APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1699–1705 Vol. 61, No. 5
0099-2240/95/$04.00+0

Influence of Nonionic Surfactants on Bioavailability and Biodegradation of Polycyclic Aromatic Hydrocarbons

FRANK VOLKERING,[1]* ANTON M. BREURE,[1] JOHAN G. VAN ANDEL,[1] AND WIM H. RULKENS[2]

Laboratory for Waste Materials and Emissions, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven,[1] and Department of Environmental Technology, Wageningen Agricultural University, 6700 EV Wageningen,[2] The Netherlands

Received 3 October 1994/Accepted 15 February 1995

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

Polycyclic aromatic hydrocarbons (PAHs) are poorly soluble, hydrophobic organic compounds which have been released into the environment on a large scale. This fact, along with the toxicity and mutagenicity of PAHs, makes these compounds priority pollutants. Although laboratory studies have revealed that virtually all PAHs are biodegradable (6), the rates of PAH degradation at contaminated sites are often much lower than the rates expected on the basis of the results of laboratory studies. It is generally accepted that a low level of bioavailability (i.e., slow release of the pollutant from solid phases to the aqueous phase) is one of the most important factors involved in the slow biodegradation of hydrophobic organic compounds in soil (16).

A possible way of enhancing the bioavailability of hydrophobic organic compounds is the application of (bio)surfactants, molecules which consist of a hydrophilic part and a hydrophobic part. Because of this property these molecules tend to concentrate at surfaces and interfaces and to decrease levels of surface tension and interfacial tension. Another important characteristic of surfactants is the fact that above a certain concentration (the critical micelle concentration) aggregates of 10 to 200 molecules, which are called micelles, are formed. The effect of a surfactant on the bioavailability of organic compounds can be explained by three main mechanisms: (i) dispersion of nonaqueous-phase liquid hydrocarbons, leading to an increase in contact area, which is caused by a reduction in the interfacial tension between the aqueous phase and the nonaqueous phase; (ii) increased solubility of the pollutant, caused by the presence of micelles which may contain high concentrations of hydrophobic organic compounds, a mechanism which has been studied extensively previously (8, 9, 15); and (iii) "facilitated transport" of the pollutant from the solid phase to the aqueous phase, which can be caused by a number of phenomena, such as lowering of the surface tension of the pore water in soil particles, interaction of the surfactant with solid interfaces, and interaction of the pollutant with single surfactant molecules.

The first mechanism is involved only when liquid-phase hydrocarbon is present and is not discussed further in this paper. Both of the other mechanisms can cause an increase in the rate of mass transfer to the aqueous phase, and therefore the relative contributions of these two mechanisms to the enhancement of bioavailability are often unknown. When biodegradation is limited by the bioavailability of the substrate, the substrate concentration in the bulk liquid is much lower than the saturation concentration. Because of this, we inferred previously (25) that in bioremediation processes the contribution of solubility enhancement is probably small. This means that facilitated transport is the most important mechanism for enhancement of biodegradation in these processes.

Many studies on the use of (bio)surfactants for soil remediation have dealt either with the effect of surfactants on desorption and biodegradation of organic contaminants sorbed onto soil (2–4, 13, 14) or with the practical problems of application of surfactants to soil (1, 22, 23). The results of these studies are often difficult to interpret because of the complexity of the interactions among soil, pollutant, surfactant, and microorganisms.

In this study, we investigated the effects of four nonionic surfactants on the bioavailability and rates of biodegradation of crystalline naphthalene and phenanthrene. Crystalline PAHs can occur in contaminated soil, but the main reason for choosing this system is its relative simplicity. Previous research has given us good insight in the biodegradation kinetics of crystalline PAHs (24, 25), and therefore the effects of the presence of surfactants on bioavailability and biodegradation can be interpreted unambiguously. Insight into these effects can be used to gain a better understanding of the more com-

* Corresponding author. Mailing address: Laboratory for Waste Materials and Emissions, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Phone: (31) 30-743544. Fax: (31) 30-293651.

plicated processes involved in the biodegradation of sorbed substrates in the presence of surfactants.

MATERIALS AND METHODS

Bacterial cultures. The isolation of strain 8909N growing on naphthalene has been described previously (25). Strain 8803F was isolated in cultures containing phenanthrene in the same way. Both of these strains are gram-negative *Pseudomonas* strains.

Media and culture conditions. Organisms were grown at 30°C in a mineral medium that was essentially the medium described by Evans et al. (10) except that the concentrations of the medium components were one-tenth those described by Evans et al. This medium contained 10 mM NH_4Cl, 1 mM NaH_2PO_4, 1 mM KCl, 0.2 mM Na_2SO_4, 0.125 mM $MgCl_2$, 2 μM $CaCl_2$, 0.01 μM Na_2MoO_4, and 0.5 ml of a spore solution per liter; the spore solution contained 0.12 mM HCl, 5 mM ZnO, 20 mM $FeCl_3$, 10 mM $MnCl_2$, 1 mM $CuCl_2$, 2 mM $CoCl_2$, and 0.8 mM H_3BO_3. No chelating agent was used. The medium was buffered at pH 7.0 with 50 mM sodium phosphate. Pure cultures were maintained on mineral medium agar slants containing 1.5% agar and the PAH required at a concentration of 0.1% (wt/vol) and were stored at 4°C. To obtain washed cells, the PAH crystals were removed from an exponentially growing batch culture by decanting, after which the broth was centrifuged for 15 min at 12,000 × *g* and washed twice with 50 mM potassium phosphate buffer (pH 7).

