CRUDE OIL BIODEGRADATION BY A MIXED BACTERIAL CULTURE

by

Jonathan Douglas Van Hamme

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ABSTRACT

Biological methods to remediate petroleum hydrocarbon-contaminated wastes typically rely on mixed cultures with broad substrate specificity. In this study, the overall objective was to gain an appreciation for microbe-microbe and microbe-substrate interactions in batch fermentation systems using Bow River crude oil as a model substrate. Due to hydrocarbon insolubility the mixed-bacterial culture was limited by substrate availability. Chemical surfactants were tested as a means to improve biodegradation. A detailed study with nonylphenol ethoxylates revealed that a hydrophile-lipophile balance of 13 was optimal for degradation enhancement at supra-critical micellization concentrations not exceeding a critical level.

The culture contained a wide variety of trypticase soy agar-culturable bacteria, and fermentations with various hydrocarbon mixtures were initially dominated by *Pseudomonas/Flavimonas* and *Stenotrophomonas* spp. A chemical surfactant increased the lag time of the *Stenotrophomonas* sp. and exposure to Bow River saturates selected for an *Acinetobacter calcoaceticus* strain. In each case, a greater variety of mainly non-hydrocarbon degrading bacteria were isolated following prolonged incubation. The culture exhibited greatest activity against the saturate and aromatic fractions, and low molecular weight volatile hydrocarbons, normally ignored in such biodegradation studies, were degraded in closed systems.

A rapid and sensitive solid phase microextraction methodology was developed for monitoring volatile hydrocarbon degradation in live cultures at 30°C. A 30 μm polydimethylsiloxane fibre extracted C_5 to C_{16} alkanes and aromatics, and C_5 to C_{11} could be quantified. Volatile hydrocarbon-degrading capabilities were retained only in cultures grown on crude oil in sealed flasks, or in open flasks amended with yeast extract. Otherwise, metabolic capacity decreased with inoculum age and correlated with reduced proportions of hydrocarbon-degrading bacteria in biodegradation flasks.

Pure and co-culture studies confirmed both the degradation hierarchy and chemical surfactant effects. Crude oil biodegradation by a non-adherent, non-emulsifying, accession-limited *Pseudomonas* sp. (strain JA5-B45) was greatly enhanced by chemical surfactant. In the absence of surfactant, degradation by this strain was slightly enhanced by an adherent, mycolic

acid-capsule producing *Rhodococcus* sp. (strain F9-D79) able to emulsify oil. The *Rhodococcus* sp. efficiently degraded alkanes up to C_{32} but was inactive against aromatics. In co-culture, it was shown that chemical surfactant enhanced biodegradation by directly increasing substrate solubility and by removing *Rhodococcus* sp. strain F9-D79 from the hydrocarbon-water interface.

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1. Introduction

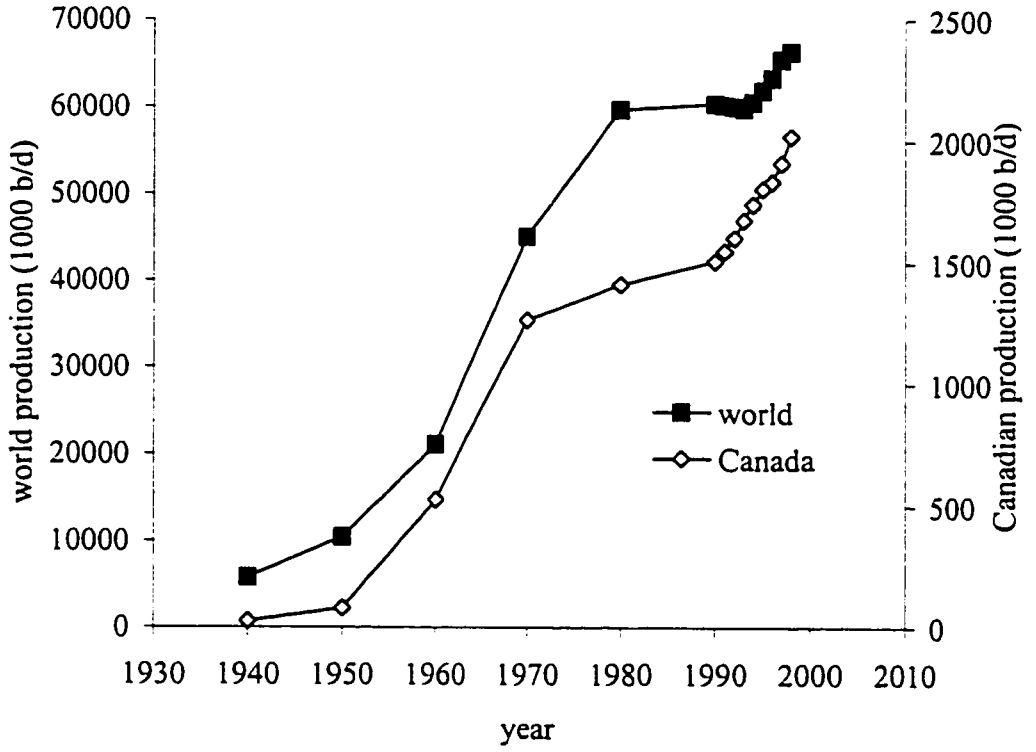
Petroleum is deeply rooted in the history of microbiology (Bushnell and Haas 1940). Long recognized as substrates supporting microbial growth, hydrocarbons seeping or intentionally recovered from reservoirs are both a target and product of microbial metabolism (Ehrlich 1995). Basic physiological, metabolic, and genetic tenets have been elucidated using this heterogeneous substrate. Applied interest in industrial and environmental fields has driven much of the fundamental research in petroleum microbiology. Heavy crude oil recovery facilitated by microorganisms was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery (MEOR) (Donaldson et al. 1989). As of 1998, there was only one producing MEOR project being carried out in the United States (Tippee 1999) although *in situ* biosurfactant/biopolymer applications continue to garner interest (Banat et al. 2000). During the 1960s, single-cell protein (SCP) production catalyzed research on microbial hydrocarbon metabolism but, due to rising oil prices and health concerns, major SCP projects were discontinued by the early 1980s (Rosenberg 1993). Since the late 1960s, interest in exploiting petroleum-degrading organisms for environmental clean up has become central to petroleum microbiology. Studies implemented by the United States Office of Naval Research led to growth of the bioremediation field (Atlas and Cerniglia 1995). Current research in petroleum microbiology encompasses oil spill remediation (Prince et al. 1999; Swannell et al. 1999), fermentor- and wetland-based hydrocarbon treatment (Geerdink et al. 1996; Jack et al. 1994; Knight et al. 1999; Rozkov et al. 1998; Simon et al. 1999), biofiltration of volatile hydrocarbons (Ergas et al. 1999), MEOR (Banat et al. 2000), oil and fuel upgrading through desulphurization (McFarland et al. 1998; Setti et al. 1999) and denitrogenation (Benedik et al. 1998), coal processing (Catcheside and Ralph 1999), fine chemical production (Mathys et al. 1999; McClay et al. 2000), and microbial community-based site assessment (MacNaughton et al. 1999).

From an environmental standpoint, the petroleum industry has global impacts through recovery, transport, refining and product usage. In Canada, crude oil production reached 2 million barrels per day (b/d) in 1998, up from 23 thousand b/d in 1940 (Figure 1.1). With reserves of 4.9 billion barrels, Canada contributes only a small fraction to worldwide

production, which surpassed 66 million b/d, or 1.05 x 10^{10} litres, in 1998 (Tippee 1999). Environmental petroleum inputs are modestly estimated at 0.1% of production or 1.05 x 10^{6} litres per day. Of this, 10% is from high profile catastrophes, 10% from natural seepage, and the remainder is from transportation, municipal, and industrial wastes and runoff (Prince 1993). A growing hydrocarbon-contaminated waste source in Canada originates in western bituminous sand deposits. Approximately 500 000 b/d of heavy oil was extracted in 1998 with projections of 1.5 million b/d by 2005. From 1996-2007, total capital investment from 21 companies in the Cold Lake, Peace River and Athabasca oil sands will be $18.8 billion (Tippee 1999). For every barrel of oil produced two tons of alkaline hydrocarbon-contaminated sand tailings are accumulated (Mossop 1980). Refineries and petroleum combustion contribute to greenhouse gas production with associated health effects (Hutcheson et al. 1996) through tropospheric ozone production and smog formation (Field et al. 1992).

Chemically, petroleum is a substrate rich in reduced carbon at 83-87% C, 10-14% H, 0.1-2% N, 0.05-1.5% O, and 0.05-6% S (Speight 1991). Found in all major geological strata dating from the Precambrian (700 million years ago) to the present, it is believed to be the product of decayed plant and animal debris which has been combined with fine grained sediments in a variety of marine, or near marine environments. Various biological compounds are recognized precursors. For example, porphyrins are chlorophyll and heme degradation products in complexes with metals such as vanadium, nickel, iron and copper. Paraffinic and naphthalenic hydrocarbons that make up to ten percent of living plant materials are thought to survive as crude oil components. Carbohydrates, sterols and lignin are possible constituents, and terpene hydrocarbons and carotenoid pigments (β-carotene) may be transformed into alkybenzenes and alkylnaphthalenes. Biological, thermal and physical (diffusion and evaporation) processes both during and after accumulation in a reservoir contribute to petroleum formation (Speight 1991).

Petroleum contains paraffins, naphthalenes, aromatics, sulfur compounds (thiols, sulfides, thiophenes), oxygen compounds (alcohols, acids, ethers, esters, ketones, furans), nitrogen compounds (pyridine, quinones, carbazoles) and metallics (iron, nickel, vanadium, copper) (Speight 1991). This broad group of chemical species is characterized by equally



(data from Tippee 1999)

Figure 1.1 Crude oil production from 1940 to 1998 in Canada and worldwide.

broad physical and chemical properties (i.e. molecular weight, solubility, melting point and boiling point) (Schwarzenbach et al. 1993; Vadas et al. 1991; Yaws et al. 1993) and crude oil diversity can be appreciated when the variety of refined products is examined (Table 1.1). These fractions are precursors for a large number of other products such as fertilizers, alcohols, weed killers, solvents, explosives, detergents, nylon, adhesives, rubber, paints and insecticides (Jones 1995).

