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The enhancement by surfactants of hexadecane degradation by *Pseudomonas aeruginosa* varies with substrate availability

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Abstract

The rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* influences various processes related to hydrocarbon degradation. However, degradation can only be enhanced by the surfactant when it stimulates a process that is rate limiting under the applied conditions. Therefore we determined how rhamnolipid influences hexadecane degradation by *P. aeruginosa* UG2 under conditions differing in hexadecane availability. The rate of hexadecane degradation in shake flask cultures was lower for hexadecane entrapped in a matrix with 6 nm pores (silica 60) or in quartz sand than for hexadecane immobilized in matrices with pore sizes larger than 300 nm or for hexadecane present as a separate liquid phase. This indicates that the availability of hexadecane decreased with decreasing pore size under these conditions. The rate-limiting step for hexadecane entrapped in silica 60 was the mass transfer of substrate from the matrix to the bulk liquid phase, whereas for hexadecane present as a second liquid phase it was the uptake of the substrate by the cells. Hexadecane degradation in batch incubations was accelerated by the addition of rhamnolipid or other surfactants in all experiments except in those where hexadecane was entrapped in silica 60, indicating that the surfactants stimulated uptake of hexadecane by the cells. Since rhamnolipid stimulated the degradation rate in batch experiments to a greater extent than any of the other 14 surfactants tested, hexadecane uptake was apparently more enhanced by rhamnolipid than by the other surfactants. Although rhamnolipid did not stimulate the release of hexadecane from silica 60 under conditions of intense agitation, it significantly enhanced this rate during column experiments in the absence of strain UG2. The results demonstrate that rhamnolipid enhances degradation by stimulating release of entrapped substrate in column studies under conditions of low agitation and by stimulating uptake of substrate by the cells, especially when degradation is not limited by release of substrate from the matrices. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Biosurfactant; Surfactant; Biodegradation; Alkane; Bioavailability

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1. Introduction

The use of surfactants to overcome bioavailability-associated limitations during soil remediation has attracted considerable attention (Miller, 1995; Volkering et al., 1998). Positive effects of surfactants may result from a stimulation of dissolution or desorption rates (Volkering et al., 1995; Grimberg et al., 1996; Mulder et al., 1998; Willumsen and Arvin, 1999) or from surfactant-mediated dispersion, solubilization, or emulsification of poorly soluble substrates (Aronstein et al., 1991; Tiehm, 1994; Miller, 1995; Volkering et al., 1998). Negative effects may also occur, however, for example because a surfactant may be toxic or due to preferential biodegradation of surfactants (Miller, 1995; Volkering et al., 1998). Furthermore, surfactants may reduce attachment of cells to substrates that are present as a separate phase, which can decrease degradation rates if attachment is needed for uptake (Churchill and Churchill, 1997; Herman et al., 1997b). Despite these general trends, the effect of surfactants and biosurfactants on the biodegradation of organic compounds is poorly predictable.

The rhamnolipid biosurfactant produced by *P. aeruginosa* can stimulate the biodegradation of long-chain alkanes by this strain, both when these compounds are present as a separate liquid phase (Itoh and Suzuki, 1972; Nakahara et al., 1981; Koch et al., 1991; Zhang and Miller, 1992) and when the substrate is present as a residual non-aqueous phase in soil (Herman et al., 1997b). Although substrate dispersion may be required, the stimulation of dispersion by rhamnolipid was not reflected by a proportional increase of the degradation rate (Zhang and Miller, 1994, 1995). Therefore, it was concluded that rhamnolipid stimulated degradation both by enhancing dispersion of substrate and by increasing cell surface hydrophobicity (Zhang and Miller, 1994, 1995; Herman et al., 1997b). In contrast, inhibition of octadecane degradation by rhamnolipid also occurred, which may have resulted from the interference of cell-hydrocarbon interaction by the surfactant (Zhang and Miller, 1994). Recently, it was shown that rhamnolipid extracts lipopolysaccharides (LPS) from cells of *Pseudomonas*, thereby

increasing the hydrophobicity of the cell surface and promoting attachment of the cells to hydrocarbon droplets (Al-Tahhan et al., 2000). It was suggested that this greater attachment stimulates hexadecane degradation (Al-Tahhan et al., 2000). When hexadecane was present as a residual liquid in soil, rhamnolipid increased degradation by some organisms but inhibited degradation by other strains (Herman et al., 1997a,b). The stimulating effect of rhamnolipid was attributed to enhanced transport of substrate to the bacteria and the inhibitory effect to rhamnolipid-induced flocculation of the cells. It is known that the stimulation of many *P. aeruginosa* strains is more pronounced for rhamnolipid than for other surfactants (Itoh and Suzuki, 1972; Nakahara et al., 1981), but the reason behind this specificity is unknown.

It has become clear that rhamnolipid stimulates different processes related to the degradation of organic substrates. Since several steps are involved in the degradation of a poorly soluble compound, a biosurfactant will only enhance degradation when the step that is stimulated is rate limiting. The step that is rate limiting may differ between different conditions. Therefore, the extent to which rhamnolipid enhances the degradation rate or even the way how the surfactant influences this rate probably depends on the form in which the substrate is present.

The goal of this work was to obtain insight into how rhamnolipid stimulates degradation of hexadecane by *P. aeruginosa* UG2 under conditions differing in the availability of the substrate. The availability varied by using hexadecane that occurs as a separate liquid phase and hexadecane present in different matrices varying in pore size. Furthermore, different hydrodynamic conditions were employed, i.e. conditions of high agitation as present during shake flask experiments and conditions of low agitation as observed during continuous flow operation of columns packed with the contaminated matrices. The approach was to determine which step in the degradation was rate limiting under these different conditions and to subsequently investigate whether rhamnolipid stimulated that step. In the analysis we discerned three basic steps: mass transfer of inaccessible

substrate from the matrix to the bulk phase, solubilization or emulsification of the substrate to a form that can be taken up by the cells, and uptake of (solubilized or emulsified) substrate by the cells. Hexadecane was used as the model substrate, since it is easily degraded by strain UG2 and has an extremely low aqueous solubility. It is anticipated that the results with this model substrate are relevant for rhamnolipid-enhanced degradation of all liquid hydrophobic compounds that are a substrate for strain UG2. To determine which step in the degradation of hexadecane was rate limiting, degradation studies in shake flasks were conducted. Subsequently, it was determined whether the degradation could be stimulated by surfactants. To better understand which step in the degradation by *P. aeruginosa* was stimulated by surfactants, the effect of rhamnolipid on degradation and emulsification was compared to the effect of several other surfactants. Column studies were conducted in the absence of cells to determine whether rhamnolipid enhanced mass transfer of hexadecane from the matrix to the aqueous under conditions of low agitation.

2. Materials and methods

2.1. Microorganisms

The hexadecane-degrading and rhamnolipid-producing bacterium *P. aeruginosa* UG2 is a soil isolate and was provided by Dr J.T. Trevors (University of Guelph, Canada) (Berg et al., 1990). *P. aeruginosa* PG201, a strain that also degrades hexadecane and produces rhamnolipid, and the mutant PG201::*rhII*, a strain deficient in rhamnolipid synthesis, were obtained from Dr U.A. Ochsner (University of Colorado, USA) (Ochsner and Reiser, 1995).

2.2. Chemicals

Hexadecane (99%) was obtained from Acros (Geel, Belgium). The surfactants used for this study were obtained from Sigma (St. Louis, MO), except for sodium dodecylbenzenesulfonate that

was obtained from Aldrich (Milwaukee, WI) and Triton X-100 that was obtained from BDH Chemicals (Vancouver, Canada). Values for the hydrophile–lipophile balances and critical micelle concentrations of the surfactants were obtained from the suppliers unless indicated otherwise.

