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ORIGINAL PAPER

Adhesion to the hydrocarbon phase increases phenanthrene degradation by *Pseudomonas fluorescens* LP6a

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Abstract Microbial adhesion is an important factor that can influence biodegradation of poorly water soluble hydrocarbons such as phenanthrene. This study examined how adhesion to an oil–water interface, as mediated by 1-dodecanol, enhanced phenanthrene biodegradation by *Pseudomonas fluorescens* LP6a. Phenanthrene was dissolved in heptamethylnonane and added to the aerobic aqueous growth medium to form a two phase mixture. 1-Dodecanol was non-toxic and furthermore could be biodegraded slowly by this strain. The alcohol promoted adhesion of the bacterial cells to the oil–water interface without significantly changing the interfacial or surface tension. Introducing 1-dodecanol at concentrations from 217 to 4,100 mg l^{-1} increased phenanthrene biodegradation by about 30% after 120 h incubation. After 100 h incubation, cultures initially containing 120 or 160 mg l^{-1} 1-dodecanol had mineralized >10% of the phenanthrene whereas those incubated without 1-dodecanol had mineralized only 4.5%. The production and accumulation of putative phenanthrene metabolites in the aqueous phase of cultures likewise increased in response to the addition of 1-dodecanol. The results suggest that enhanced adhesion of bacterial cells to the oil–water interface was the main factor responsible for enhanced biodegradation of phenanthrene to presumed polar metabolites and to CO_2.

Keywords Aerobic phenanthrene biodegradation · Bacterial adhesion · 1-Dodecanol · *Pseudomonas fluorescens* · Hydrophobicity

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Introduction

The bioavailability of hydrophobic compounds in non-aqueous phase liquids (NAPLs) is often a major barrier to biodegradation. The combination of low aqueous solubility and partitioning of these compounds into a water-immiscible phase is a challenge to microbial transformation and degradation. A classic example is polycyclic aromatic hydrocarbons, which are contaminants of concern ('priority pollutants') (Gillesby et al. 1997; Safe 1998; U.S. EPA 2002). The persistence of PAHs in the environment is mostly due to their hydrophobic character and low water solubility (Volkering et al. 1992; Woo and Park 2004), therefore, they are often found either in

NAPLs or strongly sorbed to soil organic matter (Breedveld and Karlsen 2000; Weissenfels et al. 1992).

Surfactants have long been used to enhance bioavailability and biodegradation of PAHs in water and soil by increasing their apparent solubilities and promoting mobilization by increasing desorption (Edwards et al. 1991; Mulligan et al. 2001; Volkering et al. 1997). Although surfactants can be used to enhance the biodegradation of less soluble compounds, not all reports of the effects of surfactants on bioremediation are positive (Bramwell and Laha 2000; Chen et al. 2000; Foght et al. 1989; Laha and Luthy 1991). Bacterial adhesion to oil–water interfaces or to the surface of solid organic compounds can be reduced or prevented in the presence of surfactants (Rodrigues et al. 2006; Stelmack et al. 1999). When the mechanism of bacterial uptake of low-solubility hydrophobic compounds is through direct cell attachment to the surfaces of liquid or solid organic compounds, then the addition of surfactants may inhibit degradation by dispersing the bacteria into the aqueous phase.

Adhesion of hydrocarbon-degrading bacteria to the oil phase, on the other hand, can enhance biodegradation of hydrophobic compounds in a NAPL phase (Hori et al. 2002; Ortega-Calvo and Alexander 1994). Strains of three bacteria that adhered to oil–water interfaces exhibited faster rates and higher extents of biodegradation as cell surface hydrophobicity increased (Obuekwe et al. 2007a, b). Conversely, when *Acinetobacter venetianaus* RAG-1 was mutated to minimize its adherence to the oil–water interface, it lost the ability to grow on liquid hexadecane (Rosenberg and Rosenberg 1981; Vaneechoutte et al. 1999).

Despite extensive previous research on microbial adhesion to oil–water interfaces, controlled enhancement of cell adhesion to promote biodegradation of target compounds in a NAPL has received little attention. In an earlier study we demonstrated that the addition of 1-dodecanol to a suspension of *P. fluorescens* LP6a cells increased their adhesion to an oil–water interface (Abbasnezhad et al. 2008). Our hypothesis was that increased adhesion of *Pseudomonas fluorescens* LP6a to an oil–water interface, mediated by 1-dodecanol, would enhance biodegradation of phenanthrene dissolved in a NAPL phase. Therefore in the current study we monitored the effect of 1-dodecanol on phenanthrene transformation and mineralization as well as its effects on biomass density and interfacial tension and quantified the influence of microbial adhesion on the kinetics of phenanthrene transformation.

Materials and methods

Chemicals

2,2,4,4,6,8,8-Heptamethylnonane (HMN, 98% pure; Aldrich Chemical Co., St. Louis, MO, USA) was used as a non-degradable water immiscible oil phase. 1-Dodecanol (98.0% pure) was purchased from Acros Organics (NJ, USA). Phenanthrene (98% pure) and *o*-terphenyl (>99% pure) were purchased from Aldrich Chemical Company. 2-Aminobenzoic acid was purchased from Analar (British Drug Houses Ltd, Poole, England). Naphthalene was obtained from Sigma Chemical Company (St. Louis, MO, USA). [9-^{14}C]Phenanthrene was used to measure mineralization of phenanthrene and its transformation (96.5% radiochemical purity; 19.3 mCi mmol^{-1}; Amersham, Arlington Heights, IL, USA). *N*,*N*-dimethylformamide (DMF) was purchased from Anachemica (Montréal, QC, Canada). *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA). All other reagents were laboratory grade, purchased from Fisher Scientific Co.