Crude oil is often separated into major fractions by solvent separation and column chromatography (Figure 1.2). Further characterization may use gas chromatography-flame ionization detection (Mueller et al. 1992), mass spectrometry (Venosa et al. 1991), ultraviolet luminous spectroscopy (Mille et al. 1991), infrared spectroscopy and ^{13}C-nuclear magnetic resonance (Rotnani et al. 1985; Venkateswaran 1995). Despite this analytical array, complete crude oil molecular characterizations are not available. This is highlighted by the uncharacterized complex mixture, or UCM, seen as an unresolved hump on GC chromatograms (Gough and Rowland 1990; Gough et al. 1992; Killops and Al-Juboori 1990). Characterizing the high molecular weight asphaltenic and polar resin fractions is also difficult due to their heterogeneous nature (Semple et al. 1989; Sheu and Mullins 1995; Speight 1991).

Typically, solvent extractions are used prior to petroleum analysis and, as a result, volatile fractions are lost. Thus, biodegradation studies involving crude oil and petroleum wastes often neglect low molecular weight alkanes and aromatics (Atlas 1981). However, crude oil can contain significant quantities of volatile organic compounds (Saeed and Al-Mutairi 2000). Traditional extraction methods such as purge-and-trap or headspace sampling can be expensive, time consuming, imprecise and are restricted to laboratory analyses. Solid phase microextraction (SPME) is a new solvent-free extraction method effective for inexpensive, accurate and rapid analysis of polar and non-polar analytes in air, water or soil. Governed by equilibrium thermodynamics between air, water and fibre, trace analyte amounts are removed from a sample allowing for repeated extraction (Pawliszyn 1997). With applications expanding in the environmental field, SPME has potential for lab and field based biodegradation studies. Table 1.2 summarizes recent examples of SPME use for monitoring

Table 1.1 Crude oil distillation products.

Fraction	Boiling range (degrees C)
Light naphtha	-151
Gasoline	-181
Heavy naphtha	150-205
Kerosene	205-260
Stove oil	205-290
Light gas oil	260-315
Heavy gas oil	315-425
Lubricating oil	> 400
Vacuum gas oil	425-600
Residuum	> 600

(Speight 1991)

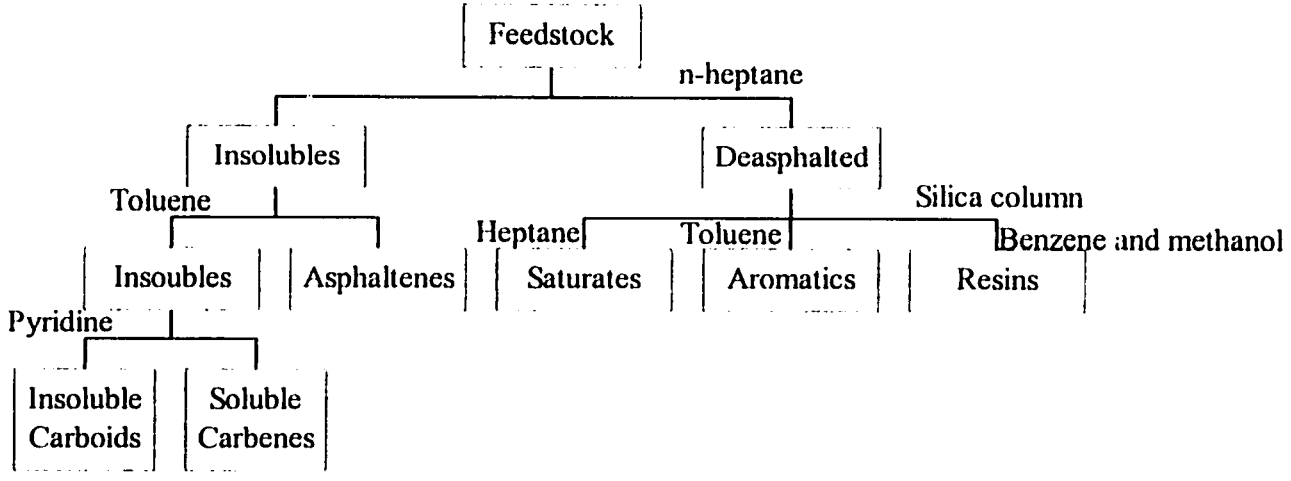


Figure 1.2 Crude oil fracationation method to separate the saturate, aromatic and asphaltene fractions (Speight 1991).

Table 1.2 Solid phase microextraction (SPME) applications in environmental petroleum hydrocarbon analysis.

Analyte	Sample matrix (sampling)	Analytical Method	Purpose	Fibre solid phase	Reference
PAHs	bitumen from road paving (headspace)	GC-MS	identification of hazardous materials	100 µm PDMS	Agozzino et al. 1999
VOCs	air	GC-FID	environmental analysis, method development, fibre comparisons	100 µm PDMS Carboxen Caropack B	Chai and Pawliszyn 1996
VOCs chlorinated alkanes, aromatics	borehole sediments (headspace)	GC-FID	quantitative analysis	85 µm polyacrylate	Dermietzel and Strenge 1999
PAHs	municipal water (headspace)	GC-FID	improved absorption on new fibre type	home-made fused silica fibre, porous activated charcoal coating	Djozan and Assadi 1999
phenanthrene 9-amino phenanthrene	water (direct)	GC-FID	investigate compound interactions with organic matter	7 µm PDMS	Doll et al. 1999

cont.

Table 1.2 cont. Solid phase microextraction (SPME) applications in environmental petroleum hydrocarbon analysis.

Analyte	Sample matrix (sampling)	Analytical Method	Purpose	Fibre solid phase	Reference
coal tar, refined petroleum products	industrial soils (headspace)	GC-MS	rapid screening, chemical characterization, method development	100 μm PDMS	Havenga and Rohwer 1999
benzene, alkylbenzenes, *n*-alkanes, PAH, creosote contamination	surface- and waste-water (direct)	GC-FID GC-MS	quantification	100 μm PDMS 7 μm PDMS	Langenfeld et al. 1996
isoparaffins, aromatics, airborne gasoline	air	GC-MS	method development, TPH quantification in environmental samples	100 μm PDMS 30 μm PDMS	Martos et al. 1997
hydrocarbon gases styrene	air	GC-FID	estimation of fibre-air partition coefficients	100 μm PDMS 30 μm PDMS	Martos and Pawliszyn 1997
PAHs	water, octanol-water (direct)	GC-FID	determine solubility, partition coefficients	100 μm PDMS	Paschke et al. 1999
Total gasoline range organics	water	Infrared spectrosopy	quantification	perfluoroalkoxy Teflon	Stahl and Tilotta 1999

petroleum hydrocarbons, mostly using polydimethylsiloxane (PDMS) fibres, in a variety of sample matricies.

Biologically, microorganisms are equipped with metabolic machinery to exploit petroleum as a carbon and energy source. The most characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas oleovorans* (van Beilen et al. 1994). A membrane-bound monooxygenase and soluble rubredoxin and rebredoxin-reductase serve to shunt electrons through NADH to the hydroxylase for conversion of an alkane into an alcohol. The alcohol can be further metabolized to an aldehyde and acid prior to proceeding into the β-oxidation and TCA cycles. Initial oxidation may be terminal, subterminal or proceed through ω-oxidation (fatty acid hydroxylation). Branched alkanes, alkenes and alkynes are metabolized (Watkinson and Morgan 1990), although the latter two are rare in crude petroleum (Speight 1991). Cycloalkanes are normally placed after aromatics in recalcitrance hierarchies (paraffins > isoparaffins > aromatics > cycloalkanes) and were originally thought to be degraded only by cometabolic and commensalistic interactions (Beam and Perry 1974; Perry 1984). A cyclohexane-degrading *Xanthobacter* sp. unable to use *n*-alkanes has been isolated (Trower et al. 1985). In this strain, a soluble NADPH-dependent enzyme systems converts cyclohexane, through an alcohol and ketone, to ε-caprolactone and then adipic acid.

Despite the importance of alkane-degradation systems, little information is available for pathways other than the aerobic monooxygenase-mediated pathway found on the OCT plasmid. Evidence for the Finnerty pathway, where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate, has been described for *Acinetobacter* sp. M1 (Sakai et al. 1996). A distinct alkane hydroxylase gene (*alk*M) linked to *alk*R, the transcriptional regulator for *alk*M, has been found in *Acinetobacter* sp. strain ADP1 (Ratajczak et al. 1998a, b). Unlike the tightly clustered *alk*BFGHJKL operon of the OCT plasmid, the system is not directly linked to rubredoxin and rubredoxin-reductase genes on the chromosome and is repressed by oxidized alkane derivatives. Two other plasmid systems have been partially characterized: the OCT plasmid in *P. maltophilia* has a distinct *alk*A gene from *P. oleovorans* (Lee et al. 1996), and the unique pDEC plasmid in

Pseudomonas sp. C12B (Kostal et al. 1998). Previously discounted, evidence for anaerobic mineralization of pure alkanes, and alkanes in crude oil, is available for sulfate-reducing and methanogenic bacteria (Aeckersburg 1998; Caldwell et al. 1998; Rueter et al. 1994; Schink 1985; So and Young 1999). The biochemical mechanisms of these reactions are unclear but appear to be oxidations or hydroxylations. Unambiguous evidence for proposed terminal desaturation pathways is not available (Aeckersburg 1998).

