Biodegradation **7**: 297–302, 1996.

Bacterial influence on partitioning rate during the biodegradation of styrene in a biphasic aqueous-organic system

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Accepted 25 May 1995

Key words: biodegradability, biosurfactants, biphasic, interface, NAPL, styrene

Abstract

The degradation by a consortium of slightly-halophile marine bacteria of styrene initially dissolved in silicone oil was monitored in batch reactors stirred at 75, 125 and 500 rpm, respectively. In the 75 and 125 rpm cases, the styrene biodegradation rate was higher than the rate of spontaneous partitioning of styrene from the oil to the water, determined under abiotic conditions. Abiotic transfer tests carried out after biodegradation runs revealed that bacterial activity had resulted in a significant increase in the rate of styrene partitioning between the two liquid phases. Even though bacterial adsorption was noticeable at the oil-water interface, this effect appeared to be due to the release by the bacteria of chemicals in the aqueous phase. Similarity with observations made with Triton X–100 suggested that the chemicals released may have been biosurfactants or solubilizing agents.

Introduction

At waste-disposal sites, in industrial effluents, or in surface waters into which petroleum has spilled, nonaqueous-phase liquids (NAPLs) frequently serve as slow-release reservoirs for hydrophobic organic pollutants (El Aalam et al. 1993; Ortega-Calvo & Alexander 1994). In the laboratory, this feature may be used to observe the biodegradation of xenobiotics that are poorly soluble in water and/or are toxic to growing bacterial cells (Ascon-Cabrera & Lebeault 1993).

The practical significance of the fate of hydrophobic organic compounds present in NAPLs has led in recent years to an important research effort. Experimental evidence suggests that in biphasic aqueous-NAPL systems, biodegradation of xenobiotics dissolved in NAPL occurs frequently at a rate higher than the rate of partitioning from nonaqueous to aqueous phases, evaluated in the absence of biodegrading microorganisms (Efroymson & Alexander 1991; Efroymson & Alexander 1994). To account for these observations, Ortega-Calvo and Alexander (1994) postulate that bacteria growing at the NAPL-water interface form a population that is physiologically distinct from that of the bacteria suspended in the aqueous phase. Some authors also consider that, via 'direct' contact to the insoluble compound, interfacial bacteria are able to transform the substrate faster than it appears in the bulk aqueous phase (Rosenberg et al. 1982; Käppeli et al. 1984). Another way to account for the experimental observations is in terms of the higher concentration gradients that are likely occuring in the vicinity of NAPL/water interfaces (Ortega-Calvo & Alexander 1994). Little information, however, is as yet available on an alternative mechanism, i.e. a possible influence of bacteria or/and bacterial metabolic by-products on the rate of partitioning of the organic xenobiotic from the NAPL to the aqueous phase. Hence, a study was conducted to determine whether bacteria indeed exert such an influence, and to explain the previously reported differences between biodegradation and abiotic transfer rate. Styrene, silicone oil and synthetic seawater, were, respectively, the organic xenobiotic, the NAPL and the aqueous phase selected for the research.

Materials and methods

All experiments were carried out in two-liter batch reactors (Biolafitte) containing 960 mL of synthetic seawater (Osswald et al. 1995) and 240 mL of silicone oil (47 V 20, Rhône Poulenc). The reactors were kept at 30 ± 1° C in a dark room (diffuse light was used when performing measurements). In successive runs, reactors were stirred with a rotating propeller at speeds of 75, 100, 125, 150 and 500 rotations per minute (rpm). At the onset of each run, before mixing the silicone oil with the aqueous phase, 7.2 g of styrene were added to the oil. This corresponded to an initial styrene concentration of 30 g L^{-1} in the NAPL. At repeated intervals during each run, the propeller was stopped, letting the two phases separate, and 20 μL aliquots of the aqueous phase were sampled and were injected in a High Performance Liquid Chromatograph (mobile phase: methanol/water; 90/10 v/v), equipped with a nucleosyl C_{18} column and a UV detector (245 nm), to evaluate the concentration of the styrene partitioned from the nonaqueous to the aqueous phase.

For the biotic experiments, batch reactors identical in all respects to those described above were inoculated with a suspension of a consortium of slightly-halophile marine bacteria obtained as described in (Osswald et al. 1995). The pH of the aqueous phase was continuously monitored and maintained at 8 by addition of 2.5 M NaOH.

