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Development of a method for the application of solid-phase microextraction to monitor biodegradation of volatile hydrocarbons during bacterial growth on crude oil

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A quantitative solid-phase microextraction, gas chromatography, flame ionization detector (SPME-GC-FID) method for low-molecular-weight hydrocarbons from crude oil was developed and applied to live biodegradation samples. Repeated sampling was achieved through headspace extractions at 30°C for 45 min from flasks sealed with Teflon Mininert. Quantification without detailed knowledge of oil–water–air partition coefficients required the preparation of standard curves. An inverse relationship between retention time and mass accumulated on the SPME fibre was noted. Hydrocarbons from C_5 to C_{16} were detected and those up to C_{11} were quantified. Total volatiles were quantified using six calibration curves. Biodegradation of volatile hydrocarbons during growth on crude oil was faster and more complete with a mixed culture than pure isolates derived therefrom. The mixed culture degraded 55% of the compounds by weight in 4 days *versus* 30–35% by pure cultures of *Pseudomonas aeruginosa*, *Rhodococcus globerulus* or a co-culture of the two. The initial degradation rate was threefold higher for the mixed culture, reaching 45% degradation after 48 h. For the mixed culture, the degradation rate of individual alkanes was proportional to the initial concentration, decreasing from hexane to undecane. *P. fluorescens* was unable to degrade any of the low-molecular-weight hydrocarbons and methylcyclohexane was recalcitrant in all cases. Overall, the method was found to be reliable and cost-effective. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 155–162.

Keywords: biodegradation; crude oil; SPME; volatile hydrocarbons

Introduction

Studies related to biological remediation of crude oil and petroleum wastes typically ignore the significant quantities of volatile hydrocarbons released despite their adverse health effects and role in tropospheric ozone production [5,11,19]. Indeed, many laboratory biodegradation studies examine only artificially weathered crude oil [27], or do not quantify the fate of volatile hydrocarbons [28]. Cost-effective remedial technologies, including biofiltration, are being developed to comply with regulated emission levels during all stages of petroleum production and use [15]. Minimizing volatile hydrocarbon losses during waste remediation is important, and schemes such as the addition of sorbing materials have been examined [23]. However, chemical analysis of petroleum hydrocarbons during biological treatment is challenging and volatile hydrocarbon analysis can be difficult and expensive.

Solid-phase microextraction (SPME) is a relatively new solventless extraction technique developed for rapid, accurate sample analysis [22]. Briefly, the sample is sealed in a vial and incubated at constant temperature under a standard set of conditions (sample volume, mixing, pH and salt concentration). A needle containing a protractable fibre is introduced into the headspace or liquid portion of the sample for a set period of time. A specific mass of analyte will be absorbed by the fibre coating based on the partition coefficients: water–air, air–fibre, and water–fibre (refer to Pawliszyn [22] for a treatment of SPME thermodynamics). Since the extraction is an equilibrium process, only a small amount of analyte will be removed onto the fibre. In contrast to exhaustive quantitative extraction methods such as liquid–liquid extraction and purge-and-trap analysis, SPME allows for repeated extraction and analysis of a single sample [14]. Following equilibration, the fibre is retracted and introduced directly into the analytical instrument of choice for quantitative analysis. Examples include HPLC [2], infrared spectroscopy [25] and gas chromatography mass spectrometry (GC-MS) [1,3,16]. No intermediate steps are required, increasing the precision and accuracy of determinations compared to conventional extraction [22].

SPME fibres can be used to extract polar and non-polar analytes from any type of sample matrix [22]. Methods are rapidly being developed to exploit SPME in environmental analysis. In a recent review [4], 24 papers were cited on the use of SPME. Aqueous samples can be analyzed by direct introduction of the fibre into the water phase [8,14,21] or via headspace sampling [7]. Complex aqueous samples can also be analyzed directly using hollow fibre membrane protection systems [31]. Analyte in solid samples must be extracted from the headspace [12] or by recovering analyte from aqueous extracts [6].

This study describes the development and application of a quantitative headspace SPME-GC-FID methodology in volatile hydrocarbon biodegradation studies during bacterial growth on crude oil. Low cost and repeated analysis of live samples were two desired methodological characteristics.

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Materials and methods

Culture medium, substrate and sample preparation

Standards, controls and cultures were prepared in 125 ml Erlenmeyer flasks containing 25 ml MT medium. MT medium contained, per litre: 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 5.0 g NaCl and 1.0 ml of trace metals solution. The trace metals solution contained, per litre: 1.5 g nitrilotriacetic acid, 5.0 g $MnSO_4 \cdot 2H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3 \cdot 16H_2O$, 0.01 g H_3BO_4 and 0.01 g $Na_2MoO_4 \cdot 2H_2O$. The initial pH of the medium was 7.0.