Aromatic hydrocarbon biodegradation has also been well characterized and described in bacteria, fungi, cyanobacteria and algae (Cerniglia 1992). The two classic routes for ring cleavage are the intradiol or *ortho*- and the extradiol or *meta*-pathways involving catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively. TOL plasmids, pWWO being the best characterized, coding for *meta*-cleavage are divided into two operons. The 'upper' operon is responsible for conversion to alcohol, aldehyde and acid, and the 'lower' pathway converts the acid to a catechol and cleaves the ring producing a semi-aldehyde prior to entry into the TCA cycle. A large plasmid in *Acinetobacter calcoaceticus* RJE74 has been found to code for *ortho*-cleavage. Alkylbenzene-degrading genes may be plasmid borne or on the chromosome and, with increasing side-chain length, are considered substituted alkanes rather than substituted aromatics (Smith 1990). Biphenyl is biodegradable but is uncommon in petroleum (Speight 1991).

Fused ring structures, naphthalene being the simplest, are attacked by dioxygenases for use as a carbon source in bacteria, and hydroxylated to arene oxides for detoxification in fungi (Cerniglia 1992). The *nah* (naphthalene to salicylate) and *sal* (salicylate to central metabolites) operons are often plasmid encoded in pseudomonads (Smith 1990). Naphthalene degradation may also occur through the phthalate pathway (Chen and Aitken 1999). Plasmids for phenanthrene metabolism have been described (Cerniglia 1992), and naphthalene dioxygenases are often referred to as PAH dioxygenases due to relaxed substrate specificity against 3, 4 and 5 ring PAHs. Detailed metabolic pathways can be found in the University of Minnesota Biocatalysis and Biodegradation Database (Ellis et al. 2000).

From a practical standpoint, current work is focused on metabolite formation and the impact on biodegradation in mixed-substrate and mixed-culture systems. Kazunga and Aitken

(2000) noted the accumulation of pyrene metabolites, *cis*-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione, in various bacterial cultures. The metabolites inhibited pyrene and benzo[*a*]pyrene biodegradation in some strains. On the other hand, low salicylate levels enhanced phenanthrene degradation in one of the *Pseudomonas* sp. examined (Chen and Aitken 1999). Salicylate also enhanced non-growth substrate biodegradation (fluroanthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene) in the same strain. Thus, benzo[*a*]pyrene recalcitrance in field situations may be due to metabolite inhibition as well as reduced bioavailability through sorption.

As with alkanes, anaerobic aromatic oxidation has not been well characterized. Anaerobic degradation has now been described in systems including sulfate-reducing and denitrifying conditions (Anderson and Lovley 2000; Coates et al. 1996; Rabus and Heider 1998). Aerobic toluene dioxygenase activity in reduced oxygen atmospheres has also been reported (Costura and Alvarez 2000).

PAHs are not the only aromatic substrates in crude oil and metabolic pathways for nitrogen, oxygen and sulfur-containing heterocycles such as fluorene, furans, thiophenes, and pyridine have been described (Bressler and Fedorak 2000; Bressler et al. 1998; Kaiser et al. 1996; Kropp and Fedorak 1998; Soto et al. 1998).

Branched alkanes from the UCM (Gough et al. 1992) and refractory biomarkers such as stearanes (Chosson et al. 1991) have been shown to be biodegradable. However, Atlas (1981) noted that relatively little evidence for resin and asphaltene biodegradation had been collected, mainly due to analytical difficulties. Twenty years later this statement is still largely true. Bertrand et al. (1983) and Rontani et al. (1985) reported asphaltene degradation during continuous crude oil fermentations, and asphaltene co-oxidation during growth on *n*-alkanes. However, the studies suffered from a lack of reproduction and appropriate controls. Growth on resins isolated from crude oil by a mixed culture and a *Pseudomonas* sp. has been reported only once (Venkateswaran et al. 1995). The most convincing evidence to emerge on biological activity against these fractions is the chloroperoxidase demetallization of petroporphyrins and asphaltenes (Fedorak et al. 1993). The authors showed that chloroperoxidase from *Caldariomyces fumago* can solubilize vanadium and nickel at pH 3. Conversion was better in

ternary solvent systems due to asphaltene insolubility and reactions were hydrogen peroxide and chloride dependent. Thus, undesireable chlorinated products probably resulted upon disruption of metal chelating systems. Obviously, this challenging research area would benefit from further mechanistic and metabolic studies due to interest in microbiological oil upgrading. In addition, asphaltenes may contribute to increased recalcitrance of biodegradable oil components by forming coats around oil droplets reducing low molecular weight hydrocarbon diffusivity at the surface (Uriazee et al. 1998).

Physiologically, hydrocarbon-degrading microorganisms are unified by their capacity to exploit water-insoluble substrates. Rather than being an exception or rarity, hydrocarbon metabolism is spread across bacterial, yeast, fungal and algal genera (Atlas 1981). From deep hydrothermal vents (Bazylinski et al. 1989) to surface soils (April et al. 2000), hydrocarbon metabolism necessarily requires interfacial interactions. Again, microbial life at boundaries is common (van Loosdrecht et al. 1990) and, while water-soluble hydrocarbon uptake does occur, decreasing solubility with increasing molecular weight restricts this effect. In the absence of oil droplets, dissolution rather than uptake has been found to be limiting for hydrocarbon biodegradation (Bury and Miller 1993). Two additional, perhaps more widespread hydrocarbon uptake modes are direct adherence to large oil droplets and interaction with pseudosolubilized oil. A more accurate nomenclature for these primary stages of hydrocarbon-interaction would be 'accession mechanisms'.

Hydrocarbon solubilization and micellar transport control hexadecane uptake rates and biodegradation during *P. aeruginosa* biosurfactant-enhanced growth (Sekelsky and Shreve 1999). Similarly, encapsulating solid *n*-C18 and *n*-C36 in liposomes increases growth and biodegradation by a *Pseudomonas* sp., indicating that cell-liposome fusion may deliver encapsulated hydrocarbons to membrane-bound enzymes (Miller and Bartha 1989). Bouchez-Naïtali et al. (1999) divided 61 hexadecane-degrading bacteria, mostly from the *Corynebacterium-Mycobacterium-Nocardia* group, based on hydrocarbon-accession mode. Direct adherence was utilized by 47% of the strains and 53% produced biosurfactants. Hydrophobicity data was used to suggest that 11% of those used biosurfactant-enhanced micellar transfer while 42% used biosurfactant-enhanced interfacial uptake.

It had been previously assumed that hydrocarbons enter bacterial cells through simple diffusion. Bateman et al. (1986) showed this to be true for naphthalene uptake by a Gram negative bacterium. However, Whitman et al. (1998) were able to show that naphthalene uptake by a *P. fluorescens* isolate was due to an active transport process. Uptake was sensitive to azide and 2,4-dinitrophenol, inhibitors of cellular energy metabolism. In addition, uptake was non-competitively inhibited by α-naphthol, a naphthalene structural analogue. Indeed, unaltered 0.2 μm hexadecane inclusions enclosed in non-unit (monolayer) membranes have been observed in adherent *Acinetobacter* sp. strain HO1-N (Kennedy et al. 1975; Scott and Finnerty 1976).

To increase contact with hydrocabons, bacteria employ various mechanisms to seek out and remain in contact with suitable substrates. Grimm and Harwood (1997) described a plasmid-encoded naphthalene chemoattraction mechanism in two motile *Pseudomonas* spp. Marx and Aitken (2000) showed that *P. putida Pp*G7, used by Grimm and Harwood (1997), showed faster naphthalene degradation rates in unmixed, heterogeneous systems than mutants deficient in either motility or naphthalene chemotaxis. Thus, chemoattraction, not just random movement afforded by motility, is important in poorly mixed systems. Adherent microorganisms such as *Acinetobacter* sp. strain RAG-1 secrete extracellular, polymeric, heteropolysaccharide capsules to coat oil droplets and free themselves from substrate-depleted interfaces (Foght et al. 1989; Ng and Hu 1989; Rosenberg 1993). To maintain contact with hydrophobic droplets cells may increase surface hydrophobicity through protein expression (Hanson et al. 1994), capsules (e.g., mycolic acids in Nocardioforms), or fibriae (Rosenberg et al. 1982). Käpelli et al. (1984) observed hair-like structures on *n*-alkane-grown *Candida tropicalis* cells that mediated hexadecane adsorption. Stress and toxicity responses, particularly to soluble low molecular weight compounds include changes in fatty acid composition, offsetting changes in membrane fluidity (Tsiko et al. 1999; Whyte et al. 1999), active excretion and immobilization (Sikkema et al. 1995).

To affect hydrocarbon pseudosolubilization (micellization) microorganisms may produce biosurfactants or bioemulsifiers. Sugar, amino acid and lipid combinations are possible in anionic, cationic, and nonionic molecules or polymers. Biosurfactants may be

classed into the following groups: glycolipids (trehalose lipids, rhamnolipids, sophorose lipids), lipopeptides, fatty acids, phospholipids, neutral lipids, polymeric biosurfactants, and particulate biosurfactants (Desai and Banat 1997; Hommel 1990; Neu 1996). As the names indicate, biosurfactants serve to lower surface and interfacial tensions, while bioemulsifiers serve to emulsify oil independently of surface tension alterations (Hommel 1990). These agents are not exclusive to hydrocarbon-degrading bacteria or hydrocarbon metabolism. Other roles include gliding motility, antibiotic effects and cell-cell interactions (Neu 1996). Indeed, biosurfactants and bioemulsifiers can limit hydrocarbon accession, especially in microorganisms using direct adherence to hydrocarbons as the primary contact mechanism (Foght et al. 1989).

