METHODS TO ASSESS THE AMENABILITY OF PETROLEUM HYDROCARBONS TO BIOREMEDIATION

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Abstract—Bioremediation has achieved acceptance as a cost-effective technique for the remediation of soils and groundwater contaminated with petroleum hydrocarbons (PHC). A range of laboratory techniques to assess the biodegradability and bioavailability of PHCs are presented. Biodegradability and bioavailability are important determinants of the bioremediation performance of PHCs. Novel methods for the assessment of the bioavailability of PHC components are described. The techniques are demonstrated for a hydraulic fluid and a spindle oil from a contaminated site. Biodegradation is measured by oxygen consumption and carbon dioxide production. Bioavailability of the PHCs is estimated based on the PHC-water partitioning of tracer compounds and a novel analysis of gas chromatograms based on Raoult’s law. The PHCs tested were only partially biodegradable (< 25% in 78 d) due to the low solubility and likely recalcitrance of some of their components. The combination of techniques outlined is expected to be of use in assessing the likely bioremediation performance of PHCs for which published data are scarce or inadequate.

Keywords—Bioavailability Biodegradability Hydraulic fluid Spindle oil Petroleum hydrocarbon

INTRODUCTION

Bioremediation is now widely accepted as a cost-effective technique for the treatment of soils and aquifers contaminated with petroleum hydrocarbons (PHC) [1,2]. During bioremediation, contaminants are transformed to metabolites or mineralized to carbon dioxide and water while soil and aquifer structures are preserved. However, bioremediation is not suitable for all contaminated sites or indeed for all contaminants. Site conditions and PHC composition are extremely important and determine the success or failure of bioremediation [3].

Most common PHCs (e.g., diesel) consist of a mixture of compounds, with the exact composition depending on the source of the crude oil and how it was refined. A number of studies have described the relative biodegradability of PHC components. Studies suggest that the order of biodegradability is \( n \)-alkanes > \( iso \)-alkanes > aromatics > cycloalkanes [4,5]. However, to estimate the biodegradability of a PHC product on this basis it is necessary to know its composition. This is made difficult by the complexity and variability of PHCs. Not only does composition vary between products, with the composition of gasoline being very different from that of lubricating oil, composition also varies seasonally, between manufacturers and countries [6]. In the many cases where the exact composition of the PHC is unknown, an experimental test of biodegradability is likely to be the most useful approach. Biodegradability is usually measured based on the extent of biodegradation of a test compound over a fixed period of time. Typically this involves measuring the disappearance of the test substance and electron acceptors (e.g., \( O_2 \)) and by the appearance of products (e.g., \( CO_2 \)) [7–9].

Bioavailability is also a major factor in determining the success of bioremediation attempts [10]. It can be defined as a measure of the rate of mass transfer to organisms relative to their degradation capabilities [11] or as the proportion of contaminants that are available for rapid transfer to the aqueous phase [12]. Bioavailability to degrader organisms usually is estimated based on either extraction procedures [10,13] or theoretical calculations, which describe mass transfer to microorganisms [11], though other novel techniques also are available (e.g., [14]). The use of extraction procedures has been focused mainly on the estimation of bioavailability in soils where contaminants are present sorbed to organic and mineral particles, and they have been used in the assessment of both risk and the potential for biodegradation. They are seldom applied to soils where significant amounts of free-phase PHCs are present.

One of the principle limits on bioavailability is the transfer of contaminants from the PHC phase, which is immiscible with water, to the aqueous phase [11,15]. Indeed, where significant amounts of free-phase PHC are present partitioning between this and the aqueous phase is likely to be the dominant control on the availability of PHC components [15]. It is for this reason that we have focused on this process as a way of predicting the bioavailability of PHC components to microorganisms.

The concentrations of PHC components in the aqueous phase at equilibrium depend on the composition of the PHC. This relationship is described by an analogy to Raoult’s law [16,17]:

\[
C_i^a = C_i^{sat} \gamma_i X_i
\]

(1)

where the subscript \( i \) refers to a single PHC component; \( C_i^a \) is the equilibrium aqueous phase concentration (mg/L); \( C_i^{sat} \) is the aqueous solubility (mg/L); \( \gamma_i \) is the activity coefficient within the PHC phase (dimensionless), which is generally assumed to be unity; and \( X_i \) is the mole fraction (dimensionless) of the component \( i \) in the PHC.