Bow River crude oil (density 0.905 g/ml; Imperial Oil, Sarnia, ON) was stored at 4°C in glass bottles sealed with Teflon Mininert caps (VICI Precision Sampling Inc., Baton Rouge, LA) to minimize volatile hydrocarbon loss. The oil could be autoclaved and stored without significant changes in compound concentrations or distribution if sealed immediately with a Teflon Mininert cap. Crude oil was added to flasks with a syringe (Hamilton Co., Reno, NV) and flasks were immediately sealed with Teflon Mininert caps prior to incubating them at 30°C on an orbital shaker set to 175 rpm.

Medium was added to flasks prior to inoculation with 25 μl of unwashed cells pre-grown for biodegradation studies. Crude oil was added after inoculation and sterile and acidified controls (0.2% v/v perchloric acid) were similarly prepared. Samples were repeatedly analyzed over time to monitor biodegradation. Since flasks were sealed, oxygen limitation was an issue. Assuming Bow River crude oil contained 85% carbon, and 50% oil degradation would occur, some theoretical oxygen demands were calculated. If 50% of the degraded oil was converted to biomass (assume $yx/s=1$) and 50% was completely mineralized, then the amount of oxygen in the headspace of a 125-ml flask (total volume 145 ml; 20% v O_2/v air) containing 25 ml medium should be sufficient to deal with 19 mg oil. Thus, 22.6 mg or 25 μl oil was chosen as the initial substrate mass.

Source and maintenance of culture

The source and the maintenance of the mixed culture have been previously described [28]. The isolates (*Pseudomonas aeruginosa*, *P. fluorescens* and *Rhodococcus globerulus*) identified by fatty acid analysis were obtained from the original mixed culture [29]. All cultures were pre-grown as a source of inoculum for 3 days in flasks stoppered with foam plugs containing MT medium supplemented with 1.0 g/l yeast nitrogen base (Difco Laboratories, Detroit, MI), 2% (w/v) Bow River crude oil and 0.625 g/l Igepal CO-630 (a chemical emulsifier).

SPME-GC-FID and SPME-GC-MS analysis

A 30-μm polydimethylsiloxane (PDMS) fibre (Supelco, Oakville, ON) was introduced into the headspace of flasks (pre-equilibrated for at least 60 min on an orbital shaker) to extract volatile hydrocarbons. Equilibration was carried out for 45 min in a 30°C waterbath. The fibre holder injection depth was set to 3.6 for sampling and desorption. Following equilibration, the fibre was immediately introduced into a Shimadzu GC-14A (Shimadzu Corp., Kyoto, Japan) equipped with the required narrow bore (0.75 mm i.d.) injector sleeve (Supelco) and a fused silica column (Restek Rtx-5MS, 5% diphenyl–95% dimethyl polysiloxane, 30 m×0.32 mm, 0.25 μ film thickness; Restek Corp., Bellefonte, PA) connected to a flame ionization detector (FID). A 3-min splitless injection (desorption) time was used. The injector was held at 225°C, the detector at 275°C, and the column was taken through the following program: 35°C for 5 min, 7.5°C/min to 225°C and 1.0 min at 225°C for a total run time of 31 min. A blank desorption was performed each morning to free the fibre of residual analyte. Fibres were conditioned in the injector port with the split valve open at 225°C overnight prior to the initial use. This overnight treatment was more effective than the suggested protocol and did not damage the sampling efficiency. Fibres are reusable between 50 and 100 times and PDMS fibres in this study were used for over 300 samples without losing extraction abilities. The GC was operated with a split of 60 ml/min and purge of 5.5 ml/min. Helium was used as carrier gas (10 ml/min) with nitrogen as makeup (40 ml/min). A horizontal baseline was used to calculate peak areas with a Shimadzu Chromatopac C-R6A.

Mass spectra were obtained using a Hewlett Packard 6973 Mass Selective detector connected to an HP 6890 gas chromatograph using helium as the carrier gas. SPME and GC temperature conditions are as described above and an HP-5 5% phenylmethylsiloxane column was used for separations (30.0 m×250 μm×0.25 μm nominal). Compounds were identified by comparing their spectra to a standard library (NIST Mass Spectral Search Program, Version 1.1a).