Similarly, chemical surfactants may have positive, negative or neutral effects on hydrocarbon-degrading organisms (Allen et al. 1999; Grimberg et al. 1996). Chemical surfactants are available in many forms, generally classed by structure, charge, hydrophile-lipophile balance (HLB), and critical micellization concentraction (cmc) (Ash and Ash 1993; Swisher 1987). Surfactant action with respect to hydrocarbon degradation include increased surface area (emulsification), increased apparent solubility (micellization), and washing from surfaces and soil pores. Negative actions include direct toxicity (particularly cationic surfactants), substrate dilution, substrate sequestration, increased hydrocarbon mobility in subsurface environments, and use a preferential carbon source (oxygen depletion, toxic byproducts) (Volkering et al. 1998). For example, Inipol EAP-22, made famous by the bioremediation efforts associated with Exxon Valdez (Prince 1993), contains oleic acid as a solubilizing agent. Oleic acid is an excellent carbon source and, as discussed above, may inhibit expression of some alkane degradation pathways (Ratajczak et al. 1998a, b). As for biosurfactants, chemical surfactants may inhibit biodegradation by preventing microbial attachment to hydrocarbon droplets (Ortega-Calvo and Alexander 1994). The reported 'confusion' in reports concerning microbial-surfactant interactions belies the apparently haphazard approach for surfactant selection during testing and experimentation as well as the varying hydrocarbon-accession modes employed by microorganisms.

Ecologically, hydrocarbon-metabolizing microorganisms are widely distributed. Ecology is the study of relationships between an organism and the surrounding environment. Autecology, prefixed by 'aut' – arising from processes within – is the study of a single species in an ecosystem. Synecology, prefixed by 'syn' – together, within or associated with – is the study of a community of organisms and their associated environment. Thus, when studying a collection of microbial populations (a community), two dichotomous approaches are possible. One is to focus on a single population and attempt to unravel events occurring within and around that population. The second is to examine bulk processes, rather than individuals contributing to those processes. The challenge is to marry these approaches, and the data derived therefrom, into a coherent, unifying story.

Three general experimental protocols have emerged in microbial ecology to study microbial communities: physiological, metabolic, and genetic. The oldest, immortalized by Koch, are now termed traditional culture methods. Briefly, organisms are cultured based on physiology and studied in isolation (Chaîneau et al. 1999). Of course, model co- and mixed-cultures can be constructed (Komukai-Nakamura et al. 1996). Whole community phosholipid fatty acid analysis (PFLA) can be used to monitor stresses and obtain a general, qualitative view of community structure (Fang and Barcelona 1998). Metabolic characterization is central to physiological studies but, in this case, metabolic protocols refer to synecological approaches through quantification of specific metabolic activities or biochemical markers in a community. Biolog breathprints are a modern example and analyses use 96-well microtitre plates containing 95 different substrates to examine community activity (Berthi-Corti and Bruns 1999). Biolog and PFLA analyses were originally intended for microbial identification but have enjoyed growing use in mixed-culture community evaluations. Kinetic approaches have been developed for Biolog studies to reduce error associated with inoculum density (Lindstrom et al. 1999). Genetic methods include probes for specific microbial species, traits or Kingdoms (Øvreås 2000), modified organisms expressing fluorescent markers (Errampalli et al. 1998), and whole community DNA or rRNA extractions (MacNaughton et al. 1999; Theron and Cloete 2000). The latter approach often involves amplifying extracted genetic material with the polymerase chain reaction (PCR) followed by electrophoretic separation on a

gel (temperature or denaturing gradient gel electrophoresis). Data can be interpreted by comparing banding patters (microbial diversity) and, if further detail is desired, by sequencing each band to determine species identity (Øvreås et al. 1998).

Each method has inherent limitations and the goal of any particular study must be considered. Metabolic and whole-community genetic approaches do not yield isolates for study, and normally do not quantify microbial numbers. Specific gene probes can allow for identification and enumeration as exemplified by reverse sample genome probing (RSGP), introduced by Voordouw for studying sulfate-reducing bacteria in oil fields (Voordouw et al. 1991). DNA extractions may not reflect active populations. RNA extractions overcome this difficulty but are more technically difficult. In addition, while DGGE and TGGE separate DNA fragments of the same length based on sequence (G + C content) each population may produce more than one band, and one band may contain several unresolved bands (Øvreås 2000). Results from Biolog analyses must be carefully interpreted, as substrate usage patterns may not reflect *in situ* metabolic activities, neglect slow-growing organisms, and are susceptible to culture bias through growth on secondary metabolites. Evidence is available to indicate that changes in community structure can take place in Biolog wells during incubation (Tate 2000). PFLA is useful for monitoring changes in physiological status, activity and shifts in community structure (Øvreås 2000).

Culture techniques, unlike genetic methods, are selective. Any given medium, or suite of media, will allow for only a small subsection of a natural microbial community to grow. This principle may be applied to non-selective methods to reduce data set complexity and must not be neglected. Indeed, applying multiple approaches in ecological studies is becoming more prevalent as techniques are standardized (Øvreås 1998; MacNaughton et al. 1999; Roony-Varga et al. 1999).

To date, community characterizations have been, for the most part, applied to field situations. Hydrocarbon-contaminated or impacted sites rather than fermentor-based treatment systems have been the target of characterization. It is possible that, following extensive selection, microbial populations can be cultured at greater frequencies than from natural communities. Regardless, while single populations have been found to carry metabolic tools

for both alkane and aromatic degradation (Whyte et al. 1997), communities are more likely to display the potential for mineralizing petroleum hydrocarbon mixtures.

The overall objective of this thesis was to characterize the physical, chemical and microbiological factors involved in crude oil biodegradation by a mixed-bacterial culture. The approach was to study both the bulk processes catalyzed by the mixed culture, and processes catalyzed by pure and co-cultures derived therefrom. Specific objectives were to:

1. relate surfactant physicochemical properties (HLB, cmc, structure) to crude oil biodegradation;
2. characterize changes in the TSA-culturable bacterial community upon exposure to different hydrocarbons and surfactant over time;
3. isolate and identify resident microorganisms and evaluate their activity against crude oil and volatile hydrocarbons in pure culture;
4. develop a rapid, cost-effective methodology for evaluating volatile hydrocarbon biodegradation in live systems during growth on crude oil;
5. evaluate the potential for volatile hydrocarbon biodegradation during growth on crude oil, and identify important factors affecting this activity;
6. construct a co-culture and examine interactions in relation to the original community with respect to biosurfactant or bioemulsifer production, and biodegradation.

The thesis is arranged in chapters, each of which is a separate publication. The common threads between the chapters are the culture, substrate and batch culture system used. The later studies are built on observations from earlier chapters and are referenced. Specifically, Chapter 2 is an examination of hydrocarbon insolubility with respect to microbial accession. Here a group of nonylphenol ethoxylate surfactants with variable physicochemical properties (HLB and cmc based on ethoxylate length) were studies. In Chapter 3, trypticase soy agar-culturable bacteria from the mixed community were isolated over time upon exposure to refinery sludge and Bow River crude oil, and fractions therefrom, in the presence and absence of surfactant. Isolates were identified with fatty acid methyl ester analysis in order to identify trends in the

community dynamics and to obtain isolates for study in pure and co-culture. Chapter 6 is an examination of two isolates, a *Pseudomonas* sp. and *Rhodococcus* sp., observed to use different hydrocarbon-accession modes. Their metabolic and physical interactions with each other, with the substrate, and with chemical surfactant were used to showcase potential interactions in the original mixed culture. Chapters 4 and 5 outline the development of a new, rapid, simple and inexpensive solid phase microextraction methodology to monitor volatile hydrocarbons. The main objective was to develop a method to be applied repeatedly to sealed biodegradation flasks over time without interrupting biological activity. Once developed (Chapter 4) the method was used to examine factors such as culture age and nutrient amendments on volatile hydrocarbon biodegradation by the original mixed culture, and pure and co-cultures. The overall conclusions from each study are summarized in Chapter 7 and a master reference list, compiled from the subsequent chapters, is provided in Chapter 8.

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2. Influence of chemical surfactants on the biodegradation of crude oil by a mixed-bacterial culture

2.1. Abstract

The effects of surfactant physico-chemical properties, such as the hydrophile-lipophile balance (HLB), and molecular structure on the biodegradation of 2% (w/v) Bow River crude oil by a mixed-bacterial culture were examined. Viable counts increased 4.6 fold and total petroleum hydrocarbon (TPH) biodegradation increased 57% in the presence of Igepal CO-630, a nonylphenol ethoxylate (HLB 13, 0.625 g/l). Only the nonylphenol ethoxylate tested with an HLB value of 13 substantially enhanced biodegradation. The surfactants from other chemical classes with HLB values of 13, (0.625 g/l) had no effect or were inhibitory. TPH biodegradation enhancement by Igepal CO-630 occurred at concentrations above the critical micelle concentration (cmc). When the effect of surfactant on individual oil fractions was examined, the biodegradation enhancement for the saturate and aromatic fractions was the same. In all cases, biodegradation resulted in increased resin and asphaltene concentrations. Optimal surfactant concentrations for TPH biodegradation reduced resin and asphaltene formation. Chemical surfactants have the potential to improve crude oil biodegradation in complex microbial systems, and surfactant selection should consider factors such as molecular structure, HLB and surfactant concentration.

2.2. Introduction

Microbiological methods to remediate crude oil contaminated wastes are often limited by low substrate solubilities which can reduce bioavailability to the degrading microorganisms (Atlas and Cerniglia 1995). Chemical and biological surfactants have been shown to have beneficial, neutral or inhibitory effects on biodegradation, highlighting the need to accurately characterize their roles so that performance in biological systems may be predicted (Bruheim et al. 1997; Deschênes et al. 1996; Nelson et al. 1996; Rocha and Infante 1997; Tiehm 1994; Zhang and Miller 1992).

Van Hamme, J.D., and Ward, O.P. 1999. Can. J. Microbiol. **45**:130-137.

Properties of chemical surfactants which influence their efficacy include charge (nonionic, anionic or cationic), hydrophile-lipophile balance (HLB - a measure of surfactant lipophilicity) and critical micelle concentration (cmc - the concentration at which surface tension reaches a minimum and surfactant monomers aggregate into micelles). Surfactants with HLB values from 8 to 15 generally form oil-in-water emulsions while surfactants with HLB values between 3 and 6 form water-in-oil emulsions (Cross 1987). The cmc is also important as solubilization and biodegradation enhancement has been shown to be both a surface tension and micellar process (Dlallo et al. 1994; Jahan et al. 1997; Pennell et al. 1997).