Dissolution experiments. The dissolution experiments were performed in 250-ml serum flasks on a rotary shaker (200 rpm) at 30°C. The experiments were started by adding 0.50 g of naphthalene (particle diameter, 1,000 to 2,400 μm) or 0.15 g of phenanthrene (particle diameter, 600 to 1,000 μm) to flasks containing 150 ml of sterile buffered mineral medium and the appropriate amount of surfactant. At regular intervals samples (0.75 ml) were filtered with a regenerated cellulose filter (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) and diluted 1:1 with acetonitrile to determine the PAH concentration in the liquid phase.

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

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

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

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

Analytical procedures. Bacterial growth was determined by measuring the optical density at 540 nm. Levels of dissolved PAHs were determined by injecting samples into a high-performance liquid chromatography apparatus (series HP 1050; Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a ChromSpher C_{18} (PAH) column (Chrompack, Middelburg, The Netherlands). The eluent used was a mixture of acetonitrile and water (85:15). Peaks were detected with a UV detector by measuring the A_{274} for naphthalene and the A_{254} for phenanthrene. The CO_2 contents of the headspace gases in serum flasks were determined with a Hewlett-Packard type 5890 gas chromatograph equipped with a thermal conductivity detector and a Hayesep Q packed stainless steel column (diameter, 0.125 in. [3.175 mm]; length, 2 m; Chrompack). Helium was used as the carrier gas, and the flow rate was 30 ml min^{-1}. The injector temperature was 150°C, the oven temperature was 80°C, and the detector temperature was 200°C. The injection volume was 250 μl with splitless injection. The dissolved oxygen contents were determined with a biological oxygen monitor as described by Dubinsky et al. (7). The viscosity of a solution was determined with a Ubelohde viscosimeter by measuring the time needed for the fluid to pass through a capillary tube at 30°C.

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

Modeling. Dissolution kinetics in the presence of surfactants were described with a model based on the model for dissolution that has been described previously (24). The model used to describe the effects of surfactants was formulated on the basis of the following assumptions: (i) at surfactant concentrations above the critical micelle concentrations, the surfactants form micelles (assumption 1); (ii) nonmicellar surfactant does not affect the solubility of PAHs (assumption 2); (iii) at equilibrium, the concentration of PAHs in the micelles is a linear function of the PAH concentration in the water phase (assumption 3); (iv) the limitation of mass transfer from the water phase to the micellar phase occurs in the water phase (assumption 4); and (v) the micelles act as a separate phase and only increase the apparent solubility of the PAHs (assumption 5). Assumptions 1 through 3 are generally accepted (9), and assumption 4 is based on the small diameter of the micelles. Assumption 5 is the central issue of the model. This assumption needs to be verified by comparing calculated values with experimental data. A model based on these assumptions was constructed (equations 1 to 5). Equation 1 gives the mass balance of a PAH in the three phases:

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

where Q_t is the amount of solid PAH (in kilograms), C_t is the aqueous PAH concentration (in kilograms per cubic meter), V is the aqueous volume (in cubic meters), $C_{\mathrm{mic,t}}$ is the micellar PAH concentration (in kilograms per cubic meter), and V_{mic} is the micellar volume (in cubic meters).

Equation 2 describes the mass transfer from crystals to the aqueous phase (dissolution):

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

where $K_{\mathrm{l,c}}$ is the crystal-water mass transfer constant (in meters per hour), A_t is the surface area of crystals (in square meters), and $C_{\max}$ is the maximum aqueous PAH concentration (in kilograms per cubic meter).

As the mass transfer limitation for transport from the aqueous phase to the micellar phase occurs in the aqueous phase (assumption 4), the mass transfer from the aqueous phase to the micelles can be described with equation 3:

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

where $K_{\mathrm{l,mic}}$ is the micelle-water mass transfer constant (in meters per hour), A_{mic} is the surface area of micelles, and m is the water-micelle partition coefficient, which is defined as the ratio of the concentration in the micellar phase to the concentration in the aqueous phase when the system is in equilibrium (in cubic meters per cubic meter). Combining these equations yields equation 4:

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

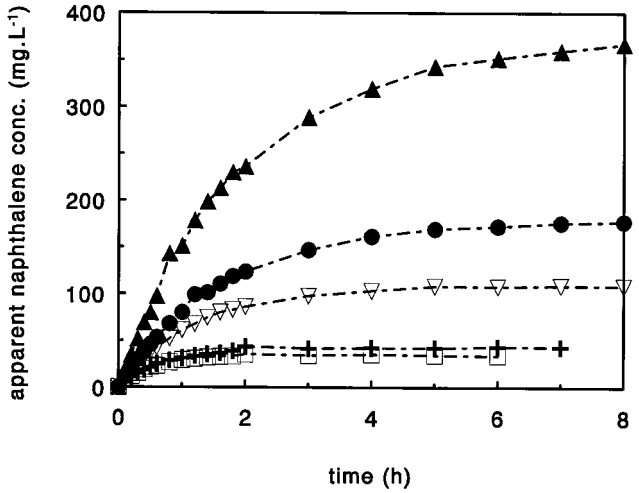

FIG. 1. Effects of different concentrations of Tergitol NPX on the dissolution kinetics of naphthalene. The preparations contained no surfactant (□) or surfactant at a concentration of 0.1 g liter^{-1} (+), 1.0 g liter^{-1} (▽), 2.0 g liter^{-1} (●), or 5.0 g liter^{-1} (▲).