Preparation of calibration curves

The original concentration of hydrocarbons in the crude oil was determined by GC-FID analysis of crude oil standards (up to 20 mg/ml) prepared in carbon disulfide (99.9%; BDH Inc., Toronto, ON). Analysis was carried out using the same instrument and column as used for SPME analysis. An AOC-17 auto injector (Shimadzu Corp.) was used to inject 1 μl samples into a wide bore inlet liner using the following conditions: column set to 35°C for 5 min, 7.5°C/min to 225°C, hold for 1 min, 10°C/min to 300°C, hold for 5 min, injector set to 225°C; and detector set to 275°C. A five-point toluene standard curve (R^2 0.9977, $n=3$) was prepared for quantifying the mass of each compound assuming an equal FID response for all hydrocarbons being analyzed. Toluene standards (31.25–500 ppm) were prepared in carbon disulfide and liquid injections were made. The column was held at 35°C for 5 min,

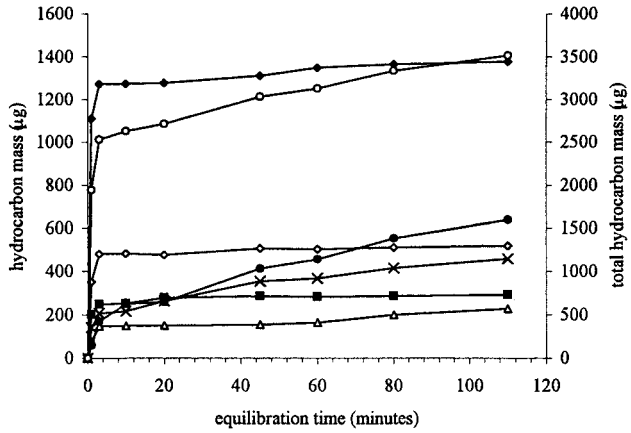

Figure 1 Effect of equilibration time on recovery of volatile compounds from headspace of Bow River crude oil samples by SPME (30°C waterbath, 145 ml total volume, 25 ml medium). Retention time ranges (min): (◇) 0–2.5, (⋄) 2.5–5, (■) 5–7.5, (△) 7.5–10, (×) 10–12.5, (z.ccirf) 12.5–15, (○) 0–15.

Table 1 GC-FID response to volatile compounds extracted with SPME from the headspace above Bow River crude oil for various retention time ranges

Retention time (min)	Elution temperature (°C)	Concentration in oil (μg/μl)	Analyte range (volume oil, μl)	Response curve (y=response; x=μg)	R^2 value
0.0–2.5	35	66.4	2.5–35	$y=30.74x-1522.1$	0.999
2.5–5.0	35	23.0	2.5–35	$y=167.4x+4020$	0.993
5.0–7.5	35–53.75	12.8	2.5–35	$y=315.86x+11{,}810$	0.983
7.5–10.0	53.75–72.5	9.7	2.5–35	$y=52{,}942\ln(x)-141{,}924$	0.984
10.0–12.5	72.5–91.25	16.4	2.5–35	$y=56{,}836\ln(x)-133{,}563$	0.999
12.5–15.0	91.25–110	18.1	2.5–35	$y=38{,}908\ln(x)-29{,}091$	0.971
Total		146.3			

increased by 1°C/min to 40°C and then by 10°C/min to 50°C. The analyte masses were summed in 2.5 min retention time windows. Twenty-five microlitres of crude oil contained the following masses of hydrocarbons in milligrams (mean±SD, n=4): 0–2.5 min, 1.50±0.12; 2.5–5.0 min, 0.50±0.05; 5.0–7.5 min, 0.26±0.04; 7.5–10.0 min, 0.22±0.02; 10.0–12.5 min, 0.32±0.07; and 12.5–15.0 min, 0.40±0.04.

Known volumes of Bow River crude oil (2.5–35 μl) were added to flasks containing MT medium followed by headspace sampling with SPME. Following FID analysis, total peak areas were calculated for 2.5 min time windows and plotted against the known hydrocarbon masses to prepare calibration curves. Six standard curves were prepared and the total mass of volatile hydrocarbons in biodegradation flasks was estimated therefrom. A control was analyzed daily and the results used to calculate percent biodegradation. Mixed alkane stock solutions (n-hexane to n-undecane) in methanol were used to prepare standards by adding 30 μl of each to sealed flasks as described above. Standards were subject to SPME-GC-FID analysis and the resulting response curves used to quantify individual alkanes.

Results

Preliminary optimization of SPME methodology

Mass transfer of analyte from the SPME fibre onto the GC column was optimized during preliminary experimentation. The fibre injection depth placed the fibre at the centre of the injector liner and a desorption time of 180 s removed >99% of the analyte. The equilibration, or sampling, time was optimized for maximum analyte recovery in the minimum time. Compounds eluting before 10 min (72.5°C, n-C_{10}) reached equilibrium in 3 min (Figure 1). Compounds eluting between 10 and 15 min (72.5–110°C, n-C_{10} to n-C_{12}) were extracted slowly, showing linear kinetics after 3 min of rapid accumulation. Overall, the initial period of rapid analyte accumulation was complete after 10 min of exposure with slower change between 30 and 60 min. Thus, a 45-min equilibration time was chosen corresponding to the total GC run time.