This means that in order to use Raoult’s law to calculate the equilibrium concentration of a component, we need to know its mole fraction within the PHC. The mass concentration of a given component within the PHC (e.g., g of component...
i per kg of PHC) is commonly measured using gas chromatography and this can easily be converted to a molar concentration by dividing by the component’s molecular weight. However, to convert this into the mole fraction \(X_i\), we need to know either the molar volume or the mean molecular weight of the PHC, which is difficult to calculate directly from the composition of PHCs due to their chemical complexity. For this reason we will describe a method that can be used to estimate the mean molecular weights and molar volumes of PHCs based on the PHC water partitioning of model compounds. In addition we will describe a method that allows the mole fractions and thus the equilibrium aqueous phase concentrations of components of concern to be estimated directly from chromatograms, obtained using gas chromatography with flame ionization detection (GC-FID).

These techniques are valuable because at the relatively low flow velocities typically found in groundwater (typically \(< 1 \text{ m/day}\)), an assumption of local equilibrium between phases is often valid [18]. In this case information on the equilibrium partitioning of components of concern can be combined with knowledge of the flow field to predict the leaching of PHC components into the aqueous phase [19]. This then provides an estimate of their rate of supply to microorganisms. Even under mass transfer limited conditions the equilibrium concentration remains of central importance because most models describe the rate of mass transfer as being proportional to the difference between the actual concentration and the equilibrium concentration [20].

To successfully assess the potential for bioremediation at a contaminated site it is necessary to examine not only the contaminants present but also a range of site-specific factors. Even for highly soluble PHCs the transfer of PHC components to the aqueous phase is highly dependant on the flow field and the physical distribution of the PHCs [21]. Where PHCs are present in high saturation pools or flow is slow due to low gradients or conductivities, dissolution and biodegradation are expected to be similarly slow. The supply of nutrients and electron acceptors also is of critical importance in determining the potential for successful bioremediation [22]. Site assessment however is outside the scope of this paper. A good set of guidelines for the execution of site assessments is given in reference [3].

Our aim is to provide a set of techniques that can be used to assess the biodegradability and likely bioavailability of complex petroleum hydrocarbons. The methods we describe can be carried out with minimal equipment and they include both standard methods and methods that we believe to be novel. The combination of techniques provides sufficient data to assess whether bioremediation of a particular PHC is likely to be successful (given suitable site conditions). Biodegradability of two PHCs (hydraulic fluid and spindle oil) are assessed using laboratory microcosm experiments and measurements of oxygen consumption and \(\text{CO}_2\) evolution. Bioavailability of PHC components is assessed based on GC-FID analysis of the PHCs and on partitioning experiments with the calculations being based on Raoult’s law.

MATERIALS AND METHODS

PHC products and their origin

The PHCs tested in this paper originate from a fibre cement plant in Niederurnen (Glarus, Switzerland). The PHC spills within the plant escaped through holes and cracks in the concrete floor to contaminate the underlying unconfined sand and gravel aquifer. Excavations at the site in 1972 found extensive PHC contamination. It is estimated that between 20,000 and 25,000 kg of PHCs are present in the soil and aquifer at the site spread over an area of approximately 4,000 m². The depth to groundwater at the site varies between 1.5 and 2 m, the hydraulic conductivity as measured by pumping test was 43.2 m/day. The PHCs usually are constrained by the foundations of the buildings but some seepage into a nearby watercourse has occurred during periods of low groundwater levels. The PHCs used in this study were recovered from the aquifer during a skimming operation to remove free-phase product. Preliminary investigations comparing GC-FID chromatograms of the PHCs (Fig. 1 A and B) with those of products used within the plant (not shown) suggested that the main contaminants are hydraulic fluid and spindle oil [23]. The chromatograms of both PHCs display between one and three humps made up of components not resolved by the chromatographic method; these are known as unresolved complex mixtures. Figures 1 C and D are cyclohexane extracts of water equilibrated with spindle oil and hydraulic fluid, respectively. They show that both PHCs contain some water-soluble components, which are mainly those with lower GC retention times and therefore lower molecular weights. Both PHCs appear also to contain a significant proportion of insoluble hydrocarbons, which are present in chromatograms of the PHCs but not in chromatograms of the extracts.