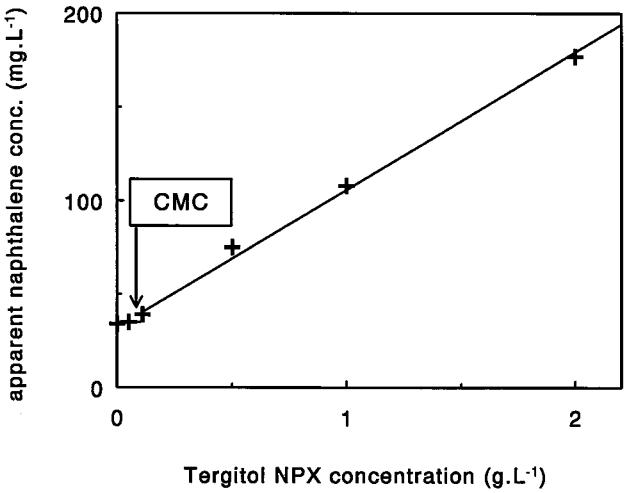

FIG. 2. Maximal solubility of naphthalene as a function of the concentration of Tergitol NPX. CMC, critical micelle concentration.

The total PAH concentration in the solution can be calculated with equation 5:

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

where C_{tot} is the overall PAH concentration in solution (in kilograms per cubic meter).

The micelle diameter was assumed to be 3×10^{-9} m (5). Because of the small micelle diameter, the water-micelle interfacial area was much larger than the water-crystal interfacial area; for example, at a surfactant concentration of 1 g liter^{-1}, the water-micelle area was 218 m^2, whereas in the dissolution experiments the water-crystal area was approximately 1.38×10^{-3} m^2 for naphthalene and 9.84×10^{-4} m^2 for phenanthrene. This means that the mass transport from the aqueous phase to the micellar phase was much faster than the mass transport from the crystals to the water phase. Thus, partitioning of the PAH molecules over the aqueous phase and the micellar phase occurs almost instantaneously compared with the dissolution process. The computer program PSI/c (BOZA automatisering, Pijnacker, The Netherlands) was used for the modeling study.

RESULTS

Dissolution experiments. The dissolution kinetics of naphthalene crystals in the presence of different concentrations of Tergitol NPX are shown in Fig. 1. As expected, surfactant at concentrations below the critical micelle concentration had no effect on the dissolution of naphthalene, whereas the presence of micelles resulted in higher apparent levels of solubility. In Fig. 2 the maximal level of apparent naphthalene solubility is plotted as a function of the surfactant concentration. From the slope of the linear part of the graph it is possible to calculate the micelle-water partition coefficient. This kind of experiment was performed with naphthalene and phenanthrene for the four surfactants. For comparison, partition was also expressed as the molar water partition coefficient ($K_{m,w}$), which was calculated as described by Edwards et al. (9). The values for the micelle-water partition coefficient and log $K_{m,w}$ resulting from the dissolution experiments are shown in Table 1.

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

Biodegradation experiments. Batchwise bacterial growth on a PAH in the presence of PAH crystals results in a typical growth curve (exponential growth, followed by a linear growth phase in which the dissolution rate of the PAH is the limiting factor) (24). To investigate the effects of surfactants on the biodegradation of PAHs, surfactants were added at different concentrations to batch cultures that were in the linear growth phase. Figure 3 shows the results of the experiments in which naphthalene was used with the surfactants Triton X-100 and Tergitol NPX. In separate experiments in which naphthalene crystals were removed before any surfactant was added, no bacterial growth was observed after the surfactant was added, and therefore the increase in the linear growth rate was not caused by the organism using the surfactant as a source of carbon and energy. Figure 4 shows the results of similar experiments performed with strain 8803F and with phenanthrene as the substrate. The PAH concentration in solution in the dissolution-limited growth phase ranged from 20 to 50 μg liter^{-1} for both naphthalene and phenanthrene. These results are in good agreement with the low aqueous PAH concentra-

TABLE 1. Water-micelle partition coefficients and log $K_{m,w}$ values of the surfactants for naphthalene and phenanthrene[a]

Surfactant	Naphthalene		Phenanthrene	
	Water-micelle partition coefficient	log $K_{m,w}$	Water-micelle partition coefficient	log $K_{m,w}$
Triton X-100	1.83×10^3	4.68	3.43×10^4	6.02
Tergitol NPX	2.19×10^3	4.79	4.12×10^4	6.20
Igepal CA-720	1.35×10^3	4.64	2.86×10^4	5.97
Brij 35	9.0×10^2	4.48	2.02×10^4	5.83

[a] $K_{m,w}$ values were calculated as described by Edwards et al. (9).