SPME response curves, quantification and reproducibility

Standard curves of total peak area *versus* total hydrocarbon mass were prepared for calibration. Six standard curves (Table 1) for 2.5 min retention time windows from 0 to 15 min were required as curves shifted from linear to logarithmic for increasing retention time ranges. Beyond 15 min (dodecane), there was no significant increase in total peak area with increasing hydrocarbon concentration. FID analysis of Bow River crude standards in CS_2 revealed that neither the column nor the detector was overloaded at these concentrations. The logarithmic curves were used although greater error was associated with higher oil concentrations.

Despite this potential for error, both peak areas and calculated hydrocarbon mass values for various retention time ranges were reproducible (Figure 2A). Note the increasing sensitivity for higher-molecular-weight compounds based on retention time. Figure 2B displays the peak area-to-total hydrocarbon mass ratio for flasks containing 2.5 and 35 μl crude oil. In the flask containing 2.5 μl crude oil, increased ratios with increasing compound molecular weight were evident. Decreased ratios were observed beyond 7.5 min for 35 μl oil.

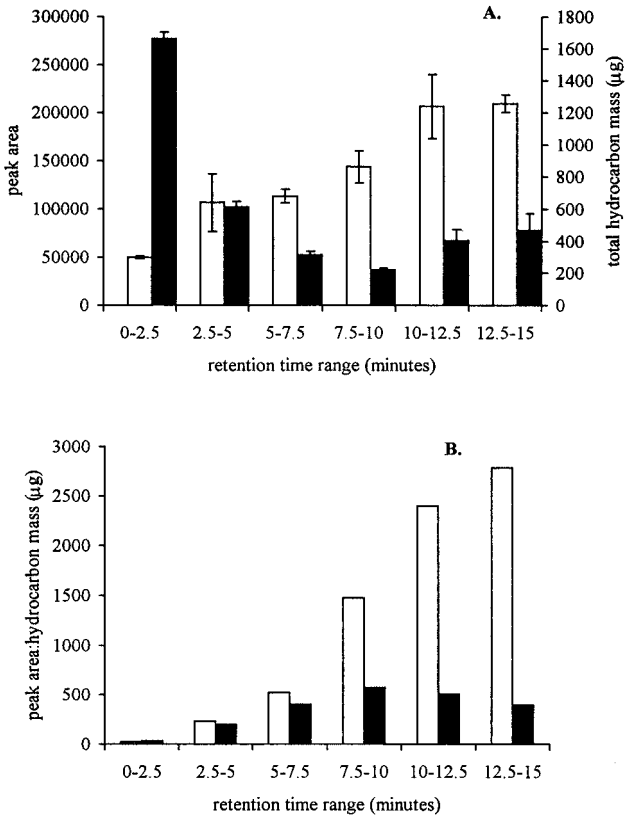

Figure 2 (A) Reproducibility and comparison of FID response compared to total analyte mass for increasing retention time windows. Open bars, peak area; filled bars, total hydrocarbon mass. Error bars show standard deviation for nine replicate samples. (B) Comparison of total peak areas to hydrocarbon mass for increasing retention time ranges. Open bars, 2.5 μl crude oil; filled bars, 35 μl crude oil.