Results and discussion

Abiotic transfert rate of styrene

A first series of laboratory experiments concerned the rate of interphase partitioning of styrene, initially present in the silicone oil, under abiotic (sterile) conditions. In contrast with results obtained earlier in 15 h-long experiments (Ortega-Calvo & Alexander 1994), the concentration of styrene in the aqueous phase did not increase linearly with time (Figure 1), regardless of the rotation speed adopted, but instead rapidly reached a plateau (for example 35 mg L^{-1} at 500 rpm) which is much lower than the solubility in synthetic seawater (205 mg L^{-1} at 20° C). Such a difference must be associated with a partition of styrene in favor of the oil.

The following Michaelis-Menten or Langmuir-like kinetic equation provided a reasonable fit to the data points in Figure 1 (r = 0.99 in all cases):

$$[\text{styrene}]_{aq} = A\frac{t}{B+t} \qquad (1)$$

In this expression, $[\text{styrene}]_{aq}$ (mg L^{-1} represents the concentration of styrene in the aqueous phase, t (s) denotes the time and A (mg L^{-1}) and B (s) are adjustable parameters. Values of A and B obtained by non-linear least-square regression are listed in Table 1 for the various experimental runs, except for the one at 500 rpm, where the initial fast rise in concentration caused optimization problems. Derivation of equation (1) with respect to time provides an expression for the rate, $V_{transfer}$, of the transfer of styrene from the silicone oil to the aqueous phase:

$$V_{transfer} = \frac{d[stryrene]_{aq}}{dt} = A\frac{B}{(B+t)^2} \qquad (2)$$

In agreement with the pattern observed in Figure 1, equation (2) predicts a progressive decrease of $V_{transfer}$ in time, starting from a maximum value $V_{transfer\ max}$ = A/B at the time t = 0. The shape of the $[\text{styrene}]_{aq}$ *vs* time curve in the 500 rpm case (Figure 1) suggests that the value of $V_{transfer\ max}$ must be much larger than 550 μg L^{-1} s^{-1}. This value and those listed in the last column of Table 1 show a monotonic increase of $V_{transfer\ max}$ with the rotation speed.

Biodegradation rate of styrene

In a second series of experiments, batch reactors identical in all respects to those described above were inoculated with a suspension of a consortium of slightly-halophile marine bacteria. The cell concentration in the inoculum was 1.6 10^7 ± 9.0 10^5 CFU mL^{-1}. Previous tests had shown that the bacterial consortium was able to degrade styrene but that it did not degrade silicone

Table 1. Maximal rate of transfer of styrene from the oil phase to the aqueous phase at different rotation speeds. The constants A and B are obtained via non-linear least square fitting of eq. (1) to experimental data. $V_{transfer\ max}$ is obtained by letting t = 0 in eq. (2).

Rotation speed (rpm)	A (mg L^{-1})	B (s)	$V_{transfer\ max}$ μg L^{-1} s^{-1}
75	24.6	4019.4	6.1
100	23.7	1999.8	11.9
125	23.7	1281.6	18.5
150	34.6	405.0	85.4