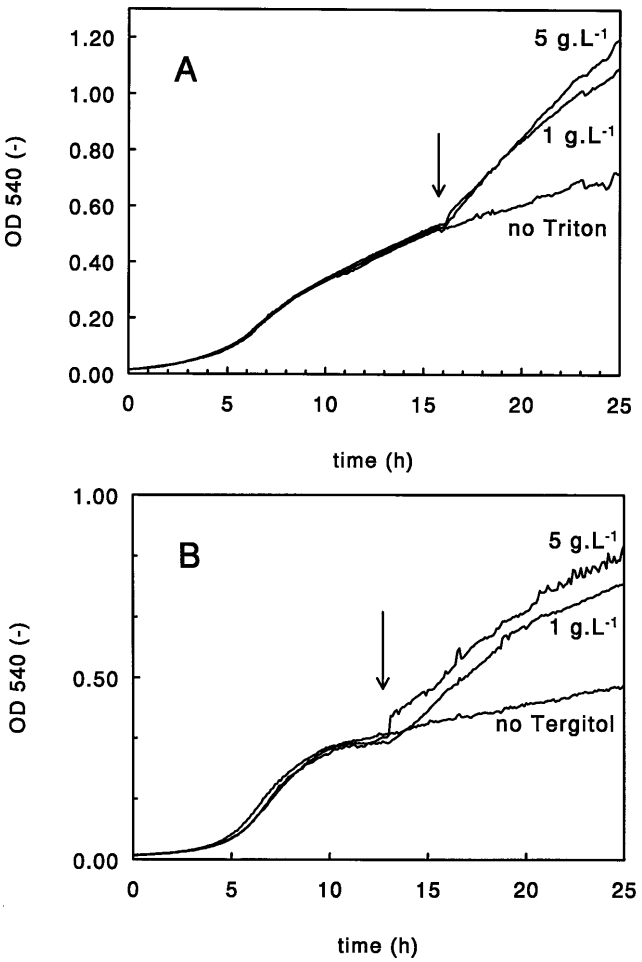

FIG. 3. Effects of adding Triton X-100 (A) and Tergitol NPX (B) on the growth of strain 8909N on crystalline naphthalene. The surfactant concentrations used are indicated on the figure; the arrows indicate when surfactant was added. OD 540, optical density at 540 nm.

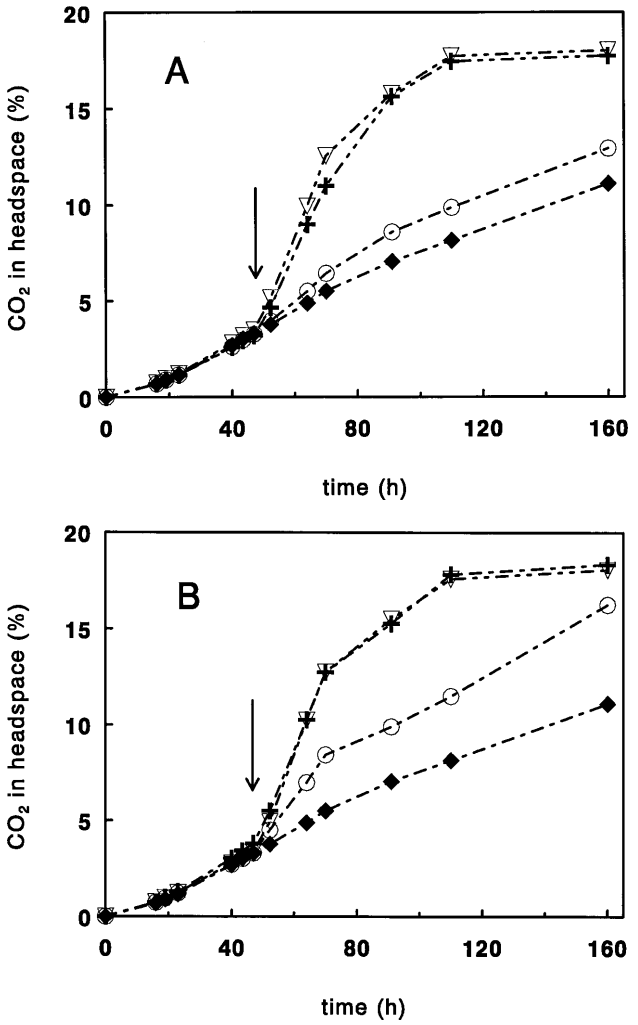

FIG. 4. Effects of adding Triton X-100 (A) and Tergitol NPX (B) on CO_2 production by strain 8803F growing on crystalline phenanthrene. Symbols: ◆, no surfactant, ○, 0.1 g liter^{-1}; +, 1 g liter^{-1}; ▽, 5 g liter^{-1}. The arrows indicate when surfactant was added.

tions measured previously in biodegradation experiments (24). As some of the PAH is present in the micellar phase, the aqueous PAH concentration is even lower. At the end of the experiment, when crystals were no longer visible and the CO_2 concentration remained constant, no difference in the CO_2 levels in the cultures with and without surfactant was detected, indicating that no surfactant biodegradation had occurred.