Data on the chemistry of the groundwater within the contaminated zone at the site are shown in Table 1 [24]. Note that the site groundwater contains significant concentrations of oxygen and nitrate, electron acceptors that we would expect to be consumed if rapid in situ biodegradation of the PHCs was occurring [25]. Preliminary experiments indicated that neither the PHCs nor the site groundwater inhibit bacterial growth significantly. The inoculum used for the inhibition tests was the same mixed inoculum used for the degradation experiments (see Biodegradability). Growth on glucose, as measured by optical density at 660 nm, was compared between site groundwater and mineral medium and only small differences were observed (data not shown). Inhibition of growth by the PHCs was tested by comparing the rate of mineralization of 14C-labelled toluene in the presence of PHCs to that in their absence. The PHCs did not significantly inhibit the mineralization of 14C-toluene (data not shown).

Biodegradability

The inoculum used for all the degradation experiments was prepared using sediment samples collected from a heating oil contaminated aquifer in Studen (Bern, Switzerland; for site description see [26]) and from pasture soil. Mixing the samples from the two sites provided a diverse inoculum with a proven capacity for PHC degradation. Inoculum was prepared using 1 g of sample (wet wt) from each site and 50 mg of yeast extract in 1 L of phosphate buffered mineral medium [27] including modifications suggested by Battersby et al. [28]. The p\(\text{H}\) was then adjusted to 7.4. To reduce lag times in PHC degradation the inoculum was exposed to the test PHCs for 14 d prior to the start of the experiments. Shortly before use the inoculum was coarse filtered using glass wool, acidified to pH 6.5 and sparged with \(\text{CO}_2\) free air.

The primary biodegradability of the PHC was measured in terms of \(\text{O}_2\) consumption in closed vessels using a Sapromat® (Voith, Heidenheim, Germany) automatic biological oxygen demand meter [9]. Sapromat experiments were conducted in
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Fig. 1. Gas chromatography with flame ionization detection chromatograms of (A) spindle oil and (B) hydraulic fluid, both of which were collected from the aquifer. Both (C) and (D) are chromatograms of the water-soluble fraction of spindle oil and hydraulic fluid, respectively.

Table 1. Chemical properties of groundwater at the contaminated site in Niederurnen (Glarus, Switzerland) as measured on May 4, 1999. Hydraulic fluid is present as free product at well S2; spindle oil as free product is present at well S5. Data reproduced from Wyssling [23]; all concentrations are mg/L unless stated otherwise.

<table>
<thead>
<tr>
<th>Observation well</th>
<th>S2</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>10.0</td>
<td>11.5</td>
</tr>
<tr>
<td>Dissolved organic carbon</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Alkalinity (mmol l⁻¹)</td>
<td>3.3</td>
<td>3.6</td>
</tr>
<tr>
<td>O₂</td>
<td>5.9</td>
<td>1.3</td>
</tr>
<tr>
<td>NO₂</td>
<td>3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>NO₃</td>
<td>0.007</td>
<td>0.013</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>1.3</td>
<td>11</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

In a second series of experiments mineralization of the test PHCs was assessed by measuring CO₂ evolution. Ten liters of phosphate-buffered mineral medium [27] were mixed with 100 ml of the inoculum and used to fill 54 serum bottles; 75 ml was added to each bottle leaving a headspace of 42 ml. Eighteen of the bottles were used as blanks; the rest were amended with PHC, half being amended with the hydraulic fluid and half with the spindle oil. The PHCs were added to the bottles adsorbed onto filter papers [28] with 1.8 mg being added to each bottle. The bottles were then crimp sealed using butyl rubber stoppers and subsequently incubated in the dark at 20°C for 87 d. Destructive sampling of three replicates per PHC and three control replicates was carried out on days 0, 7, 14, 28, 59, and 87. The mineralization was calculated from the increase in the inorganic carbon content of the PHC-amended bottles following subtraction of any increase in the blanks.

Bioavailability: Assessment based on partitioning of tracers

Investigations into the bioavailability of the PHCs began with experiments to determine their molar volumes and mean molecular weights. Previous analysis of the PHCs by GC showed that the PHCs contained only traces of ethylbenzene and xylenes; the maximum concentration measured was 1.8 mg/kg [29]. Because of this it was possible to use ethylbenzene and o-xylene as tracers to determine the molar volumes and mean molecular weights of the PHCs based on their partitioning into an aqueous phase. The two PHCs were spiked with 2% by volume of both ethylbenzene and o-xylene and mixed continuously overnight in closed bottles using a magnetic stir bar. Serum bottles with a nominal volume of 50 ml were then filled with 40 ml of 0.05 M HCl and 10 ml of spiked PHC and crimp sealed with polytetrafluoroethylene-faced butyl rubber septa. Three replicates were made for each of the test PHCs. The contents of the bottles were stirred slowly using magnetic stir bars, which served to increase the rate of equilibration without causing PHC droplets to form in the aqueous phase. The bottles were allowed to equilibrate for 7 d; preliminary experiments showed this time to be sufficient for full equilibration. They were then inverted and centrifuged at 2,000 rpm for 30 min at room temperature to allow PHC-free samples of the aqueous phase to be taken using a glass
synergized with a steel needle. Samples were then transferred without headspace to 2-mL sample vials with polytetrafluoroethylene-faced silicone septa and analyzed immediately by high-performance liquid chromatography.

