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Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on crude oil

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Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on Bow River crude oil was investigated using solid phase microextraction (SPME). Inoculum treatments were examined in relation to C_5–C_{11} hydrocarbon degradation. Up to 1600 mg/l biomass (dry weight) was tested without achieving significant volatile hydrocarbon partitioning and affecting analysis. Inoculum age rather than concentration had the most profound impact on biodegradation. When late log phase crude oil-grown inocula were used, C_5–C_{11} biodegradation reached 55–60%; methylcyclohexane and other branched compounds eluting before *n*-C_8 were recalcitrant. Increasing the late log inoculum concentration from 0.63 to 63 mg/l resulted in a twofold increase in degradation rate without improving the substrate range. Methylcyclohexane recalcitrance was correlated with reduced levels of hydrocarbon-degrading bacteria and volatile hydrocarbon evaporation from the inoculum flasks. A decreased lag phase prior to degradation was observed when using early stationary phase cultures as inocula and most compounds up to C_{11}, including methylcyclohexane, were biodegraded. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 356–362.

Keywords: crude oil; mixed-bacterial culture; methylcyclohexane; solid phase microextraction; volatile hydrocarbons

Introduction

The petroleum industry and the use of its derived products contribute significantly to volatile hydrocarbon release [5]. Emissions reduction has aroused international interest due to direct and indirect impacts on humans, plants and animals [19,26]. Biological methods to eliminate hazardous products and to achieve odour control [7,16] are an attractive alternative to phase transfer techniques such as activated carbon [24].

Novel biofiltration technologies will have application in systems currently being used to biologically treat petroleum-contaminated wastes where low molecular weight hydrocarbons are vented through high aeration. For example, during a bioventing study, Malina *et al* [9] found that while 84–98% of decane was biodegraded, only 7.5–29% of toluene was removed biologically prior to evaporation. Conversely, Dahlan *et al* [3] showed toluene removed from wastewater with a stripping tank could be biodegraded in series with a liquid bioreactor.

For mixed hydrocarbon streams such as crude oil, one compound may positively or negatively affect the biodegradation of a second compound. For example, a *Mycobacterium vaccae* strain poorly degrades styrene unless toluene is present as cosubstrate, while toluene degradation is reduced in the presence of styrene [2]. These interactions are further complicated in mixed culture systems. However, mixed cultures have the potential for broader metabolic activity, and the interaction of two or more strains is often a prerequisite for growth and biodegradation [8].

A rapid and accurate method for monitoring low molecular weight hydrocarbons during crude oil biodegradation using solid phase microextraction (SPME) has been described [21], simplifying mixed-hydrocarbon substrate analyses. In this study, we examined factors affecting volatile hydrocarbon biodegradation during growth on crude oil by a mixed culture in closed, batch systems.

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Materials and methods

Culture source and preparation

A mixed-bacterial culture isolated from petroleum-contaminated soil and maintained in cyclone fermentors on various hydrocarbon substrates (diesel fuel, crude oil, motor oil, refinery sludge) was used [20]. The culture was stored at −80°C in 20% w/v glycerol and pregrown in foam-stoppered flasks at 30°C (175 rpm orbital shaking) in 50-ml batch cultures on 2% w/v Bow River crude oil (21.7% volatiles, 24.5% saturates, 42.2% aromatics, 5.8% resins, 5.5% asphaltenes; density 0.905 g/ml; Imperial Oil, Sarnia, ON, Canada) with a chemical surfactant (0.625 g/l Igepal CO-630; Rhône-Poulenc, Cranbury, NJ) and 1.0 g/l yeast nitrogen base (Difco Laboratories, Detroit, MI). A growth curve has been published [20]; it was characterized by 24 h of rapid growth (10^8–10^9 CFU/ml) followed by a decline to stationary phase between 24 and 48 h. Biodegradation flasks were prepared immediately after reviving inoculum stored at −80°C (lag phase inoculum), and after 1 (late log), 2 (early stationary) and 7 (stationary) days of growth. In several experiments, the yeast nitrogen base was replaced with yeast extract (Difco Laboratories). The bacterial community has been described [22].