Microorganism and inoculum preparation

Pseudomonas fluorescens LP6a was chosen for this work because of its positive response to adhesion enhancement by long chain alcohols (Abbasnezhad et al. 2008) and its ability to utilize variety of PAHs such as phenanthrene as a sole source of carbon and energy (Foght and Westlake 1991). The cultures were grown overnight at 28°C in tryptic soy broth (TSB) on a rotary shaker at 150 rpm. The cells were harvested by centrifugation at 12,000×*g* and washed twice with 10 mM potassium phosphate buffer (pH 7). Washed cells were then resuspended in Bushnell Haas (BH) medium (g l^{-1}: magnesium sulfate 0.2; ammonium nitrate 1.0; ferric chloride 0.05, potassium hydrogen monophosphate 1.0, potassium dihydrogen phosphate 1.0, calcium chloride 0.02; pH adjusted to 7–7.2). This suspension was used as inoculum for biodegradation and mineralization experiments incubated at 28°C

with gyrotory shaking at 150 rpm. In all biodegradation and mineralization experiments phenanthrene was dissolved in the biologically inert, water-immiscible carrier HMN to create a NAPL phase.

Phenanthrene biodegradation assay

To prepare culture flasks, 5 ml of *P. fluorescens* LP6a inoculum was added to 45 ml of sterile BH medium in a 250-ml Erlenmeyer flask. 1-Dodecanol was added to and mixed with the inoculated medium as described below, then 1 ml of HMN containing an appropriate mass of dissolved phenanthrene was added to the culture to yield final phenanthrene concentrations of 89–891 mg l^{-1} (0.5–5 mmol l^{-1}), calculated based on the total volume of culture medium (aqueous plus NAPL). The volume ratio of the two phases, i.e., the volume of NAPL (HMN ± phenanthrene) to the total volume of aqueous phase (BH medium and inoculum), was kept constant at 10% to eliminate any surface area effects due to change of NAPL volume. To account for other factors that may contribute to phenanthrene concentration decrease during experiment and sample preparation, parallel abiotic (uninoculated) experiments were included for each condition in biodegradation and mineralization tests.

To enable accurate delivery of small volumes of 1-dodecanol (final concentrations $\leq$180 mg l^{-1}), 1-dodecanol was dissolved in DMF and 250 µl of this solution was added to each flask; the DMF, which immediately dissolved into the aqueous phase, is non-toxic to *P. fluorescens* LP6a at these concentrations (J. Foght, unpublished studies). For larger volumes, 1-dodecanol was added directly to the medium. Due to the high solubility of the 1-dodecanol in alkane solvents like HMN, the resulting culture consisted of two phases: an aqueous phase containing the suspended cells and a non-aqueous phase containing HMN with most of the phenanthrene and 1-dodecanol. Cultures were incubated at 28°C with gyrotory shaking at 150 rpm.

At the end of each experiment, a sufficient volume of concentrated HCl was added to the flask to achieve pH $<$ 1 and kill the cells. DMF containing *o*-terphenyl was added to each flask to serve as both an extraction and surrogate standard for phenanthrene quantification by gas chromatography (GC). Tetradecanol dissolved in DMF was used as the extraction standard as well as surrogate GC standard for 1-dodecanol. The flasks were placed at 4°C until they were extracted at room temperature. The extraction was performed thrice with 25 ml HPLC grade dichloromethane. The pooled solvent extract was collected in an Erlenmeyer flask and an aliquot was filtered through anhydrous sodium sulphate to remove entrained water. To reduce the polarity of alcohols and produce sharp GC peaks, all samples containing 1-dodecanol were derivatized using BSTFA as follows: 200 µl BSTFA reagent were added to 1 ml of extract in glass screw-cap vials, mixed, capped tightly with Teflon liners, heated at 70°C for 15 min then cooled to room temperature. Derivatized extracts were analyzed using an Agilent Technologies 5890 GC. An HP-1 capillary column (25 m × 0.322 mm × 0.17 µm) was used to determine the concentration of phenanthrene and 1-dodecanol. The GC running conditions have been described elsewhere (Abbasnezhad et al. 2008).

1-Dodecanol toxicity and degradation tests

To test for toxicity, inoculated TSB medium was amended with 1-dodecanol over a concentration range of 50 mg to 10 g l^{-1}. Growth of 150-µl samples of culture over 74 h was measured as optical density at 600 nm using a SPECTRAmax PLUS 384 microplate spectrophotometer (Molecular Devices Corporation, CA, USA), compared with a parallel control culture containing no 1-dodecanol.

To determine the degradability of 1-dodecanol by *P. fluorescens* LP6a, neat 1-dodecanol or solutions of 1-dodecanol in DMF were filter-sterilized (0.22 µm pore size; Millipore Corp, Billerica, MA) before addition to 50 ml of inoculated BH medium in 250-ml Erlenmeyer flasks, as described above. In these degradation tests, 1-dodecanol was the sole carbon and energy source present at initial concentrations of 820 or 4,100 mg l^{-1}. At the end of the experiment cultures were acidified, extracted and analyzed by GC as described above.