The molar volumes and mean molecular weights of the two test PHCs were estimated from the test data using an analogy to Raoult’s law (Eqn. 1). For the purposes of this calculation the mole fraction $X_i$ was defined as

$$X_i = \frac{N_i}{N_o + N_i} \quad (2)$$

where $N$ is a number of moles per unit volume (mol cm$^{-3}$) and the subscript $o$ refers to the bulk PHC phase (i.e., oil) and the subscript $i$ refers to a single PHC component. Combining Equations 1 and 2 yields

$$\frac{N_o}{N_i} = \frac{1}{X_i - 1} = \left( \frac{C_{oi}}{C_{ri} - 1} \right)$$

Noting that $N_i = \left( f_i \rho / M_r \right)$ where $f$ is the volume fraction of a component in the oil (dimensionless), $\rho$ is the component’s density (g cm$^{-3}$), and $M_r$ is a molar mass (g mol$^{-1}$), we can compute the PHC molar volume $V_{mol}$ (cm$^3$ mol$^{-1}$) using

$$V_{mol} = \frac{1}{M_r} = \frac{C_{oi}}{C_{ri} - 1} \left( f_i \rho / M_r \right)$$

and the PHC mean molar mass $M_r$ (g mol$^{-1}$) from

$$M_r = \frac{V_{mol} \rho}{f_i} \quad (5)$$

**Bioavailability: Assessment based on GC-FID analysis**

In addition to these equilibrium experiments we also present a method for estimating equilibrium aqueous concentrations of PHC components based on GC-FID chromatograms. The method relies on the proportionality of the FID signal to the number of carbon atoms passing the detector, which has been demonstrated for molecules without heteroatoms by Holm [30]. Chromatograms were obtained for both of the spiked PHCs as well as for a series of n-alkanes with carbon numbers ranging from 10 to 44. The chromatograms of the spiked PHCs were then divided into retention time sections with the retention time of an alkane peak at the center of each section. The sections were integrated and the areas divided by 8. The mole fraction of a component within the PHC is then given by

$$X_i = \frac{A_i}{A_o + A_i} \quad (6)$$

where $A_i$ is the sum of all the transformed areas of the chromatogram sections and $A_o$ is the transformed area of the component peak. The $A_o$ is present in the denominator because the spiked components had retention times significantly lower than that of the lightest alkane standard and were not part of one of the chromatogram sections. Once the mole fraction has been estimated, Raoult’s law (i.e., Eqn. 1) can be used to estimate the equilibrium aqueous phase concentration of the component. Approximate values for the mean molecular weights of the PHCs were estimated by assuming that their composition could be represented using the relationship between retention time and molecular weight from the alkane series. The mean molecular weight was then estimated as a weighted mean; this estimate is independent of that made based on the partitioning of tracer compounds into the aqueous phase.

**Analytical methods**

Inorganic carbon production and O$_2$ consumption in the mineralization experiments were measured by analysis of the headspace gas. The sealed bottles were acidified to pH 2 using orthophosphoric acid and left to equilibrate for 1 h on a shaker. Headspace samples were injected into a Carlo Erba GC 8340 (Fisons Instruments, Beverly, MA, USA) equipped with a thermal conductivity detector. The column was a HayeSep D run isothermally at 75°C, and the carrier gas was N$_2$. Identification and quantification of CO$_2$ and O$_2$ were performed by comparison of peak areas and retention times to those of external standards. Henry’s law was applied to account for O$_2$ and CO$_2$ dissolved in the aqueous phase [31].

Aqueous samples were analyzed for ethylbenzene and o-xylene by high-performance liquid chromatography using a 40:60 acetonitrile:water eluent with a flow rate of 1 mL per minute. Aqueous samples (50 μL) were separated on a Merck Lichrocart® 125-4 column packed with Lichrospher® 100 RP 18, 5μm diameter (both from Merck, Whitehouse Station, NJ, USA). Detection was by ultraviolet absorption at 260 nm; peak identification and quantification were by comparison of areas and retention times with those of external standards.