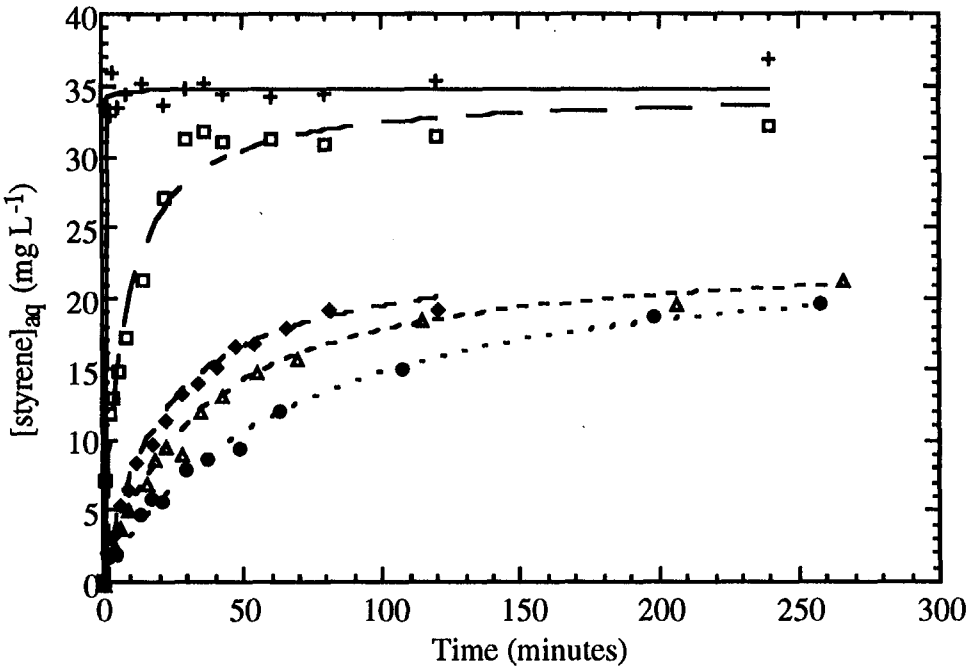

Figure 1. Evolution of the concentration of styrene in the aqueous phase, $[\text{styrene}]_{aq}$, in batch reactors operated at 75 rpm (●), 100 rpm (△), 125 rpm (♦), 150 rpm (□) and 500 rpm (+), respectively. The initial styrene concentration in the silicone oil was 30 g L^{-1} in all cases. The curves through the data points correspond to eq. (1).

oil. Three experimental runs were carried out, with rotation speeds of 75, 125 and 500 rpm. The rate of disappearance of styrene in the silicone oil was monitored by periodically interrupting the rotation of the propeller, letting the two phases in the batch reactors rest for a few minutes, sampling 1 mL aliquots in the oil phase and, finally, measuring $[\text{styrene}]_{oil}$ in these aliquots via HPLC. Under the conditions of this second series of experiments, involving biodegradation, the rotation speed does not appear to have influenced significantly the rate of biodegradation of styrene from the oil phase (Figure 2). For all three rotation speeds used, the disappearance rate started off relatively low, until approximately 21 days after the onset of the experiments, at which point it increased drastically. This abrupt rate increase was concomittant with a clear change in the general aspect of the biphasic mixture in the batch reactors operated at 75 and 125 rpm; the initially stable layering of the two liquid phases gave way to large numbers of tiny oil droplets dispersed in the aqueous phase.

Using mass balance arguments, one may calculate from the loss of styrene in the oil phase during any given time interval the corresponding rate of

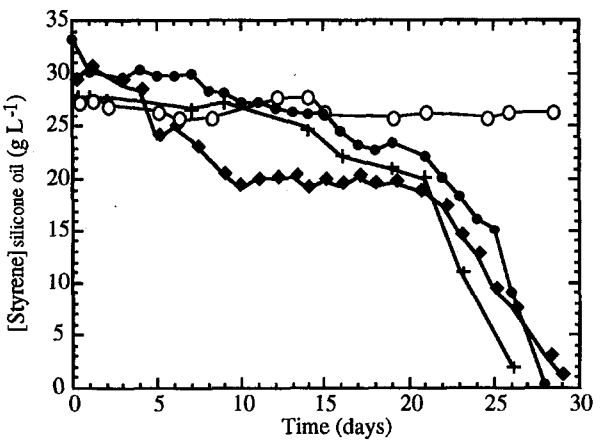

Figure 2. Concentration of styrene in the oil phase, as a function of time, in batch reactors operated at 75 rpm (●), 125 rpm (♦) and 500 rpm (+), and inoculated with a consortium of marine bacteria. An abiotic control at 500 rpm (○) was run simultaneously.

styrene partitioning from the non-aqueous to the aqueous phase. In the following, we shall consider that this rate is, to a very good approximation, equal to the rate of styrene biodegradation by the bacteria, on the grounds that variations of $[\text{styrene}]_{aq}$ are numer-

Table 2. Comparison of the rates of styrene biodegradation, V_{biodeg}, and of maximal transfer, $V_{transfer\ max}$, from oil to aqueous phase in batch reactors stirred at 75, 125, and 500 rpm, respectively. $V_{transfer\ max}$ values are provided for an initial $[styrene]_{oil}$ of 30 g L^{-1} as well as, at 125 rpm, for $[styrene]_{oil}$ = 5 g L^{-1}. The 'time interval' column corresponds to the time ranges considered in the evaluation of V_{biodeg}.

Rotation speed (rpm)	Time interval (days)	V_{biodeg}* (μg L^{-1} s^{-1})	$V_{transfer\ max}$ (abiotic) (μg L^{-1} s^{-1})	
			at 30 g L^{-1}	at 5 g L^{-1}
75 rpm	25–26	17.5	6.1	nd[a]
	25–28	14.2		
125 rpm	22–25	8.2	18.5	5.00
	22–29	6.3		
500 rpm	24–26	14.0	>550	nd[a]
	20–27	10.5		

* V_{biodeg} is calculated with the formula:

$$V_{biodeg}(\mu L^{-1}s^{-1}) = \frac{[\text{styrene degraded during the time interval } (\mu\ gL^{-1})]_{oil} \times \text{oil phase volume (mL)}}{\text{waterphase volume (mL)} \times \text{time interval (s)}}$$

