Chapter 6

Processes Involved in the (Bio)surfactant-Enhanced Biodegradation by a Rhamnolipid-Producing *Pseudomonas* of Hexadecane Present in Porous Matrices

The influence of surfactants on the biodegradation by *Pseudomonas aeruginosa* UG2 of hexadecane present in porous matrices and in two-liquid phase media was determined. The biodegradation rate of hexadecane was lower for a matrix with 6 nm pores and for quartz sand than for matrices with pore sizes of 300 nm and 16-40 µm, and for hexadecane present as a second liquid phase. For all matrices except for the matrix with 6 nm pores, biodegradation was enhanced by the addition of several surfactants at a concentration of 100 mg/L. The rhamnolipid biosurfactant produced by strain UG2 caused a larger enhancement of the biodegradation rate than any of the other 14 surfactants tested. Rhamnolipid stimulated the mass transfer of hexadecane from all matrices to the aqueous phase during column studies, but apparently failed to stimulate mass transfer from the matrix with 6 nm pores under the conditions of more intense agitation that were applied during the biodegradation experiments. The more effective stimulation by rhamnolipid of hexadecane biodegradation compared to the other surfactants tested could not be explained by its higher emulsifying activity. The effect of the surfactants was not related to their hydrophile-lipophile balances or their critical micelle concentrations. The combined results suggest that the stimulating effect of surfactants on the biodegradation of hexadecane results from an effect on uptake of the substrate by the cells.

INTRODUCTION

The use of surfactants to overcome bioavailability-associated limitations during soil remediation has attracted considerable attention (Aronstein et al., 1991; Zhang and Miller, 1992; Tiehm, 1994; Miller, 1995; Providenti et al., 1995a, b; Van Hoof and Jafvert, 1996; Churchill, P.F. and Churchill, 1997; Herman et al., 1997b; Volkering et al., 1998). Positive effects may result from the stimulation by surfactants of dissolution or desorption rates, substrate dispersion, solubilization or emulsification (Aronstein et al., 1991; Tiehm, 1994; Miller, 1995; Volkering et al., 1998). Negative effects may result from surfactant toxicity or (preferential) biodegradation of surfactants (Miller, 1995; Volkering et al., 1998). Strains with a high cell surface hydrophobicity seem to be less prone to stimulation by surfactants, probably because surfactants reduce attachment to and uptake of separate-phase substrates (Churchill, P.F. and Churchill, 1997; Herman et al., 1997b). For some strains the relative effects of different surfactants is dependent on the physico-chemical properties of the surfactants (Oberbremer et al., 1990; Bruheim et al., 1997), but for others the effect seems to be specific for a particular surfactant (Nakahara et al., 1981; Van Hoof and Jafvert, 1996).

Many studies have shown that a rhamnolipid biosurfactant produced by *Pseudomonas* sp. can stimulate the biodegradation of long chain alkanes by rhamnolipid-producing strains (Itoh and

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Suzuki, 1972; Nakahara et al., 1981; Koch et al., 1991; Zhang and Miller, 1992). A specific interaction between rhamnolipid or rhamnolipid-solubilized compounds and the rhamnolipid-producing bacteria seems to exist, since rhamnolipid enhanced the biodegradation by a rhamnolipid-producing strain to a greater extent than several other surface active compounds (Itoh and Suzuki, 1972; Nakahara et al., 1981). Furthermore, biodegradation of alkanes by other strains or mixed cultures was not stimulated by rhamnolipid (Hisatsuka et al., 1971; Providenti et al., 1995b; Bregnard et al., 1998) or was stimulated to a lesser extent by rhamnolipid than by other biosurfactants (Oberbremer et al., 1990). Rhamnolipid can also enhance the biodegradation of hexadecane by rhamnolipid-producing *Pseudomonas* strains when the substrate is present as a residual non-aqueous phase in soil (Herman et al., 1997b). Although substrate dispersion might play an important role (Zhang and Miller, 1994, 1995), it is not known how surfactants stimulate the biodegradation of hydrophobic substrates by rhamnolipid-producing strains, especially when the substrate initially is present in porous matrices. Furthermore, it is unknown why the stimulation is especially pronounced for rhamnolipid.

Different processes are involved in the biodegradation of a poorly soluble liquid substrate that is initially present in porous matrices. First, the substrate has to be transferred from the matrix to sites where it can come into direct contact with microorganisms. When this process is rate-limiting, surfactants can enhance the biodegradation rate by enhancing these mass transfer rates. Subsequently, the substrate has to be taken up by the cells. It is generally assumed that rhamnolipid-producing *Pseudomonas* strains take up hydrophobic alkanes from submicron or pseudosolubilized droplets (Nakahara et al., 1977, 1981; Bouchez-Naïtali et al., 1999). The term 'pseudosolubilized' is traditionally used to describe solubilized, emulsified, or otherwise surfactant-bound substrate. The term 'emulsified substrate' is considered to be most accurate for the hexadecane-rhamnolipid supramolecular complexes. Surfactants can stimulate uptake of separate-phase liquid substrate by enhancing mass transfer of substrate from large substrate droplets to the cells, either by emulsification of the substrate or by enhancing the subsequent uptake of emulsified substrate (Barnett et al., 1974; Velankar et al., 1975; Haferburg et al., 1986). The emulsifying effect of surfactants is solely dependent on their physico-chemical properties. The importance of droplet size, interfacial tension, specific substrate-water interfacial areas, and agitation for determining the rate of (pseudo)solubilization is well established (Gutierrez and Erickson, 1977; Reddy et al., 1983). The effect of surfactants on uptake of emulsified substrate might involve interactions between surfactant and cell (membranes) and therefore is possibly specific for a combination of organism and surfactant. After the substrate is taken up, it is converted and mineralized.