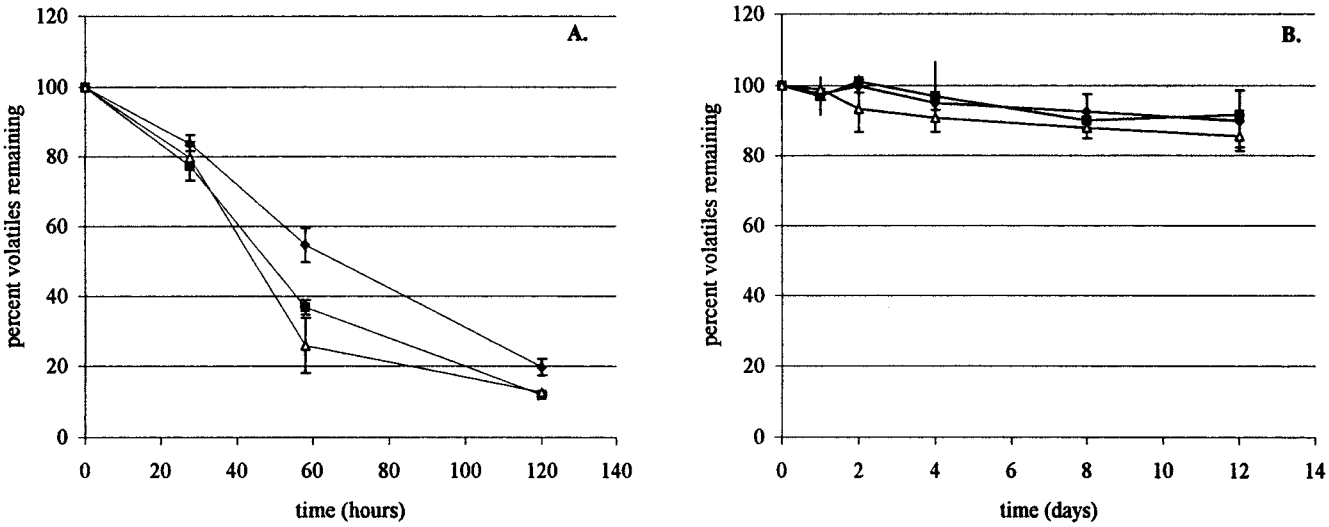

Figure 3 Comparison of capping systems for retention of volatile compounds above Bow River crude oil cultures in acidified controls for various retention time ranges (min): (◇) 0–5, (■) 5–10, (△) 10–15. (A) Teflon-lined silicone septum, repeatedly pierced. (B) Re-sealing Teflon Mininert cap. Error bars show standard deviation of triplicate samples.

Biodegradation of volatile hydrocarbons by pure and mixed cultures

Volatilization from biodegradation flasks had to be eliminated to allow for repeated sampling over time. Teflon-lined silicone septa were insufficient for retaining volatile components during biodegradation experiments if repeated sampling was carried out. Once a septum had been pierced, 80% of the compounds was lost from acidified flasks repeatedly sampled over 120 h (Figure 3A). Losses were negligible in flasks sealed with Teflon Mininert caps (Figure 3B). Killed and sterile controls were stable for longer than 20 days during biodegradation experiments.

Both sample acidification and autoclaving of crude oil for sterilization were examined for control preparation. Addition of 1.6% (v/v) perchloric acid, corresponding to a pH of 1.0, did not affect SPME analysis (Table 2A). Oil could be autoclaved and stored in a Mininert-capped vial without significant volatile hydrocarbons loss (Table 2B).

Total volatile hydrocarbon biodegradation by bacterial cultures growing on crude oil was monitored using SPME-GC-FID analysis. Biodegradation kinetics for a mixed culture, three pure cultures (*P. aeruginosa*, *P. fluorescens*, *R. globerulus*), and a co-culture (*P. aeruginosa* and *R. globerulus*) was determined. Total percent degradation was calculated relative to a control analyzed daily. *P. fluorescens* did not utilize any compounds visible on the chromatogram, while *P. aeruginosa* and *R. globerulus* displayed similar overall degradation kinetics (Figure 4). Combining *P. aeruginosa* and *R. globerulus* in co-culture did not alter the kinetics and, in each case, degradation was complete at 25–30% by day 4. The mixed culture from which the isolates were originally obtained

Table 2 Effect of (A) perchloric acid and (B) autoclaving on sampling efficiency of volatile hydrocarbons from the headspace of biodegradation flasks using SPME

(A)

Percent acid (v/v)	pH	Percent recovery[a]
0	7	100.0
0.2	2.06	97.2
0.4	1.62	98.4
0.8	1.35	98.3
1.6	1.07	98.0

(B)

Retention time range (min)	Elution temperature (°C)	Percent recovery[b]
0–2.5	35	90.1 (2.1)
2.5–5	35	95.3 (0.9)
5–7.5	35–53.8	98.9 (3.7)
7.5–10	53.8–72.5	96.5 (4.6)
10–12.5	72.5–91.3	98.8 (8.5)
12.5–15	91.3–110	98.7 (1.5)
Total	35–110	93.4 (0.4)

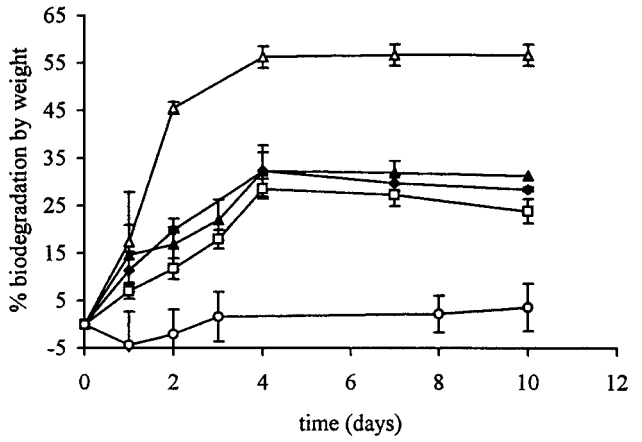

Figure 4 Biodegradation kinetics of volatile hydrocarbons during growth on Bow River crude oil. Total degradation of compounds eluting below 125°C is shown for: (△) mixed culture, (▲) *P. aeruginosa* + *R. globerulus*, (◇) *P. aeruginosa*, (□) *R. globerulus*, (○) *P. fluorescens*. Error bars show standard deviation of triplicate samples repeatedly analyzed over time.