Microbial adhesion to hydrocarbon (MATH) test

Three subsamples from cultures grown in TSB overnight to stationary phase were assayed for microbial adhesion to HMN using the MATH test, slightly modified from the original method (Rosenberg et al. 1980). Phosphate buffer (0.1 M, pH = 7) was used to wash and resuspend cultures and

HMN was used as the organic phase. Other details of the method have been described previously (Abbasnezhad et al. 2008).

Surface and interfacial tension measurements

Surface and interfacial tensions of cell-free spent culture medium were measured using a Single Fibre Process Tensiometer (K14, Krüs, USA). *P. fluorescens* LP6a cells were grown for 48 h in TSB, then 250 µl of DMF containing 1-dodecanol was added to obtain final concentrations of 50, 100 or 150 mg l^{-1} 1-dodecanol and the cultures were mixed for 10 min on a rotary shaker. The bacterial cells were removed from the spent culture broth by filtration (0.22 µm pore size; Millipore) and the surface and interfacial tensions of the cell-free filtrate were immediately measured by the De Nouy ring method (McInerney et al. 1990). Interfacial tension was measured against a thin layer of *n*-hexadecane poured on the surface of the sample liquid. All measurements were performed with the fresh interface at room temperature.

Mineralization experiments

Cultures prepared for mineralization experiments were induced using 2-aminobenzoate to decrease the lag phase and enhance the rate of phenanthrene biodegradation. Of the three chemicals previously shown to induce PAH degradation by *P. fluorescens* LP6a—salicylate, naphthalene and 2-aminobenzoate—the latter was used because unlike the other two, it is not metabolized by the bacteria but it can induce activity of all necessary enzymes (Foght 2004). A stock solution of 0.5 M 2-aminobenzoate was prepared in 95% ethanol and 250 µl of this solution was added to 50-ml cultures in BH medium to obtain a final concentration of 2.5 mM.

Phenanthrene mineralization was measured as $^{14}CO_2$ evolution from [9-^{14}C]phenanthrene in 250 ml biometer flasks containing 25 ml of culture medium with inducer. Two hundred and fifty microliters of HMN containing [9-^{14}C]phenanthrene plus unlabelled phenanthrene was added to each culture at time zero, giving the desired phenanthrene concentration and ~100,000 disintegrations per minute (dpm). Cumulative $^{14}CO_2$ production was measured as previously described (Ulrich et al. 2009). At intervals, 0.7-ml samples of culture were removed and clarified at 13,000×*g* in a microfuge. A 0.5 ml portion of supernatant (containing water-soluble metabolites) was transferred to 10 ml ACS fluor (Amersham Biosciences, UK Ltd.). The pellet (containing cell-associated radiolabel) was resuspended in 1.0 ml of 10 mM potassium phosphate buffer (pH 7) and 0.5 ml was added to 10 ml ACS fluor. Samples were counted using a Beckman LS3801 liquid scintillation counter with automatic quench correction. Samples were dark adapted for 30 min before counting to reduce chemiluminescence. The dpm measured for each sample were then used to calculate the percentage of label associated with each fraction relative to the ^{14}C added at time zero, after correcting for background radiation (typically 30 dpm, using samples of the aqueous phase and KOH (CO_2 trap) sampled at time zero).

Results

1-Dodecanol is non-toxic to and is biodegraded by *P. fluorescens* LP6a

Because alcohols and long-chain acids can be toxic to cells, we measured aerobic growth of *P. fluorescens* LP6a in the presence of 1-dodecanol. At final 1-dodecanol concentrations of 50 mg l^{-1} to 10 g l^{-1} in TSB medium, there was no inhibitory effect on growth of *P. fluorescens* LP6a as measured by optical density, compared with a control lacking 1-dodecanol (data not shown). Furthermore, 1-dodecanol was completely degraded by *P. fluorescens* LP6a within 14 days, as determined by GC analysis, either when added at 830 mg l^{-1} as sole carbon source in BH medium or when added to TSB. In BH cultures containing HMN, 890 mg l^{-1} phenanthrene and 1-dodecanol at an initial concentration of 4,100 mg l^{-1}, the average rate of 1-dodecanol biodegradation was ~130 mg day^{-1}. Thus, any effects of 1-dodecanol on *P. fluorescens* LP6a adhesion to hydrocarbons were expected to decay with time as the alcohol was biodegraded.

1-Dodecanol increases adhesion of cells to hydrocarbons

To confirm previous observations (Abbasnezhad et al. 2008) that long-chain alcohols can influence cell surface hydrophobicity, we assessed the influence

of 1-dodecanol (180 mg l^{-1}) on the adhesion of *P. fluorescens* LP6a to the NAPL–water interface during incubation in BH medium with phenanthrene dissolved in HMN. Samples of culture were taken at intervals from the aqueous phase for measurement of adhesion using the MATH test. Cells incubated with 1-dodecanol showed an average of 86 ± 6% adhesion to the oil phase, remaining fairly constant during the incubation period (Fig. 1). Cells incubated in parallel without 1-dodecanol showed much lower (35%) adhesion to HMN initially, increasing during incubation to reach 82% by 60 h, comparable to the test cells. Thus, treatment of the cells with 1-dodecanol caused a persistent effect despite presumed biodegradation of the alcohol during incubation.