Gas chromatography of PHC samples was carried out using a Trace 2,000 gas chromatograph fitted with a 7-m BGB-5 column (internal diameter 0.25 mm, film thickness 0.25 μm) and a 15-m precolumn (internal diameter 0.53 mm including 3 m coated with SE54 0.45-μm film thickness). The temperature program was 60°C for 5 min then 50°C per min followed by 5 min at 330°C. The injection method was large volume (100 μL) on column, and the solvent was cyclohexane. Carrier gas flow was 3 mL/min of hydrogen.

Total PHC content within the Sapromat flasks was measured at the end of the incubation period by IR-spectroscopy. As a comparison the analysis was also carried out with 1 g of each PHC, which is the same mass initially present in the Sapromat experiments. The contents of the Sapromat flasks for each PHC were pooled, their pH adjusted to two with HCl, prior to extraction with CCl$_4$. Polar hydrocarbons were then separated from the raw extract by column fractionation with aluminum oxide. Total PHCs were subsequently quantified in both the raw extract and in the fractionated extract. The IR spectroscopy was performed at specific wavelengths from 2,800 to 3,200 nm and compared with the Simard standard [32].

**RESULTS AND DISCUSSION**

**Biodegradability**

A short lag phase of 2 to 3 d was observed at the start of the Sapromat tests with hydraulic fluid and spindle oil (Fig. 2). The rate of oxygen consumption then increased and became steadily slower such that after approximately 65 d it reached a maximum of 3.3 mg d$^{-1}$. The values for oxygen consumption, percentage biodegradation, and PHC loss at the end of the experiment are given in Table 2. The disparity between the calculated extent of primary biodegradation and the measured disappearance of PHCs might be due to losses of volatile compounds into the stopper of the flask or the formation of polar compounds that are not extracted efficiently by CCl$_4$. 

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**Equations:**

1. $V_{mol} = \frac{1}{M_r} = \frac{C_{oi}}{C_{ri} - 1} \left( f_i \rho / M_r \right)$
2. $M_r = \frac{V_{mol} \rho}{f_i}$
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Fig. 2. Oxygen consumption during the Sapromat® (Voith, Heidenheim, Germany) experiments, two replicates each of hydraulic fluid, spindle oil, and the inoculum blank. Due to the large number of measurements only every 200th data point is shown.

Mineralization of the two PHCs was calculated based on measurements of CO₂ production in microcosms over a period of 87 d (Fig. 3). The (corrected) rate of CO₂ production from both PHCs shows a short lag phase between 0 and 7 d, followed by a period of increased mineralization between 7 and 28 d; thereafter the rate of mineralization declined. The extent of mineralization was calculated by comparing the actual amount of CO₂ produced to the theoretical amount released by full mineralization of all the PHC present, assuming a PHC carbon content of 84.5% by weight [33]. The maximum degree of mineralization was 12% for the hydraulic fluid and 10% for the spindle oil. These relative values for mineralization are lower than those for O₂ consumption; this may be attributable to the formation of partially oxidized intermediates in the Sapromat experiments and to the different experimental conditions, i.e., the bioavailability may have been greater in the Sapromat experiments because the PHCs were present as an emulsion rather than sorbed onto filter paper. Features common to both sets of biodegradation experiments were that both PHCs underwent considerable biodegradation, but that the rate of biodegradation declined over time. This suggests that both PHCs possess a biodegradable fraction and another larger fraction that is either recalcitrant or insoluble, such that biodegradation of these PHCs is likely to be incomplete. However, it is possible despite the considerable duration of these experiments that the limited extent of biodegradation observed is due in part to the inoculum not being fully adapted to the PHCs or the laboratory conditions.

