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ORIGINAL PAPER

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Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent

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Abstract *Mycobacterium* PYR-1 was used in a two-phase partitioning bioreactor (TPPB) to degrade low and high molecular weight polycyclic aromatic hydrocarbons. TPPBs are characterized by a cell-containing aqueous phase, and an immiscible and biocompatible organic phase that partitions toxic substrates to the cells based on their metabolic demand and the thermodynamic equilibrium of the system. A bioavailable solvent, that is, a solvent usable as a carbon source, was used as the organic layer. Although bioavailable solvents are traditionally deemed unsuitable for use in TPPBs, bis(ethylhexyl) sebacate had superior chemical properties to other solvents examined and was cost-effective. In this system, 1 g of phenanthrene and 1 g of pyrene were completely degraded within 4 days, at rates of 168 mg l^{-1} day^{-1} and 138 mg l^{-1} day^{-1}, respectively, based on a 3-l aqueous volume. This is the highest pyrene degradation rate reported in the literature to date. Significant degradation of naphthalene and anthracene was also obtained. This work demonstrates that bioavailable solvents can be successfully used in TPPB systems, and may change the protocols commonly used to select solvents for TPPBs in the future.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous chemicals composed of two or more fused aromatic rings, which are produced via natural and anthropogenic sources. Several PAHs are known to possess mutagenic, teratogenic and/or carcinogenic properties, and 16 are listed on the US Environmental Protection Agency priority pollutant list (Kanaly and Harayama 2000). Their toxic properties have prompted several studies examining their destruction, recently focussing on their degradation in two-phase partitioning bioreactors (TPPBs) (Bouchez et al. 1997; Daugulis and Janikowski 2002; Guiysse et al. 2001; Janikowski et al. 2002; Marcoux et al. 2000; Villemur et al. 2000).

As noted by Guiysse et al. (2001), the low solubility of PAHs makes them excellent candidates for treatment in TPPBs, and, in fact, the highest PAH degradation rates recorded to date have been achieved in TPPB systems (Janikowski et al. 2002; Marcoux et al. 2000). In a TPPB system, an organic phase is loaded with the target substrate, which then partitions to the biomass in the aqueous phase, based on equilibrium considerations and the real-time demand of the microorganisms. The organic phase permits much higher quantities of hydrophobic compounds such as PAHs to be dissolved in the system and, if substrate–interface uptake mechanisms are present, it may enhance degradation rates (Déziel et al. 1999; Guiysse et al. 2001).

When designing any TPPB system, solvent selection is a critical consideration, as experiments have demonstrated that the identity and quantity of solvent used can impact mass transfer and degradation rates in the TPPB (Bouchez et al. 1995; Déziel et al. 1999; Marcoux et al. 2000; Ortega-Calvo 1995; Villemur et al. 2000). The selected solvent must not be toxic to the organism, should have suitable physical and chemical properties (i.e. be immiscible, non-volatile, etc.), should be inexpensive and be readily available. In addition to these requirements, non-bioavailable solvents (i.e. solvents that the organism cannot use as a carbon source) have always been used, as it has traditionally been thought that additional carbon sources will interfere with the degradation of the target substrates (Déziel et al. 1999; Malinowski 2001).

The aim of this study was to investigate the use of a TPPB for the degradation of mixtures of PAHs by *Mycobacterium* PYR-1, an organism known to degrade several PAHs (Heitkamp and Cerniglia 1988). In contrast to all previous work, a bioavailable solvent, bis (ethylhexyl) sebacate (BES), was selected for its superior

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physical properties relative to other potential solvents. The degradation of four PAHs, naphthalene, anthracene phenanthrene and pyrene, was examined in the presence of this bioavailable solvent.

Materials and methods

Organism and growth conditions

Mycobacterium PYR-1 (available as NRRL B-24157) was obtained from Dr. C.E. Cerniglia, Division of Microbiology, National Center for Toxicological Research, FDA, Jefferson, Ark. Stock cultures were maintained on agar plates coated with phenanthrene crystals. The agar contained, per litre of dH_2O: 15 g bacto-agar, 1 g glucose, 0.38 g tryptone, 0.38 g yeast extract, 0.05 g $MgSO_4.7H_2O$, 0.12 g NaCl, 0.48 g $(NH_4)_2SO_4$, 0.40 g $NaNO_3$, 0.90 g KH_2PO_4, four drops of 0.01% $FeCl_3$ solution and 2 ml of trace element solution containing, per litre: 0.30 g KI, 0.43 g $SnCl_2.2H_2O$, 0.20 g LiCl, 0.80 g $CuSO_4.5H_2O$, 2.10 g $AlK(SO_4)_2.H_2O$, 0.55 g $NiCl_2$, 0.85 g $CoCl.6H_2O$, 0.60 g boric acid, 0.37 g $MnSO_4.H_2O$ and 0.30 g $FeSO_4.7H_2O$. These plates were streaked with *Mycobacterium* PYR-1 cells and coated with 1 ml of a saturated solution of phenanthrene in iso-octane. The iso-octane was allowed to evaporate, leaving a lawn of phenanthrene crystals on the plate.