Bioavailability of micellar PAHs. The first step in the biodegradation of PAHs is an oxidation step; therefore, the initial oxygen uptake rate of washed cells can be used as a measure of bacterial activity on PAHs. Because of the short time needed for the measurements, the possibility that growth occurred can be eliminated, and Michaelis-Menten kinetics can be used to describe the relationship between activity and substrate concentration (27). Figure 5 shows the results of oxygen uptake experiments performed with strain 8909N and naphthalene with and without Triton X-100 and Tergitol NPX. The K_m values obtained by fitting the data with Michaelis-Menten kinetics are also shown. The K_m found in this way can be used as a measure of bacterial activity, but has no physiological background and therefore cannot be used as a characteristic for microorganisms.

DISCUSSION

Effects of surfactants on dissolution kinetics. The increase in the apparent level of solubility due to the presence of surfactant micelles which we observed is consistent with the results of Edwards et al. (9), who obtained slightly lower values for log $K_{m,w}$, probably because of their use of 1% methanol in the aqueous phase. Although the water-micelle partition coefficient is a useful parameter, it provides no information about dissolution kinetics. Therefore, a dissolution model was constructed in which it was assumed that the increase in the apparent level of solubility was the only effect of the micelles. As an example, the results of modeling for dissolution of naphthalene when Tergitol NPX was the surfactant are shown in Fig. 6. The factor $K_{l,c} \cdot A_t$, a measure of the maximal dissolution rate, was calculated from the experiment performed without surfactant as described previously (24); the same value was used for modeling the experiments with surfactant (Fig. 6 and Table 2).

The model predictions for dissolution kinetics clearly underestimate the dissolution rates of PAHs at surfactant concentrations higher than the critical micelle concentration. By in-

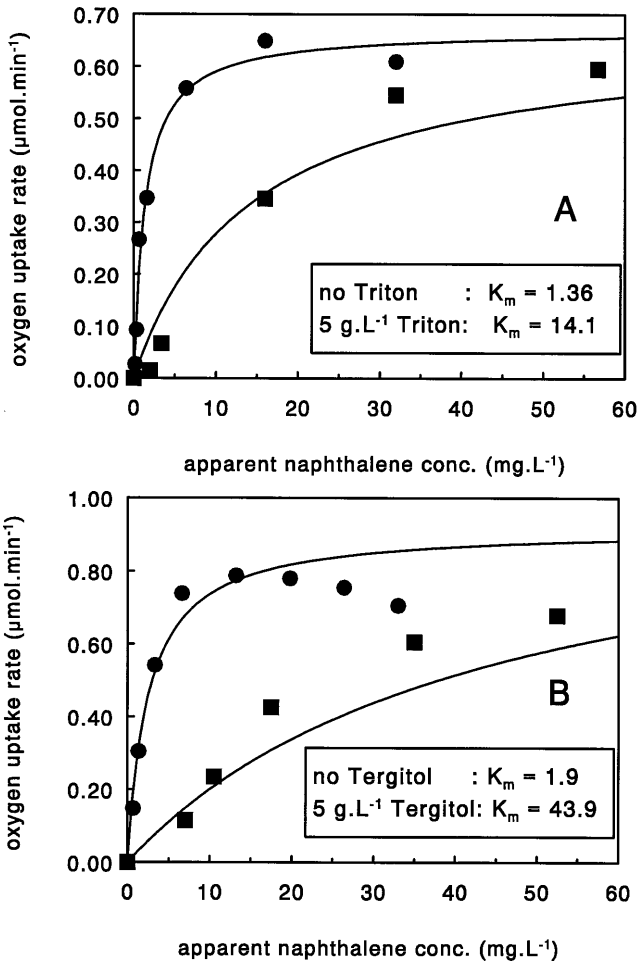

FIG. 5. Oxygen uptake rate of strain 8909N on naphthalene as a function of the apparent naphthalene concentration with and without the surfactant Triton X-100 (A) or Tergitol NPX (B). Symbols: ●, no surfactant, ■, 5 g of surfactant per liter. The curves represent fitted Michaelis-Menten kinetics.

creasing the factor $K_{l,c} \cdot A_t$, however, it was possible to obtain a good description of the dissolution kinetics. The maximal dissolution rates, which were found by increasing the $K_{l,c} \cdot A_t$ values for the different surfactants, are given in Table 2. The effects of the surfactants were more pronounced for phenanthrene than for naphthalene; the reason for this difference is not clear. The high values for $K_{l,c} \cdot A_t$ cannot be explained by decreases in viscosity, as viscosity measurements revealed that the viscosity of a surfactant solution increased slightly as the surfactant concentration increased; this is consistent with the observations of Vigon and Rubin (23). Therefore, the presence of micelles affects the dissolution rate, and the assumption that the micelles act only as a separate phase is not valid. Two possible mechanisms for the effect of surfactant micelles on the dissolution rate can be put forward. In the first mechanism the micelles are present not only in the aqueous bulk phase, but also in the diffusion layer which surrounds the PAH crystals. This should decrease the PAH concentration in the layer and thus increase the $K_{l,c}$ value. More likely, however, is the possibility that a hemicellular layer of surfactant molecules is formed around the crystals. This allows a dynamic exchange of the micellar PAH to occur. The latter mechanism is very similar to the interactions of surfactant molecules with soil interfaces (17) and has also been proposed by Gerson (11), although Gerson presented no evidence. On the basis of the results of the experiments described above, no distinction between the two mechanisms can be made.