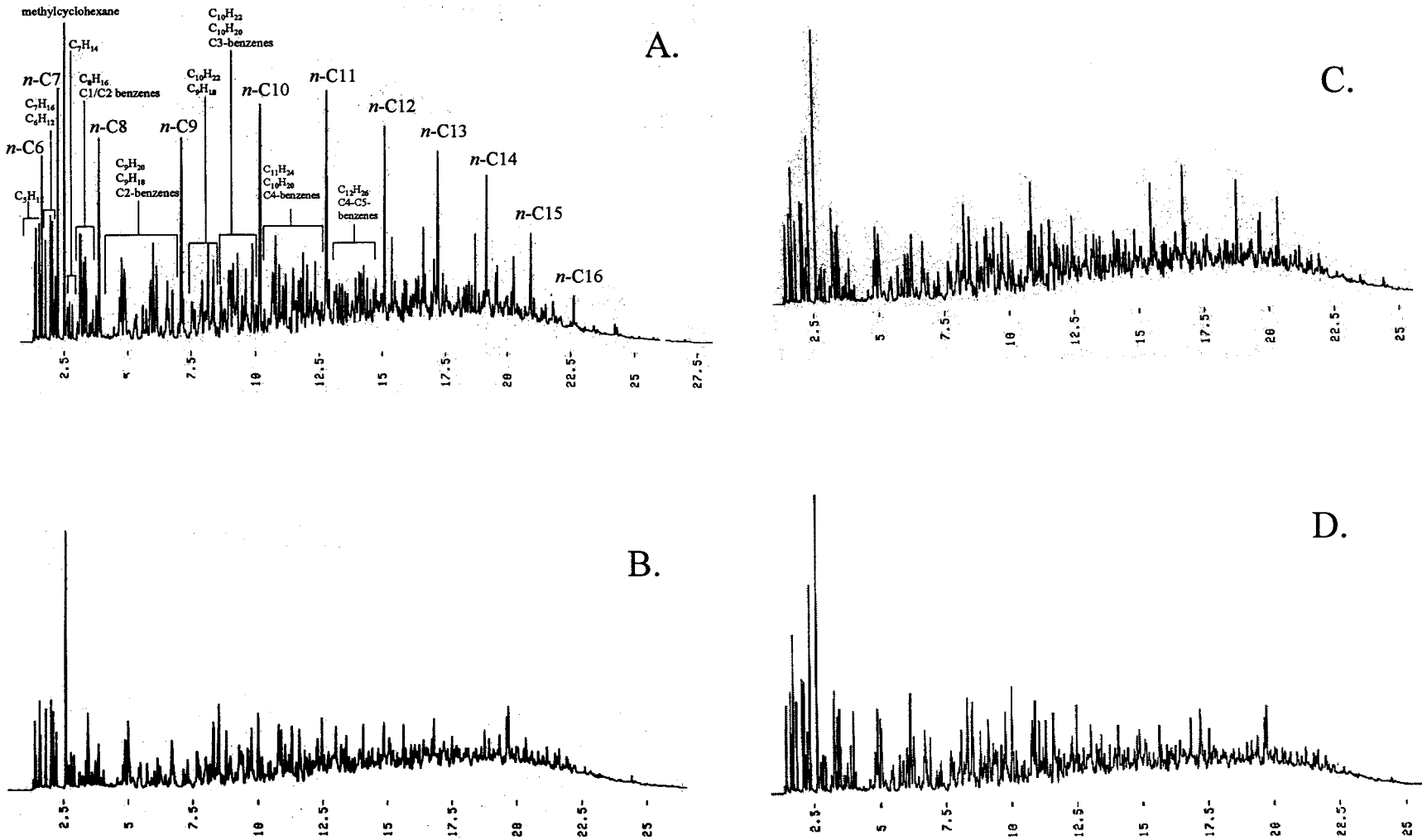

Figure 5 Representative chromatograms from SPME-GC-FID analysis of volatile hydrocarbons in the headspace of biodegradation flasks containing Bow River crude oil after 10 days of incubation. (A) Control, (B) mixed culture, (C) *P. aeruginosa*, (D) *R. globerulus*, (E) co-culture.

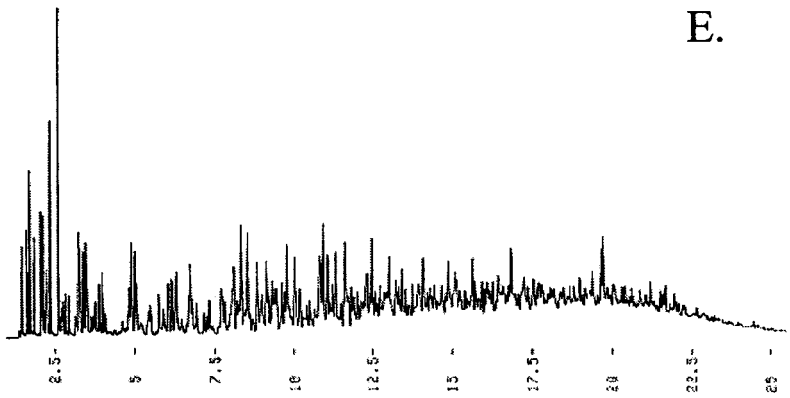

Figure 5 (Continued).

displayed a faster degradation rate and eliminated 55% of the low-molecular-weight hydrocarbons in 4 days.