From the foregoing, it is concluded that biosurfactants might influence the biodegradation of poorly soluble hydrocarbons present in porous matrices by enhancing the mass transfer of substrate from the matrices to sites where it is accessible to the microorganisms, by emulsifying the substrate, or by stimulating the uptake of emulsified substrate. The goal of this work was to determine how (bio)surfactants enhance biodegradation of a very hydrophobic substrate by the rhamnolipid producing bacterium *P. aeruginosa* UG2, both when this substrate is present in porous matrices and when it occurs as a second liquid phase. To determine the cause of the earlier observed specific stimulation of the biodegradation rate by rhamnolipid, the effect of rhamnolipid was compared to the effect of several synthetic surfactants. Hexadecane was used as the model-substrate, since it is easily degraded by strain UG2 and has an extremely

low water solubility. Matrices with different pore sizes were used to determine whether the effects of rhamnolipid on biodegradation of hexadecane and on mass transfer of hexadecane from the matrix to the aqueous phase were dependent on the pore size. The effect of rhamnolipid on these mass transfer rates was investigated in more detail by using column studies. Furthermore, the emulsification of hexadecane by different surfactants was studied.

MATERIALS AND METHODS

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

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, Wis.) and Triton X-100 that was obtained from BDH Chemicals (Vancouver, Canada).

Rhamnolipid. Rhamnolipid was produced by *P. aeruginosa* UG2 and was purified as described in Chapter 4.

Matrices. The model materials used were Silica 60, quartz sea sand, controlled pore glass, P3 VitraPOR filter candles (3 mm in diameter and 3 mm in height, pore size 16-40 µm, Elgebe, Leek, The Netherlands), and coarse glass beads. Supliers are given in Chapter 5. Physical properties are given in Table 5.1.Matrices were contaminated with hexadecane as described in Chapter 5.

Media. The mineral salts medium either contained 0.53 g $Na_2HPO_4.12H_2O$, 0.14 g KH_2PO_4, 0.2 g $MgSO_4.7\ H_2O$, 0.1 g $(NH_4)_2SO_4$, and 5 mL of a trace element solution (Janssen et al., 1984) per liter of doubly distilled water or contained ten-fold less trace elements and magnesium and was made up in demineralized water. 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.

Biodegradation experiments. For the biodegradation experiments with substrate coated to matrices, 250 mg contaminated matrix was aseptically placed in 8 mL tightly closed incubation vessels. For the experiments employing substrate present as a second liquid phase, the required amount of a 1:10 (v:v) solution of hexadecane in pentane was added to each 8 mL incubation vessel, after which the pentane was allowed to evaporate by incubation for at least 5 h in a sterile cabinet. A preculture was grown for three days with hexadecane and was used to prepare the inoculated culture with a culture density of 2 mg protein/L. 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 incubation vessel, and the closed vessels were incubated at 30 ^oC by end-over-end rotation at 1.4 or 39 rpm. This difference in mixing rates had no effect on the biodegradation rate of hexadecane coated to silica and of hexadecane present as a second liquid phase. The amount of residual substrate (C, mg hexadecane / L culture fluid) was monitored by sacrificing separate vessels of the incubated parallel cultures at least in duplicate (substrate depletion curves) or triplicate (when the effect of added surfactants was determined). The amount of hexadecane was determined by extracting the contents of the incubation vessels with 0.05 mL of 10% HCl, 2 mL of ethanol, and 1 mL of isooctane. After vigorous agitation for 5 min and centrifugation at 4000 rpm when necessary to separate phases, the isooctane phase was analyzed by GC. Controls showed that no hexadecane disappeared from the incubation vessels by abiotic processes. The

amount of oxygen in the vessels 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.

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 supernatant was obtained by centrifugation of 1 mL sample for 5 min at 6000 rpm and filtration of the supernatant using a nylon disposable filter (4 mm in diameter, 0.2 µm pore size, Alltech). The toxicity of the surfactants was tested by cultivating strain UG2 with 5 mM citrate in the absence presence of 500 mg/L 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 rhamnolipid. For testing of 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 on hexadecane was determined by measuring the surfactant concentration in a filtered culture supernatant after five days of cultivation. The initial surfactant concentration was 100 mg/L (synthetic surfactants) and 20, 100, and 500 mg/L (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 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 hexadecane. All experiments were performed in 100 mL flasks containing 20 mL mineral salts medium at 30 ^{o}C on a rotary shaker (200 rpm), unless mentioned otherwise. Precultures were grown on the substrate used for the main culture (*i.e.* hexadecane, citrate or pyruvate).

Column studies. Stainless steel preparative HPLC columns (length 7.0 cm, i.d. 2.2 cm) were dry packed in incremental steps with matrices contaminated with approximately 6 mg hexadecane/g matrix (Chapter 5). Although the bulk densities and porosities differed widely (Table 5.1), the initial amount of hexadecane in the columns differed by less than a factor 3 (Table 6.1). The electrolyte solution used for the column studies was mineral salts medium supplemented with 0.2 g/L NaN_3 to prevent biodegradation of the substrate and surfactants. The columns were placed vertically and elution was performed with upward flow. After saturation of the columns by elution with 100 mL electrolyte solution using a flow rate of 0.1 mL/min, the concentration of the hexadecane was determined by GC analysis of five 7 mL effluent fractions. Subsequently, a tracer experiment with conservative, partitioning and interfacial tracers was performed which lasted up to 20 pore volumes (Chapter 5). The breakthrough profiles of the conservative tracer potasium bromide indicated that the columns were packed homogeneously and that physical nonequilibrium effects were absent. After the tracer experiment, the hexadecane-containing columns were eluted with 400 mL electrolyte solution containing 500 mg/L rhamnolipid using a flow rate of 0.1 mL/min. Samples of 7 mL of the effluent were collected in extraction tubes. These fractions were either analyzed for hexadecane by GC after extraction of the samples with 1 mL isooctane and 0.05 mL 10% HCl (>25

fractions) or for rhamnolipid by HPLC (>6 fractions). The residual concentration of hexadecane in the column at the end of the experiments was 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 isooctane. 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 ^{o}C. In this way, the hexadecane concentration in the matrices was determined with an accuracy of 5% (initial concentration) or 10% (end concentration). Mass balances for hexadecane were 85%-104%.