Culture medium, substrate and biodegradation setup

The basal salts medium (pH 7.0) contained per litre: 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7\,H_2O$, 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 $Na_2MoO_4 \cdot 2H_2O$.

Bow River crude oil was stored in Mininert capped (VICI Precision Sampling, Baton Rouge, LA) bottles at 4°C. Unwashed culture (25 μl) was added to a final concentration of 0.63 mg/l (wet weight) in 25 ml medium in 125-ml flasks with a total volume of 145 ml for biodegradation experiments. Crude oil (25 μl) was added with a syringe (Hamilton, Reno, NV) immediately before sealing flasks with Teflon Mininert caps. Flasks were incubated at 30°C on an orbital shaker (175 rpm). For biomass titration

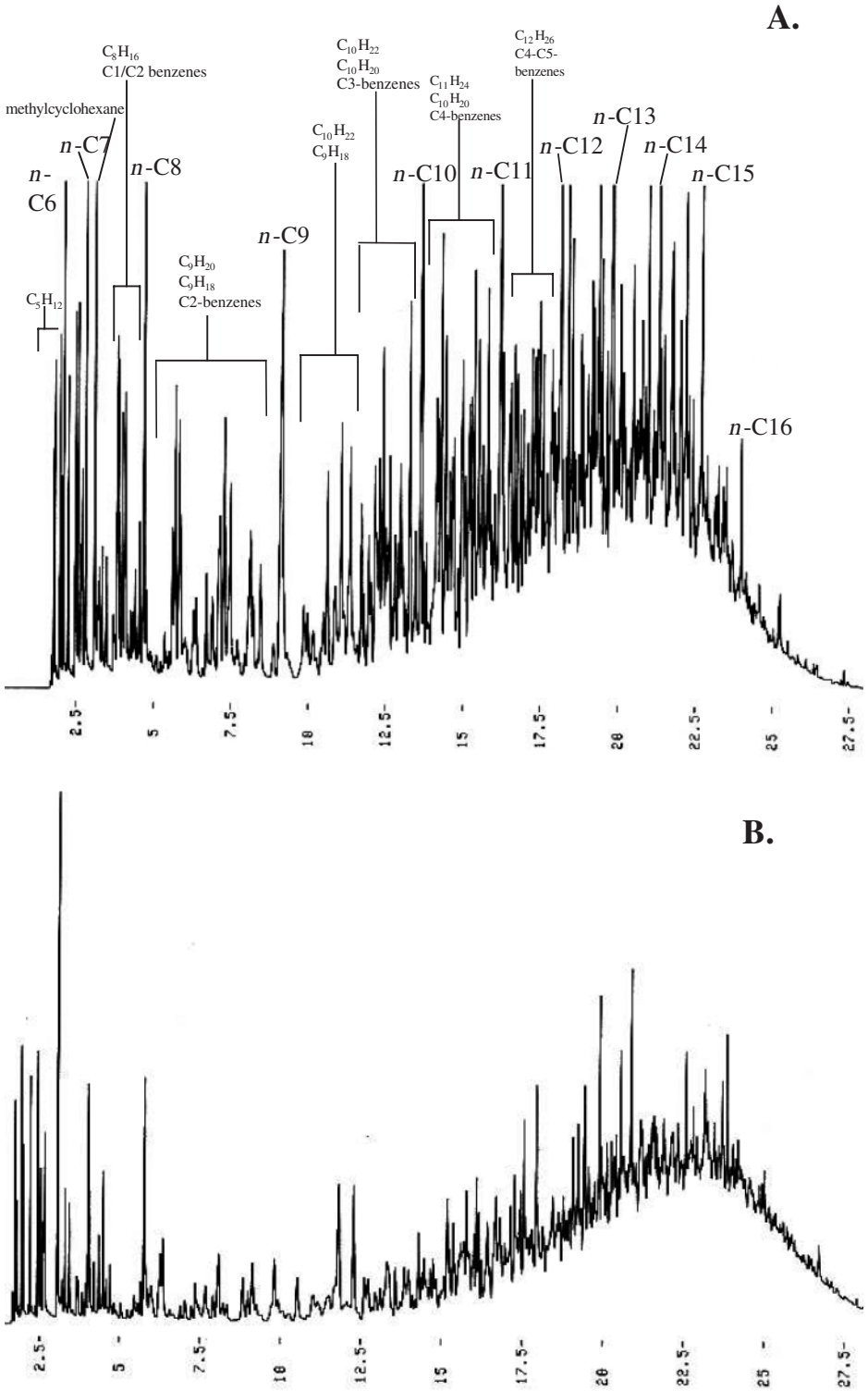

Figure 1 (A) Headspace SPME-FID chromatogram from a 7-day old sterile control flask containing Bow River crude oil. (B) Representative chromatogram following biodegradation by culture initiated with 7-day-old mixed-bacterial inoculum.