Bioavailability: Qualitative assessment

Chromatograms of the extracts of the aqueous phase show that the lighter components within both PHCs display significant solubility within the aqueous phase (Fig. 1. C and D). However, a large proportion of the components of both PHCs appear to be essentially insoluble. Comparing the aqueous phase extracts with the original PHCs (Fig. 1 A and B) it can be seen that the components with longer GC retention times are largely absent. This is in line with what we would expect given the inverse correlation between molecular mass and water solubility [34]. The fact that both PHCs appear to have a significant insoluble fraction also is in agreement with the findings of the biodegradation experiments i.e., that a large proportion of both PHCs are subject to very slow biodegradation due to limited degradability or availability.
Bioavailability: Assessment based on tracer partitioning

Following equilibration aqueous phase concentrations of ethylbenzene and o-xylene were measured and used to estimate the molar volumes and mean molecular masses of the two PHCs (Table 3) The relative standard deviation of the aqueous phase concentration within three replicates was 10% or less in all cases. Both of the test PHCs have relatively high molecular masses (276–362 g mol$^{-1}$) and molar volumes (310–416 cm$^3$ mol$^{-1}$) and there is some variation between the estimates based on the o-xylene data and those based on the ethylbenzene data. This variation can be explained by uncertainty in the aqueous solubility [35] of the compounds, analytical errors, and possible deviations of the PHC phase activity coefficients from the assumed value of unity. As a further test of the method the values of molar volume and molecular weight gained from the partitioning of o-xylene were used to predict the aqueous phase concentration of ethylbenzene and vice versa. The largest error in these predictions was 23%, which demonstrates that the values of mean molecular weight and molar volume obtained from the partitioning of one component can be used to predict the aqueous phase concentration of others with reasonable accuracy. The method assumes that the mole fraction of each component within the PHC phase remains unchanged throughout the experiment. This is not strictly the case because some finite mass of the tracer compounds will be lost to the aqueous phase. However, the relatively large volume of PHC compared to water and the limited aqueous solubility of the tracer compounds used ensures that the change in their concentration within the PHC phase is small enough to be negligible. Using dilute HCl as the aqueous phase inhibited the growth of bacteria while avoiding the need for toxic growth inhibitors (e.g., mercuric chloride) and is expected to have had no significant effect on the partitioning behavior of the ethylbenzene and o-xylene. The extent of any salting-out effect was estimated using the salting out coefficient for a NaCl/benzene system [35] and was found to reduce aqueous phase concentrations by less than 3%. Overnight mixing of the PHCs following the addition of the tracer compounds to them ensured that mixing was complete despite the high viscosity of the PHCs involved.

Empirical correlations developed by Eastcott et al. [34] predict that PHCs with high molecular weights and molar volumes will display low solubility and volatility. In addition calculations based on the universal functional activity coefficient (UNIFAC) method [36] suggest that low molecular weight components within such high molecular weight PHCs are likely to have activity coefficients less than one. The xUNIFAC program (Engelen HK, Randhol P. 2000, xUNIFAC free download from: http://www.nt.ntnu.no/users/randhol/xlunifac/) predicted that for a model mixture of a C30 alkane with ethylbenzene and o-xylene (mole fractions of both spiked compounds set to 0.05, which is representative of mole fractions occurring in the experiments) the activity coefficients of ethylbenzene and o-xylene were 0.70 and 0.72, respectively. For a low molecular weight alkane (i.e., pentane-containing ethylbenzene and o-xylene at the same mole fractions), activity coefficients are predicted to be 1.38 and 1.40, respectively. This suggests that for a given component its bioavailability will be lower in a high molecular weight PHC than in a low molecular weight PHC. This effect is likely to have an adverse effect on the time required for cleanup of heavy PHCs, including those studied here.

Bioavailability: Assessment based on GC-FID chromatograms

The GC-FID chromatograms of the spiked PHCs (Fig. 4A and B) and a series of n-alkanes (Fig. 4C) were used to make predictions of the equilibrium aqueous phase concentrations of ethylbenzene and o-xylene. Comparison of the estimated aqueous phase concentrations (Table 4) to the experimental values (Table 3) shows that there is a good correspondence between the two. The largest error in the estimates, for o-xylene in hydraulic fluid, was 26%. This degree of accuracy may be sufficient to make useful predictions about equilibrium aqueous concentrations of specific components. These can then be used in assessing likely biodegradation performance or the potential impact on downgradient receptors. In addition the estimate can be made using only the GC-FID chromatogram and readily available solubility data, with components of interest being identified by comparison of retention times to external standards. Although there will be many components that will be difficult to identify in this way, this does little to detract from the usefulness of the method because many of the components of greatest concern (e.g., benzene, toluene, ethylbenzene, and xylenes [BTEX]) can be identified readily.