Emulsions. Emulsions of hexadecane and water were prepared using a phase ratio (v:v) of 1:2 (experiment reported in Fig. 6.8) or 1:8 (other experiments) by vortexing for 30 s. 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 incubated upside down for 1 h to allow the largest droplets to float. Subsequently, triplicate samples of 1 mL were taken from the aqueous phase and were analyzed by GC. The particle size and size distribution for 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.

***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 hydrolysis of the cell suspension. Pyruvate was determined using a colorimetric assay with KOH and salicylic aldehyde in samples of 200 µL (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 ^{o}C and increased with 8 ^{o}C per min to 200 ^{o}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 with evaporative light scattering detection (Chapter 4), SDBS was analyzed by HPLC with UV detection at 228 nm (Chapter 5), and the nonionic surfactants Brij 30, Brij 35, and polyoxyethylene(10)dodecanol were analyzed using HPLC-ELSD using gradient elution. For the latter analysis, the gradient 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 rate was 0.5 mL/min. The HPLC setup was described in Chapter 4 and 5.

RESULTS AND DISCUSSION

The biodegradation of hexadecane by the rhamnolipid-producing organism *P. aeruginosa* UG2 was determined in batch incubations where hexadecane was either present in porous model matrices or present as a second liquid phase. The initial amount of hexadecane present in the cultures (C_0) was 30-2000 mg hexadecane/L culture fluid or 0.004-0.3% (v/v). These amounts exceeded the aqueous solubility of hexadecane, which is 0.0036 mg/L (Schwarzenbach et al., 1993). The culture density in the two-liquid phase experiments with C_0 = 350 mg/L increased 40-fold over the incubation time, indicating that biodegradation of hexadecane resulted in growth. The yield was 0.75 mg protein/mg hexadecane, which is similar to previously determined values for aliphatics (Bailey and Ollis, 1986).

The biodegradation rate of hexadecane present as a second liquid phase was the same for *P. aeruginosa* PG201 and the rhamnolipid-deficient mutant PG201::*rhlI*, either when hexadecane was present as a second liquid phase or coated to silica.

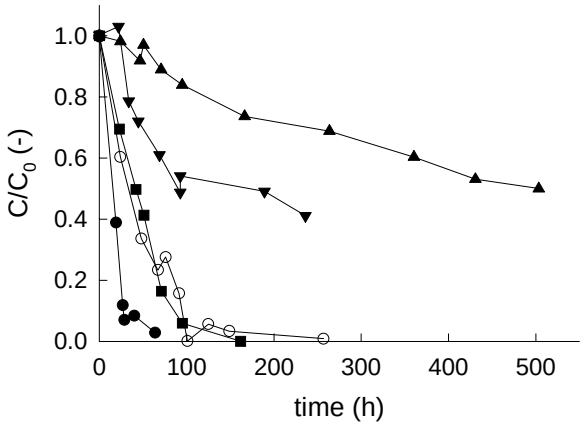

Figure 6.1. Biodegradation of hexadecane by *P. aeruginosa* UG2. Substrate was initially coated to silica (▲); sea sand (▼), CPG-10-3000 (■), VitraPOR P3 (●), or was present as a second liquid phase (O). Batches were sacrificed in time in duplicate.

Therefore, it can be assumed that the amount of rhamnolipid that was produced by the wild type strains in these experiments (< 2 mg/L) had no effect on the biodegradation rate and that the cultures to which no surfactant was added represented a situation where rhamnolipid was absent.

Biodegradation of hexadecane present in porous matrices. Pronounced differences in biodegradation rates of hexadecane were observed between the different porous matrices (Fig. 6.1). The biodegradation rate of substrate coated to silica 60 (pore size 6 nm) and sea sand was lower than the biodegradation rate of substrate present as a second liquid phase, showing that the bioavailability of hexadecane coated to these matrices was relatively low (Fig. 6.1). Furthermore, the biodegradation rate of hexadecane present in silica decreased in time (Fig. 6.1, 6.2) and increased with increasing C_0 between 32 and 372 mg/L (Fig. 6.2A), indicative of a process that is first order in the concentration of coated hexadecane. Indeed, a data set consisting of four substrate depletion curves with C_0 of 372, 268, 105, and 32 mg/L could be accurately fitted to an exponential decay function with a first order rate constant of $(1.49\pm0.05)\cdot10^{-3}$ h^{-1} (r^2=0.998, n=25, Fig 2A). The biodegradation rate of substrate coated to CPG-10-3000 (pore size 300 nm)

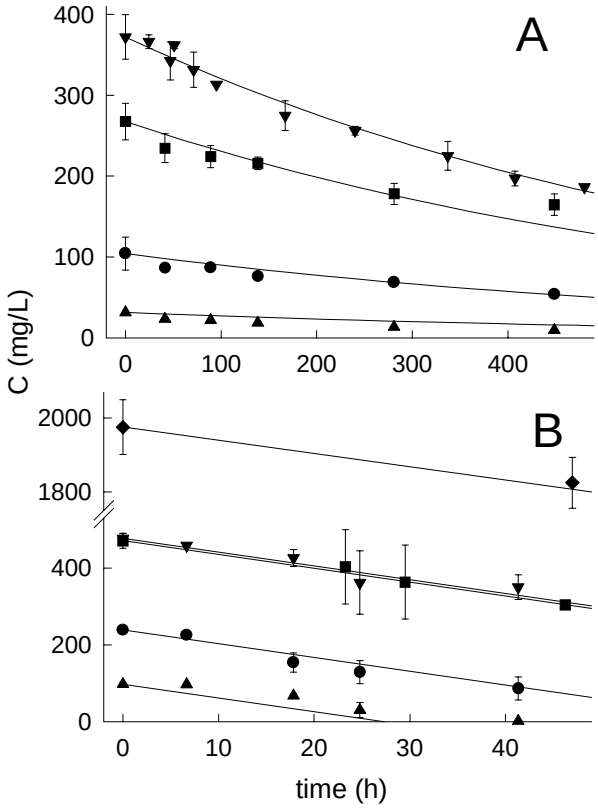

Figure 6.2. Effect of C_0 on the biodegradation rate of hexadecane by *P. aeruginosa* UG2. (A) Substrate was coated to silica, C_0 was 372 mg/L (▼); 268 mg/L (■); 105 mg/L (●); and 32 mg/L (▲). (B) Substrate was present as a second liquid phase, C_0 was 1976 mg/L (◆); 477 mg/L (▼); 471 mg/L (■); 239 mg/L (●); and 98 mg/L (▲). Batches were sacrificed in time in duplicate (A, ▼ and B, ■; error bars indicate measured values) or triplicate (all other data; error bars indicate standard deviation).