To evaluate the potential impact of surface active properties of 1-dodecanol we measured the surface and interfacial tensions of cell-free culture medium in the presence or absence of 100 or 200 mg l^{-1} 1-dodecanol (Table 1). The surface and interfacial tension measurements of deionized water were comparable to previous literature values (Aratono et al. 2001; Dorobantu et al. 2004). Surface and interfacial tensions of uninoculated TSB and spent cell-free TSB medium were slightly lower than this control in the absence of 1-dodecanol. Addition of 100 or 200 mg l^{-1} 1-dodecanol to spent cell-free medium further reduced the surface tension by 3 and 7 mN m^{-1} respectively, compared to the same spent medium without 1-dodecanol. Interfacial tension measurements for the same samples revealed decreases of 5 and 12 mN m^{-1} respectively, compared to culture medium without 1-dodecanol. Thus, the presence of 1-dodecanol had little effect on surface tension and only a moderate effect on interfacial tension.

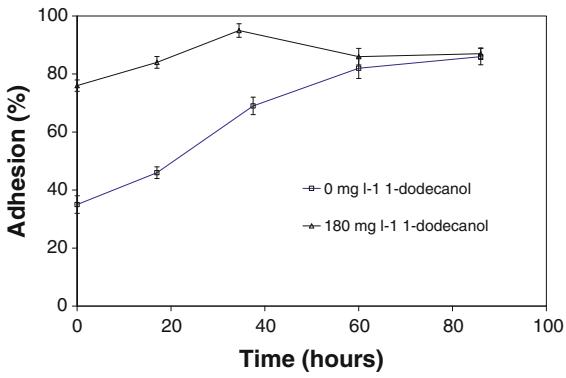

Fig. 1 Effect of 1-dodecanol on the adhesion of *P. fluorescens* LP6a cells to HMN, assayed at intervals during incubation in the absence or presence of 180 mg l^{-1} 1-dodecanol. Adhesion is reported as the percentage of total cells associated with HMN, per the MATH method (Rosenberg et al. 1980). Data points represent the mean of three samples from single culture flasks; error bars show the standard deviation

1-Dodecanol enhances biodegradation of phenanthrene dissolved in HMN

Given the observed enhancement of cell adhesion to the NAPL phase without toxicity, we tested the effect of 1-dodecanol on phenanthrene biodegradation, measured by GC as removal of the parent compound. This was tested by varying the concentration of 1-dodecanol at a constant phenanthrene concentration (891 mg l^{-1}) or, reciprocally, by varying the concentration of phenanthrene in the NAPL phase at a constant 1-dodecanol concentration (217 mg l^{-1}). In both trials residual phenanthrene was extracted and measured after 120 h incubation and the mean rate of biodegradation over this interval was calculated.

Without 1-dodecanol present, after 120 h incubation strain LP6a had degraded only 5% of the phenanthrene initially present, whereas addition of as little as 217 mg l^{-1} 1-dodecanol improved

Table 1 Effect of 1-dodecanol on surface and interfacial tensions of deionized water, uninoculated TSB and spent cell-free TSB culture medium amended with 1-dodecanol at final concentrations of 0, 100 or 200 mg l^{-1}

Aqueous phase analyzed	Dodecanol (mg l^{-1})	Surface tension (mN m^{-1})	Interfacial tension (mN m^{-1})
Deionized water	0	72.0 ± 0.41	48.9 ± 0.46
Uninoculated TSB	0	70.3 ± 0.50	46.5 ± 0.67
Spent cell-free TSB culture medium	0	64.7 ± 0.70	44.5 ± 0.77
	100	61.3 ± 0.76	39.2 ± 0.46
	200	57.9 ± 0.72	32.6 ± 0.66

phenanthrene biodegradation to 33% (Fig. 2a). Higher initial concentrations of 1-dodecanol (up to 4,100 mg l^{-1}) did not substantially increase either the extent or rate of phenanthrene biodegradation. Thus, a 1-dodecanol concentration of 217 mg l^{-1} was selected for the reciprocal experiment (Fig. 2b) with different initial phenanthrene concentrations and a constant volume ratio of NAPL:water. As the total initial phenanthrene concentration increased from 89 to 891 mg l^{-1} the total phenanthrene loss dropped from circa 90 to 32% by 120 h, but the average rate of biodegradation over that interval increased from 16 to 56 mg day^{-1}. These data illustrate that at a constant initial dodecanol concentration, the rate of biodegradation increases with the initial concentration of phenanthrene.