Surfactant toxicity. It is well known that surfactants can be toxic to bacteria. Although nonionic surfactants are generally less toxic than ionic surfactants and although gram-negative bacteria are generally less sensitive than gram-positive bacteria (20), it seemed likely that the presence of surfactants at the high concentrations used in this study (up to 10 g liter^{-1}) could have a negative effect on the bacterial strains that were used. However, in all of the growth and activity experiments that were performed, no toxic effect of the surfactants on the microorganisms was found.

Biodegradation experiments. Addition of surfactant to batch cultures growing on crystalline naphthalene and phenanthrene during the dissolution-limited phase results in an increase in the linear growth rate. The effect is more pronounced for phenanthrene than for naphthalene. This effect cannot be caused by an increase in PAH solubility since the aqueous PAH concentration in the dissolution-limited phase is virtually zero. This implies that the maximal dissolution rate increases when surfactant is present (facilitated transport). These results confirm the increase in the dissolution rate which we observed in the dissolution experiments. However, the increase in the growth rate was less than the increase in the maximal dissolution rate that was found in the dissolution experiments. This can be explained by the fact that there was less PAH present in the biodegradation experiments than in the dissolution experiments. An additional explanation involves sorption of the surfactant onto the bacteria, a common phenomenon (20) which results in lower aqueous surfactant concentrations. When surfactant was added at the beginning of batch growth experiments with naphthalene, the exponential growth phase was longer, but the maximal growth rate on naphthalene did not increase. In contrast, Bury and Miller (5) observed higher maximal growth rates on *n*-decane and *n*-tetradecane in the presence of surfactants than in the absence of surfactants. The surfactants which these authors used, however, were degraded along with the alkanes, and the increase could have been caused by direct bacterial uptake of micelles filled with alkanes.

Bioavailability of micellar PAHs. In several studies micellar

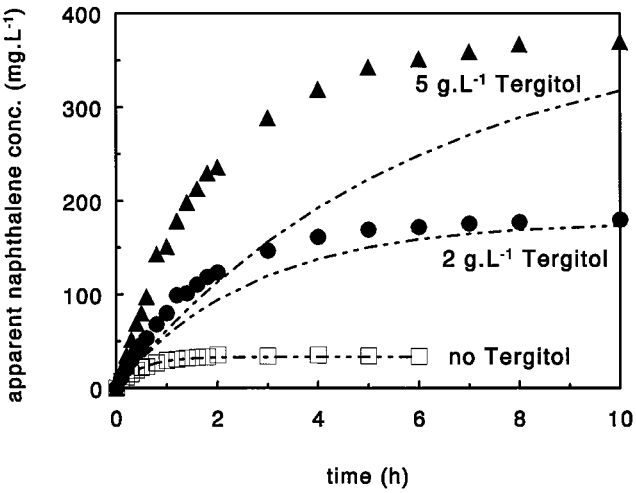

FIG. 6. Modeling of dissolution kinetics in the presence of different concentrations of Tergitol NPX. The symbols represent measured data and the dashed lines represent model predictions.

TABLE 2. Maximal dissolution rates calculated from dissolution experimental data obtained with crystalline naphthalene and phenanthrene in the presence of different concentrations of nonionic surfactants

Surfactant concn (g liter^{-1})	Maximal dissolution rate (g liter^{-1} h^{-1}) for:							
	Naphthalene				Phenanthrene			
	Triton X-100	Tergitol NPX	Igepal CA-720	Brij 35	Triton X-100	Tergitol NPX	Igepal CA-720	Brij 35
0	0.013	0.013	0.013	0.012	0.001	0.001	0.001	0.001
0.1	0.014	0.013	0.014	0.011	ND[a]	ND	ND	ND
0.5	0.019	0.015	0.017	0.013	0.010	0.010	0.009	0.008
1	0.025	0.016	0.022	0.015	0.017	0.016	0.017	0.013
2	0.029	0.019	0.026	0.017	0.031	0.027	0.028	0.022
5	0.035	0.034	0.037	0.024	0.068	0.060	0.072	0.047

[a] ND, not determined.