2.3. Rhamnolipid

Rhamnolipid was produced by *P. aeruginosa* UG2 during growth on glucose in a mineral salts medium (Van-Dyke et al., 1993), isolated from the culture broth by a series of consecutive steps of acid precipitation and dissolution in 50 mM NaHCO_3 , and purified by column chromatography over Sephadex LH20 with methanol as the eluent (Noordman et al., 2000a).

2.4. Matrices

The matrices used were silica 60 (particle size 40–63 μm , pore size 6 nm, specific surface area $4.9 \times 10^2 \text{ m}^2 \text{ g}^{-1}$, Merck, Darmstadt, Germany), quartz sea sand (particle size 100–300 μm , Merck), controlled pore glass (CPG-10-3000, particle size 60–125 μm , pore size 300 nm, specific surface area $7.8 \text{ m}^2 \text{ g}^{-1}$, Electro Nucleonics Inc., Fairfield, NJ), VitraPOR P3 filter candles (3 mm in diameter and 3 mm in height, pore size 16–40 μm , Elgebe, Leek, The Netherlands), and coarse glass beads (particle size 1000 μm , Fisher Scientific, Den Bosch, The Netherlands). The specific surface area of sea sand was $0.74 \text{ m}^2 \text{ g}^{-1}$, as determined by gas-physisorption using nitrogen (Chiou et al., 1990). The specific surface area of the glass beads was calculated to be $2.6 \times 10^{-3} \text{ m}^2 \text{ g}^{-1}$ from $4\pi r^2$, assuming perfect spherical geometry and using 0.64 as the fraction of the total volume occupied by the spheres (random close packing).

Matrices were contaminated by adding 70–930 mg hexadecane to 50 g matrix suspended in 100 ml pentane. After incubation for approximately 12 h, the pentane was slowly removed using a rotary evaporator. Since the volatility of pentane is high (boiling point under ambient pressure 35–36 °C) and reduced pressure was used, it can be assumed that pentane was completely removed.

The initial concentration of hexadecane in the matrices was determined by extraction of 100 mg matrix with 2 ml isooctane and analysis of the isooctane layer by gas chromatography (GC). The analysis was performed in triplicate and the standard error was 5%.

2.5. Media and growth conditions

The mineral salts medium was made up in doubly distilled water and contained 0.53 g l^{-1} $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.14 g l^{-1} KH_2PO_4 , 0.2 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$, and 5 ml l^{-1} of a trace element solution (Janssen et al., 1984) or was made up demineralized water and contained 10-fold less trace elements and magnesium. The pH was adjusted to 7.0. No differences between the media were observed with respect to the biodegradation of hexadecane or glucose by strain UG2. All experiments were performed in 100 ml flasks containing 20 ml mineral salts medium at $30 \text{ }^\circ\text{C}$ on a rotary shaker (200 rpm), unless mentioned otherwise. Precultures were grown on the same substrate as that used for the main culture (i.e. hexadecane, citrate or pyruvate).

2.6. Biodegradation experiments

For the biodegradation experiments with substrate entrapped in matrices, 250 mg of contaminated matrix was aseptically placed in 8 ml tightly closed culture flasks. For the experiments employing substrate present as a separate liquid phase, the required amount of a 1:10 (v:v) solution of hexadecane in pentane was added to each 8 ml culture flask, after which the pentane was allowed to evaporate by incubation for at least 5 h in a sterile cabinet. This period should result in complete removal of pentane due to its high volatility. However, any residual pentane would unlikely have influenced the experiments since UG2 cannot utilize pentane as growth substrate. A preculture was grown for three days with hexadecane to a protein content of $30\text{--}70 \text{ mg protein l}^{-1}$ and was used to prepare the inoculated culture with a culture density of $2 \text{ mg protein l}^{-1}$. Surfactants were added to portions of this inoculated culture from sterile aqueous stock solutions. A portion of

1 ml of inoculated culture was added to each culture flask, and the closed flasks were incubated at $30 \text{ }^\circ\text{C}$ under end-over-end rotation at 1.4 or 39 rpm. This difference in mixing rates had no effect on the biodegradation rate of hexadecane entrapped in silica 60 and hexadecane present as a separate liquid phase. The amount of residual substrate (C, mg hexadecane l^{-1} culture fluid) was monitored by sacrificing separate flasks of the incubated parallel cultures at least in duplicate (substrate depletion curves) or triplicate (when the effect of added surfactants was determined). Student's *t*-test was used to determine whether the differences between the amount of residual substrate for the different surfactants additions were significant. The amount of hexadecane was determined by extracting the contents of the culture flasks with 0.05 ml of 10% HCl, 2 ml of ethanol, and 1 ml of isooctane. The isooctane phase was analyzed by GC after vigorous agitation for 5 min and centrifugation at 4000 rpm when necessary to separate phases. Controls showed that no hexadecane disappeared from the culture flasks by abiotic processes. The amount of oxygen in the flasks was calculated to be sufficient to allow complete oxidation of hexadecane. The fact that hexadecane biodegradation or growth was not faster in the presence of additionally added oxygen or by using a mineral medium containing a higher concentration of inorganic nutrients also showed that biodegradation was not limited by a deficiency in oxygen or inorganic nutrients.

The production of rhamnolipid during degradation of hexadecane was determined by analyzing the rhamnolipid concentration in a filtered culture supernatant after 5 days of growth. Filtered culture supernatants were obtained by centrifugation of 1 ml samples for 5 min at 6000 rpm and filtration of the supernatants using nylon disposable filters (4 mm in diameter, $0.2 \text{ } \mu\text{m}$ pore size, Alltech). The toxicity of the surfactants was tested by cultivating strain UG2 with 5 mM citrate in the absence or presence of 500 mg l^{-1} surfactants and following the optical density of the culture in time. The effect of rhamnolipid on growth with pyruvate was determined by following the pyruvate concentration in time of cultures growing with 3 mM pyruvate in the absence and presence

of 500 mg l^{-1} rhamnolipid. For testing the toxicity associated with the matrices, strain UG2 was grown with 3 mM pyruvate in 10 ml medium with 1.5 g of a matrix material and the pyruvate concentration was monitored in time. Since biodegradation of pyruvate was unaffected by the presence of any of these matrices, it can be concluded that no toxicity was associated with them. The biodegradation of surfactants during growth with hexadecane was determined by measuring the surfactant concentration in a filtered culture supernatant after 5 days of cultivation. The initial surfactant concentration was 100 mg l^{-1} (synthetic surfactants) or 20, 100, or 500 mg l^{-1} (rhamnolipid). The use of surfactants as sole source of carbon and energy was determined by following the optical density in time of cultures containing 500 mg l^{-1} surfactant in the absence of any other potential carbon source. Growth curves for strain UG2 with hexadecane were determined in 1 l flasks filled with 200 ml mineral medium and 50 μl of hexadecane.

