results obtained for biosurfactants are mixed. For instance, a rhamnolipid biosurfactant stimulated the biodegradation of sorbed phenanthrene by the indigenous populations in a creosote-contaminated soil but inhibited biodegradation of sorbed phenanthrene in a sandy loam soil (Providenti et al., 1995a). However, two additional interesting phenomena have been observed. First, a specific interaction has been found between organisms and biosurfactant, and second, a specific interaction is reported between substrate and biosurfactant (Hommel, 1990). These observations will be briefly described.

Several reports have suggested that a specific interaction exists between a biosurfactant and the biosurfactant-producing microorganism. However, this effect is well documented only for rhamnolipid and sophorolipid. Rhamnolipid enhanced the biodegradation by a rhamnolipid-producing strain to a greater extent than several other synthetic surfactants or biosurfactants (Itoh and Suzuki, 1972; Nakahara et al., 1981). Similarly, alkane utilization by *T. bombicola* was stimulated to a greater extent by the sophorolipid biosurfactant produced by the yeast than by other surfactants (Hommel, 1990).

The solubilizing or emulsifying activity of several biosurfactants was specific for certain substrates. For instance, a biosurfactant produced by *P. maltophilia* CSV89 had higher emulsifying activity for 1-naphthylaldehyde and substituted aromatics than for aliphatic compounds (Phale et al., 1995), the biosurfactant emulsan had highest emulsifying activity for a mixture of aliphatic and aromatic hydrocarbons (Rosenberg et al., 1979a), an emulsifying agent produced by *P. cepacia* AC1100 had a high specificity for 2,4,5-trichloroacetic acid and for chlorinated aliphatics (Banerjee et al., 1983), a biosurfactant produced by *Pseudomonas PG-1* showed a selectivity for pristane (Reddy et al., 1983), and the pseudosolubilizing activity in culture fluid of *Pseudomonas N1* was the highest for hexadecane (Goswami and Singh, 1991). The specificity of the emulsifying and solubilizing factors produced by *Endomycopsis lipolytica* was dependent on and highest for the growth substrate, suggesting that different biosurfactants are produced by this organism dependent on the substrate (Roy et al., 1979). The suggested substrate-specificity conveyed by these biosurfactants is intriguing, since these results suggest that molecular recognition occurs between the biosurfactant and the substrate. It is unlikely that the hydrophobic interior of a surfactant micelle can convey any solubilizing specificity. Specificity could arise when the substrate is encaptured in a three dimensional cavity of a (high molecular weight) biosurfactant (Roy et al., 1979), similar to the way substrate specificity is determined by the structure of an enzyme. This is a very inefficient manner of accommodating substrate, however. Furthermore, the specificity of the surfactant
was usually found for the compound used as growth substrate in the experiments (Roy et al., 1979; Reddy et al., 1983; Goswami and Singh, 1991; Phale et al., 1995), which must not be the physiological substrate.

**Knowledge gaps.** Despite the general trends outlined above, the effect of surfactants on the biodegradation of organic compounds is poorly predictable. Opposed effects are frequently observed. To advance the understanding of surfactant-enhanced biodegradation of organic compounds, three topics have to be further investigated. First, it needs to be established which of the rate-limiting processes are stimulated by surfactants and which of them are relevant. Second, insight is required in the interaction between a biosurfactant and the biosurfactant-producing microorganism. Third, it needs to be determined which factors determine whether a strain can profit from surfactant addition or not.

**OUTLINE OF THIS THESIS**

The work described in this thesis was aimed at establishing whether and how biosurfactants or biosurfactant-producing microorganisms stimulate the biodegradation of hydrophobic organic contaminants in soil. More specifically, it was focussed on obtaining more insight into the processes that can be stimulated by surfactants and the interaction between a biosurfactant and the biosurfactant-producing microorganism.