Crude oil may be characterized by separation into its SARA fractions: saturates, aromatics, resins and asphaltenes (Speight 1991). Since a broad range of chemical species exists within each fraction which have the potential to interact differently with surfactant micelles, selection of suitable chemical surfactants from the thousands of commercially available products (Ash and Ash 1993) is challenging. For example, with ethoxylated surfactants, nonpolar saturated compounds are thought to partition into the micellar core, while aromatic hydrocarbons may partition between the core and outer ethoxylate shell (Dlallo et al. 1994; Pennell et al. 1997). Surfactant selection to enhance biodegradation is further complicated by the use of mixed cultures containing microorganisms that may employ different modes to access hydrophobic compounds. Microorganisms have been shown to interact directly with soluble hydrocarbons, assimilate surfactant pseudosolubilized hydrocarbons, or increase their cell surface hydrophobicity in order to adhere directly to large hydrocarbon droplets (Singh and Desai 1986). While studying a pure culture system simplifies research, mixed cultures have been shown to be more effective for degrading mixed substrates (Sugiura et al. 1997).

In this paper, the effects of surfactant physico-chemical properties and molecular structure on crude oil biodegradation by a mixed-bacterial culture were examined.

2.3. Materials and Methods

2.3.1. Chemicals

Bow River crude oil (d 0.889 g/ml) was obtained from Imperial Oil (Sarnia, ON) and stored in a tightly sealed container to prevent evaporation. It contained 25.5% volatile material, 24.6% saturates, 39.0% aromatics, 5.4% resins and 5.5% asphaltenes. No changes in density or chemical composition (based on SARA analysis) were noted in the stock oil over the course of the study.

A range of nonionic nonylphenol ethoxylates (Table 2.1) was chosen to evaluate surfactant HLB effects. Different chemical classes were represented by a variety of nonionic, anionic and cationic formulations, each with an HLB value of 13. The surfactants, obtained directly from the manufacturer, were as follows: Alkamuls 600 DO, Antarox P-104, Igepal CO series (Rhône-Poulenc, Cranbury, NJ); Chemal TDA-9/85, Chemeen DT-15 (Chemax, Greenville, SC); Hamposyl L (Hampshire Chemical Corporation, Lexington, MA); and Sandopan DTC Acid Liquid (Clariant, Lachine, PQ). All surfactant samples were 94 to 100% pure except for Chemal TDA-9/85 (85% in water) which was prepared to give a known concentration of the active ingredient. Surfactants were used without additional purification and 10% (w/v) stock solutions were prepared in deionized water. Igepal CO-210, Igepal CO-430 and Hamposyl L were insoluble in water, so stock solutions were prepared in dichloromethane (DCM) immediately prior to use.

2.3.2. Surface tension measurements

Surface tension measurements were made by the du Nouy method (Shaw 1992) using a Fisher Scientific Surface Tensiomat 21 equipped with a platinum-iridium ring. For cmc determinations, serial dilutions of chemical surfactants were prepared in distilled water in detergent-free 50-ml beakers, and 10 ml was retained for measurements. Critical micelle concentrations were estimated by noting the point at which further increases in surfactant concentration no longer decreased the surface tension. Culture samples were prepared by

Table 2.1 Properties of nonylphenol ethoxylates.

Surfactant	Approx. No. of ethoxylate units	HLB*	cmc** (mg/l)
Igepal CO-210	1.5	4.6	15.2
Igepal CO-430	4.0	8.5	24.8
Igepal CO-520	5.0	10.0	29.4
Igepal CO-530	6.0	10.8	34.5
Igepal CO-610	7.5	12.2	43.3
Igepal CO-630	9.0	13.0	53.6
Igepal CO-720	12.0	14.2	79.3
Igepal CO-730	15.0	15.0	113.7
Igepal CO-850	20.0	16.0	197.7
Igepal CO-880	30.0	17.2	535.4
Igepal CO-970	50.0	18.2	3149.5

* from trade literature, and Ash and Ash (1993)

** Estimated values for Igepal series from Hsiao et al. (1956)

centrifuging 15 ml of broth at 12 000 *g* for 10 minutes prior to removing the cell-free supernatant, taking care not to transfer any of the upper oil layer, to a clean beaker. To ensure that the broth did not contain residual hydrocarbons 10 ml of the supernatant was transferred to a second clean beaker and allowed to sit in a fume hood for 15 minutes prior to measuring the surface tension. The average of 5 readings was calculated and, between each reading, the platinum-iridium ring was passed through a flame after rinsing with dichloromethane, water and acetone to remove residual hydrocarbons. The experiments were carried out at 30°C with 175 rpm orbital shaking in 2-l flasks containing 500 ml of medium and 2% (w/v) Bow River crude oil with and without 0.625 g/l Igepal CO-630. A non-inoculated control was included for comparison to account for weathering of the oil. The data for the culture without surfactant added is the average of three separate experiments.

2.3.3. Culture medium

The phosphate-buffered mineral salts medium used to evaluate effects of surfactants was adapted from Lal and Khanna (1996) and amended with 0.5 g/l Bacto yeast nitrogen base (Difco Laboratories, Detroit, MI). The medium contained per litre: 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 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 $MnSO_4 \cdot 2\ H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $ZnSO_4 \cdot 7\ H_2O$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3 \cdot 16\ H_2O$, 0.01 g H_3BO_4 and 0.01 g $Na_2MoO_4 \cdot 2\ H_2O$. Medium was prepared fresh for each experiment and, if necessary, the pH was adjusted to 7.0 with 2.5 N NaOH.

The mineral salts medium used for selection and maintenance of the mixed culture contained per litre (Ward and Singh 1997): 1.0 g KH_2PO_4, 1.5 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g Na_2CO_3, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 0.005 g $FeSO_4$, 0.02 g $MnSO_4$ and 1.0 ml of trace metals solution. The trace metals solution contained per litre: 0.0144 g $ZnCl_2 \cdot 4\ H_2O$, 0.012 g $CoCl_2$, 0.012 g $Na_2MoO_4 \cdot 2\ H_2O$, 1.9 g $CuSO_4 \cdot 5\ H_2O$, 0.05 g H_3BO_4 and 35 ml HCl.

2.3.4. Culture selection, maintenance and inoculum preparation

A mixed microbial population from hydrocarbon contaminated soil was inoculated into a cyclone fermentor (Liu et al. 1981) containing mineral salts medium (Ward and Singh 1997) supplemented with 60 g/l refinery sludge (25% hexane extractable material, 13% hexane insoluble material, 62% water) as the sole carbon and energy source. The cyclone had a 1.0 l working volume and 10% by volume of the reactor was replaced with fresh medium and sludge every day for five months. Similar cultures, none of which were supplemented with surfactant, from the same source were maintained on diesel (50% by volume replaced with fresh medium and 5 ml diesel twice weekly) and motor oil (50% by volume replaced with fresh medium and 3 ml 10W30 twice weekly). Samples from each fermentor were combined, inoculated to 10% (v/v) and grown for one week at 30°C on 10% (w/v) Bow River crude oil with 1.25 g/l Igepal CO-630 at 30°C on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) set at 175 rpm. Twenty percent (w/v) glycerol was added to the culture and 1.0 ml aliquots were flash frozen on dry ice in 1.5 ml tubes before storing at minus 80°C.

Prior to an experiment, aliquots of culture were thawed in a 30°C water bath and inoculated into medium containing 2% (w/v) Bow River crude oil and 0.625 g/l Igepal CO-630. The culture was grown for one week at 30°C and was used as the source of inoculum for experiments. Culture activity was stable over the period of study as determined by repeatedly measuring the biodegradation of Bow River crude oil under a standard set of conditions.

2.3.5. Growth and oil degradation conditions

For all experiments, medium was prepared fresh and 50 ml was added directly to 250-ml Erlenmeyer flasks followed by the addition of crude oil (1.0 g) and surfactant if necessary. In the case of Igepal CO-210, Igepal CO-430 and Hamposyl L, surfactant was added to the flask and the solvent was evaporated under nitrogen prior to adding the medium (the surfactants are non volatile). Following inoculation, flasks were incubated at 30°C on an orbital shaker set to 175 rpm, and pH was maintained at 7.0 with 2.5 N NaOH as required. Biodegradation of the non-volatile fractions of crude oil was determined by comparing to a time zero or autoclaved control. Error bars in all figures indicate the standard deviation of triplicate samples.

2.3.6. Microbial counts

Total bacterial counts were determined by removing 0.5 ml of culture and preparing serial dilutions in 50 mM phosphate buffer (pH 7.2) prior to spreading onto trypticase soy agar (40 g/l) plates (soybean-casein digest agar, Becton Dickinson, Cockeysville, MD). Plates were incubated at 30°C for three days prior to counting colonies on a Quebec colony counter.

2.3.7. Oil extraction and fractionation

Following incubation, 20 ml of DCM was added to flasks to obtain a total petroleum hydrocarbon extract. The aqueous and solvent phases were transferred to four 50 ml Corex centrifuge tubes, and flasks were rinsed three more times to bring the total volume of DCM used to 50 ml. Tubes were centrifuged at 12,000 *g* (International Equipment Co., Needham Heights, MA) to break oil in water emulsions. The upper layer was discarded, and the lower oil-containing phase was filtered into a round bottom flask through sodium sulfate to remove residual water. The majority of the solvent was removed under vacuum with an Evapotec Rotary Evaporator (Haake Buchler, Saddlebrook, NJ), and the remainder was transferred to a pre-weighed, 50-ml glass beaker which was allowed to dry to a constant weight in a fume hood prior to gravimetrically measuring the TPH extract.

