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 C5 to C16 were detected and those up to C11 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 methycyclohexane 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.

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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 troposphere 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 protratable 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.
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 KH₂PO₄, 1.5 g K₂HPO₄, 1.0 g NH₄Cl, 0.5 g MgSO₄·7H₂O, 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 MnSO₄·2H₂O, 0.01 g FeSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g ZnSO₄·7H₂O, 0.01 g CuSO₄·5H₂O, 0.1 g CoCl₂, 0.01 g Al₂(SO₄)₃·16H₂O, 0.01 g H₂BO₃ and 0.01 g Na₂MoO₄·2H₂O. 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 Mininet 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 Mininet cap. Crude oil was added to flasks with a syringe (Hamilton Co., Reno, NV) and flasks were immediately sealed with Teflon Mininet 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 (assumed x/v = 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 O₂/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 globulatus) 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 μm 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% phenylmethyl-silicone column was used for separations (30.0 m×0.25 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 (R² = 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,