experiments, 7-day inoculum cultures were centrifuged at 12 000×*g* for 15 min, weighed, and resuspended in 50 mM phosphate buffer (pH 7.0) to the desired concentration. Controls were prepared by autoclaving oil and adding it to sterile flasks or by acidifying cultures with 0.2% v/v perchloric acid [21]. Triplicate flasks were repeatedly analyzed over time.

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

As previously described [21], a 30-μm polydimethysiloxane (PDMS) fibre (Supelco, Oakville, ON, Canada) was used for headspace sampling from biodegradation flasks equilibrated in a 30°C waterbath. The needle housing was set to 3.6 and a 45-min equilibration time was used. A narrow bore (0.75 mm ID) 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, Bellefonte, PA) were used with a Shimadzu GC-14A (Shimadzu, Kyoto, Japan) for FID analysis. For sample injections, the split valve was closed and the fibre held in place for 3 min (injector 225°C, detector 275°C). The column temperature program was 5 min at 35°C, 1°C/min to 40°C, 10°C/min to 225°C and hold for 1.5 min. Horizontal baseline integration was used during peak area determinations (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 were as described above and an HP-5 5% phenylmethylsiloxane column was used (30.0 m×250 μm×0.25 μm nominal). Compounds were identified (Figure 1A) by comparing spectra to a standard library (NIST Mass Spectral Search Program, Version 1.1a).

Calibration curves

As previously described [21], hydrocarbons in Bow River crude oil were quantified by analyzing crude oil standards prepared in carbon disulfide (99.9%; BDH, Toronto, ON, Canada) with GC-FID using the above temperature program extended to 300°C. FID response was standardized with a five-point toluene calibration curve. Mass values for short retention time ranges were calculated and used to prepare SPME standard curves (Table 1). For biodegradation samples analyzed with SPME, mass values were calculated from these curves and summed to yield a total mass. Biodegradation was calculated with respect to abiotic or sterile controls analyzed daily throughout experimental time courses.

Methylcyclohexane (99%+ anhydrous; Aldrich Chemical, Milwaukee, WI) stock solutions were prepared in HPLC-grade methanol (EM Science, Gibbstown, NJ). Standards for SPME were prepared by introducing 40 μl of each stock solution into 125-ml flasks as described for biodegradation studies. A standard curve for 50 μg to 400 μg methylcyclohexane was prepared (R^2 0.998) and used for quantitation.

Growth curves

For growth curve experiments, triplicate flasks were prepared for each time point. Serial dilutions prepared in 50 mM phosphate buffer (pH 7.0) were plated onto trypticase soy agar plates (soybean–casein digest agar, Becton Dickinson, Cockeysville, MD) and incubated for 3 days. Further incubation was not required prior to counting total heterotrophs as colony-forming units per milliliter (CFU/ml). Oil-degrading bacteria were enumerated using a miniaturized five-tube most probable number (MPN) approach with Sweet Mixed crude oil (density 0.827 g/ml; Imperial Oil) as the sole carbon and energy source [22].

Results

Volatile hydrocarbon (C_5 to C_{11}) biodegradation by a mixed-bacterial culture during growth on crude oil was monitored using SPME. Only compounds eluting before dodecane exhibited a concentration response (Table 1) and compounds above hexadecane were not extracted (Figure 1A). Figure 1A is a chromatogram showing volatile hydrocarbons extracted from a 7-day sterile control (identical to time zero sample), and a typical chromatogram following 7 days of biodegradation by the mixed-bacterial culture is shown in Figure 1B. Methylcyclohexane and other low molecular weight branched alkanes, cycloalkanes and aromatics were recalcitrant.