and VitraPOR P3 (pore size 16-40 μm) was similar to the rate of biodegradation of substrate present as a second liquid phase (Fig. 6.1). These results indicate that the rate-limiting process in the experiments with hexadecane coated to silica was the release of substrate from the matrix, whereas biodegradation of hexadecane coated to CPG-10-3000 and VitraPOR P3 was not limited by mass transfer of the substrate from the matrix to the aqueous phase. The fact that biodegradation was limited by mass transfer for silica with 6 nm pores but not by the matrices with pores of 300 nm and 16-40 μm suggests that the mass transfer under the conditions of the biodegradation experiments

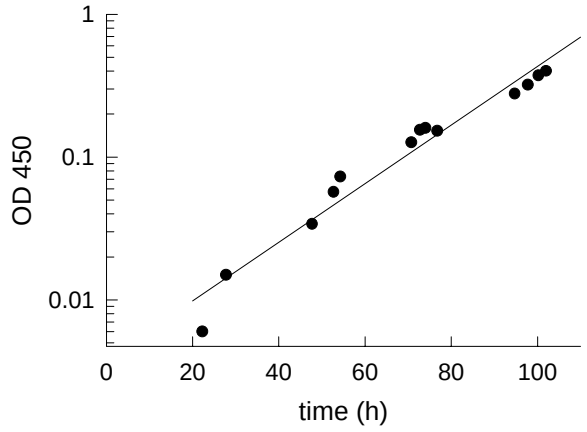

Figure 6.3. Growth curve for *P. aeruginosa* UG2 when grown with hexadecane in two-liquid phase media. Data represent the average of two parallel grown cultures.

was determined by the pore size of the matrices.

Biodegradation of hexadecane in two-liquid phase media. The initial biodegradation rate for hexadecane present as a second liquid phase was independent on C_0 in the range of 100 to 2000 mg/L. Analysis of the combined data of five substrate depletion curves yielded an initial rate of 3.6±0.1 mg L^{-1} h^{-1} (0-45 h, r^2=0.9994, n=16, Fig. 6.2B). Growth curves of strain UG2 were exponential up to an OD_{450} of 0.4, with a growth rate of 0.02 h^{-1} (Fig. 6.3). The results suggest that the rate-limiting process in the biodegradation of hexadecane in two-liquid phase media was the uptake or conversion of substrate, which is related to the culture density, and not the dissolution or emulsification of substrate, which would have caused linear growth and would have caused degradation to be related to the initial amount of substrate.

Effect of surfactants on the biodegradation of hexadecane present in porous matrices. When hexadecane initially was present in silica, the addition of 20, 100 or 500 mg/L rhamnolipid to the cultures did not enhance the rate of biodegradation (Fig. 6.4A). A more extensive determination of the effect of rhamnolipid and other surfactants on the biodegradation of hexadecane present in silica, sea sand, CPG-10-3000, and VitraPOR P3 was accomplished by measuring the

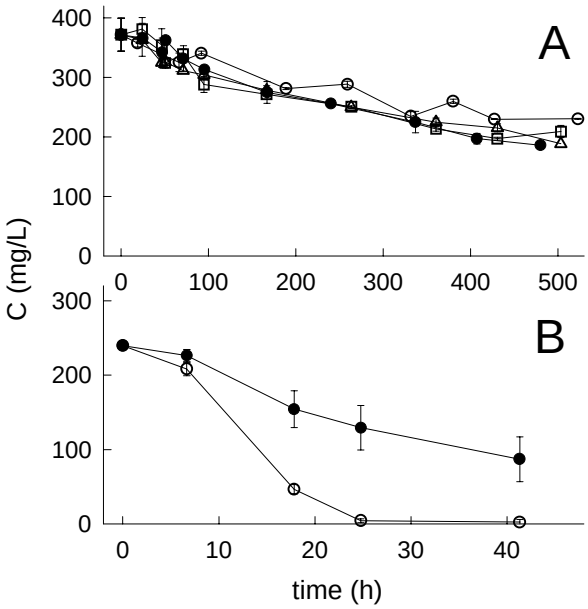

Figure 6.4. Effect of rhamnolipid on biodegradation of hexadecane initially present in silica (A) or present as a second liquid phase (B). Batches were sacrificed in time in duplicate. The concentration of rhamnolipid added was 0 mg/L (●), 20 mg/L (□), 100 mg/L (Δ), or 500 mg/L (**O**).

amount of residual hexadecane (C, mg hexadecane/L culture fluid) in batch cultures with and without added surfactant (100 mg/L). 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 coated to silica, the amount of hexadecane remaining at this time was independent of the presence of rhamnolipid and the nonionic alcohol ethoxylates Brij 30, Brij 35, and polyoxyethylene(10)dodecanol ($C_{12}E_{10}$) (Fig. 6.5A). The addition of the anionic surfactant sodium dodecylbenzenesulfonate (SDBS) also had no effect on hexadecane biodegradation, as was determined after 250 h of growth. Biodegradation of hexadecane coated to sea sand and CPG-10-3000, which had larger pore sizes, was enhanced by rhamnolipid at all concentrations tested, including the submicellar concentration of 20 mg/L (Fig. 6.5BC). The nonionic alcohol ethoxylates (100 mg/L) also enhanced biodegradation of hexadecane initially coated to sea sand (Fig. 6.5B). However, SDBS retarded hexadecane biodegradation when the