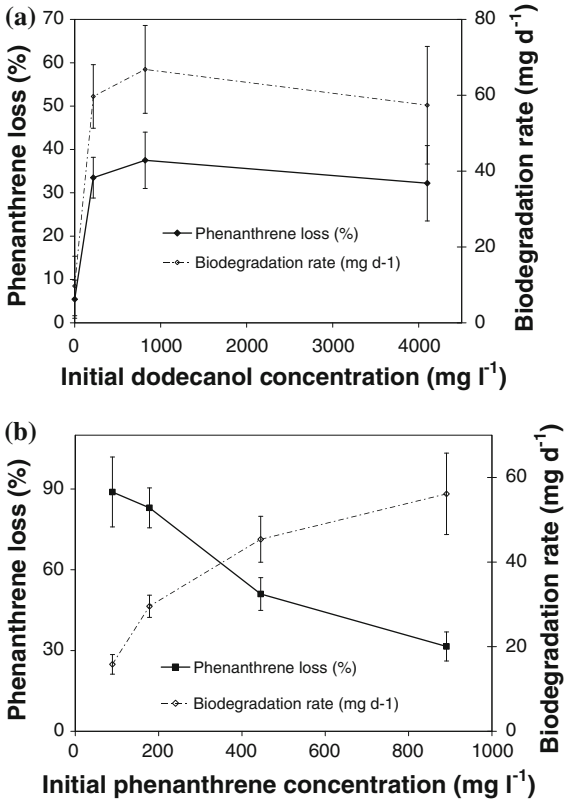

Fig. 2 Effect of 1-dodecanol on the biodegradation of phenanthrene in NAPL after 120 h incubation with *P. fluorescens* LP6a. **a** Cultures with different initial concentrations of 1-dodecanol (0–4,100 mg l^{-1}) and constant phenanthrene concentration (891 mg l^{-1}). **b** Cultures with different initial phenanthrene concentrations (89–891 mg l^{-1}) and constant 1-dodecanol concentration (217 mg l^{-1})

To examine the effect of 1-dodecanol on phenanthrene biodegradation over a longer incubation period, LP6a cultures were incubated in BH with HMN and phenanthrene plus 1-dodecanol at concentrations of 0, 820 or 4,100 mg l^{-1}. At intervals of 120, 240 and 360 h, triplicate flasks were sacrificed and residual phenanthrene was measured. Over the first 240 h, phenanthrene losses followed the same trend in Fig. 2a, but by 360 h all cultures exhibited equivalent phenanthrene losses of 53–60% (Fig. 3). These experiments show that longer incubation times allowed the cultures to approach a comparable level of phenanthrene degradation (55 ± 15% after 360 h) regardless of initial 1-dodecanol concentration; that is, 1-dodecanol was effective at stimulating biodegradation of phenanthrene in a NAPL phase only in the initial stages of incubation, possibly because it was being biodegraded during the incubation period.

Mineralization versus transformation of ^{14}C-phenanthrene in NAPL phase

The previous experiments measured loss of phenanthrene using GC analysis. However, this loss of the parent compound can represent complete oxidation to CO_2 (mineralization) or partial oxidation to polar

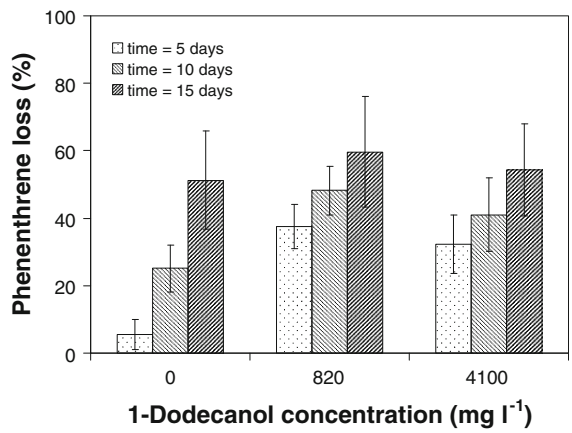

Fig. 3 Effect of 1-dodecanol concentration on phenanthrene degradation by *P. fluorescens* LP6a after incubation in BH + NAPL for 120, 240 or 360 h. Each column represents the mean value of three independent replicates; error bars show the standard deviation. Initial phenanthrene concentration was 891 mg l^{-1}. The culture:NAPL ratio was constant in all experiments. The data for 120 h incubation are the same as those shown in Fig. 2a

metabolites (transformation) or different ratios of both fates. To discriminate between these environmentally important fates, a series of experiments was conducted using induced *P. fluorescens* LP6a cultures to determine the effect of inoculum size and dodecanol concentration on mineralization of ^{14}C-phenanthrene in the NAPL phase. The use of radiolabeled phenanthrene and biometer flasks allowed repeated measurement of mineralization without sacrificing the whole culture (as required for GC analysis).

The data in Fig. 4 reveal that addition of 120 or 160 mg l^{-1} of 1-dodecanol enhanced the extent of mineralization of phenanthrene in the NAPL phase compared to a control lacking 1-dodecanol. Initial rates of $^{14}CO_2$ production were comparable for all cultures but after ~24 h the rate of mineralization slowed in the cultures lacking 1-dodecanol, whereas those containing 1-dodecanol continued at the same rate until ~40 h incubation. By 100 h, cultures initially containing 120 or 160 mg 1-dodecanol l^{-1} achieved similar extents of mineralization (10–13% of the initial phenanthrene), whereas the culture without 1-dodecanol reached a plateau at <5% mineralized.