[a] not determined.

ically insignificant (Figure 1) compared to those of $[styrene]_{oil}$ (Figure 2). The absence of a rational trend in the resulting V_{biodeg} values (Table 2) suggests that these values are affected by experimental errors or are naturally erratic, and that consequently their mean, equal to 11.8 Cμg L^{-1} s^{-1}, should be used in comparisons with $V_{transfer\ max}$. Whatever viewpoint is adopted, the conclusions are identical. For the experimental runs with rotating speeds of 125 and 500 rpm, V_{biodeg} is lower than $V_{transfer\ max}$. However, for the run at 75 rpm, the situation is opposite; V_{biodeg} is about 2.5 times larger than $V_{transfer\ max}$. This observation is similar to that, made by other authors (Ortega-Calvo & Alexander 1994), of a four-fold increase in the partitioning rate of naphthalene, initially present in heptamethylnonane, after inoculation with a naphthalene-degrading strain of *Arthrobacter* sp.

One possible criticism of the rate comparisons is that V_{biodeg} is estimated at a time when $[styrene]_{oil}$ is much lower than its initial value of 30 g L^{-1}, and when therefore the actual value of the styrene partitioning rate might be much smaller than that listed in Table 1 (or in third column in Table 2). To assess the effect of $[styrene]_{oil}$, a complementary abiotic transfer test was carried out with an initial $[styrene]_{oil}$ of 5 g L^{-1}. Only one rotation speed was considered, 125 rpm, because $V_{transfer\ max}$ at 75 rpm is already smaller than V_{biodeg} when the initial $[styrene]_{oil}$ was 30 g L^{-1}, and because $V_{transfer\ max}$ at 500 rpm is so high at 30 g L^{-1} that it is unlikely to become smaller than V_{biodeg} even when $[styrene]_{oil}$ drops to a few grams per liter. Interestingly, at 125 rpm, $V_{transfer\ max}$ decreases enough with decreasing $[styrene]_{oil}$ that when the latter equals 5 g L^{-1}, $V_{transfer\ max}$ is smaller than V_{biodeg}, as in the 75 rpm case (Table 2, last column).

The case for biosurfactant production

Direct feeding by the interfacial bacteria off of the non-aqueous phase was suggested to explain the fact that observed V_{biodeg} values are larger than $V_{transfer\ max}$ in reactors run at 75 and 125 rpm (Ortega-Calvo & Alexander 1994). Another explanation is related to the probably steeper styrene concentration gradient in the immediate vicinity of the oil/water interface. Adsorption of bacterial cells at the interface would seem to play a key role (Ascon-Cabrera & Lebeault 1993; Ortega-Calvo & Alexander 1994). Interfacial adsorption of bacteria was clearly noticeable in our experiments as well. The significant biomass accumulation we observed via light-microscopy would require adsorbed bacteria to have a marked hydrophobic character. This was indeed evinced by the results of a standard hydrophobicity test (Rosenberg et al. 1980) performed after centrifugal separation of adsorbed- and bulk aqueous-phase bacteria. The former were approximately 67% hydrophobic, whereas the aqueous-phase bacteria were only 12% hydrophobic.

These observations would appear to confirm the significant role of the adsorption of bacterial cells