solubilization has been used to enhance the availability of poorly soluble substrates (5, 12, 21), and the role of solubilization by microbial excretion products in growth on alkanes has been studied extensively (19). The high exit rates of micellar substrate allow exponential growth at high cell densities. It should be noted that this is no evidence that the substrate in the micelles is readily available to the microorganisms. The oxygen uptake rate of strain 8909N on naphthalene was affected by the presence of surfactants, as shown in Fig. 5 for Triton X-100 and Tergitol NPX. This effect cannot be attributed to toxicity of the surfactants. One explanation for this observation is the possibility that the PAHs were partitioned between the water phase and the micellar phase. Figures 5 and 7 show the results of the same experiments but in Fig. 7 the *x* axis shows the aqueous naphthalene concentration which was calculated from the total naphthalene concentration with the partition coefficients in Table 1. The K_m values obtained by fitting these results with Michaelis-Menten kinetics are also shown in Fig. 5 and 7. From the corresponding lines and values for K_m, it is clear that the naphthalene concentration in the water phase controls the bacterial activity. More evidence for this was obtained from growth experiments performed with strain 8909N cells, which were not adapted to high concentrations of naphthalene. We observed that in batch growth experiments with naphthalene (data not shown) inoculation with active cells that originated from batch cultures containing low naphthalene concentrations (linear growth phase) resulted in a lag phase of 10 to 24 h, whereas after inoculation with active cells that originated from cultures containing high naphthalene concentrations (exponential growth phase) there was no lag phase. This indicates that high concentrations of naphthalene can be toxic to the bacteria and that the bacteria can adapt to these high concentrations. This is not an uncommon phenomenon for gram-negative bacteria (26). Growth experiments performed with naphthalene and unadapted strain 8809N cells showed that in cultures to which 1 to 5 g of Triton X-100 per liter was added before inoculation no lag phase occurred. This illustrates that the toxicity of naphthalene is reduced when it is present in micelles. This is a good explanation for the fact that adaptation times are shorter in the presence of biosurfactants than in the absence of biosurfactants, as described by Oberbremer et al. (18).

The most important conclusion that can be drawn from the data described above is that PAHs in the micellar phase are not readily available to microorganisms. Thus, micellar PAH is a protected reservoir that may replenish aqueous-phase PAH when it is depleted by biodegradation. This has been stated previously by Zhang and Miller (28), but the data which these authors presented do not justify their conclusion, as they compared equilibrium solubility with growth kinetics. The fact that micellar substrate is not readily bioavailable has important consequences for the application of surfactants in bioremediation. First, the presence of micelles may lower the concentration of contaminant in the water phase, thereby reducing the bacterial activity or growth. This was seen in the oxygen uptake

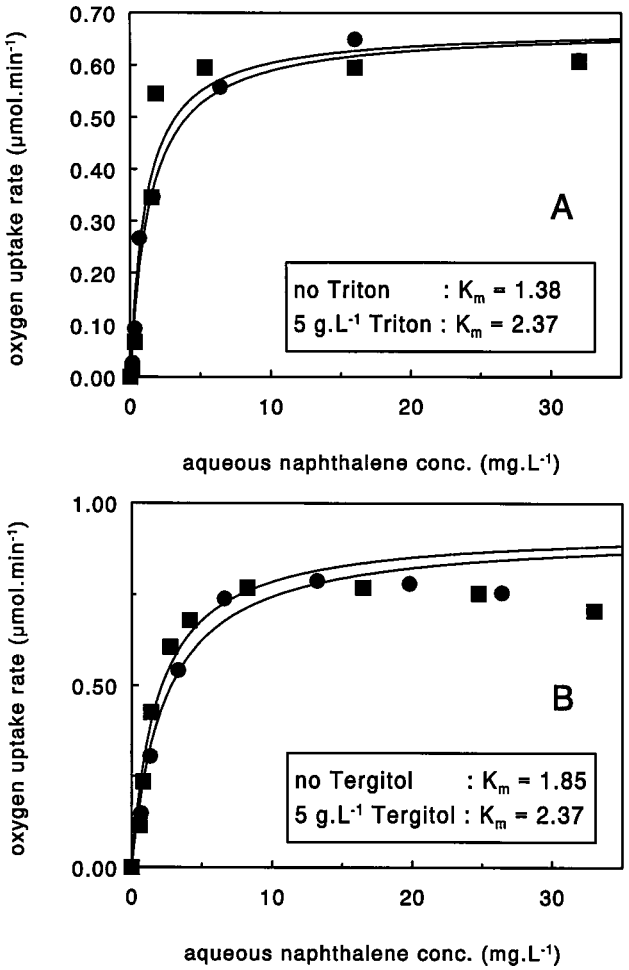

FIG. 7. Oxygen uptake rate of strain 8909N on naphthalene as a function of the aqueous naphthalene concentration with and without the surfactant Triton X-100 (A) or Tergitol NPX (B). Symbols: ●, no surfactant; ■, 5 g of surfactant per liter. The curves represent fitted Michaelis-Menten kinetics.

experiments whose results are shown in Fig. 5. At the same total naphthalene concentration, the activity of cells is reduced by the presence of a surfactant. This effect, combined with the toxicity of the surfactants, could explain the inhibition of phenanthrene mineralization by micellar surfactant, as described by Laha and Luthy (13, 14). Second, in in situ processes, the nonavailable substrate may be washed out and thus cause unwanted contamination of groundwater. For the reasons described above, careful study is needed before the use of surfactants in biological soil treatment can be recommended.