Hydrocarbon partitioning, inoculum and substrate concentration

Since SPME analysis relies on analyte partition coefficients, increases in biomass will potentially affect quantification during biodegradation studies. A biomass titration was carried out and Figure 2 illustrates that the FID response decreased 25–40% with 1600 mg/l biomass. It is clear that higher molecular weight compounds were more susceptible to partitioning, and partitioning of low molecular weight hydrocarbons did not occur at practical biomass concentrations. A 100-fold inoculum concentration increase (from 0.63 to 63 mg/l) doubled degradation in the initial 24 h from 20% to 40% but did not affect total degradation (55–60%) or substrate range.

Table 1 Summary of gas chromatography conditions and SPME response curves for volatile hydrocarbons from Bow River crude oil

Retention time (min)	Elution temperature (°C)	Mass in 25 μl oil (μg)	Analyte range (volume oil, μl)	Response curve (y=response; x=μg)	R^2 value
0.0 to 2.5	35	856	2.5 to 35	$y=29.74x+3195.4$	0.970
2.5 to 5.0	35	587	2.5 to 35	$y=182.2x-440.6$	0.994
5.0 to 7.5	35 to 37.5	125	2.5 to 35	$y=515.9x+4554.4$	0.995
7.5 to 10.0	37.5 to 40	117	2.5 to 35	$y=552.75x+9816.3$	0.951
10.0 to 12.5	40 to 65	142	2.5 to 35	$y=41468\ \ln(x)-105038$	0.975
12.5 to 15.0	65 to 90	208	2.5 to 35	$y=64468\ \ln(x)-165766$	0.984
15.0 to 18.5	90 to 125	486	2.5 to 35	$y=54825\ \ln(x)+4556.2$	0.965
Total		2521			

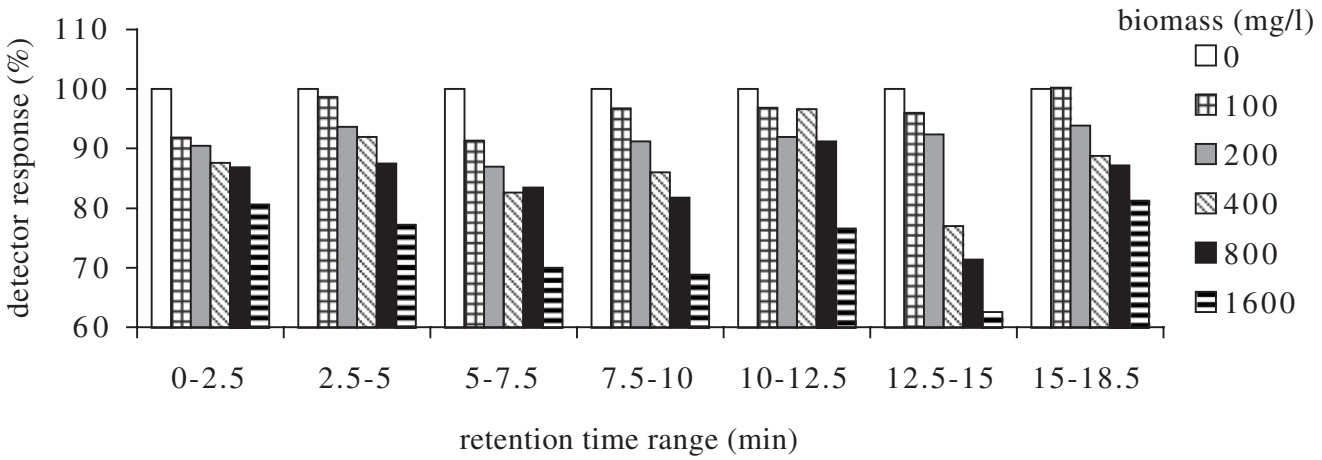

Figure 2 Volatile hydrocarbon partitioning by mixed culture biomass (mg/l).