This method requires that the component peak can be identified, because the aqueous concentration is calculated from the mole fraction and the aqueous solubility of the component. Also it requires that the chromatographic system displays minimal discrimination. In addition, it is essential that the chromatograms are run until all the PHC components have passed the detector (i.e., the signal has returned to baseline); failure to do this will result in an overestimation of the mole fraction of light components within the PHC. The accuracy of the method might be improved by allowing activity coefficients to have values other than unity, but this would add additional complexity and in many cases an assumption of unit activity should suffice. However, significant error is likely to occur if

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Table 3. Measured equilibrium aqueous phase concentrations of o-xylene and ethylbenzene and estimates of molar volumes and mean molecular weights based on them. Errors are given as ±1 standard deviation and do not include uncertainty in activity coefficients or solubility data

<table>
<thead>
<tr>
<th>Petroleum hydrocarbon (PHC)</th>
<th>Equilibrium aqueous phase concentration mg/L</th>
<th>Aqueous solubility mg/L</th>
<th>Estimated PHC molar volume cm$^3$/mol</th>
<th>PHC density g/cm$^3$</th>
<th>Estimated PHC mean molecular mass g/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydraulic fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o-xylene</td>
<td>10.8 ± 0.65</td>
<td>184$^a$</td>
<td>376 ± 23</td>
<td>0.87</td>
<td>327 ± 20</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>10.7 ± 0.38</td>
<td>168$^a$</td>
<td>416 ± 15</td>
<td>0.87</td>
<td>362 ± 13</td>
</tr>
<tr>
<td>Spindle oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o-xylene</td>
<td>9.0 ± 0.63</td>
<td>184$^a$</td>
<td>310 ± 22</td>
<td>0.89</td>
<td>276 ± 19</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>9.8 ± 1.01</td>
<td>168$^a$</td>
<td>381 ± 38</td>
<td>0.89</td>
<td>339 ± 34</td>
</tr>
</tbody>
</table>

$^a$ Data from Schwarzenbach et al. [35].
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**Fig. 4.** Gas chromatography with flame ionization detection chromatograms: (A) and (B) are of the spindle oil and hydraulic fluid, both spiked with ethylbenzene and o-xylene. (C) is a series of n-alkanes with carbon numbers 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 40, 44. (D) shows the chromatogram of the spiked spindle oil in the form of a bar graph. Bar heights are the areas of the chromatogram sections. (E) shows the same chromatogram following transformation by dividing the areas of each chromatogram section by their representative carbon number. Note that (D) and (E) are intended to be purely illustrative; some distortion along the x axis occurs in these diagrams due to the fixed width of the categories; this does not occur in the actual calculations.

This method is used to predict the partitioning of polar additives e.g., ethanol, methyl tertiary butyl ether. Their activity coefficients within the PHC phase are expected to be greater than one and the presence of heteroatoms is likely to reduce the response of the FID toward these compounds causing underestimation of their mole fraction. It is possible in principle to compensate for both of these effects by estimating the PHC phase activity coefficient and using the concept of effective carbon number [30] to account for differences in FID response. To further illustrate how the method operates we include sample data for the spindle oil including the areas of the chromatogram sections and the areas of the component peaks (Table 5). Figures 4 D and E supplement this example by showing graphically the transformation of the chromatogram into sections and the effects of the subsequent transformation.

**Implications for the field**

Despite the fact that the PHCs studied have been present in the subsurface for more than 30 years, the chromatograms of the aqueous phase extract have shown that they still possess a soluble fraction. This may imply that there has been limited contact between the PHC phase and mobile groundwater, i.e., that dissolution has been limited by the flow regime at the site. However, this is not necessarily the case. The PHCs studied have considerably smaller soluble fractions than fuels do; for example they contain only traces of the BTEX compounds that form a significant proportion of many fuels. The small size of the soluble fraction is significant because the rate of loss of soluble components from the PHC phase is an exponential function of the mole fraction: The rate of wash out is low if the mole fraction is low [37]. The highest content of a BTEX compound is 9 mg/kg of toluene within the spindle oil. Using the larger of the two estimates for the mean molecular weight of the spindle oil we can make a conservative estimate of the equilibrium aqueous phase concentration of toluene 0.017 mg/L. This is smaller by a factor of six than the lowest $K_s$ (half saturation constant) value given for consumption of toluene by *Pseudomonas putida* in [38]. This indicates that biodegradation of the BTEX compounds is likely to be slow due to their low aqueous phase concentrations, and will become slower over time. Low aqueous phase concentrations may well be a major limitation on biodegradation at the field site. It also has been observed that the rate of wash out declines further at very low mole fractions, possibly due to mass transfer limitation within the PHC phase [37]. This effect could be pronounced in the PHCs studied here as they are relatively viscous (the manufacturer quotes a viscosity of 68 cSt at 40°C, for the hydraulic fluid) and diffusion coefficients within liquids are known to be inversely proportional to the viscosity of the liquid; this is described by the generalized Stokes–Einstein relation [39]. In addition the UNIFAC model suggests that light components within heavy PHCs are likely to have activity coefficients below one, which is expected to further slow their dissolution.