The effect of different concentrations of phenanthrene on mineralization was examined by incubating cultures with ^{14}C-phenanthrene in NAPL at concentrations of 178, 214, 356 or 445 mg l^{-1}. 1-Dodecanol was added at initial concentrations of 120 or 180 mg l^{-1} so as to limit changes in the ratios of

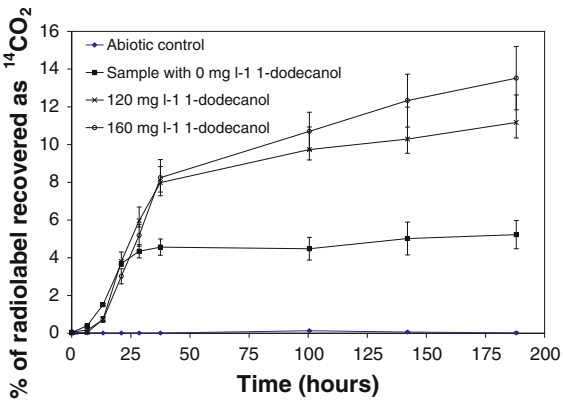

Fig. 4 Effect of 1-dodecanol concentration on mineralization of ^{14}C-phenanthrene in NAPL, calculated as the percentage of initial radiolabel recovered as $^{14}CO_2$. The initial phenanthrene concentration was 178 mg l^{-1}. Data points represent the mean of three independent replicates and error bars show the standard deviation

NAPL to 1-dodecanol, and a control culture lacking 1-dodecanol at each concentration of phenanthrene was included for comparison. After 150 h all cultures had stopped producing $^{14}CO_2$ (not shown), and the plateau was taken to be the final extent of phenanthrene mineralization (Table 2). The extent of phenanthrene mineralization increased in the presence of 1-dodecanol compared to cultures lacking 1-dodecanol. This improvement in mineralization was proportional to the initial phenanthrene concentration, ranging from 2-fold to ~4-fold.

The extent of mineralization was low in all cultures (<12%) yet the previous biodegradation experiments with unlabelled phenanthrene, determined by GC analysis of extracted residual phenanthrene, had revealed losses of 40–80% over the same phenanthrene concentration range (Fig. 2b). To resolve this contradiction we conducted a ^{14}C-phenanthrene mineralization experiment using induced inoculum and performed partial radiochemical balances during incubation to determine additional fates of the radiolabelled substrate. This was achieved by measuring radioactivity in the aqueous phase (i.e., conversion to partially oxidized polar metabolites) and the cell pellet (i.e., incorporation into cell mass or sorption to the cell surface) in addition to cumulative $^{14}CO_2$ production. The ^{14}C measured in the aqueous phase followed the same trend as the ^{14}C mineralized (Fig. 5) but represented a much larger proportion of the radiolabel. With addition of 1-dodecanol, only 6% of phenanthrene was mineralized after 140 h whereas 85% of the ^{14}C was recovered in the aqueous phase. In contrast, the sample without 1-dodecanol showed mineralization of 2.3% in the same time period, and only 40% of the radiolabel was recovered in the aqueous phase. In both the presence and absence of 1-dodecanol the ^{14}C associated with the biomass was <10% after 140 h (data not shown). Consequently, in the presence of 1-dodecanol, the large majority (>90%) of the phenanthrene was transformed (mineralized, converted to water-soluble metabolites, or associated with biomass). In the sample without 1-dodecanol, almost 50% of ^{14}C remained in the HMN phase, presumably as non-transformed phenanthrene, as determined by difference. These data suggest that most non-mineralized ^{14}C partitions into the aqueous phase as polar metabolites of incomplete phenanthrene biodegradation.

Table 2 Mineralization of ^{14}C-phenanthrene dissolved in NAPL phase, after 150 h incubation

Initial phenanthrene concentration (mg l^{-1})[a]	Initial 1-dodecanol concentration (mg l^{-1})	Proportion of ^{14}C-phenanthrene mineralized (%)	Total mass of phenanthrene mineralized (mg)[b]	Increase in mineralization (fold-increase)
178	0	5.0	0.23	–
214	0	4.9	0.26	–
356	0	2.2	0.20	–
445	0	1.0	0.11	–
178	120	10.3	0.46	2.0
214	120	10.8	0.58	2.2
356	180	5.8	0.52	2.6
445	180	3.8	0.43	3.9

[a] Total radiolabelled and unlabelled phenanthrene dissolved in HMN, expressed as mass per total volume of culture, with a constant activity of ~100,000 dpm $flask^{-1}$