Representative chromatograms for control and biodegradation flasks containing the mixed culture, *P. aeruginosa*, *R. globerulus*, and the co-culture are shown in Figures 5A–E. Chromatograms from the *P. fluorescens* culture were unchanged when compared to the control. The most obvious undegraded compound in each case was identified by MS as methylcylcohexane. While degradation of compounds eluting after 15 min is evident, the values could not be quantified due to poor response with increasing hydrocarbon concentrations. Linear alkanes from hexane were easily removed by capable cultures and many substituted cycloalkanes, alkanes and benzenes were degraded. Table 3 summarizes the mass removal and maximum biodegradation rate for *n*-alkanes between hexane and undecane by each culture. The pure and co-cultures of *P. aeruginosa* and *R. globerulus* did not remove *n*-C_6 and *n*-C_7 while *n*-C_9 to *n*-C_{11} were eliminated. *R. globerulus* and the co-culture removed 50% of the *n*-C_8, compared to *P. aeruginosa* alone which completely removed this compound. The original mixed culture exhibited higher maximum removal rates for *n*-alkanes although a 24-h lag period that was not evident for pure cultures was observed. The mixed culture degraded hexane and heptane and degradation rates declined with increasing molecular weight for all cultures. This phenomenon was a function of the initial substrate concentration.

Discussion

SPME was explored as a low-cost, reproducible and sensitive technique to monitor low-molecular-weight hydrocarbon biodegradation during growth on crude oil in a closed system. This is the first application of SPME technology for the repeated quantification of volatile hydrocarbons below *n*-C_{11} in live bacterial cultures.

SPME method development is straightforward for pure compounds or mixtures of pure compounds in liquid matrices. Additional care is required when analyzing heterogeneous mixtures such as petroleum hydrocarbons. Determination of the extraction time required examination of different retention time ranges as low-molecular-weight compounds in the mixture equilibrated faster than high-molecular-weight species. Indeed, higher-molecular-weight compounds (>*n*-C_{10}) displayed relatively slow,

Table 3 Extent and rate of linear alkane (hexane to undecane) biodegradation for the mixed and pure cultures

Culture	*n*-C_6	*n*-C_7	*n*-C_8	*n*-C_9	*n*-C_{10}	*n*-C_{11}
Mass remaining (μg) [a]						
Control	339.2 (12.2)	240.5 (10.4)	97.2 (6.9)	49.5 (1.7)	20.6 (0.9)	10.3 (0.5)
P. aeruginosa	335.9 (21.6)	221.4 (10.6)	3.8 (0.0)	9.4 (0.1)	0.9 (0.0)	0.2 (0.0)
P. fluorescens	356.9 (25.1)	255.4 (11.9)	112.6 (6.4)	52.8 (2.1)	21.6 (0.2)	12.5 (1.0)
R. globerulus	377.1 (82.6)	224.8 (6.6)	65.8 (13.4)	1.5 (0.0)	0.9 (0.0)	0.2 (0.0)
P. aeruginosa+*R. globerulus*	331.9 (13.7)	187.6 (52.5)	49.8 (0.9)	1.5 (0.0)	0.9 (0.0)	0.2 (0.0)
Mixed culture	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Maximum degradation rate (μg/h) [b]						
Control	–	–	–	–	–	–
P. aeruginosa	–	–	0.83	0.54	0.22	0.06
P. fluorescens	–	–	–	–	–	–
R. globerulus	–	–	0.58	0.83	0.41	0.21
P. aeruginosa+*R. globerulus*	–	–	0.65	0.83	0.27	0.12
Mixed culture	6.18	5.02	4.05	1.97	0.46	0.05

[a]Mean±SD (n=3).
[b]Rate from 24 to 48 h.

linear accumulation rates and did not approach equilibrium under the conditions used. This observation relates to the higher partition coefficients for higher-molecular-weight compounds and is in agreement with Langenfeld et al. [14] who examined the extraction of pure alkanes and polycyclic aromatic hydrocarbons with a 100-μm PDMS fibre. The authors also suggested that the increased extraction time was due to the low diffusion coefficients of high-molecular-weight species.

A 45-min extraction was chosen in this study as the period of rapid analyte accumulation, where reproducibility is difficult, had passed. This time was convenient as it matched the total time between GC runs. An infinite extraction time is theoretically required to reach equilibrium [12] although sufficient levels of analyte were extracted here in short periods of time. Faster equilibration times would be achievable at higher temperatures and with vigorous sample mixing [22], although this was not desirable for live samples. If more rapid kinetics were being investigated, then killed samples for each time point may be required. Equilibrium is not a prerequisite for SPME as long as the extraction time is fixed. Havenga and Rohwer [12] developed a method for screening hydrocarbons in industrial soils under non-equilibrium conditions.