Volatile hydrocarbon removal kinetics were examined to verify that sufficient oxygen was available for aerobic biodegradation. With 362, 724 or 1448 μg/ml oil per liquid volume, C_5–C_{11} biodegradation reached 60% after 5 days (Figure 3). During the initial 24 h, biodegradation rates were similar for all three concentrations at 0.40–0.56 μg h^{-1} ml^{-1}. The maximum degradation rates for the period between 24 and 48 h were correlated to initial oil concentration (Figure 3 inset). The total mass degraded was also linearly related to the initial substrate concentration.

Inoculum preparation

Biodegradation in cultures initiated with inocula from different growth stages on 20 g/l Bow River crude oil in foam-plugged flasks was examined. Cultures initiated with early stationary or stationary phase inocula displayed a 24-h lag period followed by 24 h of rapid degradation, ultimately removing 60% of the volatile hydrocarbons (Figure 4A). The lag period prior to degradation was eliminated in cultures initiated with both lag and late log inocula with 30% degradation after 24 h. After 4 days, activity in cultures initiated with late log inoculum slowed and ultimately reached 70% degradation. Conversely, cultures initiated with lag phase inoculum were active until day 7 with greater than 90% removal. A sample chromatogram (Figure 4B) shows that cultures initiated with lag phase inoculum removed all compounds eluting below 37.5°C (before 7.5 min.).

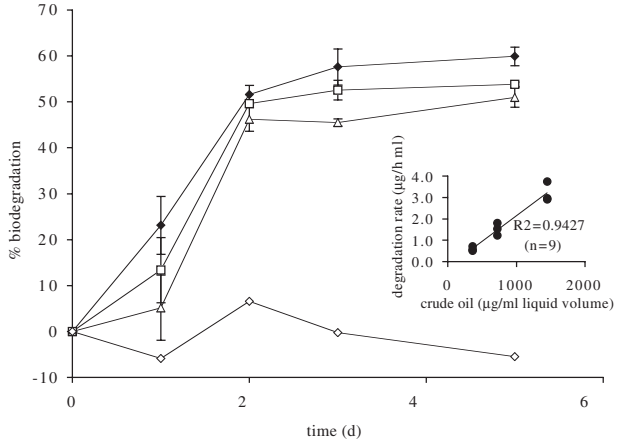

Figure 3 Effect of a crude oil concentration on volatile hydrocarbon biodegradation kinetics: (✧) abiotic control, (◆) 362 μg/ml, (□) 724 μg/ml, (△) 1448 μg/ml (error bars SD, $n = 3$). Inset: Relationship between oil concentration and degradation rate between 24 and 48 h.

Methylcyclohexane was removed after a 4-day acclimation period in flasks initiated with lag phase inoculum (Figure 5A). Growth curves, total heterotrophs and hydrocarbon-degrading bacteria were prepared for cultures initiated with lag phase and stationary phase (7-day) inocula (Figure 5B). Total heterotrophic growth was similar in both culture systems. Conversely, the percentage of hydrocarbon degraders was higher and more stable when lag phase inoculum was used (10^7 MPN/ml) compared to stationary phase inoculum (maximum 10^4 MPN/ml at 2 days).

Preservation of enhanced activity

Enhanced methylcyclohexane biodegradation was not observed when the original inoculum was subcultured after 7 days to produce a second inoculum culture (Table 2, row A). However, sealed biodegradation cultures initiated with lag phase inoculum fed with additional crude oil or subcultured after 14 days did retain enhanced activity (Table 2, row B). Culture aliquots stored at −80°C in 20% w/v glycerol and grown in foam-plugged flasks lost enhanced activity after prolonged incubation (Table 2, row C); boosting the crude oil concentration in foam-plugged inoculum flasks from 20 to 100 g/l was not effective (Table 2, row D). The C_5 to C_{11} hydrocarbon concentration in the aqueous phase of both control and inoculum flasks declined rapidly from 5700 μg/ml (100 g/l oil) and 1500 μg/ml (20 g/l oil) to less than 300 μg/ml in 24 h (Figure 6). Enhanced activity was retained in foam-plugged inoculum cultures if yeast nitrogen base was replaced with yeast extract (Table 2, row E). Adding either of these to biodegradation flasks had no effect on biodegradation kinetics.