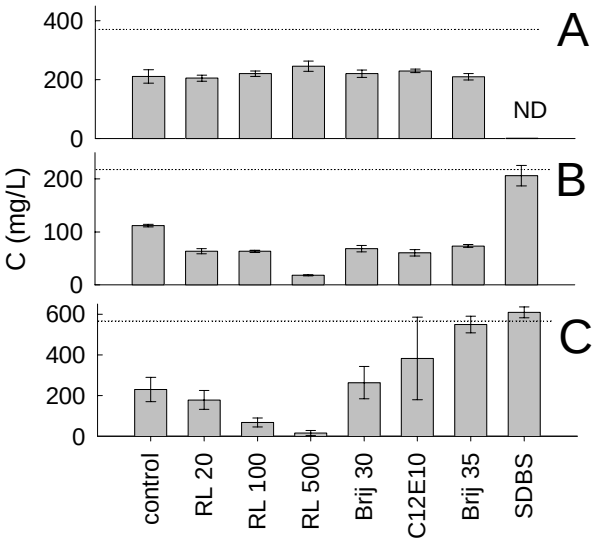

Figure 6.5. Effect of surfactants on biodegradation of hexadecane present in matrices by *P. aeruginosa* UG2. No surfactant was added to the control. Rhamnolipid was added to a concentration of 20, 100, or 500 mg/L for the bars labeled RL 20, RL 100, and RL 500, respectively. Other surfactants were added at a concentration of 100 mg/L. Substrate was coated to silica (A), sea sand (B), or CPG-10-3000 (C). Batches were analyzed in triplicate for residual hexadecane after 433, 93, or 51 h of cultivation for silica, sea sand, and CPG-10-3000, respectively. Dotted line indicates C_0.

substrate was coated to sea sand and CPG-10-3000 (Fig. 6.5BC). For hexadecane coated to VitraPOR P3, biodegradation was stimulated by rhamnolipid (100 and 500 mg/L) but not by Brij 30 (100 mg/L). These results indicate that biodegradation of hexadecane was stimulated by rhamnolipid and by the nonionic surfactants only for matrices where biodegradation was not limited by mass transfer of hexadecane to the aqueous phase. These were the matrices with larger pore sizes. Furthermore, it can be concluded that none of the surfactants used stimulated the mass transfer of hexadecane from silica to the aqueous phase under these experimental conditions. The stimulation by rhamnolipid always exceeded the stimulation by any of the other surfactants ($P<0.05$).

Effect of surfactants on the biodegradation of hexadecane in two-liquid phase media. The effect of rhamnolipid and fourteen synthetic surfactants on the biodegradation of hexadecane in two-liquid phase media was determined. Mixed effects were observed (Fig. 6.4B, 6.6). Rhamnolipid and almost all ethoxylated nonionic surfactants stimulated biodegradation, including Brij 30, Brij 35, $C_{12}E_{10}$, and Triton X-100 (Fig. 6.6). The anionic surfactant SDBS and the nonionic surfactant Brij 78 inhibited hexadecane biodegradation, whereas the carbohydrate-containing nonionic surfactants did not affect biodegradation (Fig. 6.6). Most strikingly, rhamnolipid (100 mg/L) stimulated biodegradation to a greater extent than any of the other surfactants at a concentration of 100 mg/L ($P<0.05$, Fig. 6.6). Since it is unlikely that rhamnolipid stimulated the metabolism of the substrate, it must be concluded that rhamnolipid enhanced uptake and that uptake was the rate-limiting step.

Degradation of surfactants. Biodegradation of surfactants may impact their effect on contaminant degradation (Volkering et al., 1998). Therefore, it was determined whether surfactants were degraded. The amounts of rhamnolipid and SDBS degraded during growth of *P. aeruginosa* on hexadecane were negligible compared to the amount of hexadecane degraded in this period. Substantial amounts (10-90%) of Brij 30, Brij 35, and $C_{12}E_{10}$ were degraded during growth with hexadecane for five days but *P. aeruginosa* could not grow with these surfactants as sole source of carbon and energy. Therefore, it can be assumed that the effects of these surfactants on the biodegradation of hexadecane were not caused by an increase in culture density resulting from surfactant biodegradation. However, the biodegradation of these surfactants could have masked their stimulating effect on hexadecane biodegradation.

Effects of surfactants on growth with citrate. Rhamnolipid, Brij 30, Brij 35, and $C_{12}E_{10}$ (500 mg/L) had no effect on the growth of *P. aeruginosa* on citrate or degradation of pyruvate. SDBS caused retarded growth of strain UG2 on citrate and