The heterogeneity of the analyte mixture resulted in the requirement for several standard curves to calculate the total volatile hydrocarbon concentration and the concentration of individual compounds. Direct calculation of biodegradation kinetics from peak areas was inappropriate due to the shift in response from linear to logarithmic and the observed increase in peak area-to-analyte concentration ratio, or sensitivity, for increasing molecular weight. Two reports on the use of SPME-GC-MS with 30- and 100-μm PDMS fibres to monitor biodegradation of diesel fuel and components (C_{14} to C_{17} *n*-alkanes, *p*-xylene and naphthalene) in water and soil have appeared [9,10]. However, Ericksson et al. [9] reported that the percentage of the total area in SPME and pentane extractions was equivalent for C_{11} to C_{17} *n*-alkanes. Increasing partition coefficients with increasing molecular weight within a homologous series of compounds demands greater SPME sensitivity with increasing carbon number [18].

Fortunately, the substrate in this study could be well characterized and external calibrations were performed. Quantification of poorly characterized hydrocarbon mixtures with SPME is difficult, although for air samples containing mixtures of volatile hydrocarbons, TPH estimations can be made without external calibration using the linear temperature program retention index (LTPRI) approach [18]. This approach was considered for this study, but the added complication of the oil and water phase precluded its use as Henry's constant corrections would be required [22].

Since repeated analysis of live samples was a requirement of the developed method, internal standard addition was inappropriate. Thus, external controls were of utmost importance. Oil could be autoclaved without losing significant quantities of volatile hydrocarbons, and acidified controls could be prepared without altering substrate partitioning. This allowed for pure and mixed cultures to be used. If target analytes are dissociable, then acidified controls may not be appropriate due to the expected changes in partition coefficients [24]. Teflon Mininert caps were required as, once pierced, the silicone backing of Teflon-coated septa quickly absorbed low-molecular-weight hydrocarbons. The closed nature of the system is a disadvantage for aerobic biodegradation studies, but an open system would result in volatilization as well as biodegradation. Ericksson et al. [9] used open flasks loosely sealed with aluminum foil to monitor diesel fuel biodegradation and detected only compounds eluting after nonane. Their controls showed significant evaporative losses and the data were normalized to an undegraded internal standard which evaporated at different rates than other compounds in the mixture.

Biodegradation kinetics was easily monitored using the developed methodology. Not surprisingly, the mixed culture exhibited a faster rate and greater extent of degradation. Mixed cultures are often used for biodegradation of mixed substrates due to the potential for greater metabolic diversity [13,26]. While the biodegradation data shown are for all of the materials eluting from the GC below 110°C (i.e. dodecane), the fate of individual compounds within the mixture could be quantified with appropriate standard curves. The maximum degradation rate for *n*-C_6 to *n*-C_{11} by the mixed culture was proportional to the initial substrate concentration. The same was true for *P. aeruginosa*, but only for *n*-C_8 to *n*-C_{11}, and for *R. globerulus*, *n*-C_9 to *n*-C_{11}. Neither of these two pure strains utilized *n*-C_6 nor *n*-C_7. The observed recalcitrance of methylcyclohexane in all cases is useful information for the development of biofiltration strategies. The use of a different fibre thickness would have allowed for a more detailed analysis of higher-molecular-weight compounds [17]. No metabolites were detected in extractions using the PDMS fibre as the fibre has stronger affinity for non-polar analytes. In other biological systems, Nilsson et al. [20] detected a variety of alcohols, ketones, and terpenoid hydrocarbons — many of them novel — with headspace sampling of *Penicillium* sp. with 100 μm PDMS and 85 μm polyacrylate fibres. Metabolites of hexadecane degradation, specifically 1- and 2-hexadecanol, by a psychrotrophic *Rhodococcus* sp. have been detected with GC-MS using a 75-μm polyacrylate fibre [30] which is more suitable for polar analytes [22]. It would be feasible to apply several fibre types to a single sample if more information is desired.

Overall, SPME is a rapid and accurate method for monitoring biodegradation of volatile hydrocarbons in crude oil biodegradation systems. Samples can be repeatedly analyzed over time under mild conditions similar to those used for routine incubation. In addition, the method is easily adapted to standard GC systems at limited cost. The principal disadvantages are the requirement for external calibration, the closed nature of the described system, and the need for several standard calibration curves. A variety of applications for SPME including electron acceptor studies, partitioning studies and rapid assessment of treatment options at bench scale can be envisioned. Air streams are amenable to SPME analysis and biofiltration studies could be simplified with this novel and sensitive analytical tool.

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