Discussion

The SPME methodology used for volatile hydrocarbon quantitation allowed for rapid and repeated volatile hydrocarbon analysis in live cultures growing on Bow River crude oil. As previously

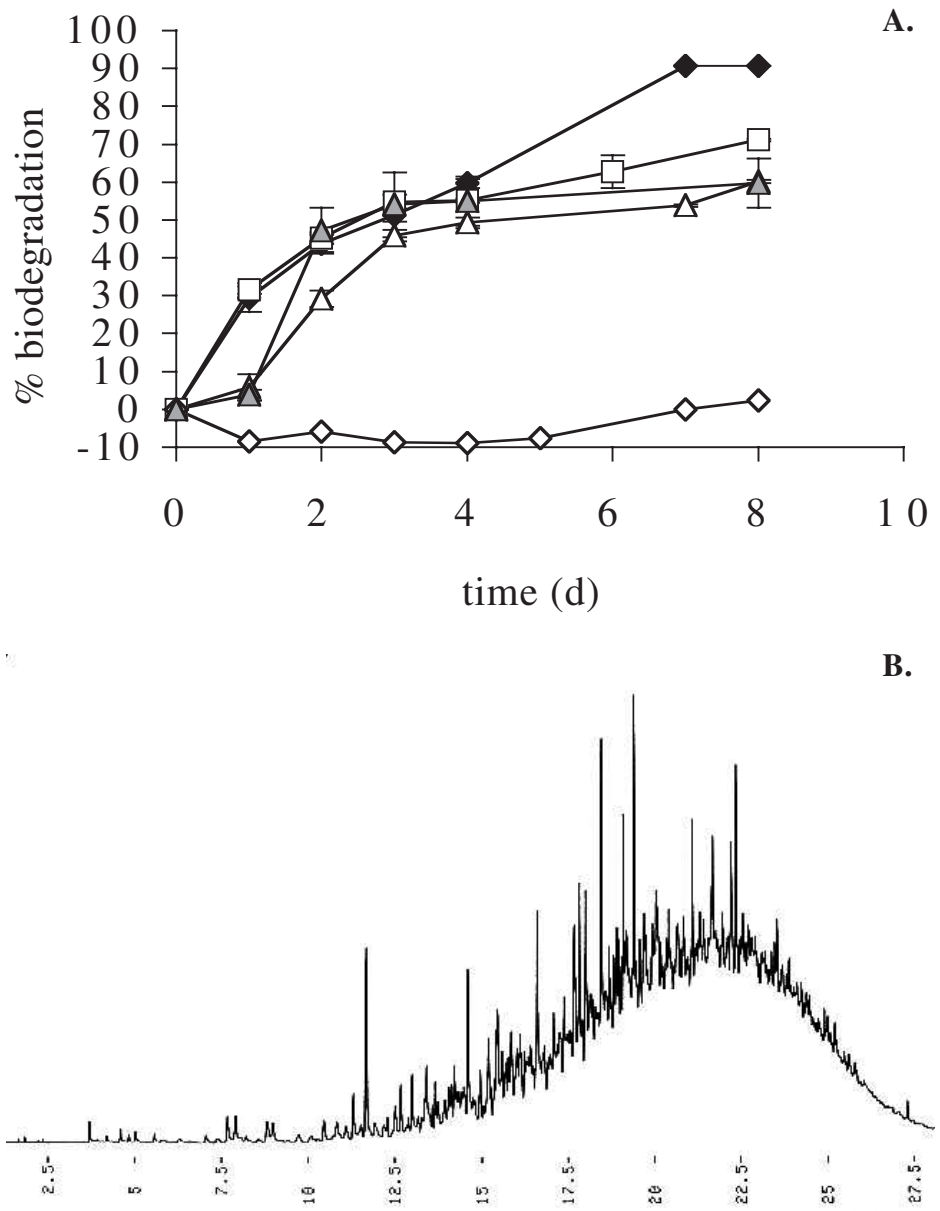

Figure 4 (A) Inoculum age (0.1% v/v) effect on volatile hydrocarbon biodegradation kinetics during growth on crude oil: (✧) abiotic control, (◆) lag phase (0 h), (□) late log phase (1 day), (△) early stationary phase (2 days), (▲) late stationary phase (7 days) (error bars SD, $n = 3$). (B) Chromatogram following biodegradation in culture initiated with lag phase inoculum after 7 days.