[b] Calculated from the proportion of ^{14}C-phenanthrene mineralized

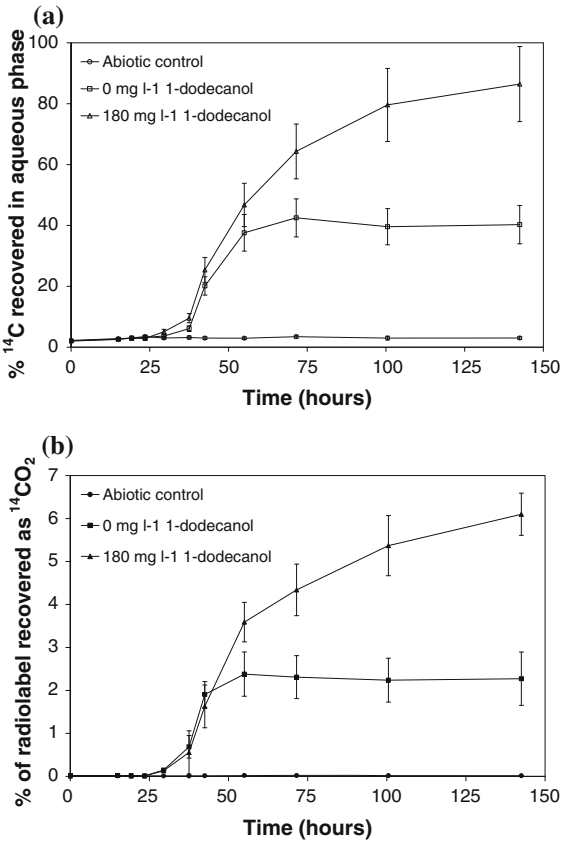

Fig. 5 Radiolabel recovery in different fractions of cultures incubated with 356 mg l^{-1} total phenanthrene and 0 or 180 mg l^{-1} 1-dodecanol, expressed as a percentage of the initial radioactivity added (~100,000 dpm). **a** Culture aqueous phase, representing partially metabolized phenanthrene metabolites; **b** cumulative $^{14}CO_2$ trapped in KOH

Discussion

The addition of 1-dodecanol to cultures at the start of incubation with phenanthrene increased the ability of growing *P. fluorescens* LP6a cells to adhere to the NAPL–water interface, as measured by the MATH test. This observation is consistent with the ability of 1-dodecanol to enhance bacterial adhesion by increasing the three-phase contact angle between cell surface, oil and water (Abbasnezhad et al. 2008). In contrast, adhesion of cells to the oil–water interface only gradually increased with time in the dodecanol-free control, in agreement with reports that hydrocarbon-degrading bacteria exhibit an increase in cell surface hydrophobicity and adhesion to oil–water interfaces during growth (Al-Tahhan et al. 2000; Norman et al. 2002; Prabhu and Phale 2003; Wick et al. 2003). The observed change in adhesion with time led us to expect that any influence of 1-dodecanol on biodegradation due to enhancement in cell surface hydrophobicity would be significant initially, then diminish with time as the bacteria become more hydrophobic and as the 1-dodecanol was biodegraded. We also hypothesized that, as the NAPL phase was depleted in phenanthrene through biodegradation, the advantage of greater adhesion to the NAPL phase would decrease. In contrast, higher initial phenanthrene concentrations in the NAPL phase should provide adherent cells with more substrate for a longer time, resulting in prolonged substrate availability and increased biodegradation rate.

Addition of 1-dodecanol increased biodegradation of phenanthrene by *P. fluorescens* LP6a, as shown by GC analysis of residual substrate and radiometric analysis of phenanthrene oxidation. As hypothesized, mineralization experiments revealed that the initial phase of biodegradation was prolonged in the presence of 1-dodecanol, resulting in a greater total production of $^{14}CO_2$. This effect was pronounced at higher phenanthrene concentrations where the presence of 1-dodecanol increased mineralization by ~4-fold during the early phase of biodegradation.

The mineralization experiments offer additional insight into the pattern of phenanthrene degradation during the first 200 h of incubation. The extent of mineralization by cultures grown in the presence of 1-dodecanol was almost twice that of cells grown without 1-dodecanol, and this increase was proportional to the initial phenanthrene concentration.

In all mineralization experiments conducted, the proportion of radiolabel recovered as $^{14}CO_2$ did not exceed 15%. Interestingly, experiments conducted to determine the distribution of the remaining ^{14}C revealed that much of the enhanced biodegradation resulted from incomplete oxidation of phenanthrene to water-soluble metabolites rather than mineralization to CO_2. Accumulation of the polar metabolites may be due to inhibition, as has been reported previously for PAHs (Bouchez et al. 1996; Heitkamp and Cerniglia 1988; Kazunga and Aitken 2000). In this study the metabolites were not characterized and further research is required to clarify the details of this phenomenon.

Several mechanisms could explain the positive influence of 1-dodecanol on phenanthrene biodegradation. One explanation is induction of the phenanthrene biodegradation pathway by the addition of 1-dodecanol. Given that the pathways for PAHs and long-chain alcohols share no common enzymes (Ludwig et al. 1995; Peng et al. 2008) this mechanism is highly unlikely. A second potential mechanism is that 1-dodecanol, being biodegradable by strain LP6a, served as an additional carbon source and increased the biomass available for phenanthrene biodegradation. However, increasing initial 1-dodecanol concentrations from 217 to 4,100 mg l^{-1} did not enhance phenanthrene biodegradation. Thus, although the observed slow biodegradation of 1-dodecanol might have increased the total biomass of strain LP6a in the cultures, this apparently did not affect mineralization.