The processes that determine the bioavailability of contaminants are different for contaminants present in different physical state (Fig. 1.2). Therefore, we determined the effect of biosurfactants on processes that may limit the biodegradation rate both for adsorbed contaminants (Chapter 2 and 3) and for liquid contaminants (Chapter 6 and 7). The model compounds used in studies with adsorbed contaminants were the polycyclic aromatic hydrocarbons phenanthrene, fluorene, and naphthalene. The aliphatic compound n-hexadecane was used as a model substrate in the studies with liquid contaminants. Most of the work was performed with the rhamnolipid biosurfactant produced by *P. aeruginosa* (Fig. 1.4A). This biosurfactant was chosen since it is well characterized and because its stimulating effect on the biodegradation of hydrophobic substrates by *P. aeruginosa* was described in literature (Hitsasuka, 1971; Zhang and Miller, 1992). The results obtained for rhamnolipid cannot necessarily be generalized. However, the general understanding of biosurfactant-enhanced bioavailability can only result from insight in the function and performance of several specific biosurfactants.

In *Chapter 2*, the effect of rhamnolipid biosurfactants on the desorption of phenanthrene from four different soils is described. Column studies were used to determine the partitioning equilibria and desorption kinetics of phenanthrene in the presence of rhamnolipid. A two-component advective-dispersive model was developed that describes the enhanced removal of contaminants by solubilizing agents. The model accounts for sorption of the contaminant and the biosurfactant, for micellar solubilization, and for admicellar sorption. The effect of rhamnolipid on the removal of phenanthrene was satisfactorily simulated with this model by using independently obtained parameters. The results show that rhamnolipid enhanced the removal of phenanthrene mainly by micellar solubilization and also by influencing sorption kinetics.

*Chapter 3* describes the facilitated transport by rhamnolipid of the polycyclic aromatic hydrocarbons naphthalene, fluorene, and phenanthrene. The study was aimed at obtaining a more detailed insight into the importance of the interactions between (adsorbed) surfactant, contaminant, and soil. Therefore, three types of matrices were used to represent soil fractions. These matrices were silica, octadecyl-coated silica, and humic acid-coated silica. The model matrices allowed determination of the sorptive interactions in well defined, homogeneous
situations in the absence of non-equilibrium effects. Retardation factors for the contaminants were reduced by rhamnolipid at concentrations exceeding the CMC due to solubilization. At low rhamnolipid concentrations, retardation factors were increased due to admicellar sorption. However, adsorbed rhamnolipid reduced the affinity of the compounds to the surfactant-modified octadecyl-coated silica. Linear free-energy relations were used to make a general prediction of surfactant-enhanced transport based on the results determined for rhamnolipid. It was concluded that surfactants stimulate removal the more hydrophobic components to the greatest extent. Admicellar sorption is expected to be relatively pronounced for the less hydrophobic components.

Adsorption of rhamnolipid to soil is described in Chapter 4. The understanding of rhamnolipid adsorption is of importance for its application in soil (bio)remediation since adsorption results in surfactant loss and a concomitant loss in (solubilizing) activity, in a reduced surfactant mobility, and in admicellar sorption of contaminants. Adsorption of rhamnolipid to soil is especially of interest since rhamnolipid is a multicomponent surfactant. Adsorption of multicomponent surfactants is incompletely understood (Somasundaran and Krishnakumar, 1997). Most studies on adsorption of multicomponent surfactants have employed homogeneous sorbents. This study focuses on adsorption of individual surfactant components to the heterogeneous sorbent soil. It was found that the more hydrophobic components adsorbed preferentially. Therefore, the rhamnolipid composition changed during adsorption and transport. This resulted in changes in the CMC of the surfactant mixture and potentially in changes in other surface active properties.