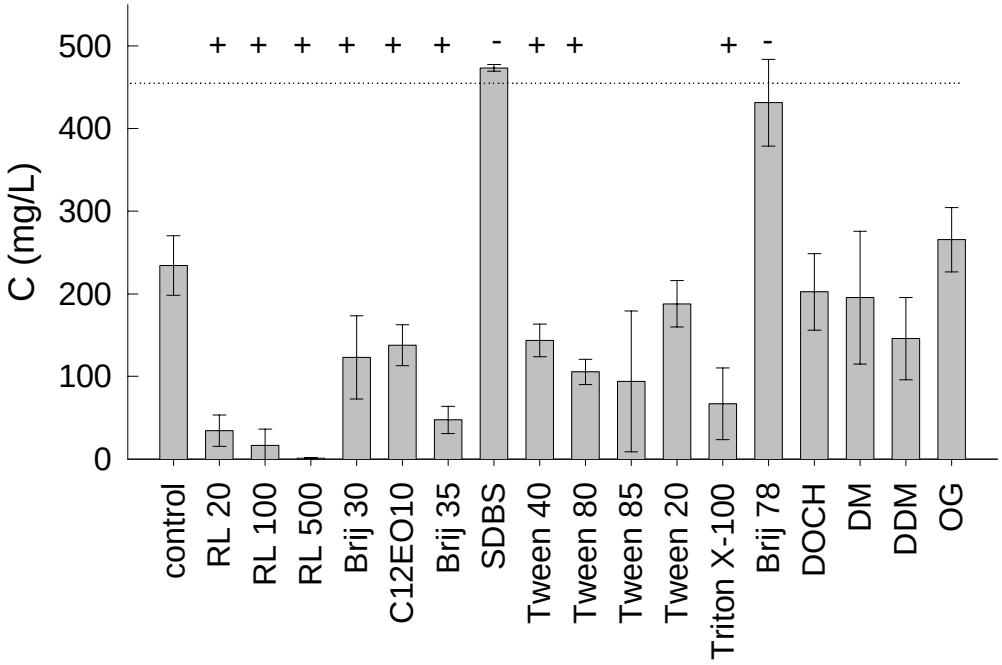

Figure 6.6. Effect of surfactants on biodegradation of hexadecane present as a second liquid phase by *P. aeruginosa* UG2. For each surfactant, five parallel grown cultures were sacrificed after 45 h of cultivation and analyzed for residual hexadecane. Rhamnolipid was added to a concentration of 20, 100, or 500 mg/L for the bars labeled RL 20, RL 100, and RL 500, respectively. Synthetic surfactants were added at a concentration of 100 mg/L. DOCH, DM, DDM, OG indicate sodium deoxycholate, *n*-decyl-β-D-maltoside, *n*-dodecyl-β-D-maltoside, and *n*-octyl-β-D-glucoside, respectively. The symbol '+' indicates that biodegradation was stimulated compared to the control ($P<0.05$), the symbol '-' indicates that biodegradation was inhibited compared to the control ($P<0.05$). Dotted line indicates C_0.

reduced the final optical density of the culture compared to growth in its absence. These results show that the surfactants were non-toxic, except for SDBS. This toxicity could have been the cause of the inhibition of hexadecane biodegradation by SDBS (Fig 4B, 4C, 5). The results also suggest that the stimulation of biodegradation by surfactants was due to their stimulation of a process that was limiting for poorly soluble substrates but not for soluble substrates.

Effect of rhamnolipid on mass transfer of hexadecane from the matrix to the aqueous phase. Column studies were used to determine the effect of rhamnolipid on mass transfer of hexadecane present in porous matrices to the bulk aqueous phase in the absence of biodegradation. Columns were packed with 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 determined directly after saturation of the columns with electrolyte solution (Table 6.1). Subsequently, the columns were eluted with electrolyte solution containing rhamnolipid. A rhamnolipid concentration of 500 mg/L was used since this concentration was found to be optimal for stimulating removal of hexadecane from sand (Bai et al., 1997). On breakthrough of rhamnolipid, the amount of hexadecane in the column effluent increased by up to three orders of magnitude (Fig. 6.7, Table 6.1). Since the amount of hexadecane in the column effluent exceeded its aqueous solubility ($3.6 \cdot 10^{-6}$ g/L (Schwarzenbach et al., 1993)) for columns packed with CPG-10-3000, sea sand, and silica, removal is caused by processes such as dissolution, detachment of droplets, or mobilization. The term removal is used here to describe the elution of hexadecane from a column packed with a

Table 6.1. Effect of rhamnolipid (RL) on removal of hexadecane from columns packed with hexadecane-contaminated matrices.

matrix	hexadecane in column	amount of hexadecane in column effluent		breakthrough of RL	
	initial amount	before application of RL	after breakthrough of RL[a]	time	pore volumes
	(mg)	(g/L)	(g/L)	(h)	(-)
silica	92	$(3.8\pm1.0)\cdot10^{-4}$	$(5.6 \text{ to } 2.0)\cdot10^{-2}$	33	13
sea sand	238	$(3.1\pm0.7)\cdot10^{-4}$	$(3.6 \text{ to } 1.6)\cdot10^{-2}$	6	3
CPG-10-3000	64	$(7.7\pm1.2)\cdot10^{-6}$	$(2.4 \text{ to } 0.5)\cdot10^{-2}$	6	2.5
glass beads coarse	179	$(1.2\pm1.1)\cdot10^{-6}$	$(4.0 \text{ to } 1.8)\cdot10^{-3}$	5	4

[a] observed range in the amount of hexadecane in the column effluent observed after breakthrough of rhamnolipid

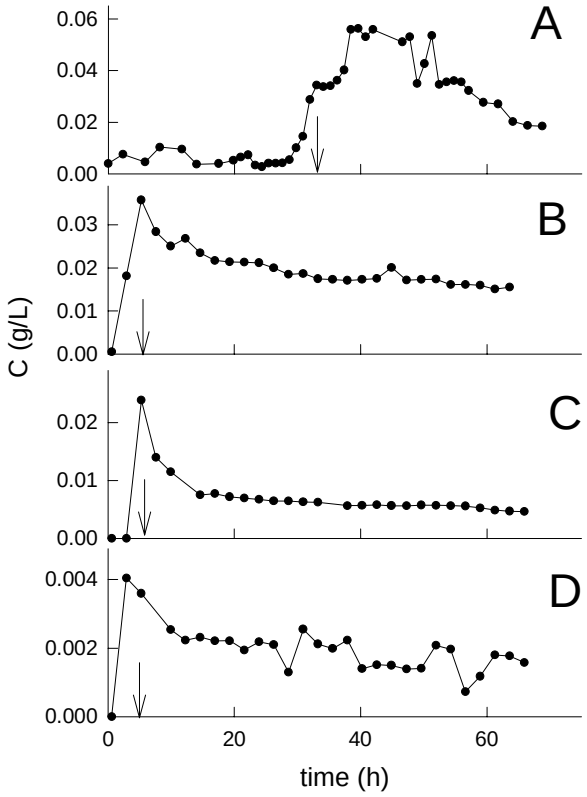

Figure 6.7. Rhamnolipid-enhanced removal of hexadecane from a column packed with hexadecane-contaminated silica (A), sea sand (B), CPG-10-3000 (C), or coarse glass beads (D). Columns were eluted with a solution containing 500 mg rhamnolipid/L. The amount of hexadecane in the column effluent (C) is shown as a function of elution time, where t=0 corresponds to the time where elution with rhamnolipid was started.

hexadecane-contaminated matrix. The removal rate decrease in time, indicating that the mass transfer rate of hexadecane from the matrix to the mobile phase decreased with decreasing residual amount of hexadecane in the columns (Powers et al., 1994).