Asphaltenes were precipitated by adding 30 times (v/w) hexane to the TPH extract, mixing with a glass rod, and allowing to sit for 8 hours. The contents of the beaker were then filtered through a pre-weighed Whatman GF/A glass microfibre filter (Whatman International Ltd., Maidstone, England) and allowed to dry before determining the weight of asphaltenes. The filtrate was collected in a round bottom flask, concentrated under vacuum, transferred to a pre-weighed 18-ml vial and dried before determining the weight of the hexane soluble maltenes. The maltenes were brought to 5 ml in a volumetric flask and 1.25 ml was removed into a pre-weighed 2.0-ml vial for SARA analysis.

After evaporating solvent and weighing the maltenes in the vial, separation of the saturate, aromatic and resin fractions was performed. Ten grams of 70-230 mesh silica powder (activated at 100°C for 24 hours) was loaded into a glass column (11 x 300 mm, 250-ml reservoir) plugged with glass wool and washed sea sand using hexane as the mobile phase.

The column was topped with approximately 0.5 g sodium sulfate before loading the sample which was dissolved in hexane. The column was eluted with 30 ml hexane, 40 ml DCM and 40 ml chloroform-methanol (1:1). The saturate fraction was eluted in the first 30 ml (as determined by gas chromatography analysis of 5 ml fractions), followed by the aromatics in the next 50 ml (monitored by UV 280 nm and GC), and the resins. Recovery was generally no less than 95%. Once again, the weight of the fractions was determined gravimetrically.

2.4. Results

2.4.1. Surfactant effects on growth and degradation

The influence of a non-ionic surfactant, the nonylphenol ethoxylate Igepal CO-630 (HLB = 13, 0.625 g/l), on the growth of, and TPH degradation by, a mixed culture was investigated in a yeast nitrogen base salts medium containing Bow River crude oil (2% w/v). The culture was incubated at 30°C on an orbital shaker set at 175 rpm. Time courses for bacterial counts (microbes other than bacteria were not observed) and TPH biodegradation in cultures with and without the addition of surfactant are presented in Figure 2.1a and b, respectively. A 4.6 fold increase in viable counts (1.9×10^9 CFU/ml) was observed with surfactant supplementation as compared with the no-surfactant control. High levels of viability ($> 10^9$ CFU/ml) were maintained through day 14 in surfactant-containing cultures whereas counts declined in the control to less than 10^8 CFU/ml after 4 days. The initial rapid growth in cultures with Igepal CO-630 is not considered to be due to the culture preferentially using the surfactant as a carbon source. When control cultures were incubated with surfactant but without crude oil, no increase in microbial counts was observed in the initial 24-hour period. A higher initial rate of TPH biodegradation was observed in the no-surfactant control with maximum degradation being observed after 48 hours (159 mg). From 15 to 24 hours, during the degradation period

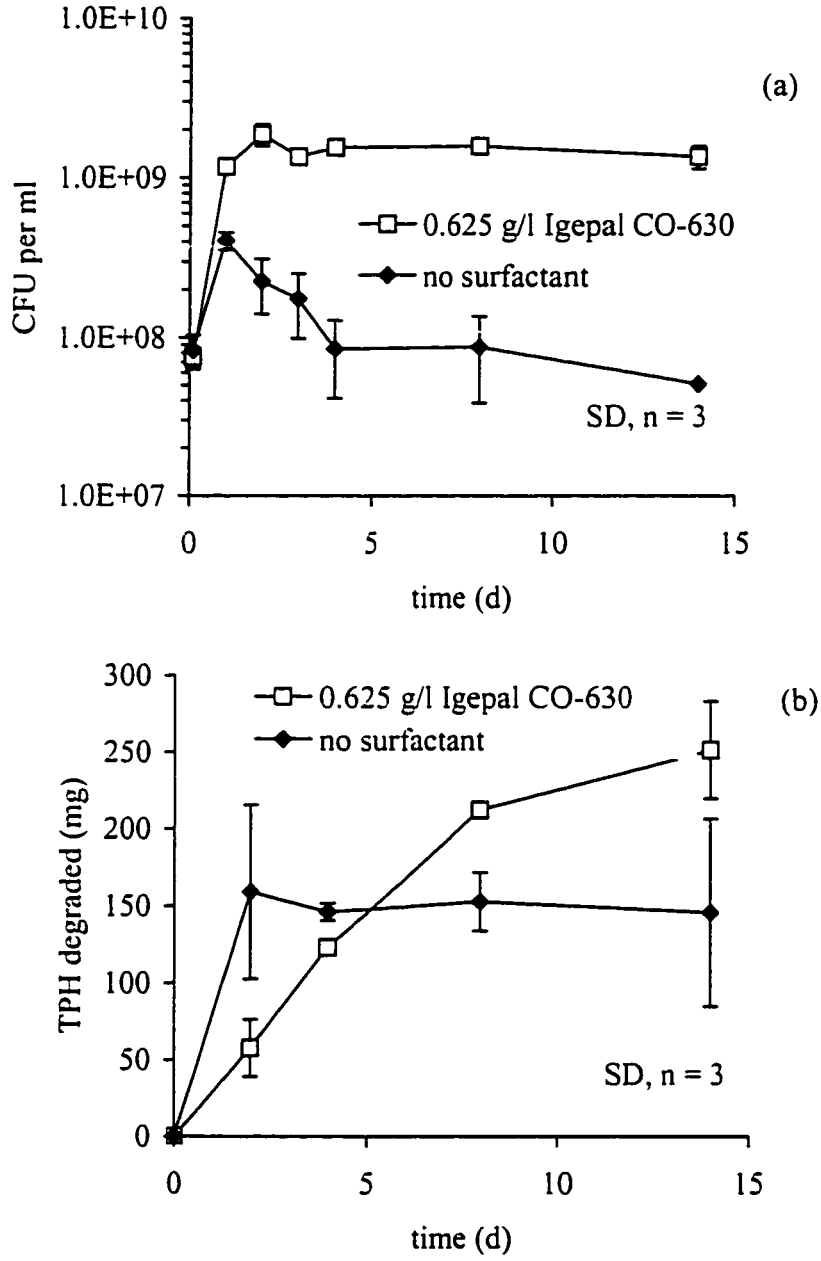


Figure 2.1 Igepal CO-630 (0.625 g/l) effect on (a) growth of a mixed culture on 2% w/v Bow River crude oil, and (b) biodegradation of 2% w/v Bow River crude oil.

and prior to decline in viable counts, the surface tension of the cell-free culture broth dropped from 65 to 50 mN/m (Figure 2.2). The non-inoculated control remained between 70 and 75 mN/m throughout the experiment. In addition, the drop in surface tension was accompanied by emulsification of the oil, which indirectly implies the production of a biosurfactant or mixture of biosurfactants. In the presence of surfactant, the extent of degradation was 250 mg after 14 days and the surface tension remained constant at approximately 32 mN/m (Figure 2.2). Adding 0.625 g/l Igepal CO-630 to the no-surfactant control after 48 hours of growth had no effect on the degradation kinetics.

2.4.2. Effect of HLB among nonylphenol ethoxylates

Surfactant properties such as the HLB and cmc are manipulated during synthesis by varying the length of the hydrophilic ethoxylate chain (Table 2.1). The influence of surfactant HLB value on crude oil biodegradation was examined by incorporating eleven structurally similar nonylphenol ethoxylates (0.625 g/l) with a range of HLB values (4.6 to 18.2) into the culture medium, which was incubated with the mixed culture for 14 days. The surfactant with an HLB of 13 the most effective with respect to enhancing TPH degradation (Figure 2.3).

2.4.3. Effect of oil to surfactant ratio

Since molar concentrations of surfactant varied from 0.26 to 2.18 mM in the above experiment, the relationship between surfactant concentration and TPH degradation was investigated for both Igepal CO-630 (HLB = 13) and Igepal CO-850 (HLB = 16). The results indicate an increase in TPH degradation at surfactant concentrations above 0.078 g/l, or 0.101 mM and 0.568 mM for Igepal CO-630 and Igepal CO-850, respectively (Figure 2.4). The cmc for Igepal CO-630 (0.087 mM) is lower than that of Igepal CO-850 (0.179 mM). Thus, enhancement of degradation occurred at surfactant concentrations greater than the cmc. In the case of Igepal CO-630, the ratio of oil to surfactant (g/l oil to g/l surfactant) which resulted in the greatest enhancement of degradation was 32 to 1.

The observed enhancement in degradation decreased at a surfactant concentration above 0.625 g/l for Igepal CO-630 (Figure 2.4). Two oil concentrations, each supplemented

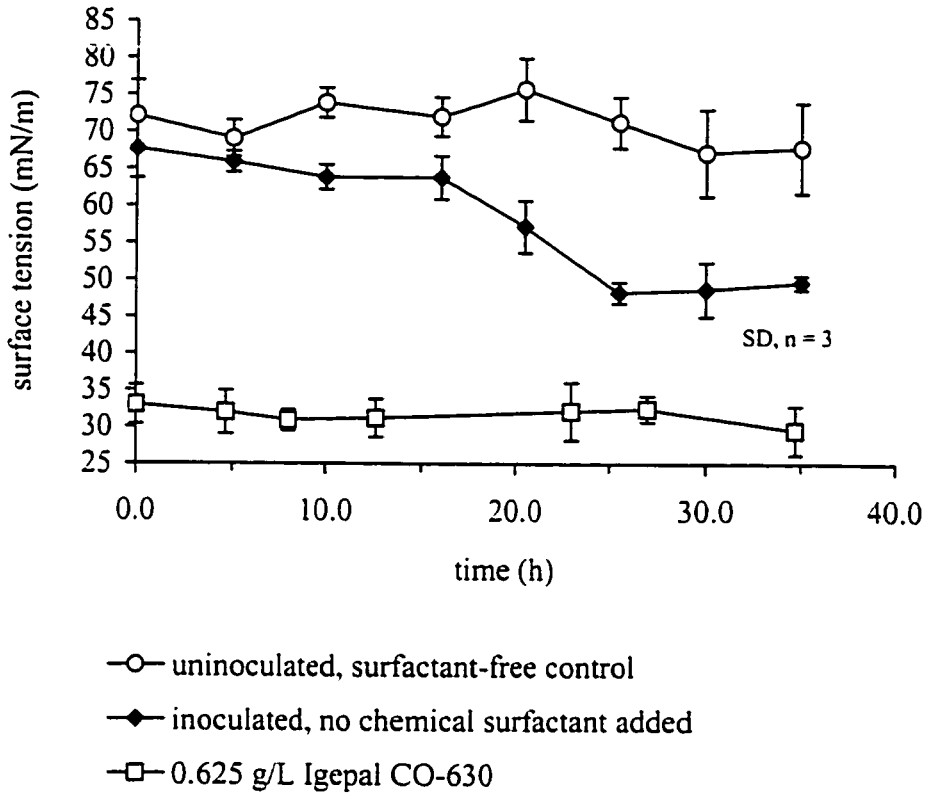


Figure 2.2 Cell free culture broth surface tension during mixed culture growth on crude oil in the presence and absence of Igepal CO-630.