Liquid medium for inoculum purposes was the same composition as the agar, with no bacto-agar and no phenanthrene. For bioreactor experiments, 250 ml of medium was inoculated with a single colony, incubated for 3 days at 30°C and 165 rpm. This was then used to inoculate two 500 ml portions of medium which were then incubated under the same conditions for an additional 3 days, then combined, centrifuged at 3,000 *g* for 5 min, resuspended in 200 ml dH_2O and added to the reactor. The large inoculum was necessary as *Mycobacterium* PYR-1 grows relatively slowly on glucose, resulting in low cell densities.

Solvent selection

Solvent biocompatibility

The growth of *Mycobacterium* PYR-1 on corn oil was examined in the presence of solvents with different values for logarithm of partition coefficient (log P). The purpose of this experiment was to determine the critical log *P* of this organism; that is, the log *P* of candidate solvents above which *Mycobacterium* PYR-1 is unaffected when grown in the presence of the solvent. The concept of a critical log *P* for organisms, and the use of solvent log *P* values, is the most accepted measure of assessing solvent biocompatibility (Bruce and Daugulis 1991; Déziel et al. 1999; Inoue and Horikoshi 1990; Laane et al. 1986; van Sonsbeek et al. 1993). Corn oil was used, rather than glucose, because it was observed that *Mycobacterium* PYR-1 does not consume glucose in the presence of many solvents. Thirteen 125 ml flasks were prepared, each containing 40 ml of aqueous medium (formulation as previously outlined but with no glucose), 5 ml of inoculum, 5 ml of one of the subsequently listed solvents and 2 ml of corn oil. One flask contained no solvent and served as the positive control. One flask contained no solvent and no corn oil and served as the negative control. The solvents examined and their estimated log *P* values (in brackets) were: iso-octane (4.36), tri(ethylene glycol) bis(2-ethyl hexanoate) (5.60), bis(2-butoxyethyl) sebacate (5.75), methyl myristate (6.27), butyl laurate (6.76), isopropyl myristate (7.17), 2,2,4,4,6,8,8-heptamethylnonane, oleyl alcohol (7.87), butoxyethyl oleate (9.21), bis(ethylhexyl) sebacate (10.08), trioctyl trimellitate (11.59). The flasks were incubated at 30°C for 5 days and the biomass was collected, washed to remove residual solvent and then dried at 100°C to constant weight. The relative metabolic activity in each flask was calculated by dividing the biomass from each flask by the biomass in the positive control.

Solvent bioavailability

Biocompatible solvents were then tested for their use as a carbon source by *Mycobacterium* PYR-1. The procedure was the same as outlined for biocompatibility experiments, except corn oil was omitted from all flasks, aside from the positive control.

PAH solubility

The solubility of high molecular weight PAHs in potential solvents was also examined. Known masses of either pyrene or fluoranthene were added to 25 ml of each solvent in small increments until no more dissolved. Increments of 1 ml of the solvent were then added until the remaining solid dissolved. The total mass of PAH added, divided by the total volume of solvent used, resulted in the solubility.