2.7. Column studies

Column studies with hexadecane-contaminated matrices were performed as described previously (Noordman et al., 2000b). Stainless steel preparative HPLC columns (length 7.0 cm, i.d. 2.2 cm) were packed with matrices that were contaminated with approximately $6 \text{ mg hexadecane g}^{-1}$ matrix. The initial amount of hexadecane in the columns differed by less than a factor 3 (Table 1). The electrolyte solution used for the column studies was mineral salts medium supplemented with $0.2 \text{ g l}^{-1} \text{ NaN}_3$ to prevent biodegradation of the substrate and surfactants. The columns were placed vertically and elution was performed with an upward flow. After saturation of the columns by elution with 100 ml min^{-1} electrolyte solution using a flow rate of 0.1 ml min^{-1} , the concentration of the hexadecane was determined by GC analysis of five 7 ml effluent fractions. Subsequently, tracer experiments were done which lasted for up to 20 pore volumes (Noordman et al., 2000b). The breakthrough profiles of the conservative tracer potas-

sium bromide indicated that the columns were packed homogeneously and that physical non-equilibrium effects were absent. After the tracer experiment, the hexadecane-containing columns were eluted with 400 ml electrolyte solution containing 500 mg l^{-1} rhamnolipid using a flow rate of 0.1 ml min^{-1} . In the experiment with hexadecane-contaminated silica, the column was eluted with rhamnolipid-containing solution for 69 h after which the flow was interrupted for 13 h. Subsequently, after continuation of the flow, the amount of hexadecane in the effluent was determined for an additional period of 6 h.

Samples of 7 ml of the column effluents were collected in extraction tubes. These fractions were analyzed either for hexadecane by GC after extraction of the samples with 1 ml iso-octane and 0.05 ml 10% HCl or for rhamnolipid by HPLC. The residual concentrations of hexadecane in the columns at the end of the experiments were determined by GC after extraction of samples taken from the top, from the bottom, and from the mixed content of the column (approximately 200 mg, in triplicate) with 2 ml ethanol, 150 μl water and 2 ml iso-octane. The exact amount of matrix that was used for the extraction was determined gravimetrically after filtration and drying of the extracted suspension for at least 16 h at $80 \text{ }^\circ\text{C}$. In this way, the residual hexadecane concentration in the matrices was determined with a standard error of 10%. Mass balances for hexadecane were 85–104%.

2.8. Emulsification of hexadecane

Emulsions of hexadecane and water were prepared by vortexing for 30 s using a phase ratio of 1:8 (v:v). The amount of hexadecane present in the aqueous phase was determined by placing 9 ml of these emulsions in 20 ml vials closed with septa. The vials were placed upside down. After incubation for 1 h, the largest droplets were floated and triplicate samples of 1 ml were taken from the aqueous phase and analyzed by GC. The particle size and size distribution for

Table 1
Effect of rhamnolipid (RL) on the elution of hexadecane from columns packed with hexadecane-contaminated matrices

Matrix	Bulk density (kg l ⁻¹)	Porosity	Hydrodynamic residence time (h per pore volume)	Initial amount of hexadecane present (mg)	Amount of hexadecane in column effluent (mg l ⁻¹)		Breakthrough of rhamnolipid	
					Before application of RL	After breakthrough of RL ^a	Time (h)	Pore volumes
Silica 60	0.49	0.70	3.1	92	0.38 ± 0.1	56 to 20	33	11
Sea sand	1.40	0.44	2.0	238	0.31 ± 0.07	36 to 16	6	3
CPG-10-3000	0.34	0.76	3.8	64	0.0077 ± 0.0012	24 to 5	6	2
Coarse glass beads	1.60	0.40	1.5	179	0.0012 ± 0.0011	4.0 to 1.8	5	3

^a Range in the amount of hexadecane observed in the column effluent after breakthrough of rhamnolipid.

these emulsions were determined 15 min after their preparation using a Nicomp submicron particle sizer (model 370, Particle sizing systems, Santa Barbara, CA) with NICOMP software.

2.9. Analytical procedures

Culture densities were determined by measuring optical densities at 450 nm (OD_{450} , Hitachi 100-60 spectrophotometer) or by determining the protein concentration with the Folin reagent after alkaline solubilization of the cell suspension. Pyruvate was determined using a colorimetric assay with KOH and salicylic aldehyde in samples of 200 μ l (Snell and Snell, 1953). Hexadecane was analyzed by gas chromatography (Hewlett–Packard model 6890) using a HP5 capillary column (Hewlett–Packard) and a flame ionization detector. The carrier gas (He) pressure was 97 kPa. The temperature program started at 120 °C and increased with 8 °C min^{-1} to 200 °C. Splitless injection with a 1 min pressure ramp of 250 kPa was used for analyzing samples with a low hexadecane concentration. Rhamnolipid was analyzed by HPLC using evaporative light scattering detection (ELSD) (Noordman et al., 2000a). SDBS was analyzed by HPLC using UV detection at 228 nm (Noordman et al., 2000b). The nonionic surfactants Brij 30 (polyoxyethylene(4)dodecyl ether), Brij 35 (polyoxyethylene(23)dodecyl ether), and polyoxyethylene(10)dodecyl ether were analyzed using HPLC-ELSD with gradient elution. For the latter analysis, a gradient was used that started at 20% acetonitrile (A), 60% water containing 0.05% trifluoroacetic acid (B), and 20% isopropanol (C). This composition was changed linearly to 20% A, 35% B, and 45% C from 5 to 20 min, and was subsequently kept constant for 3 min. The flow rates were 0.5 ml min^{-1} . The HPLC setup consisted of a Spark Basic Marathon autosampler (Spark Holland BV, Emmen, The Netherlands), a Merck L-6200 pump (Hitachi, Ltd., Tokyo, Japan), a Chromosphere PAH 100 mm column (Chrompack, Bergen op Zoom, The Netherlands), a Merck L-4200 UV–VIS detector (Hitachi), and an evaporative light scattering detector (MARK III, Varex, Burtonsville, USA).

3. Results

3.1. Degradation of hexadecane by *P. aeruginosa* in shake flasks

To determine which step in the degradation of hexadecane by the rhamnolipid-producing organism *P. aeruginosa* UG2 was rate limiting under conditions differing in substrate availability, degradation of hexadecane was followed in batch incubations where the substrate was either present as a separate liquid phase or present in various porous matrices. The initial amount of hexadecane (C_0) was 30–2000 $\text{mg hexadecane l}^{-1}$ culture fluid or 0.004–0.3% (v/v). These amounts exceeded the aqueous solubility of hexadecane, which is 0.0036 mg l^{-1} (Schwarzenbach et al., 1993). The culture density in the experiments with hexadecane present as a separate liquid phase at $C_0 = 350 \text{ mg l}^{-1}$ increased 40-fold over the incubation time, indicating that biodegradation of hexadecane resulted in growth. The yield was 0.75 $\text{mg protein mg}^{-1}$ hexadecane, which is similar to previously determined values for aliphatic hydrocarbons (Bailey and Ollis, 1986).

The initial degradation rate of hexadecane present as a separate liquid phase was determined as a function of the initial amount of substrate present to determine which step is rate-limiting under these conditions. The initial degradation rate was independent of C_0 in the range of 100–2000 mg l^{-1} for cultures with identical initial cell densities of 2 mg protein l^{-1} (Fig. 1A). Analysis of the combined data of five substrate depletion curves yielded an initial rate of $3.6 \pm 0.1 \text{ mg l}^{-1} \text{ h}^{-1}$ (0–45 h, $r^2 = 0.9994$, $n = 16$, Fig. 1A). This rate is expected to be linearly dependent on the culture density. Growth curves of strain UG2 indeed were exponential up to an OD_{450} of 0.4, with a growth rate of 0.02 h^{-1} (Fig. 2). When dissolution or emulsification of substrate would have been rate limiting, linear growth would be expected and the degradation rate would be related to the amount of substrate present (Volkering et al., 1992, 1995). Since this was not observed, the rate-limiting step under these conditions was not the dissolution or emulsification of substrate but rather the uptake or the degradation

thereof. However, at very high cell densities the rate-limiting step eventually will be the dissolution or emulsification of the substrate (Nakahara et al., 1981).