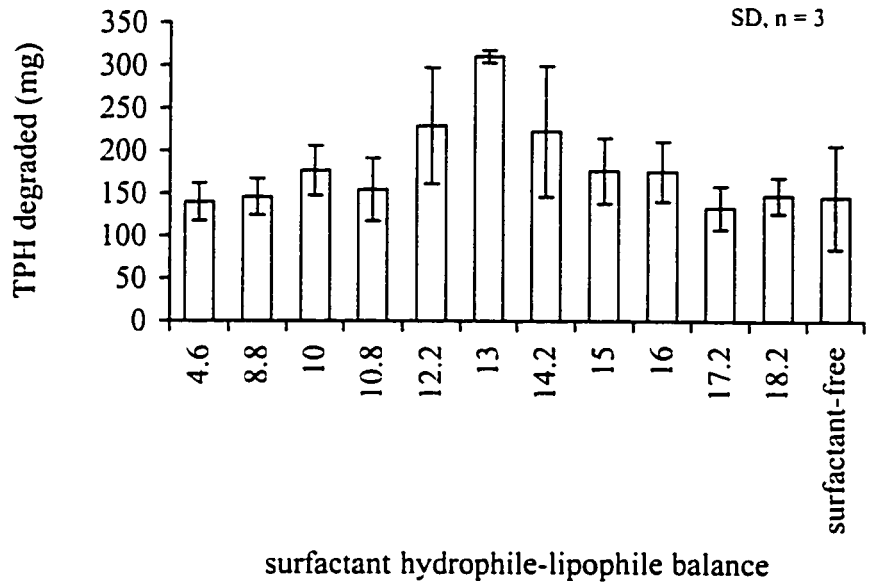


Figure 2.3 Nonylphenol ethoxylate hydrophile-lipophile balance effect on 2% w/v Bow River crude oil biodegradation by the mixed-bacterial culture (14-day incubation).

with two surfactant concentrations, were tested by measuring TPH degradation after 14 days to determine the influence of the ratio of oil to surfactant on degradation (Table 2.2). It was noted that a ratio of 32 was ideal for both 1% and 6% crude oil.

2.4.4. SARA fractions

Bow River crude oil contains 24.6% saturates, 39.0% aromatics, 5.4% resins and 5.5% asphaltenes. In order to more fully characterize the influence of surfactants on petroleum degradation the changes in the SARA fractions in 14 day cultures supplemented with five nonylphenol ethoxylates (HLB values from 10.8 to 15) were determined (Table 2.3). The general hierarchy of degradation (% of each fraction degraded) was saturates followed by aromatics (more aromatics were degraded on a total weight basis) while the resin and asphaltene fractions increased slightly. The influence of Igepal CO-630 concentration on degradation of the individual components exhibited a similar degradation pattern (Table 2.3). Conditions resulting in higher degradation of saturates and aromatics also resulted in lower yields of resins and asphaltenes.

2.4.5. Effect of HLB 13 surfactants

From these data, an HLB value of 13 was chosen to investigate the influence of surfactants (0.625 g/l) from different chemical classes on the biodegradation of Bow River crude oil after a 14-day incubation with the mixed culture (Table 2.4). Only one surfactant substantially increased TPH degradation over the control. Igepal CO-630 enhanced biodegradation while Alkamuls 600DO inhibited oil degradation. Chemal TDA-9/85, which is structurally similar to Igepal CO-630 but lacks an aromatic ring in the hydrophobe, increased biodegradation only slightly. Biodegradation was not strictly related to surface tension and cmc values (Table 2.4).

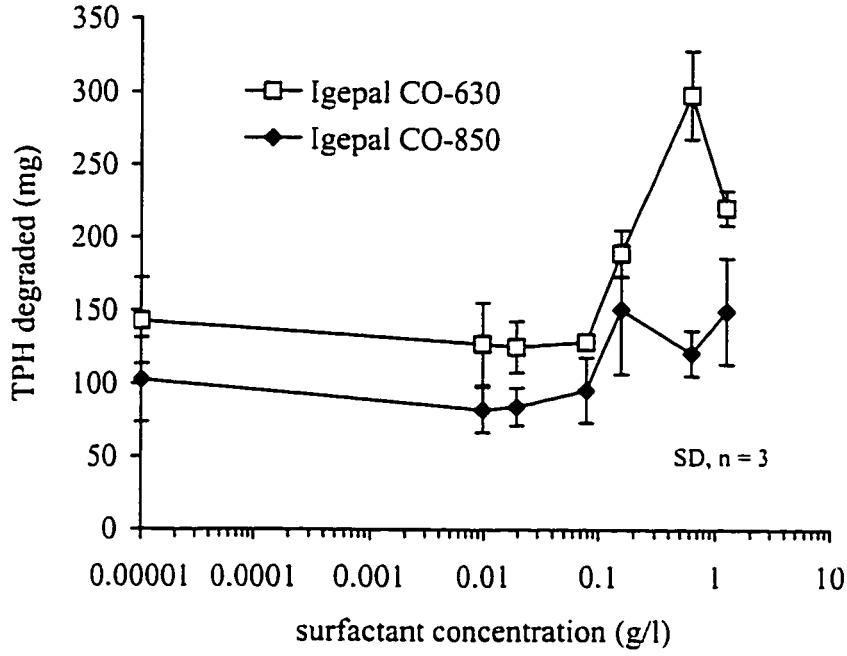


Figure 2.4 Igepal CO-630 (HLB 13) and Igepal CO-850 (HLB 16) concentration effect on the biodegradation of 2% w/v Bow River crude oil by the mixed-bacterial culture (14 day incubation).

Table 2.2 Oil-to-surfactant ratio effect on Bow River crude oil biodegradation by the mixed-bacterial culture (14 day incubation).

Igepal CO-630 concentration (g/l)	Oil concentration (g/l)	Oil to surfactant ratio	TPH degraded %
0.3125	10	32	22.3
0.625	10	16	13.6
0.625	20	32	38.3
0.625	60	96	6.8
1.875	60	32	30.4

Notes: Data are averages of triplicate samples.

2.5. Discussion:

2.5.1. Surfactant effects on growth and degradation

Addition of the nonylphenol ethoxylate Igepal CO-630 enhanced both the extent of growth and degradation of Bow River crude oil. It should be noted that a detailed investigation of the culture composition is being undertaken, including examining changes in the community structure during the biodegradation process. In the absence of a chemical surfactant, the initial burst of degradation was accompanied by the formation of a strong, stable emulsion with a significant reduction in surface tension which is indirect evidence that a biosurfactant, or more likely a mixture of biosurfactants, was produced. The loss of activity and population decline after two days may be due to the nature of the biosurfactant(s) being produced and was not reversed by the addition of Igepal CO-630 to 48-hour cultures originally free of chemical surfactant. The well characterized extracellular bioemulsifier emulsan, produced by *Acinetobacter calcoaceticus* RAG-1, has been shown to reduce crude oil biodegradation (Foght et al. 1989). Emulsan appears to coat oil droplets with a hydrophilic shell allowing hydrophobic cells to detach from the interface and onto a fresh source of easily metabolized substrate (Rosenberg 1993). The results in Figure 1b indicate that the organisms were mainly limited by uptake and not the lack of metabolic ability.

2.5.2. Effect of HLB among nonylphenol ethoxylates

Varying the length of the hydrophilic ethoxylate tail within a homologous series of nonylphenol ethoxylates changes the HLB and ultimately the solubilizing properties of the surfactant (Dlallo et al. 1994; Pennell et al. 1997). In this study, nonylphenol ethoxylates with HLB values between 4.6 and 18.2 were tested and, while none were inhibitory, the surfactant with an HLB of 13 in particular was demonstrated to enhance biodegradation. While this surfactant was included during inoculum growth it should not have biased the results as it was not included during the extended phase of culture enrichment and selection. Bruheim et al.

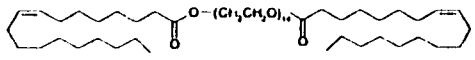
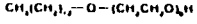
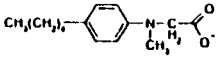
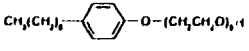
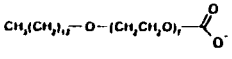
Table 2.3 Bow River crude oil (1.0 g) SARA fraction weights following a 14-day incubation. Values for various Igepal CO-630 concentrations and for nonylphenyl ethoxylates with different HLB values (0.625 g/l) shown.

		Fraction weight (mg)								
		(a) Igepal CO-630 (g/l) HLB = 13				(b) HLB of nonylphenol ethoxylate				
Fraction	Initial (mg)	0.078	0.156	0.625	1.25	10.8	12.2	13	14.2	15
Saturates	253.1	163.8	147.5	104.9	131.4	164.3	146.0	131.1	120.9	156.0
Aromatics	407.6	325.0	311.3	240.4	284.1	330.8	290.9	260.7	251.8	322.4
Resins	49.5	79.6	72.4	63.9	80.9	77.5	65.4	59.1	63.2	75.4
Asphaltenes	56.3	63.7	71.0	65.7	57.7	56.9	62.9	58.1	73.8	66.1
Total:	766.6*									

Notes: Data are averages of triplicate samples.