To check whether hexadecane removal was rate-limited, the flow was temporarily interrupted during several experiments with hexadecane-contaminated silica. It was observed that the amount of hexadecane in the column effluent transiently increased from $1.9\cdot10^{-2}$ to $7.5\cdot10^{-2}$ g/L in the experiment with an aqueous phase that contained rhamnolipid, and from $(3.8 \pm 1.0)\cdot10^{-4}$ g/L to $4\cdot10^{-3}$ g/L in the experiment with an aqueous phase that did not contain rhamnolipid. This shows that removal of hexadecane was rate-limited (Pennell et al., 1993). The residual amounts of hexadecane in the matrix samples taken after the experiment from the top and bottom of the columns were not significantly different from the amounts in samples from the mixed column content for the columns packed with silica and sea sand. This indicated that hexadecane was removed from these columns to the same extent in all positions, and implies that the removal was determined by mass transfer of hexadecane from the matrix

to the mobile phase and not by its subsequent transport. Therefore, the amounts of hexadecane in the column effluent directly reflect these mass transfer rates. The observation that rhamnolipid enhanced removal thus indicates that rhamnolipid stimulated the mass transfer of substrate from the matrices to the aqueous phase under the hydrodynamic conditions of the column experiments.

The mass transfer rates in the column studies both in the absence and in the presence of rhamnolipid increased in the order of glass beads < CPG-10-3000 < sea sand ≈ silica. Mass transfer rates thus increased with increasing specific surface area of the matrices, except for sea sand. This correlation likely results 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. Therefore, this correlation suggests that mass transfer during continuous flow conditions was determined by the hexadecane-water interfacial area (Chapter 5). An opposite dependency of the mass transfer rate on the type of matrix was observed for the batch experiments, since the mass transfer rate seems to be related to the pore size under the conditions of end-over-end mixing in the batch experiments. For the matrices used in this study, the pore size and the specific surface area of the matrix were inversely correlated.

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 of the biodegradation experiments. For instance, 0.2% of the hexadecane initially present in the columns was removed from the column packed with silica during 70 h of continuous elution, whereas 11% was degraded in this period during the biodegradation experiments (Fig. 6.1). For sea sand and CPG-10-3000 the difference in these rates was even greater. This implies that mass transfer of hexadecane from the matrices to the aqueous phase was enhanced by end-over-end mixing and that the effect of mixing 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 the matrix to the bulk solution apparently could not be further enhanced by rhamnolipid or another surfactant. Addition of surfactant also failed to further stimulate the biodegradation of phenanthrene present as crystals or dissolved in an organic phase by *P. aeruginosa* under conditions of high agitation (Köhler et al., 1994).

Emulsification. Hexadecane emulsification by several surfactants was investigated since it is one of the steps that is thought to be involved in the mass transfer of substrate from a separate liquid phase to cells of *P. aeruginosa*. The emulsifying activity of the surfactants was determined by visual inspection 1 hour after emulsion preparation by vortexing. Rhamnolipid, SDBS, all the nonionic polyoxyethylene surfactants, and dodecylmaltoside formed stable emulsions (Fig. 6.8) 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 brought into the aqueous phase by vortexing a two-phase mixture of hexadecane and an aqueous surfactant solution containing rhamnolipid, Brij 30, $C_{12}E_{10}$, Brij 35, or SDBS was 52 ± 19, 46 ± 22, 105 ± 49, 67 ± 61, and 139 ± 27 mg/L, respectively. All surfactants increased the amount of hexadecane in the aqueous phase compared to the control without surfactant (11 ± 6 mg/L), but the values were not highest for rhamnolipid. The number-averaged size and size distribution of hexadecane droplets in aqueous solution containing rhamnolipid, Brij 30, $C_{12}E_{10}$, Brij 35, SDBS, and Triton X-100 was 0.4 ± 0.3, 1.2 ± 0.9, 1.3 ± 1.0, 2.1 ± 2.8, 0.2 ± 0.2, and 0.8 ± 0.7 μm, respectively. Rhamnolipid

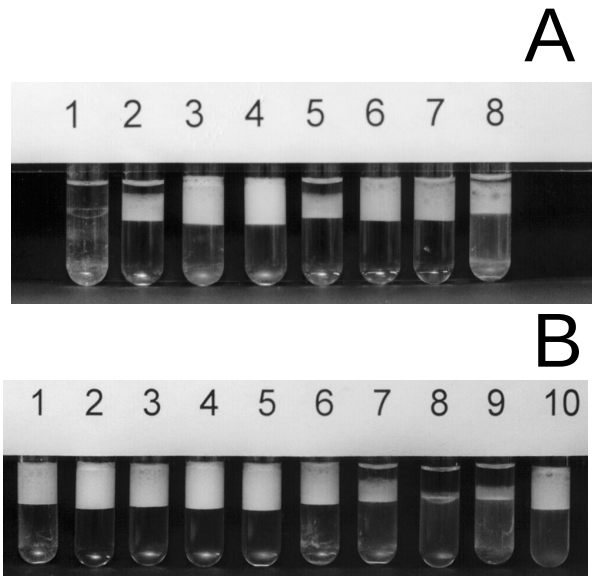

Figure 6.8. Emulsification of hexadecane by various surfactants (100 mg/L unless indicated otherwise). Pictures of emulsions were taken 1 h after preparation. (A) 1, no surfactant; 2, rhamnolipid (20 mg/L); 3, rhamnolipid (100 mg/L); 4, rhamnolipid (500 mg/L); 5, Brij 30; 6, $C_{12}E_{10}$; 7, Brij 35; 8, SDBS. (B) 1, Triton X-100; 2, Brij 78; 3, Tween 20; 4, Tween 40; 5, Tween 80; 6, Tween 85; 7, Sodium deoxycholate; 8, *n*-octyl-β-D-glucoside; 9, *n*-decyl-β-D-maltoside; 10, *n*-dodecyl-β-D-maltoside.

formed smaller emulsion droplets than the nonionic surfactants, although SDBS formed even smaller droplets. The combined results show that rhamnolipid had strong emulsifying activity towards hexadecane, as had the surfactants SDBS, Brij 35, Triton X-100, Brij 78, and dodecylmaltoside.