Biodegradation experiments showed that the PHCs also have a significant biodegradable fraction (>10% mineralization over 87 d). Given the favorable chemistry of the site groundwater (oxygen and nitrate are present) and the fact that the contaminants have been in the subsurface for decades, we might have expected the biodegradable fraction to be depleted already, but this clearly is not the case. This further suggests that the transfer of the biodegradable components to the aqueous phase may be limiting biodegradation at the site. This
could be due to phase separation at the site (i.e., the PHCs may be present largely in the form of high saturation pools that have only limited contact with mobile groundwater), which, along with the nature of the PHC involved, has the potential to seriously limit the rate of dissolution and therefore biodegradation of the PHCs at this site. Taken with the fact that the PHCs have a significant recalcitrant fraction this suggests that bioremediation is unlikely to be successful in significantly reducing the mass of contaminants at the site under the present conditions. However, it might be possible to achieve enhanced biodegradation if steps were taken to improve the bioavailability of the PHCs by changing the flow regime or by the use of surfactants to increase the solubility of the PHCs.

CONCLUSION

The biodegradability and bioavailability of two PHCs have been examined using a combination of simple tests. Both of the PHCs examined showed significant biodegradability, but also appear to have a large fraction that is poorly bioavailable and/or poorly biodegradable. However, we cannot exclude the possibility that greater biodegradation could have been achieved with a different inoculum. We evaluated bioavailability using two novel approaches, namely an approach based on the partitioning of tracer compounds and an approach based on the analysis of GC-FID chromatograms, and found reasonable agreement between the two methods. This should facilitate the assessment of the potential for bioremediation at PHC-contaminated sites and also may be valuable in assessing potential risks to downgradient receptors. The tests we have performed do not fully explain the apparently poor in situ biodegradation performance of these PHCs. Biodegradation at the site may have been limited by the hydraulic conditions (e.g., phase separation and limited water movement through the contaminated zone), which were not within the scope of this paper.

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REFERENCES


Table 4. Estimated values for ethylbenzene and o-xylene aqueous phase concentrations and petroleum hydrocarbon mean molecular weights based on analysis of (gas chromatography with flame ionization detection) chromatograms; petroleum hydrocarbon (PHC)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Area of peak or chromatogram section</th>
<th>Area divided by carbon number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylbenzene (C8)</td>
<td>291,108</td>
<td>36,388</td>
</tr>
<tr>
<td>O-xylene (C8)</td>
<td>361,463</td>
<td>45,182</td>
</tr>
<tr>
<td>N-decane (C10)</td>
<td>451,105</td>
<td>45,111</td>
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<tr>
<td>N-dodecane (C12)</td>
<td>2,118,092</td>
<td>176,508</td>
</tr>
<tr>
<td>N-tetradecane (C14)</td>
<td>421,154</td>
<td>30,082</td>
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<tr>
<td>N-hexadecane (C16)</td>
<td>144,076</td>
<td>9,004</td>
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<tr>
<td>N-octadecane (C18)</td>
<td>1,377,741</td>
<td>76,541</td>
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<tr>
<td>N-eicosane (C20)</td>
<td>1,401,240</td>
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<tr>
<td>Docosane (C22)</td>
<td>2,057,169</td>
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<tr>
<td>N-tetracosane (C24)</td>
<td>1,608,443</td>
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<td>N-hexacosane (C26)</td>
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<tr>
<td>Octacosane (C28)</td>
<td>906,439</td>
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<tr>
<td>N-triacontane (C30)</td>
<td>1,201,216</td>
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<td>Dotriacontane (C32)</td>
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<td>N-hexatriacontane (C36)</td>
<td>1,656,792</td>
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<td>N-tetracosane (C40)</td>
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<tr>
<td>N-tetracontane (C44)</td>
<td>197,504</td>
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<tr>
<td>Total</td>
<td>17,243,601</td>
<td>872,756</td>
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</table>
Methods to assess the amenability of PHCs to bioremediation