To determine the rate-limiting step in the degradation of hexadecane under conditions where its availability was lower than when it was present as a separate liquid phase, the rate of degradation was determined for hexadecane that was present in porous matrices. Pronounced differences between biodegradation rates were observed for different porous matrices (Fig. 3). The degradation rates of substrate entrapped in silica 60 (pore size 6 nm) and sea sand were lower than when substrate was present as a separate liquid phase, showing that the availability of hexadecane en-

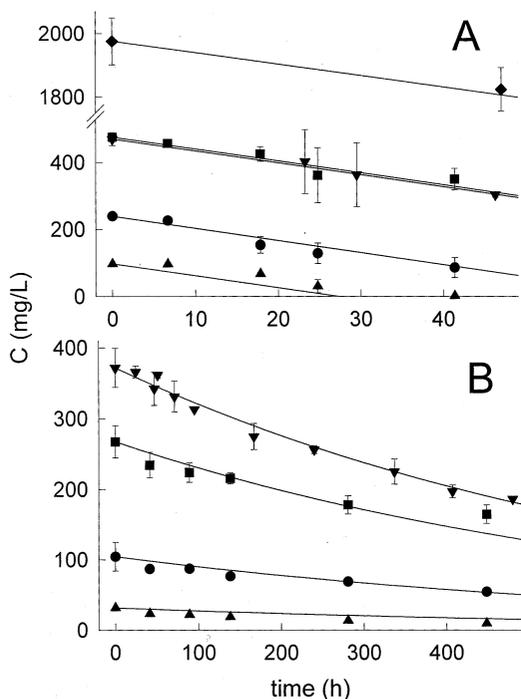


Fig. 1. Effect of C_0 on the initial biodegradation rate of hexadecane by *P. aeruginosa* UG2 in shake flask cultures. (A) Substrate present as a separate liquid phase. C_0 was 1976 mg l⁻¹ (◆); 477 mg l⁻¹ (■); 471 mg l⁻¹ (▼); 239 mg l⁻¹ (●); and 98 mg l⁻¹ (▲). (B) Substrate entrapped in silica 60. C_0 was 372 mg l⁻¹ (▼); 268 mg l⁻¹ (■); 105 mg l⁻¹ (●); or 32 mg l⁻¹ (▲). Batches were sacrificed in time in triplicate (error bars indicate standard deviation), except for the data labeled with ▼ where batches were sacrificed in duplicate (error bars indicate spread in measured values).

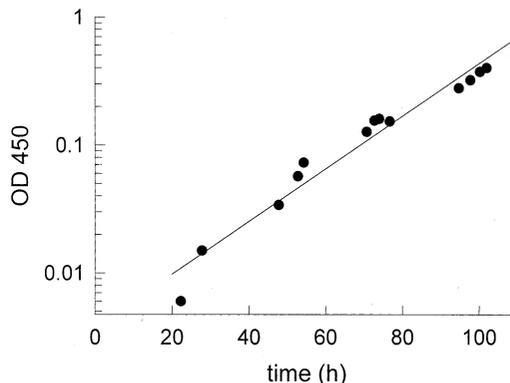


Fig. 2. Growth curve for *P. aeruginosa* UG2 growing with hexadecane in two-liquid phase medium in shake flask cultures. Data represent the average of two parallel cultures. C_0 was 188 mg l⁻¹.

trapped in these latter matrices was relatively low (Fig. 3). The degradation rate of hexadecane present in silica 60 decreased in time (Figs. 1B and 3) and increased with increasing C_0 (Fig. 1B). A series of four substrate depletion curves could be fitted to an exponential decay function with a first-order rate constant of $(1.49 \pm 0.05) \times 10^{-3} \text{ h}^{-1}$ ($r^2 = 0.998$, $n = 25$, Fig. 1B). This indicates that the rate-limiting step in the degradation of hexadecane entrapped in silica 60 was the mass transfer of substrate from the matrix to the aqueous phase. In contrast, the degradation rate of hexadecane entrapped in CPG-10-3000 (pore

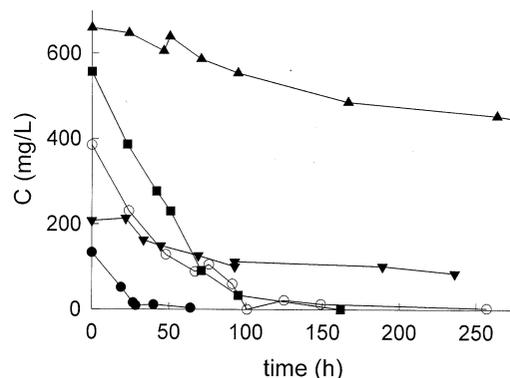


Fig. 3. Biodegradation of hexadecane by *P. aeruginosa* UG2 in shake flask cultures. Substrate was initially entrapped in silica 60 (▲); sea sand (▼), CPG-10-3000 (■), VitraPOR P3 (●), or was present as a separate liquid phase (○). Batches were sacrificed in time in duplicate.

size 300 nm) and VitraPOR P3 (pore size 16–40 μm) was similar to when it was present as a separate liquid phase (Fig. 3), showing that degradation was not limited by mass transfer of the substrate from the matrix to the aqueous phase. The relatively low degradation rate of hexadecane present in sea sand will be discussed after describing the column experiments. The fact that biodegradation was limited by mass transfer for silica with 6 nm pores but not for the matrices with pores of 300 nm and 16–40 μm suggests that under the conditions of intensive mixing mass transfer was limiting only when the pore size of the matrix was small.

3.2. Effect of surfactants on the degradation of hexadecane in shake flasks

The effect of rhamnolipid and 14 synthetic surfactants on the biodegradation of hexadecane in two-liquid phase media was determined. Since the rate-limiting step under these conditions either was the uptake or conversion of substrate, any stimulation of the degradation rate by surfactants should result from a stimulation of this step. Mixed effects were observed (Figs. 4A and 5). Rhamnolipid and almost all ethoxylated nonionic surfactants stimulated degradation of hexadecane, including Brij 30, Brij 35, $\text{C}_{12}\text{E}_{10}$, and Triton X-100 ($P \leq 0.05$, Fig. 5). The anionic surfactant SDBS and the nonionic ethoxylated surfactant Brij 78 inhibited hexadecane degradation, whereas the carbohydrate-containing nonionic surfactants did not affect degradation (Fig. 5). Most strikingly, rhamnolipid stimulated degradation to a greater extent than any of the other surfactants at the same concentration ($P < 0.05$, Fig. 5). No inhibitory effects of rhamnolipid were observed (Fig. 4A), in accordance with results with strain *P. aeruginosa* ATCC 9027 (Zhang and Miller, 1992), but in contrast to later work with the same strain (Zhang and Miller, 1994). Since it is unlikely that rhamnolipid stimulated the intracellular metabolism of hexadecane, it is suggested that uptake was the rate-limiting step under these conditions of high agitation and that rhamnolipid enhanced degradation by stimulating the uptake of the substrate by the cells.