Correlation of the emulsifying activity of the surfactants with their effect on hexadecane biodegradation. It was determined whether the outstanding effect of rhamnolipid could be understood from its emulsifying activity. A correlation between the emulsifying activities of the surfactants and their effects on hexadecane biodegradation must however be made with caution since the experimental setup of both types of experiments differed in the phase ratios, mixing intensities and presence of cells. These parameters can directly influence the type of emulsions formed. Emulsification experiments showed that rhamnolipid was among the best surfactants to emulsify hexadecane. However, it was at par with SDBS, which is a surfactant that inhibited biodegradation of hexadecane. Also other surfactants with favorable emulsifying activities, such as Brij 78 and dodecylmaltoside, failed to stimulate biodegradation. Therefore, no direct relation was found between the emulsifying activities of the surfactants and their effect on biodegradation.

Correlation of the effects of surfactants on biodegradation with their HLB. The effects of surfactants on biodegradation of hexadecane were correlated with their hydrophile-lipophile balance (HLB). The HLB of a surfactant is an empirical parameter that describes the affinity of the surfactant for the oil-water interface (Becker, 1984). The HLB of the surfactants used in the present study ranged from 9.7 for Brij 30 (Sigma) to 23.4 for sodium deoxycholate (Kunieda and Sato, 1992). We estimated an HLB of rhamnolipid of 24.1 by using group contributions (Lin, I.J. et al., 1973), and of 17.0 by using a correlation of HLB with CMC for sodium carboxylic acids (Lin, I.J. 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 correlated to the HLB value of the surfactants. Previously, the surfactant HLB was observed to correlate with their effect on biodegradation for a mixed culture (Oberbremer et al., 1990) and a *Rhodococcus* sp. grown to stationary phase (Bruheim et al., 1997), but such a correlation was absent for a rhamnolipid-producing *P. aeruginosa* (Nakahara et al., 1981), a *Deleya salina* strain (Bruheim and Eimhjellen, 1998), and another mixed culture (Van Hoof and Jafvert, 1996).

Correlation of the effects of surfactants on biodegradation with their CMC. The

critical micelle concentration (CMC) of rhamnolipid is approximately 50 mg/L (Zhang and Miller, 1995; Herman et al., 1997b; Chapter 2, 3, and 4). Of the surfactants with CMC values similar to rhamnolipid, some stimulated biodegradation (Brij 35, CMC 72 mg/L; $C_{12}E_{10}$, CMC 46 mg/L (Guha and Jaffé, 1995); Tween 40, CMC 55 mg/L estimated from HLB; and Triton X-100, CMC 43 mg/L (Guha and Jaffé, 1995)) but one inhibited biodegradation (Brij 78, CMC 55 mg/L estimated from HLB). Several surfactants with CMC values lower than rhamnolipid stimulated biodegradation (Brij 30, 1.4 mg/L; Tween 80, 16 mg/L). Surfactants with CMC values higher than 100 mg/L (SDBS, octylglucoside, deoxycholate, and decylmaltoside) did not stimulate hexadecane biodegradation. Since all surfactants were used at a concentration of 100 mg/L, 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 (this study) or even at 10 or 5 mg/L (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). The absence of a correlation with surfactant CMC for our data indicates that the CMC *per se* is not of importance for determining the effect of surfactants in the biodegradation experiments.

Processes involved in the rhamnolipid-enhanced biodegradation of hexadecane. None of the surfactants that were tested stimulated the biodegradation of hexadecane initially present in silica. Apparently, the transfer of hexadecane from the matrix to the aqueous phase that limited the biodegradation rate was not stimulated by any of the surfactants used under conditions of end over end mixing. In contrast, the degradation of hexadecane initially present in VitraPOR P3 and in CPG-10-3000 and of hexadecane present as a second liquid phase media was stimulated by rhamnolipid and other surfactants.

Since it is generally assumed that *P. aeruginosa* takes up hexadecane in pseudosolubilized or emulsified form (Nakahara et al., 1977, 1981; Bouchez-Naïtali et al., 1999) and it is unlikely that rhamnolipid influences the metabolism of hexadecane, the above implies that surfactants either enhanced the rate of emulsification or the rate of uptake of (emulsified) substrate. Two observations suggest the latter to be the case. First, experiments without rhamnolipid present indicated that the rate-limiting process was uptake rather than solubilization of substrate. Second, no obvious relation was found between the effects of the surfactants on the biodegradation of hexadecane and their emulsifying activities, their CMC, or their HLB. The stronger stimulation by rhamnolipid than by other surfactants might arise from the existence of a specific cell-biosurfactant interaction that increases flux of organic compounds over the membrane. The existence of such a specific uptake mechanism has been suggested, however without conclusive proof (Hisatsuka et al., 1971; Ratledge, 1988).

CONCLUSIONS

The results show 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 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 under continuous flow operation but not under conditions of high agitation. This effect is expected to be important during *in situ* bioremediation when biodegradation is limited by transport of the contaminant from soil to the site where bacterial activity takes place (Herman et al., 1997a), and when non-

aqueous phase liquids are to be removed by surfactant-enhanced pump and treat technology (Pennell et al., 1993; Bai et al., 1997). The stimulation by rhamnolipid of the biodegradation of substrate present as a separate liquid phase seems to result from an effect on uptake of substrate. This effect was of importance under well-mixed conditions.