observed [21], compounds up to C_{11} could be quantified using a 30-μm PDMS fibre at 30°C. A closed system was necessary due to rapid volatilization, precluding oxygen exchange. The observation that the degradation rate and extent increased linearly over the substrate range used and results from the fed-batch experiment appeared to indicate sufficient oxygen was present. Increasing substrate concentrations did reduce the fraction degraded in the first 24 h, possibly a toxicity effect as volatile hydrocarbons may negatively affect microbial activity by damaging membrane integrity or altering enzyme structure and function [15].

High biomass levels were required to effect low molecular weight hydrocarbon partitioning. Had alterations in analyte partitioning occurred, quantitation with the prepared SPME standard curves would have been inaccurate [10,12]. On the other hand, to reduce volatilization in large open systems, partitioning by biomass would be desirable. This may be improved by using more hydrophobic cultures or cultures producing capsular material. Nocardioforms, which form mycolic acid capsules [17] have high sorptive capacity for phenanthrene, regardless of biodegradative capabilities [18]. Binding would increase with molecular weight [14] and may reduce bioavailability of high molecular weight substrates. However, adding recalcitrant sorbing agents [6,11] or sorbing agents acting as cosubstrates [13] can enhance crude oil biodegradation. Further work in this area is warranted and SPME would be a suitable methodology [4].

The mixed culture used in this study is inactive against methylcyclohexane and other branched hydrocarbons eluting

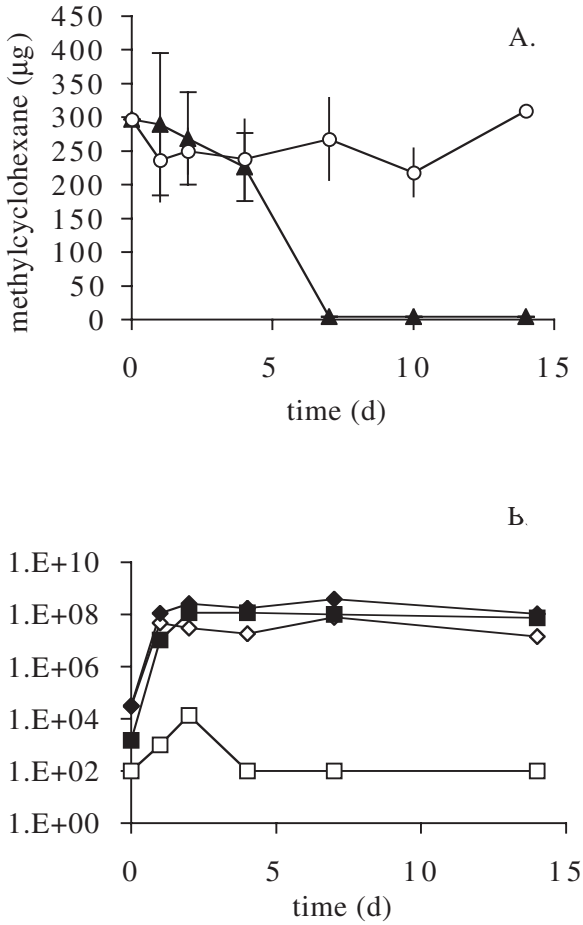

Figure 5 (A) Methylcyclohexane removal in flasks initiated with: (▲) lag phase (0 h), and (○) stationary phase (7-day) inoculum. (B) Inoculum age effect on oil-degrading bacterial levels in biodegradation flasks: (◆,◇) lag phase; and (■,□) stationary phase inoculum. Filled symbols — total heterotrophs (CFU/ml), open symbols — oil degraders (MPN/ml).