Bioreactor experiments

Fermentations were carried out in New Brunswick Scientific Bioflo III fermentors. The fermentors were equipped with Broadley James D100 Series Oxyprobes and FermProbes for monitoring dissolved oxygen and pH, respectively. The pH was controlled to within ±0.1 of the set point with 2 M KOH and H_2SO_4 solutions. The reactors were operated at 30°C, a pH of 7.4, an agitation rate of 300 rpm and an aeration rate of 2.0 l min^{-1}. Under these conditions the bioreactor contents were completely dispersed, and a bioreactor sample yielded both an organic phase and an aqueous phase for analysis. Biomass was measured as the cell dry weight of a 15 ml sample of well-mixed reactor broth. To remove residual solvent prior to drying and weighing, the biomass pellets were washed seven times with isooctane, a technique that we had previously shown to be >97% effective (data not shown). Aqueous medium (3 l) was prepared, with the masses of $MgSO_4.7H_2O$, NH_4SO_4 and $NaNO_3$ tripled. Both the aqueous medium and organic layer were autoclaved at 121°C and 15 psig(~100 kPa)for 20 min. In the first bioreactor experiment, 500 ml of BES was loaded with 1.001 g pyrene and 0.989 g phenanthrene. In the second bioreactor experiment, 500 ml of BES was loaded with 0.513 g anthracene and 0.494 g naphthalene, and a spike of 0.515 g of phenanthrene was added to the reactor after 48 h. Nutrient boluses, containing all of the components of 3 l of aqueous medium in 100 ml of dH_2O, were added periodically to each fermentation.

Analytical methods

PAHs in the organic layer were quantified using fluorescence spectroscopy. PAH concentrations in the aqueous phase were assumed negligible. Fluorescence spectra were collected using a QuantaMaster QM1 fluorescence spectrometer (Photon Technology International, London, Ontario, Canada) equipped with a 75 W xenon arc lamp and Czerney-Turner excitation and emission monochromators. Excitation and emission slits were set to 2 nm bandpass for all measurements. A solution sample holder was used to hold the quartz cuvettes in the path of the excitation radiation. The quartz cuvettes used were type 3H, with a path length of 10 mm (NSG Precision Cells, Farmingdale, N.Y.)

Data were collected from this system using the Felix software package, version 1.1. In order to be in the linear range of detection for PAHs, all samples taken from the organic layer were diluted by a factor of 10,000 in anhydrous ethanol. Synchronous scans were performed to generate unique peaks for each PAH. The detection conditions for the synchronous scan of each PAH [change in wavelength ($\Delta\lambda$), peak maximum, integration area] were, in nanometres: naphthalene (35.0, 320.0, 315–330), anthracene (171.0, 422.0, 409–434), phenanthrene (53.5, 345.2, 342–350), pyrene (36.0, 369.7, 366–375).

Chemicals

PAHs were purchased from Sigma-Aldrich Canada, Oakville, Ontario. With the exception of trioctyl trimellitate (TOTM) and BES, all solvents were obtained from Sigma-Aldrich Canada. TOTM was obtained from L.V. Lomas, Brampton, Ontario. BES and all other chemicals used were obtained from Fisher Scientific, Ottawa, Ontario.

Results

Solvent selection

In order to identify biocompatible solvents, the metabolic activity of *Mycobacterium* PYR-1 relative to a control flask was measured in the presence of various solvents (Fig. 1). An essentially sigmoidal-shaped curve was obtained, indicating a critical log *P* value of approximately 7.0 for this organism. Three points on the curve vary from the sigmoidal pattern. At a log *P* of 4.36, there is a point generated in the presence of iso-octane, with a relative metabolism of 36%, which is much higher than expected for points below the critical log P of the organism. Two other points, at log *P* values of 7.87 and 9.21, generated in the presence of oleyl alcohol and butoxyethyl oleate, respectively, show relative metabolisms of only 49% and 56%, which is much lower than anticipated for solvents above the critical log *P* of the organism.

The next step in solvent selection was to examine the bioavailability of different chemicals with log *P* values greater than 7.0. Previous results (unpublished data) indicated that alkanes, alkenes, alkynes and alcohols were bioavailable to *Mycobacterium* PYR-1. Therefore the bioavailability of other chemical classes was examined (Fig. 2). The two esters, ispropyl myristate (IM) and BES, show relative metabolic activities greater than 100%, indicating that they are bioavailable to *Mycobacterium* PYR-1. In contrast, the two highly branched compounds, 2,2,4,4,6,8,8-heptamethylnonane (HMN) and TOTM had relative metabolic activities of 8% and 13% respectively, indicating that they are essentially not bioavailable to *Mycobacterium* PYR-1.