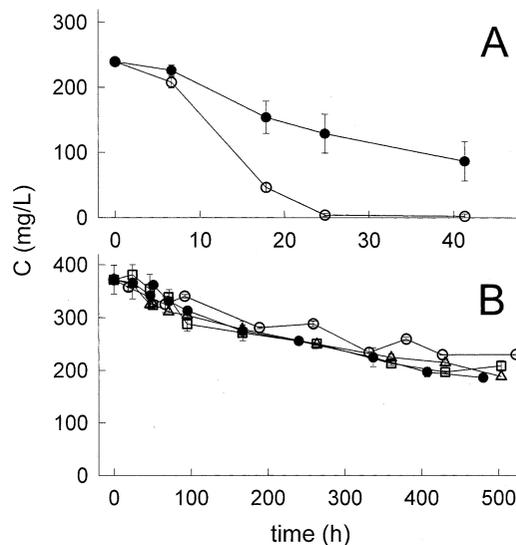


Fig. 4. Effect of rhamnolipid on the biodegradation of hexadecane initially present as a separate liquid phase (A) or in silica 60 (B) in shake flask cultures. Batches were sacrificed in time in duplicate. The concentration of rhamnolipid added was 0 mg l^{-1} (●), 20 mg l^{-1} (□), 100 mg l^{-1} (△), or 500 mg l^{-1} (○).

It was determined whether rhamnolipid could stimulate the degradation of hexadecane present in porous matrices, in order to reveal whether rhamnolipid stimulated the mass transfer under the conditions of intense agitation. The effect of rhamnolipid was compared to the effect of synthetic surfactants. When hexadecane initially was present in silica 60, the addition of rhamnolipid to the cultures did not enhance the rate of biodegradation (Fig. 4B). The effect of rhamnolipid and other surfactants on the biodegradation of hexadecane present in silica 60, sea sand, CPG-10-3000, and VitraPOR P3 was determined by measuring the amount of residual hexadecane in shake flasks with and without added surfactant. Cultures were analyzed at the time where approximately half of the substrate was degraded in the control incubations to which no surfactant had been added.

For hexadecane entrapped in silica 60, the rate of degradation was not enhanced by addition of rhamnolipid and the nonionic alcohol ethoxylates Brij 30, Brij 35, and polyoxyethylene(10)dodecyl ether ($\text{C}_{12}\text{E}_{10}$) (Fig. 6A). The addition of the

anionic surfactant sodium dodecylbenzenesulfonate (SDBS) also did not stimulate degradation of hexadecane entrapped in silica 60, as determined after 250 h of incubation. Apparently, the mass transfer of hexadecane from the matrix to the aqueous phase was not stimulated by any of the surfactants used under the conditions of intensive mixing as applied in these experiments. In contrast to the results with silica 60, degradation of hexadecane entrapped in sea sand and CPG-10-3000 was enhanced by rhamnolipid at all concentrations tested, including the submicellar concentration of 20 mg l⁻¹ (Fig. 6B and C). The nonionic alcohol ethoxylates also enhanced biodegradation of hexadecane entrapped in sea sand (Fig. 6B) but SDBS retarded hexadecane biodegradation when the substrate was entrapped in sea sand or CPG-10-3000 (Fig. 6B and C). For hexadecane entrapped in VitraPOR P3, biodegradation was stimulated by rhamnolipid (100 and 500 mg l⁻¹) but not by Brij 30 (100 mg l⁻¹). The stimulation by rhamnolipid exceeded the stimula-

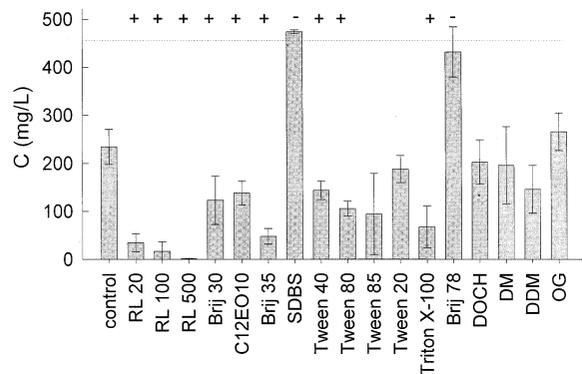


Fig. 5. Effect of surfactants on the degradation of hexadecane present as a separate liquid phase in shake flask cultures. Rhamnolipid was added to a concentration of 20, 100, and 500 mg l⁻¹ for the bars labeled RL 20, RL 100, and RL 500, respectively. Synthetic surfactants were added at a concentration of 100 mg l⁻¹. DOCH, DM, DDM, OG indicate sodium deoxycholate, *n*-decyl-*b*-D-maltoside, *n*-dodecyl-*b*-D-maltoside, and *n*-octyl-*b*-D-glucoside, respectively. For each surfactant, five parallel cultures were sacrificed after 45 h of cultivation and analyzed for residual hexadecane. The symbol '+' indicates that biodegradation was stimulated compared to the control ($P < 0.05$) and '-' indicates that biodegradation was inhibited ($P < 0.05$). The dotted line indicates the initial substrate concentration.

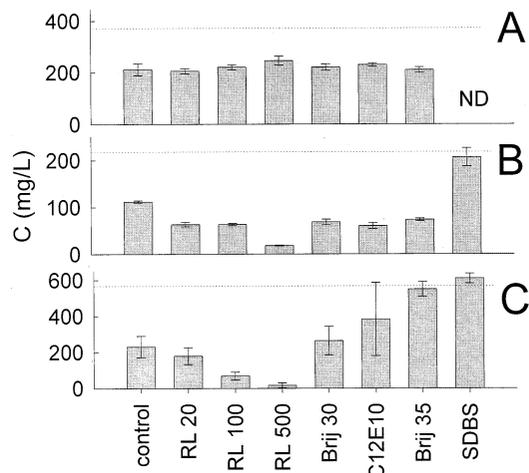


Fig. 6. Effect of surfactants on the biodegradation by *P. aeruginosa* UG2 of hexadecane present in matrices in shake flask cultures. No surfactant was added to the control. Rhamnolipid was added to a concentration of 20, 100, and 500 mg l⁻¹ for the bars labeled RL 20, RL 100, and RL 500, respectively. Other surfactants were added at a concentration of 100 mg l⁻¹. Substrate was entrapped in silica 60 (A), sea sand (B), or CPG-10-3000 (C). Batches were analyzed in triplicate for residual hexadecane after 433, 93, and 51 h of cultivation for silica 60, sea sand, and CPG-10-3000, respectively. The dotted line indicates the initial substrate concentration.

tion by any of the other surfactants for all matrices ($P < 0.05$).

A comparison of the effects of surfactants on the degradation of hexadecane in different matrices shows that surfactants stimulated the biodegradation of hexadecane to a greater extent for matrices where the availability of the substrate was relatively high and biodegradation was fast. This suggests that the stimulation by surfactants of the mass transfer of hexadecane from the matrix to the aqueous phase was not the most important effect of the surfactants under the conditions of these experiments.

Only low amounts of rhamnolipid were produced by strain UG2 in our mineral salts medium, either when growing with glucose or with hexadecane. Rhamnolipid production by this strain is higher in a medium with low concentrations of trace elements (Van-Dyke et al., 1993). However, to determine whether the amount of rhamnolipid that was produced by the wild-type strains in

these experiments ($< 2 \text{ mg l}^{-1}$) had any effect on the biodegradation rate, hexadecane degradation by *P. aeruginosa* strain PG201 was compared to degradation by the rhamnolipid-deficient mutant PG201::*rhlI*. The rate of biodegradation of hexadecane by both strains was the same, both when hexadecane was present as a separate liquid phase and when it was entrapped in silica 60. Therefore, it can be assumed that the cultures to which no surfactant was added represented a situation where rhamnolipid was absent.

It was determined whether the surfactants used here were degraded by *P. aeruginosa*, to reveal whether the observed stimulation of hexadecane degradation was caused by an increase in culture density resulting from surfactant biodegradation (Volkering et al., 1998). The amounts of rhamnolipid and SDBS degraded during growth with hexadecane were negligible compared to the amount of hexadecane degraded in this period. Substantial amounts (10–90%) of Brij 30, Brij 35, and $\text{C}_{12}\text{E}_{10}$ were degraded during growth with hexadecane for five days, but *P. aeruginosa* could not use these surfactants as sole source of carbon and energy. Therefore, the stimulation of hexadecane degradation was not caused by growth with these surfactants. However, the biodegradation of these surfactants could have masked a stimulating effect on hexadecane biodegradation.