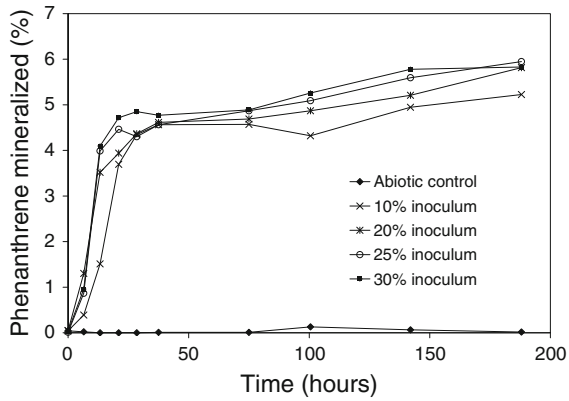

Fig. 6 Effect of the inoculum size on the mineralization of phenanthrene by *P. fluorescens* LP6a. Means of triplicate cultures are shown. Error bars are omitted for clarity

Preliminary experiments showed that varying inoculum size (from 10% up to 30%) did not affect total $^{14}CO_2$ production (Fig. 6). The results in Fig. 6 combined with the data in Fig. 2a indicate that inoculum size does not have a significant impact on biodegradation or mineralization of phenanthrene, thus substantiating this conclusion. Therefore, catabolism of dodecanol, and any associated increase in biomass, was not an important factor in biodegradation of phenanthrene.

A third mechanism could be emulsification of the NAPL phase by 1-dodecanol, giving a greater surface area for dissolution of phenanthrene from smaller droplets. We did not observe any difference in the appearance of the droplets of HMN phase from the medium in shake flasks due to the presence of 1-dodecanol and no emulsification was observed. These observations are consistent with the literature describing surfactant behavior. According to Bancroft's rule (Langevin 2006) oil-soluble surfactants, such as 1-dodecanol, are not expected to stabilize oil-in-water emulsions. Our data confirmed that a concentration of 200 mg l^{-1} 1-dodecanol reduced the interfacial tension of growth medium by at most 27% and resulted in a surface tension of 57 mN m^{-1}. Other investigators have reported that this magnitude of reduction in surface and interfacial tension by surfactants could not result in any significant emulsification (Huang et al. 2009; Willumsen and Karlson 1997; Youssef et al. 2004). A study of oil-soluble surfactants in hexadecane-water emulsions (Lobo and Svereika 2003) showed that even larger reductions of interfacial tension had no significant effect on droplet size

distribution. In another study on the characteristics of surfactant producing bacteria (Huang et al. 2009), it was observed that bacterial cultures with surface tensions above 50 mN m^{-1} did not give any emulsification of oil, as measured by a drop-collapse test. We conclude, therefore, that changes in the interfacial area due to the size of HMN were unlikely to be caused by addition of 1-dodecanol.

The enhanced phenanthrene biodegradation observed in this study in the presence of 1-dodecanol was consistent with the positive influence of 1-dodecanol on microbial adhesion to the oil–water interface (Abbasnezhad et al. 2008), thereby providing more rapid uptake of phenanthrene by the bacteria. Adhesion can facilitate the diffusion of substrate from the organic phase to the cells (Ortega-Calvo and Alexander 1994; Rosenberg and Rosenberg 1981; Weisz 1973). In our experiments, improved adhesion of LP6a to the HMN–water interface was the most likely explanation for the enhanced degradation and mineralization. Importantly, concentrations of 1-dodecanol as low as 200 mg l^{-1} were sufficient to stimulate this enhanced activity.

The importance of adhesion in biodegradation processes may be underestimated in many studies. Most studies in research laboratories have focused on the role of surface active compounds to increase mass transfer, without considering their counter-effect on detaching cells from the interface (Churchill et al. 1995; Singh et al. 2007; Volkering et al. 1997). Laboratory experiments are usually conducted at a small scale where sufficient mixing is available. In large scale bioremediation applications this is rarely the case. Studies determining the effectiveness of full scale bioremediation projects are usually focused on the role of factors such as nutrients, temperature and surfactants (Bragg et al. 1994; Gallego et al. 2007). The role of adhesion typically is not considered in field biodegradation studies, mostly because there is not a well established connection between adhesion and biodegradation in laboratory studies. When there is a contamination incident, such as the Exxon Valdez oil-spill (Shaw 1992), the contaminant is usually spread over an extended area and economic considerations prevent using any artificial mixing facilities. These conditions emphasize the importance of adhesion to the oil–water interface for the biodegradation of contaminants. In general, the role of diffusion of substrate to the cells over short distances is expected to become more dominant as the aqueous concentration of the substrate of interest decreases (Weisz 1973). The aqueous concentration of target compounds is further reduced by dilution in a non-aqueous phase, as in this study. Consequently, microbial adhesion may offer significant advantages for large-scale hydrocarbon bioremediation, in order to overcome bioavailability limitations and play a more important role in increasing the mass transfer of hydrocarbons to the microorganisms. Development of bioremediation approaches that use adhesion as an asset to benefit process performance may be an attractive strategy.

Several studies have established some connection between adhesion and its possible role in biodegradation of poorly water soluble compounds (Bouchez et al. 1999; Chakraborty et al. 2010; Obuekwe et al. 2007a, b, 2008, 2009; Ortega-Calvo and Alexander 1994; Rosenberg and Rosenberg 1981; Suchanek et al. 2000). Despite these studies, to the best of our knowledge the direct use of adhesion-promoting agents to improve biodegradation of poorly water soluble hydrocarbons such as alkanes and PAHs has not been reported. The present report is the first definitive example demonstrating enhanced biodegradation of a PAH using an additive to improve the adhesion of bacteria to the oil–water interface. Due to the importance of the oil–water interface in biodegradation and bioprocessing of hydrocarbons, this study suggests new strategies for bioremediation and bioprocessing and for optimizing existing processes. It may also help further our fundamental understanding of mechanisms and methods involved in such processes.

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