The broad substrate spectrum of *Mycobacterium* PYR-1 substantially complicated final solvent selection. That is, although bioavailable solvents would typically be eliminated at this point, with only two non-bioavailable solvents identified, the two esters were maintained as potential candidates based on other desirable properties (Table 1). All solvents listed in Table 1 have a high enough log *P* to be biocompatible with *Mycobacterium* PYR-1 and all have sufficiently high boiling points such that volatilization during prolonged fermentor operation would not be a problem. However, significant differences in cost and PAH solubility are noticeable between the solvents. The esters, IM and BES, have much greater PAH solubilities than the branched compounds, HMN and TOTM, by a factor of 10 or more. Additionally, HMN is the most expensive solvent, making it unfavourable for bioreactor applications, and TOTM is very viscous, making it difficult to work with. Although not listed in

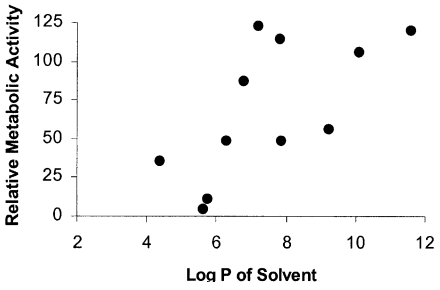

Fig. 1 Growth of *Mycobacterium* PYR-1 on corn oil in the presence of solvents with various logarithm of partition coefficient (*log P*) values

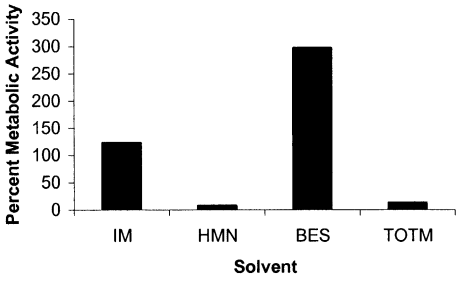

Fig. 2 Metabolism of *Mycobacterium* PYR-1 in the presence of various solvents, with log *P* values greater than 7.0, relative to growth in the presence of corn oil. *IM* Isopropyl myristate, *HMN* 2,2,4,4,6,8,8-heptamethylnonane, BES bis(ethylhexyl) sebacate, *TOTM* trioctyl trimellitate

Table 1 Properties of various biocompatible solvents with logarithm of partition coefficient (*log* P) greater than 7.0. *BES* Bis (ethylhexyl) sebacate, *TOTM* trioctyl trimellitate, *IM* ispropyl myristate, *HMN* 2,2,4,4,6,8,8-heptamethylnonane, *CAS* Chemical Abstracts Service. Costs of BES, IM and HMN were taken from Sigma-Aldrich, cost of TOTM was taken from L.V. Lomas Brampton, Ontario, Canada

Property	TOTM	HMN	IM	BES
CAS no.	3319-31-1	4390-04-9	110-27-0	133-62-3
Log *P*	11.59	7.79	7.17	10.08
Specific gravity	0.984	0.793	0.853	0.914
Boiling point (°C)	414	240	193	248
Pyrene solubility (g l^{-1})	3.3±0.1	3.9±0.1	51.0±2.0	62.9±2.5
Fluoranthene solubility (g l^{-1})	4.2±0.2	7.5±0.3	76.7±3.1	63.0±2.4
Cost (US$ l^{-1})	17.36	338.49	107.75	28.57
Bioavailable ?	No	No	Yes	Yes

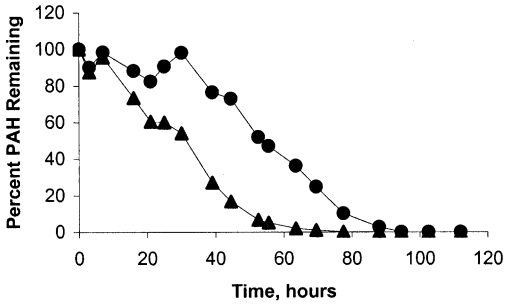

Fig. 3 Degradation of 1.001 g pyrene and 0.989 g phenanthrene. Nutrient boluses were added at 25, 52.5, 77.5 and 101.5 h. Per cent pyrene remaining (●), per cent phenanthrene degraded (▲)

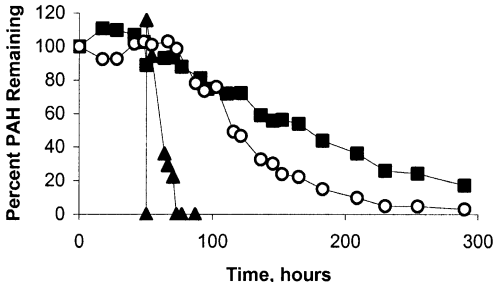

Fig. 4 Degradation of 0.513 g anthracene and 0.494 g naphthalene with a phenanthrene spike (0.515 g) at 48 h. Nutrient boluses were added at 28, 47.5, 72, 102, 139, 172, 216 and 336 h. Per cent anthracene degraded (◯), per cent naphthalene degraded (■), per cent phenanthrene remaining (▲)

the Table 1, it is important to note that none of the listed solvents were of any concern from a safety standpoint, which is another key factor when selecting a solvent.