To determine whether the inhibition of hexadecane biodegradation by some of the surfactants was caused by surfactant toxicity, the effect of several surfactants on growth of strain UG2 with well-soluble and easily degradable substrates was determined. Rhamnolipid, Brij 30, Brij 35, and $\text{C}_{12}\text{E}_{10}$ had no effect on the growth of *P. aeruginosa* with citrate or on the degradation of pyruvate. SDBS caused retarded growth of strain UG2 with citrate and reduced the final optical density of the culture compared to growth in its absence. These results show that the surfactants were not toxic, except for SDBS. The toxicity of SDBS could have been the cause of the inhibition of hexadecane biodegradation by this surfactant (Figs. 5 and 6B, C). It furthermore seems that the biosurfactant only stimulates degradation of hydrophobic compounds.

3.3. Column studies

Since it was surprising that rhamnolipid did not stimulate mass transfer of hexadecane that was entrapped in silica 60 to the aqueous phase during conditions of intense agitation that were applied in the shake flask experiments, it was determined whether the biosurfactant enhances mass transfer during column studies. Packed bed columns present conditions of less agitation and hydrodynamic conditions that are similar to those during *in situ* soil remediation. Due to the fact that no cells were present, the effect of rhamnolipid on mass transfer could be determined independent from its effect on degradation of hexadecane. Columns were packed with hexadecane-contaminated silica 60, sea sand, CPG-10-3000, or coarse glass beads, and were first eluted with electrolyte solution. The amount of hexadecane in the column effluent in the absence of rhamnolipid was measured directly after saturation of the columns with electrolyte solution (Table 1). Since the amount of hexadecane in the column effluent exceeded its aqueous solubility ($3.6 \times 10^{-3} \text{ mg l}^{-1}$ (Schwarzenbach et al., 1993)) for columns packed with CPG-10-3000, sea sand, and silica 60, hexadecane was eluted from these columns partly in solubilized or emulsified state (Table 1). Flow interruption experiments have shown that hexadecane elution from the columns packed with contaminated silica 60 was limited by mass transfer rates of hexadecane from the matrix to the aqueous phase (Noordman et al., 2000b). Furthermore, since the residual amounts of hexadecane in the matrix samples taken after the experiment from the top, bottom, and from the mixed column content of columns packed with silica 60 and sea sand were equal, hexadecane was removed from the column material to the same extent in all positions (Noordman et al., 2000b). This implies that the rate with which hexadecane was removed from the columns was dependent on the mass transfer rate of hexadecane from the matrix to the mobile phase and not on its subsequent transport velocity.

After saturation of the columns with electrolyte solution, they were eluted with electrolyte solution containing rhamnolipid. A rhamnolipid concen-

tration of 500 mg l^{-1} was used since this was optimal for stimulating the elution of hexadecane from columns packed with contaminated sand (Bai et al., 1997). After elution with 2–11 pore volumes with the biosurfactant solution, rhamnolipid was detected in the effluent (Table 1). Differences in breakthrough times for rhamnolipid were likely caused by differences in hexadecane-water interfacial areas between the contaminated matrices (Noordman et al., 2000b). Adsorption of the surfactant to the hexadecane-water interface in the columns will cause retarded transport, in the same way as observed for other anionic surfactants (Kim et al., 1997; Noordman et al., 2000b).

On breakthrough of rhamnolipid, the amount of hexadecane in the column effluents increased by up to three orders of magnitude (Fig. 7, Table 1). The amount of hexadecane in the column effluent decreased in time (Fig. 7), indicating that the mass transfer slowed down with a decreasing residual amount of hexadecane in the columns (Powers et al., 1994). To check whether hexadecane elution from a column packed with hexadecane-contaminated silica 60 in the presence of rhamnolipid was limited by mass transfer of hexadecane from the matrix to the aqueous phase, as was found for elution in the absence of rhamnolipid (Noordman et al., 2000b), the flow was temporarily interrupted after 69 h of elution with rhamnolipid. It was observed that the amount of hexadecane in the column effluent after continuation of the flow transiently increased from 19 to 75 mg l^{-1} (Fig. 7A). This observation shows that elution of hexadecane from this column was limited by mass transfer of substrate from the matrix to the aqueous phase (Pennell et al., 1993). It is shown that rhamnolipid indeed stimulated the mass transfer of hexadecane from matrices to the aqueous phase under the hydrodynamic conditions of the column experiments, not only for sand (Bai et al., 1997) but also for the four matrices used in the current study.

The mass transfer rates of hexadecane from the matrix to the bulk aqueous phase in the column studies increased in the order of glass beads < CPG-10-3000 < sea sand \approx silica 60, both in the absence (also see Noordman et al., 2000b) and in

the presence of rhamnolipid. Mass transfer rates thus increased with increasing specific surface area of the matrices, except for sea sand. The higher mass transfer rate for sea sand than expected based on its specific surface area and the relatively low degradation rates (Fig. 3) show that this material has properties similar to a matrix containing pores with small pore sizes. The presence of pores could not be detected using gas-physorption with nitrogen. However, the properties might be caused by the surface of the beads being rough or patchy, as was shown with electron micrographs for another type of matrix (Kim et al., 1999). The observed correlation between mass transfer rates and specific surface area likely re-

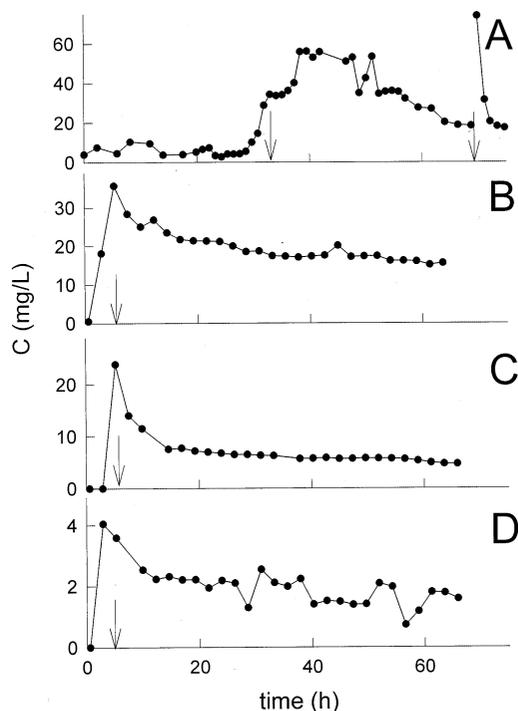


Fig. 7. Rhamnolipid-enhanced elution of hexadecane from a column packed with hexadecane-contaminated silica 60 (A), sea sand (B), CPG-10-3000 (C), or coarse glass beads (D). Columns were eluted with a solution containing 500 mg l^{-1} rhamnolipid. The amount of hexadecane in the column effluent (C) is shown as a function of elution time, where $t=0$ corresponds to the time where elution with rhamnolipid was started. The (first) arrow in each panel indicates the time of breakthrough of rhamnolipid. The second arrow in panel A indicates the time where the flow was interrupted for 13 h.