In the work described in Chapter 5 it is assessed whether tracers can be used to determine the amount of liquid contaminants in soil and to determine their removal rates. Determination of these properties is complicated due to the heterogeneous distribution of these compounds in soil. However, determination of the amount of contaminant present and of their removal rates is of importance for estimating cleanup times, for risk assessment, or for following soil remediation in time. Tracers are compounds that are used to obtain information on a given system from the analysis of their transport properties. For this study, tracers were used that either partition between aqueous and nonaqueous phases (partitioning tracers) or that adsorb to the nonaqueous-aqueous interface (interfacial tracers). Due to this partitioning or adsorption, respectively, these tracers are transported more slowly through soil than a non-retarded compound. The retardation of the tracers compared to a non-retarded compound should therefore be dependent on the amount of liquid contaminant present in the soil and the nonaqueous-aqueous interfacial area, respectively. Hexadecane was used as a model compound. The results described in Chapter 5 show that retardation factors for partitioning tracers were indeed determined by the amount of hexadecane in the matrices, and that this was largely independent on the type of porous material. Furthermore, retardation factors for interfacial tracers were higher for columns where the removal rate of the hexadecane was higher. The results corroborate the possibility to use tracers to determine the amount of liquid contaminant in porous matrices and their removal rate. The contaminated matrices that were characterised in Chapter 5 were subsequently used in Chapter 6 to determine the effect of rhamnolipid on removal of liquid contaminants from the matrix and on their biodegradation.

In Chapter 6, the influence of rhamnolipid on the biodegradation of hexadecane by *P. aeruginosa* UG2 is reported. Hexadecane initially was present in porous matrices or as a second liquid phase.
The biodegradation rate of hexadecane present in silica and sea sand was limited by mass transfer of the contaminant from the matrix to the aqueous phase. For two other matrices with larger pore sizes, biodegradation was equally fast as biodegradation of hexadecane present as a second liquid phase. For the silica matrix, neither rhamnolipid nor any other surfactant tested could enhance these mass transfer rates during these experiments. For the other matrices and for hexadecane present as a second liquid phase, rhamnolipid and other surfactants increased the biodegradation rates. However, rhamnolipid caused a larger enhancement of the biodegradation rate than any of the 14 other surfactants tested. The effect of rhamnolipid on the mass transfer of hexadecane from the matrices to the aqueous phase was determined by using column studies. The outstanding stimulation by rhamnolipid of hexadecane biodegradation compared to the other surfactants tested could not be directly explained from its exceptional emulsifying activity, or from other physicochemical properties of the surfactants. The combined results suggest that the stimulating effect of surfactants on the biodegradation of hexadecane results from their specific effect on uptake of the substrate by the cells.

The objective Chapter 7 was to obtain insight in the specific interaction observed between rhamnolipid and \( P. \) aeruginosa strains. In the first part of this work it was determined whether hexadecane biodegradation by other biosurfactant-producing and hexadecane-degrading bacteria is also stimulated by their biosurfactants. The other strains used for this work were \( Acinetobacter \) calcoaceticus RAG1, \( Rhodococcus \) erythropolis DSM 43066, \( Arthrobacter \) paraffineus ATCC 19588, and an unidentified strain BCG112. These strains produce the biosurfactants emulsan, trehaloselipid (Fig. 1.4B), a membrane-associated biosurfactant, and a high molecular weight biosurfactant, respectively. None of these strains were stimulated by addition of their own biosurfactants or by addition of rhamnolipid to the same extent as \( P. \) aeruginosa UG2 was. This shows that the specific interaction that exits between rhamnolipid and \( Pseudomonas \) is not observed for the other strains. The second part of this work was aimed at identifying this specific interaction. This was accomplished by studying the effect of rhamnolipid of the uptake of hydrophobic compounds by \( P. \) aeruginosa UG2. The epoxidation rate of the model substrate \( \alpha,\omega \)-tetradecadiene by a whole-cell suspension of \( P. \) aeruginosa was enhanced by rhamnolipid. Stopped flow fluorescence analysis of the uptake of the hydrophobic probe 1-naphthylphenylamide by whole cell suspensions showed that the rhamnolipid-enhanced uptake was specific for rhamnolipid and energy-dependent. These results reveal the existence of a novel mechanism by which hydrophobic compounds are taken up. The enhancement of substrate uptake by rhamnolipid probably is the physiologically most important effect of rhamnolipid.

Chapter 8 provides a synthesis of the results from the previous chapters. It is discussed how the research described in this thesis contributes to attain the central objective of the project, namely to determine whether and how biosurfactants enhance the biodegradation of hydrophobic organic compounds in soil. Furthermore, implications are discussed of these results for application of biosurfactants for soil remediation.