Based on its superior PAH solubility and low cost, and in contrast to all previous work done in TPPB systems, the bioavailable solvent BES was selected for this TPPB system.

PAH degradation in a TPPB system

Bioreactor experiments were conducted to quantify the degradation of PAHs in the presence of BES, a bioavailable solvent. In the first experiment, the degradation of phenanthrene and pyrene was examined. These are two PAHs that *Mycobacterium* PYR-1 is known to readily degrade, and which serve as model PAHs for low molecular weight and high molecular weight PAHs, respectively (Fig. 3).

Phenanthrene and pyrene were completely degraded 78 h and 103 h into the fermentation, respectively. The percentage of dissolved oxygen fell slowly over the fermentation to a final value of 69%, and at no time did oxygen become limiting. Biomass increased steadily during the fermentation, to a final concentration of 1.7 g l^{-1}. It is apparent that pyrene and phenanthrene were degraded simultaneously by *Mycobacterium* PYR-1 at rates of 138 mg l^{-1} day^{-1} and 168 mg l^{-1} day^{-1}, respectively. These rates, and others reported here, were determined for the entire data set using a least squares fit, and in all cases the correlation coefficient was greater than 95%. Also, at no time were PAH degradation products detected during this experiment and the other bioreactor studies.

A second bioreactor experiment was conducted to examine degradation of two other low molecular weight PAHs, naphthalene and anthracene, in the presence of BES (Fig. 4). Both naphthalene and anthracene were significantly degraded, although degradation was not obvious initially. A bolus of phenanthrene was added 48 h into the fermentation to attempt to stimulate degradation. It was successful as, after the spike, there was a noticeable decline in anthracene levels that was not present prior to the spike.

Naphthalene degradation was slow and constant. It began within the first 24 h of the fermentation, and continued in a linear fashion until the 250-h point. Degradation of both anthracene and naphthalene levelled off at approximately 250 h into the fermentation, at values of 95% and 75% degradation, respectively. The linear sections of the anthracene and naphthalene degradation curves correspond to degradation rates of 40 mg l^{-1} day^{-1} and 14.5 mg l^{-1} day^{-1}, based on a 3-l aqueous volume. The phenanthrene spike was degraded at a rate of 210 mg l^{-1} day^{-1}.

Despite the cessation of PAH degradation at the 250-h mark, base continued to be consumed for the duration of the fermentation. The biomass curve rose steadily in the early portions of the fermentation, to a final concentration of 3.5 g l^{-1}. The percentage of dissolved oxygen decreased to a minimum of 52% at 70 h, corresponding to the point of highest biomass concentration, after which point it levelled off. At no point was oxygen limiting.

Discussion

Biocompatibility experiments for *Mycobacterium* PYR-1 established a critical log *P* of approximately 7.0. This is quite high, relative to other bacteria, but agrees with reported critical log *P* values for other members of the suborder *Corynebacterineae*, including *Corynebacterium* spp., *Rhodococcus* spp. and some *Brevibacterium* spp., which are between 6.0 and 7.0 (Inoue and Horikoshi 1990). These bacteria all have distinct bacterial cell walls containing mycolic acids (Prescott et al. 1996), which may influence the way they interact with solvents, resulting in higher critical log *P* values.

The sigmoidal curve obtained during the biocompatibility experiment (Fig. 1) contained three points that were outliers. One point at log P 4.36 generated in the presence of iso-octane was higher than anticipated and may be a result of a tolerance developed by this organism for iso-octane, as *Mycobacterium* PYR-1 was isolated from below an oil field (Heitkamp and Cerniglia 1988) where iso-octane would likely have been present. Two other points generated in the presence of oleyl alcohol

(log *P* 7.87) and butoxyethyl oleate (log *P* 9.21), had lower than anticipated relative metabolisms. As 100% relative growth was observed in the presence of other esters and alcohols, it is likely that the presence of impurities in the solvents was the cause, as both of these solvents were less than 90% pure.