Table 2

Physico-chemical properties of surfactants used in this study and their effect on the biodegradation of hexadecane by *P. aeruginosa* UG2

	RL	Brij 30	C ₁₂ E ₁₀	Brij 35	SDBS	Triton X-100	Water
Amount of hexadecane emulsified (mg l ⁻¹) ^a	52 ± 19	46 ± 22	105 ± 49	67 ± 61	139 ± 27	ND ^b	11 ± 6
Particle size (QELS, μm)	0.4 ± 0.3	1.2 ± 0.9	1.3 ± 1.0	2.1 ± 2.8	0.2 ± 0.2	0.8 ± 0.7	ND ^b
CMC (mg l ⁻¹)	20–200	7–10	50	40–100	500	100	NA ^c
HLB	17–24	9.5	14.6	16.9	10.6	13.4	NA ^c
Effect on biodegradation ^d	++	+	+	+	–	+	NA ^c

^a See Section 2 for protocol.

^b ND: not determined.

^c NA: not applicable.

^d Effect of the surfactant on the rate of biodegradation by *P. aeruginosa* UG2 of hexadecane present as a separate liquid phase (Fig. 6). ++ indicates the highest degree of stimulation observed; + indicates stimulation compared to control ($P \leq 0.05$); – indicates inhibition compared to control ($P < 0.05$).

sults from the procedure used to contaminate the matrices, which created thin films with hexadecane-water interfacial areas that depended on the specific surface areas of the matrices, and suggests that mass transfer during continuous flow conditions was determined by the hexadecane–water interfacial area, both in the absence of rhamnolipid (Noordman et al., 2000b), as in its presence (this study).

The mass transfer rates in the absence of rhamnolipid under continuous flow conditions were orders of magnitude lower than those under the conditions of higher agitation during end over end mixing as in the biodegradation experiments. For instance, 0.2% of the hexadecane initially present in the columns was removed from the column packed with silica 60 during 70 h of continuous elution, whereas 11% was degraded in this period during the biodegradation experiments (Fig. 1). For sea sand and CPG-10-3000 the difference in these rates was even greater.

3.4. Emulsification of hexadecane

It was determined whether the effect of rhamnolipid on hexadecane biodegradation in the shake flask experiments could be explained by its emulsifying activity. Emulsification of hexadecane under the identical conditions as employed during the degradation experiments was not visible. Therefore, conditions had to be used for the

emulsification studies that differed with the degradation studies in the ratios between aqueous and organic phases, mixing intensities, and presence of cells. Since these parameters can directly influence the type of emulsions formed, the emulsifying activities of the surfactants as determined here are only indicative of their activities during the degradation studies. Visual inspection at 1 h after preparation of the emulsions by vortexing indicated that rhamnolipid, SDBS, all the nonionic polyoxyethylene surfactants, and dodecylmaltoside formed stable emulsions but only the emulsions formed by Brij 35, SDBS, Triton X-100, Brij 78, and dodecylmaltoside were stable up to 7 days. The amount of hexadecane that was emulsified by a surfactant was measured quantitatively by vortexing of a two-phase mixture of hexadecane and an aqueous surfactant solution. All surfactants stimulated emulsification compared to the control without surfactant but the values were not highest for rhamnolipid (Table 2). Rhamnolipid formed smaller emulsion droplets than the nonionic surfactants, although SDBS formed even smaller droplets, as determined with light scattering (Table 2). These results show that rhamnolipid had a strong emulsifying activity towards hexadecane, as had the surfactants SDBS, Brij 35, Triton X-100, Brij 78, and dodecylmaltoside. Of these good emulsifiers, the rhamnolipid biosurfactant as well as Brij 35 and Triton X-100 stimulated the degradation of hexadecane when

present as a second liquid phase but Brij 78 and dodecylmaltoside failed to stimulate biodegradation (Fig. 5). This indicates that no direct relation existed between the emulsifying activities of the surfactants and their effect on biodegradation.

In several studies, the relative effects of different surfactants on degradation of hydrophobic compounds were found to be correlated with the surfactant hydrophile–lipophile balance (HLB) (Oberbremer et al., 1990; Bruheim et al., 1997). The HLB of a surfactant is an empirical parameter that describes the affinity of the surfactant for the oil–water interface (Becker, 1984). To determine whether such a correlation can also be found for *P. aeruginosa* UG2, the effects of surfactants on the biodegradation of hexadecane were correlated with their HLB. The HLB of the surfactants used in the present study ranged from 9.7 for Brij 30 to 23.4 for sodium deoxycholate (Kunieda and Sato, 1992). We estimated an HLB of rhamnolipid of 24.1 by using group contributions (Lin et al., 1973), and of 17.0 by using a correlation of HLB with CMC for sodium carboxylic acids (Lin et al., 1973). Not all surfactants with similarly high HLB values (deoxycholate, HLB 23.4; Brij 35, HLB 16.9; Tween 40, HLB 15.6; Tween 20, HLB 16.7) stimulated biodegradation. Moreover, some surfactants with much lower HLB values (e.g. Brij 30, HLB 9.7) stimulated biodegradation. Therefore, the effect of surfactants on biodegradation was not directly correlated with their HLB values, as was previously shown for *P. aeruginosa* strain S7B1 and a set of nonionic surfactants with HLB values between 4 and 17 (Nakahara et al., 1981). Such a correlation also was absent for a *Deleya salina* strain (Bruheim and Eimhjellen, 1998) and a mixed culture (Van Hoof and Jafvert, 1996).

When a surfactant stimulates degradation by solubilizing the substrate, the surfactant should be present above its critical micelle concentration (CMC). The CMC of rhamnolipid is approximately 50 mg l^{-1} (Zhang and Miller, 1995; Herman et al., 1997b; Noordman et al., 2000a). Of the surfactants with CMC values similar to rhamnolipid, some stimulated biodegradation (Brij 35, CMC 72 mg l^{-1} ; $\text{C}_{12}\text{E}_{10}$, CMC 46 mg l^{-1} (Guha and Jaffé, 1995); Tween 40, CMC 55 mg l^{-1}

estimated from HLB; and Triton X-100, CMC 43 mg l^{-1} (Guha and Jaffé, 1995)) but one inhibited biodegradation (Brij 78, CMC 55 mg l^{-1} estimated from HLB (Becker, 1984)). Several surfactants with CMC values lower than rhamnolipid stimulated biodegradation (Brij 30, CMC 1.4 mg l^{-1} ; Tween 80, CMC 16 mg l^{-1}). Surfactants with CMC values higher than 100 mg l^{-1} (octylglucoside, deoxycholate, and decylmaltoside) did not stimulate hexadecane biodegradation. Since all surfactants were used at a concentration of 100 mg l^{-1} , these latter surfactants were solely present as monomers in our experiments. However, since rhamnolipid stimulated biodegradation already at a submicellar concentration of 20 mg l^{-1} (this study) or even at 10 or 5 mg l^{-1} (Hisatsuka et al., 1971, 1972; Zhang and Miller, 1995; Herman et al., 1997b), the presence of micelles is not strictly required. Surfactants at submicellar concentrations also enhanced the biodegradation of phenanthrene by a mixed culture (Aronstein et al., 1991). A possible explanation for the surfactant-enhanced degradation at submicellar concentrations may be found in the extraction of LPS from the cells by the surfactants occurring already at these low concentrations (Al-Tahhan et al., 2000). We found no correlation between the effect of surfactants on the degradation of hexadecane in shake flask experiments and the CMC of the surfactants. This supports the conclusion that rhamnolipid did not enhance degradation of hexadecane present as a second liquid phase under these conditions of high agitation by solubilizing the substrate.