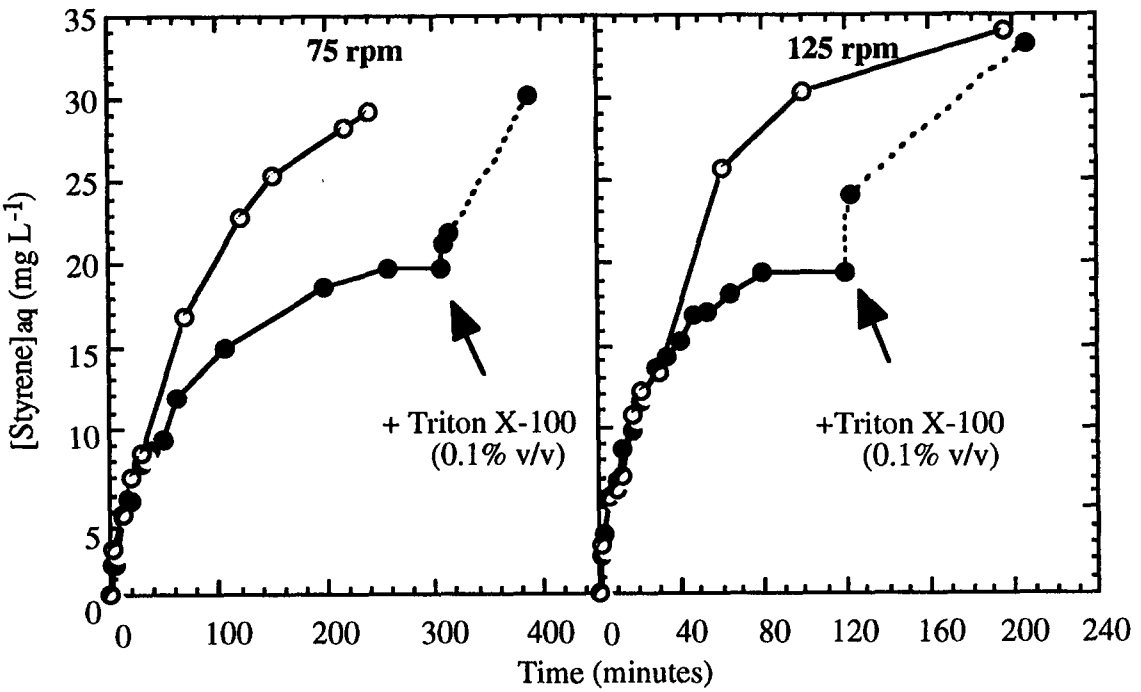

Figure 3. Evolution of the concentration of styrene in the aqueous phase, $[styrene]_{aq}$, in batch reactors operated under abiotic conditions at 75 rpm (left) and 125 rpm (right). The initial styrene concentration in the oil was 30 g L^{-1} The filled circles (●) correspond to tests prior to biodegradation, whereas the open circles (○) are associated with tests run after styrene biodegradation, elimination of bacteria via centrifugation and addition of mercuric chloride.

at the oil/water interface. Nevertheless, there is still the possibility that this adsorption is a coincidental occurence, resulting from an initial diversity in the hydrophobic character of the cells in the bacterial population. An alternative mechanism by which bacteria could consume the substrate (styrene) at a rate higher than $V_{transfer\ max}$ would consist of a modification by the bacteria, i.e. surfactant production, of the rate of styrene partitioning from the nonaqueous to the aqueous phase. To assess whether this mechanism may have played a role in the biodegradation experiments described above, the contents of the reactors stirred at 75 and 125 rpm, respectively, were poured into 2 L burettes. After equilibration in a cold room (4° C) for about 7 h, during which a sharp interface developed between the oil and water phases, the silicone oil was removed by applying suction. The water was centrifuged at 12,000 g for 15 minutes to remove bacteria. In addition, 40 mg of $HgCl_2$ were added to the supernatant to prevent further styrene uptake by the few cells that may have remained after centrifugation. An assessment of the abiotic rate of transfer of styrene, under exactly the same conditions as in the first series of experiments (cf above), was performed with this supernatant as the aqueous phase.

The results obtained in these second abiotic transfer tests show that the appearance of styrene in the supernatant aqueous phase is significantly faster than during the first abiotic experiments (Figure 3). The activity of bacteria clearly modified the characteristics of the medium and, consequently, the rate of styrene partitioning from the nonaqueous to the aqueous phase. The general appearance of the reactors during the biodegradation experiments, as described above, suggests a change in the properties such as surface tension of the oil/water interface. This change may have been caused by the adhesion of biomass, by the progressive accumulation of metabolic by-products in the aqueous phase, or may have resulted from the synthesis by the bacteria of surfactants or solubilizing agents (Zhang & Miller 1992, 1994). Production and presence of biosurfactants is strongly supported by the fact that addition of 1.2 mL of a surfactant, Triton X–100, in abiotic transfer tests run prior to styrene biodegradation led to $[styrene]_{aq}$ levels similar to those obtained with the aqueous extract, after biodegradation (Figure 3).

Conclusions

The results reported in the present paper indicate that besides adsorbing at the NAPL/water interface in biphasic reactors, bacteria are able to actively modify the rate of styrene transfer from the oil- to the aqueous phase. Circumstancial evidence suggests that biosurfactants or solubilizing agents, produced by the bacteria, may be responsible for this rate change. Whatever mechanism is involved, the influence exerted by bacteria on the partitioning rate of organic xenobiotics dissolved in NAPLs may have important environmental consequences, particularly in terms of the mobility of these compounds.

Acknowledgment

The authors would like to express their gratitude to J. Soler for her technical assistance and J.M. Lebeault for stimulating interest in this work.

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