Results of bioavailability experiments revealed *Mycobacterium* PYR-1 is able to metabolize a variety of chemical types, including, alkanes, alkenes, alkynes, alcohols and esters. Only branched chemicals, including HMN, appeared to resist degradation, which is not surprising. The terminal methyl groups of HMN make it resistant to degradation by most organisms (Déziel et al. 1999) and it has been used in many TPPB systems (Bouchez et al. 1997; Efroymson and Alexander 1991; Kohler et al. 1994; Marcoux et al. 2000). Unfortunately, although HMN is resistant to degradation, it is not an ideal solvent for TPPB systems involving PAHs, as it is expensive relative to other solvents and has a poor capacity for PAHs.

Bioavailable solvents are traditionally eliminated from the list of possible solvents for a TPPB system because they may interfere with the degradation of the target substrates. However, unusual constraints have been encountered in this particular system involving *Mycobacterium* PYR-1: it has a very high critical log P value of 7.0, significantly limiting the range of solvents that are not toxic, and it is able to degrade a wide range of chemical types. The only two non-bioavailable compounds identified had relatively poor solubilities for pyrene and phenanthrene, and HMN is too expensive to be feasible in a TPPB. A search of chemical catalogues for other available and affordable highly branched compounds yielded no additional candidates.

While two substrates can interfere with each other's degradation patterns (e.g. diauxie), they may also be cometabolized, or degraded independently of one another. Bauer and Capone (1988), who examined mixtures of PAHs, concluded that, once cross-acclimation has occurred, degradation of individual components can occur independently of other components. With such a limited list of non-biodegradable solvents from which to select, it raised the question—could a bioavailable solvent be suitable for a TPPB system? Using BES as the bioavailable solvent, the results of this study (Fig. 3 and Fig. 4) indicate that a bioavailable solvent can in fact be used in a TPPB system and allow for degradation of the target substrates. Four PAHs, naphthalene, anthracene, phenanthrene and pyrene were all degraded in the presence of a bioavailable solvent. It is likely that the BES was simultaneously metabolized along with the PAHs as high biomass concentrations were attained in both fermentations, including a final biomass of concentration of 3.5 g l^{-1} in the second fermentation, which contained a total of only 1.5 g of PAHs.

In addition to permitting PAH degradation, there is evidence that the presence of the bioavailable solvent did not impact the rate of PAH degradation. The pyrene degradation rate of 138 mg l^{-1} day^{-1} is the highest reported in the literature to date, and the phenanthrene degradation rate of 210 mg l^{-1} day^{-1} is second only to the rates achieved by Janikowski et al. (2002), suggesting that the BES is not adversely affecting PAH degradation. Additionally, in other experiments conducted in our laboratory (data not shown), pyrene degradation rates were very consistent, ranging from 84 mg l^{-1} day^{-1} to 99 mg l^{-1} day^{-1} under identical conditions, whether pyrene was present as the only carbon source or as a mixture of PAHs—a result consistent with the findings of Bauer and Capone (1988). The fact that the degradation rates achieved are very high, that *Mycobacterium* PYR-1 can simultaneously metabolize PAHs, and that the presence of additional PAHs does not affect the rate of degradation of individual PAHs, suggests that having the solvent as an additional carbon source will not impact PAH degradation rates.

Recent reviews of TPPB technology by Déziel et al. (1999) and Malinowski (2001) stress the importance of finding solvents that are biocompatible and non-biodegradable for use in TPPB systems, but the results presented here suggest that the latter constraint may not be necessary. This would allow selection from a wider range of solvents, increasing the likelihood of identifying a solvent with more desirable properties. In this study, retaining bioavailable solvents as an option allowed for the selection of a solvent that was less expensive and had greater than ten times the capacity for PAHs than HMN or TOTM, the non-bioavailable alternatives. An increased capacity for the target substrate is particularly relevant if the solvent can be used to extract the xenobiotic from polluted sites, directly linking the extraction and treatment processes, as has recently been demonstrated (Janikowski et al. 2002).

While the results of this study are promising, it is important to note that they may not be universally applicable. There are many forms of substrate competition and inhibition which may make using a bioavailable solvent problematic, as has traditionally been thought. For example, while *Mycobacterium* PYR-1 appears to simultaneously metabolize several substrates independently of one another, many organisms use carbon sources in a sequential fashion (Guha et al. 1999; Marcoux et al. 2000), and may consume a bioavailable solvent prior to degrading the target substrates. To avoid complications, potential bioavailable solvents for TPPB systems should be tested for these phenomena prior to use.

In conclusion, the results presented here indicate that, in contrast to traditional TPPB practice, bioavailable solvents should not be eliminated as potential solvents on the basis of this criterion alone. This will allow for a much broader range of potential solvents in any TPPB system.

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