4. Discussion

4.1. Release of hexadecane from the matrices

Rhamnolipid and several other surfactants stimulated the degradation of hexadecane to a greater extent when it was entrapped in matrices with pores larger than 300 nm (CPG-10-3000 and VitraPOR P3) than when it was entrapped in the matrix with small pores of 6 nm (silica 60) or in sea sand. In other words, surfactants had the largest effect for hexadecane entrapped in ma-

trices where biodegradation was relatively fast. This shows that the stimulation by surfactants of the mass transfer of substrate from the matrices to the aqueous phase was not the most important effect of the surfactants under the conditions of intense agitation. It was even shown that rhamnolipid could not stimulate mass transfer in the shake flask experiments when hexadecane was present in silica 60. However, under conditions of no agitation during the column experiments, mass transfer was enhanced by rhamnolipid by orders of magnitude. Therefore it is expected that rhamnolipid enhances the degradation of a hydrophobic liquid entrapped in matrices under conditions similar to these column experiments by enhancing mass transfer of the substrate from the matrix to the bulk, which indeed was found to be true (Herman et al., 1997a).

Under continuous flow conditions the mass transfer rate was much lower than in the batch experiments and was correlated with the specific surface area of the matrices, whereas during the batch experiments it depended on the pore size of the matrices. Presumably, mass transfer of hexadecane from the matrix to the bulk solution is enhanced by mixing, especially for matrices with large pores, but cannot be further enhanced by rhamnolipid or another surfactant under conditions of intense agitation. A similar dependence on hydrodynamic conditions was found for the effect of surfactants on the biodegradation of phenanthrene present as crystals or dissolved in an organic phase by *P. aeruginosa* (Köhler et al., 1994). Under conditions of low agitation, surfactant addition stimulated degradation but under conditions of high agitation surfactants failed to further stimulate biodegradation.

Strikingly, an opposite dependency of the mass transfer rate on the type of matrix was observed for the batch and column experiments. Whereas mass transfer during the column studies correlated with the specific surface area of the matrix, the mass transfer rate under the conditions of end-over-end mixing in the batch experiments seems to be related to the pore size. For the matrices used in this study, the pore size and the specific surface area of the matrix were inversely correlated. This implies that mass transfer of hex-

adecane from the matrices to the aqueous phase was stimulated by end-over-end mixing in the shake-flask experiments and that the stimulation was greatest for the matrices with larger pores. Larger pores offer greater exposure of substrate that is present in these pores to the turbulent bulk solution. Under conditions of high agitation, the mass transfer of hexadecane from silica 60 to the bulk solution apparently could not be further enhanced by rhamnolipid or another surfactant.

The stimulation by rhamnolipid of hexadecane degradation under conditions of high agitation exceeded the stimulation by any of the other surfactants studied, either when the substrate was present as a second liquid phase or when it was entrapped in sea sand, CPG-10-3000, or VitraPOR P3. Probably, the step that was stimulated by surfactants was the same for all these conditions. Since under conditions of intense agitation surfactants did not stimulate degradation of hexadecane merely by enhancing mass transfer of the substrate from the matrix or liquid phase to the bulk, this implies that surfactants enhanced degradation under these conditions either by promoting emulsification of the substrate or by stimulating the uptake of the substrate by the cells.

4.2. Uptake

Two observations suggest that surfactants enhanced degradation during the conditions of high agitation as applied in the batch degradation experiments by stimulating the uptake of the substrate by the cells rather than by promoting emulsification of the substrate. First, since exponential growth was observed for the biodegradation experiments in the absence of rhamnolipid with hexadecane present as a separate liquid phase and since the degradation rate under these conditions was independent on the amount of substrate present, uptake (or metabolism) rather than solubilization of substrate was the rate-limiting process. Second, no correlation was found between the stimulating activity of the surfactants and their emulsifying activities, CMC value, or their HLB. The fact that dispersion of octadecane is stimulated by the biosurfactant to a much greater extent than mineralization (Zhang and

Miller, 1992) is in accordance with our present conclusion that uptake and not dispersion is rate limiting. Apparently, rhamnolipid can enhance uptake more efficiently than any of the other surfactants tested. The enhancement of the uptake rate is striking, since it has been shown that micellar-solubilized substrate often is not available to bacteria or is less available than dissolved substrate (Volkering et al., 1995; Guha and Jaffé, 1996; Zhang et al., 1997; Willumsen and Arvin, 1999).

Rhamnolipid may have enhanced uptake in the batch experiments either by enhancing uptake of dissolved, solubilized, or emulsified substrate by the cells or by enhancing uptake of substrate from hydrocarbon droplets after promoting attachment of the cells to these droplets (Miller, 1995; Bouchez-Naitali et al., 1999). The first mechanism seems plausible since it is generally assumed that rhamnolipid-producing *Pseudomonas* strains take up hydrophobic alkanes from submicron or emulsified droplets (Nakahara et al., 1977, 1981; Churchill and Churchill, 1997; Bouchez-Naitali et al., 1999). The mechanism could involve a specific cell-surfactant interaction or constitute of a surfactant-mediated uptake pathway (Hisatsuka et al., 1971; Rattlege, 1988). This interaction or pathway may be specific for rhamnolipid, which would explain the stronger stimulation by rhamnolipid as compared to other surfactants. Alternatively, rhamnolipid may extract LPS from the outer membrane and thereby increase attachment of cells to hydrocarbon droplets (Al-Tahhan et al., 2000). Therefore, extraction of LPS may indirectly enhance uptake. Cells of *P. aeruginosa* indeed became more hydrophobic after incubation with rhamnolipid (Zhang and Miller, 1994; Al-Tahhan et al., 2000), although the rhamnolipid concentration used was very high compared to the concentration that is produced by the organism and was approximately 10-fold higher than the concentration of rhamnolipid used in our experiments. The latter mechanism does explain the observed aggregation of cells of certain strains of *P. aeruginosa* during growth on hexadecane in the presence of rhamnolipid (Herman et al., 1997a) but it does not readily explain why

rhamnolipid enhances degradation of poorly soluble substrates better than other surfactants. Furthermore, it is not very efficient for a bacterium to reduce its cell surface hydrophobicity by extraction of LPS from its cellular envelope using extracellular rhamnolipid produced by the organism itself. In order to obtain insight into the mechanism by means of which mechanism rhamnolipid increases uptake, it is necessary to investigate in greater detail how different strains of *P. aeruginosa* take up hydrophobic substrates, to determine whether rhamnolipid can directly enhance uptake of dissolved or solubilized substrate by *P. aeruginosa* without prior attachment of the cells to liquid substrate, and to determine whether a causal relation exists between the extraction of LPS and the rate of hexadecane degradation.

This study shows that the way how and the extent to which degradation of hexadecane is enhanced by rhamnolipid depends on the availability of the substrate. It was demonstrated that rhamnolipid enhanced two different processes that are relevant for remediation of soil contaminated with non-aqueous phase liquids. These processes are the mass transfer of entrapped or residual substrate from matrices to the aqueous phase and the biodegradation of substrate present as a separate liquid phase. Stimulation of the former process was observed in column studies under continuous flow operation but not in batch experiments under conditions of high agitation. This effect is expected to be important during in situ bioremediation when degradation is limited by transport of the contaminant from soil to the site where bacterial activity takes place (Herman et al., 1997a), and during removal of non-aqueous phase liquids by surfactant-enhanced pump and treat technology (Pennell et al., 1993; Bai et al., 1997) where the mass transfer rate also often is limiting the remediation rate (Powers et al., 1994). The stimulation by rhamnolipid of the biodegradation of substrate present as a separate liquid phase seems to result from surfactant-enhanced uptake of emulsified substrate. This effect was of importance under well-mixed conditions when uptake was rate-limiting.

Acknowledgements

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