ACKNOWLEDGMENT

This work was supported by grant 8977 from the Netherlands Integrated Soil Research Program.

REFERENCES

1. **Abdul, A. S., T. L. Gibson, and D. N. Rai.** 1990. Selection of surfactants for the removal of petroleum products from shallow sandy aquifers. Ground Water **28:**920–926.
2. **Aronstein, B. N., and M. Alexander.** 1992. Surfactants at low concentrations stimulate the biodegradation of sorbed hydrocarbons in samples of aquifer sands and soil slurries. Environ. Toxicol. Chem. **11:**1227–1233.
3. **Aronstein, B. N., and M. Alexander.** 1993. Effect of a nonionic surfactant added to the soil surface on the biodegradation of aromatic hydrocarbons within the soil. Appl. Microbiol. Biotechnol. **39:**386–390.
4. **Aronstein, B. N., Y. M. Calvillo, and M. Alexander.** 1991. Effect of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. Environ. Sci. Technol. **25:**1728–1731.
5. **Bury, S. J., and C. A. Miller.** 1993. Effect of micellar solubilization on biodegradation rates of hydrocarbons. Environ. Sci. Technol. **27:**104–110.
6. **Cerniglia, C. E.** 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351–368.
7. **Dubinsky, Z., P. G. Falkowski, A. F. Post, and U. M. van Hes.** 1987. A system for measuring phytoplankton photosynthesis in a defined light field with an oxygen electrode. J. Plankton Res. **9:**607–612.
8. **Edwards, D. A., Z. Liu, and R. G. Luthy.** 1992. Interactions between nonionic surfactant monomers, hydrophobic organic compounds and soil. Water Sci. Technol. **26:**147–158.
9. **Edwards, D. A., R. G. Luthy, and Z. Liu.** 1991. Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. Environ. Sci. Technol. **25:**127–133.
10. **Evans, C. G. T., D. Herbert, and D. W. Tempest.** 1970. The continuous cultivation of microorganisms. II. Construction of a chemostat. Methods Microbiol. **2:**277–327.
11. **Gerson, D. F.** 1993. The biophysics of microbial surfactants: growth on insoluble substrate, p. 269–286. *In* N. Kosaric (ed.), Biosurfactants. Marcel Dekker, Inc., New York.
12. **Guerin, W. F., and G. E. Jones.** 1988. Mineralization of phenanthrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **54:**937–944.
13. **Laha, S., and R. G. Luthy.** 1991. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. Environ. Sci. Technol. **25:**1920–1930.
14. **Laha, S., and R. G. Luthy.** 1992. Effects of nonionic surfactants on the mineralization of phenanthrene in soil-water systems. Biotechnol. Bioeng. **40:**1367–1380.
15. **Liu, Z., D. A. Edwards, and R. G. Luthy.** 1992. Sorption of non-ionic surfactant onto soil. Water Res. **26:**1337–1345.
16. **Mihelcic, J. R., D. R. Lueking, R. J. Mitzell, and J. M. Stapleton.** 1993. Bioavailability of sorbed- and separate-phase chemicals. Biodegradation **4:**141–153.
17. **Nayyar, S. P., D. A. Sabatini, and J. H. Harwell.** 1994. Surfactant adsolubilization and modified admicellar sorption of nonpolar, polar, and ionizable organic contaminants. Environ. Sci. Technol. **28:**1874–1881.
18. **Oberbremer, A., R. Mueller-Hurtig, and F. Wagner.** 1990. Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl. Microbiol. Biotechnol. **32:**485–489.
19. **Reddy, P. G., D. Singh, P. K. Roy, and J. N. Baruah.** 1982. Predominant role of hydrocarbon solubilization in the microbial uptake of hydrocarbons. Biotechnol. Bioeng. **24:**1241–1269.
20. **Swisher, R. D.** 1987. Surfactant biodegradation. Surfactant science series 18. Marcel Dekker, Inc., New York.
21. **Thiem, A.** 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. **60:**258–263.
22. **Van Dyke, M. I., S. L. Gulley, H. Lee, and J. T. Trevors.** 1993. Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. J. Ind. Microbiol. **11:**163–170.
23. **Vigon, B. W., and A. J. Rubin.** 1989. Practical considerations in the surfactant-aided mobilization of contaminants in aquifers. J. Water Pollut. Control Fed. **61:**1233–1240.
24. **Volkering, F., A. M. Breure, A. Sterkenburg, and J. G. van Andel.** 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. **36:**548–552.
25. **Volkering, F., A. M. Breure, and J. G. van Andel.** 1993. Effect of microorganisms on the bioavailability and biodegradation of crystalline naphthalene. Appl. Microbiol. Biotechnol. **40:**535–540.
26. **Weber, F., L. P. Ooijkaas, R. W. Schemen, S. Hartmans, and J. A. M. de Bont.** 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl. Environ. Microbiol. **59:**3502–3504.
27. **Wilkinson, T. G., and D. E. F. Harrison.** 1973. The affinity for methane and methanol of mixed cultures grown on methane in continuous culture. J. Appl. Bacteriol. **36:**309–313.
28. **Zhang, Y. M., and R. M. Miller.** 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. **58:**3276–3282.