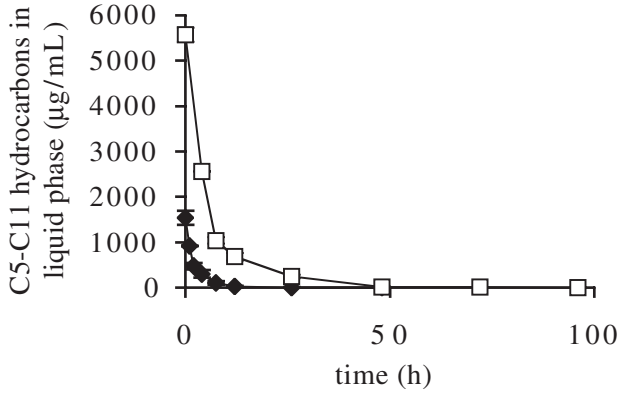

Figure 6 Volatilization of C_5 to C_{11} hydrocarbons from foam-plugged inoculum flasks. (◆) 20 g/l Bow River crude, (□) 100 g/l Bow River crude (error bars SD, $n = 4$).

before n-C_8 [21]. Increasing the inoculum concentration increased degradation rates, but did not induce methylcyclohexane removal. Inoculum age, not concentration, had the greatest impact on degradation rate and extent. Rather than observing greater activity in cultures initiated with mid- to late log inocula, increasing lag times and methylcyclohexane recalcitrance were observed.

Cultures grown in closed flasks retained the capability to degrade methylcyclohexane, indicating that stable selection depended on low molecular weight compounds being present and available for uptake. Lloyd-Jones and Trudgill [8] showed that a *Rhodococcus* sp., a *Flavobacterium* sp. and a *Pseudomonas* sp. grew on branched cyclohexanes, alcohols, ketones and acids only when inoculated as a coculture. They also noted that if precultured in nutrient broth, growth on methylcyclohexane as the sole carbon and energy source continually declined over time and was eliminated after 72 h. This phenomenon correlated with the observation that two unidentified plasmids were lost from the consortium [8]. In this study, replacing yeast nitrogen base with yeast extract in inoculum flasks eliminated the loss of activity, although it had no effect when added to biodegradation flasks. Yeast extract has been shown to have positive effects on the degradation of C_{10}–C_{21} alkanes, branched alkanes and a substituted cyclohexane in diesel fuel by a psychrotrophic *Rhodococcus* sp. [25].

Overall, biodegradation rates and extents were higher for low molecular weight compounds in capable cultures. However, a diauxic phenomenon was observed for methylcyclohexane and other C_5–C_7 branched compounds. Similar behaviour has been

Table 2 Inoculum preparation effects on enhanced volatile hydrocarbon degradation capabilities in biodegradation flasks

Inoculum	Inoculum age (days)	Degradation lag (days)	% Degradation (SD, n=3)
(A) Subculture of original	lag (0)	2	62.1 (2.4)
	late log (1)	1	62.4 (0.7)
	early stationary (2)	0	61.6 (1.6)
(B) 14-day degradation culture initiated with lag phase inoculum	fed batch	0	90 (0.4)
	subculture	0	89.2 (1.2)
(C) Culture in (B) frozen	lag (0)	0	89.4 (0.0)
	stationary (7)	1	59.6 (1.4)
(D) 100 g/l Bow River crude in inoculum flask	lag (0)	0	89.6(0.0)
	late log (1)	0	70.4 (9.7)
	early stationary (2)	1	59.5 (0.7)
(E) Original inoculum			
Yeast nitrogen base	lag (0)	0	92.2 (0.0)
	stationary (7)	1	59.9 (1.7)
Yeast extract	lag (0)	0	89.8 (0.0)
	stationary (7)	0	90.7 (0.0)

described for biofilters treating toluene, ethylbenzene and xylene mixtures [23]. Biofilters [27] or batch systems with volatile hydrocarbon recycling may allow for more effective biodegradation during heterogeneous oily waste treatment. Berthe-Corti *et al* [1] described a closed, three-stage bioreactor for treating petrochemical wastes. A stirred tank, trickle-bed reactor for exhaust gas treatment, and a photobioreactor for CO_2 scrubbing were combined, producing a system with no requirements for additional oxygen.

In this study, profound effects of long-term inoculum preparation and maintenance on volatile hydrocarbon biodegradation by a mixed-bacterial culture were observed. Since methylcyclohexane-degrading retention required that cultures be maintained in a closed system or supplemented with yeast extract there is a need to develop methods for retaining volatile compounds in the aqueous phase using partitioning agents with high sorption coefficients.

Acknowledgements

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