* the remainder of the 1.0 g Bow River crude is composed of volatile hydrocarbons

Table 2.4 Surfactant (0.625 g/l; HLB 13) structural effects on Bow River crude oil biodegradation (2% w/v) by the mixed bacterial culture, and surfactant properties.

Surfactant	TPH degraded (mg)	cmc (mg/l)	Surface tension (dynes/cm)	Class and charge	Structure
Alkamuls 600 DO	46	61.2	45.7	polyethylene glycol diester of oleic acid nonionic	
Antarox P-104	111	19	45.5	block copolymer of ethoxylate and polyoxypropylene nonionic	
Chemal TDA-9	180	21.5	34.6	alkyl ethoxylate nonionic	
Chemeen DT-15	158	64.7	41.7	PEG 15 N-tallow-1,3-diaminopropane mild cationic	
Hamposyl L	141	not determined		N-lauroyl sarcosine anionic	
Igepal CO-630	277	53.6	27	nonylphenol ethoxylate nonionic	
Sandopan DTC-acid	133	23.7	29.8	PEG-7 tridecyl ether carboxylic acid anionic	
No surfactant	145				

Notes: Data determined experimentally. 14-day incubation.

(1997) found surfactants, mainly polyglycol ethers, with HLB values between 10 and 12 to be most effective for enhancing respiration and release of $^{14}CO_2$ from [1-^{14}C]hexadecane by stationary *Rhodococcus* sp. 094 cells. Interestingly, in some cases exponential phase cells were negatively affected by the same surfactants. Lupton and Marshall (1978) evaluated a series of polysorbates (Tween) and found that growth on, and degradation of, a mixture of alkanes by an *Acinetobacter* sp. was most effectively enhanced by the surfactant with an HLB of 10. A study of a *Mycobacterium* sp. capable of degrading a variety of polyaromatic hydrocarbons (PAHs) revealed that growth on acetate was inhibited in the presence of an alkane ethoxylate with an HLB of 13 and an alkylphenol ethoxylate with an HLB of 14.6 (Tiehm 1994). The author also noted that inhibitory effects on both the *Mycobacterium* and a mixed culture only ceased when the HLB was above 17, apparently due to the lipophilicity of surfactants with 6 to 13 ethylene groups (Tiehm 1994). While the lack of an inhibitory effect in this study was unexpected, perhaps due to the adaptability of the mixed culture, the data show that intermediate HLB values are best for biodegradation enhancement.

2.5.3. Effect of oil to surfactant ratio

The enhancement of degradation by Igepal CO-630 occurs at surfactant concentrations greater than the cmc value. Since micelle formation is known to occur at these surfactant concentrations, degradation appears to be promoted by micellar solubilization and not strictly surface tension reductions. In addition, when the ratio of oil to surfactant was lower than 32, the level of enhancement decreased. Since the surfactant was not inhibitory at even higher concentrations when the oil concentration was increased, dilution of usable substrate may have occurred across an increased number of micelles. Alternately, the surfactant may have been used as a preferred growth substrate. Tiehm (1994) noted that SDS was used preferentially by a mixed culture during biodegradation of PAHs, and surfactant biodegradation has been extensively documented (Swisher 1987). In order to support the results from the HLB scan, Igepal CO-850 was titrated and, although there was a slight increase in degradation above the cmc, it was not as effective as Igepal CO-630. It is clear that surfactants must be tested at a range of concentrations surrounding the cmc in order to maximize their effectiveness.

2.5.4. Changes in SARA fractions

During the HLB scan of nonylphenol ethoxylates and titration of Igepal CO-630, the degradation of saturates and aromatics was equally improved under optimal conditions. Similarly, the increase in the resin fraction, and to a lesser extent the asphaltene fraction, was lower. The reduction in resin formation is somewhat unexpected, as this fraction has been shown to increase during treatment of oil wastes (Ferrari et al. 1996), presumably due to the production of polar metabolites. There has been one report of the isolation of a mixed culture and a *Pseudomonas* sp. capable of utilizing resins as sole carbon and energy source (Venkateswaran et al. 1995). The results may indicate that polar metabolic products were subsequently metabolized under optimal conditions.

2.5.5. Effect of surfactants with HLB values of 13

The data presented (Table 2.4) clearly illustrate that HLB cannot be used as the sole determinant for surfactant selection. This is in agreement with the general conclusion reached by Bruheim and Eimhjellen (1998) who used respirometry to evaluate the effects of a variety of surfactants on crude oil biodegradation. Any given surfactant, aside from its emulsion forming ability, is also a potential carbon source or toxic agent in a microbial system (Swisher 1987). All of the surfactants with HLB values of 13 were tested above their cmc and no apparent relationship between degradation and surface tension was noted. In terms of emulsification, only Alkamuls 600 DO was ineffective, and weak emulsions were formed by Antarox P-104 and Chemeen DT-15. Since Alkamuls 600 DO consists of two moieties of oleic acid, it is conceivable that it was used as a carbon source resulting in the inhibition of crude oil biodegradation. Igepal CO-630, the most effective surfactant, was initially chosen due to its widespread availability and ability to form oil in water emulsions. Chemal TDA-9, an alkyl ethoxylate with the same number of ethoxylate units as Igepal CO-630, lacks an aromatic ring in the hydrophobic moiety and enhanced degradation slightly. This may be due to faster surfactant biodegradation, although the emulsion was stable over 14 days. The results indicate that surfactant structure, not just the HLB value, should be considered during the selection

process. We are unable to speculate on which surfactant structural properties are important based on the data available. Bruheim and Eimhjellen (1998) indicated that the effects of surfactants for a group of polyoxyethylenes on *Deleya salina* in the stationary phase depended more on the properties of the hydrophobic head group. However, they also noted that stimulatory surfactant effects became inhibitory when the number of ethoxylate groups attached to a hydrophobic laureate moiety was increased from 4 to 23.

Overall, chemical surfactants have the potential to improve microbial growth and degradation of crude oil in fermenter based systems, even when complex consortia are used. When selecting a chemical surfactant, numerous factors including concentration, chemical structure and HLB, as well as the physiology of the microorganisms involved, must be considered. In addition, the lifetime of the surfactant, both during and after treatment, as well as the toxicity of the surfactant and its breakdown products should be evaluated during practical applications.

2.5.6. Acknowledgements

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3. Community dynamics of a mixed-bacterial culture growing on petroleum hydrocarbons in batch culture

3.1. Abstract

The effects of various hydrocarbon substrates and a chemical surfactant, capable of enhancing crude oil biodegradation, on the community structure of a mixed-bacterial inoculum were examined in batch culture. Of 1000 TSA-culturable isolates, 68.6% were identified at the genus level or better by phospholipid fatty acid analysis over 7-day time course experiments. Cultures were exposed to 20 g/l Bow River crude oil with and without 0.625 g/l Igepal CO-630 (a nonylphenol ethoxylate surfactant), 5 g/l saturates, 5 g/l aromatics, or 125 g/l refinery sludge. A group of six genera dominated the cultures: *Acinetobacter*, *Alcaligenes*, *Ochrobactrum*, *Pseudomonas/Flavimonas*, *Stenotrophomonas*, and *Yersinia*. Species from four of the genera were shown to be capable of hydrocarbon degradation, and counts of hydrocarbon degrading and total heterotrophic bacteria over time were nearly identical. *Pseudomonas/Flavimonas* and *Stenotrophomonas* normally dominated during the early portions of cultures, although the lag phase of *Stenotrophomonas* appears to have been increased by surfactant addition. *Acinetobacter calcoaceticus* was the most frequently isolated microorganism during exposure to the saturate fraction of crude oil. Regardless of substrate, the culture medium supported a greater variety of organisms during the latter portions of cultures. Understanding the community structure and dynamics of mixed-bacterial cultures involved in treatment of heterogeneous waste substrates may assist in process development and optimization studies.

3.2. Introduction

Complex mixtures of petroleum hydrocarbons, such as crude oil and refinery sludge, can be expected to alter the activity and structure of natural microbial communities. A variety of physiological and molecular approaches have been applied to evaluate selective pressures caused by hydrocarbons in the environment. For example, Langworthy et al. (1998) evaluated

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Van Hamme, J.D., Odumeru, J.A., and Ward, O.P. Can. J. Microbiol. 46:441-450.

phenotypic and genotypic changes in a microbial community exposed to polyaromatic hydrocarbons in freshwater sediment by phospholipid fatty acid (PFLA) analysis and nucleic acid analysis. PFLA profiles showed a shift in community structure from pristine to contaminated sites. Although PFLA profiles also showed marked seasonal variation, the frequency of *nah*A and *alk*B gene sequences and the degradative capabilities of communities was greater at contaminated sites. In general. it has been found that the proportion of hydrocarbon degrading organisms increases substantially upon exposure to hydrocarbons, reflecting the selectivity of the new carbon source (Song and Bartha 1990).

Due to the expected superiority and metabolic versatility of mixed-cultures over pure cultures, they are being applied for the treatment of petroleum wastes in fermentor-based systems. In such a situation, a pre-acclimated hydrocarbon-degrading culture may be sequentially exposed to a variety of heterogeneous hydrocarbon-contaminated waste streams. Different wastes may affect the structure and metabolic abilities of the community. Venkateswaran and Harayama (1995) tracked changes in a crude oil-degrading mixed culture through six transfers onto the residual hydrocarbon extracts of the previous ten-day fermentation. They found that the proportion of aromatic degrading organisms increased with each transfer and an *Acinetobacter* sp., capable of degrading only saturated compounds, was not isolated after the third transfer. However, a limited number of strains were selected from agar plates based on morphological characteristics.

The goals of this study were to: identify, using fatty acid analysis, the TSA-culturable isolates in a bacterial community shown to degrade petroleum hydrocarbons; evaluate the hydrocarbon-degrading capacities of these isolates in pure culture; and characterize changes in the community structure over time under a variety of conditions. Observing how members of a community react to various substrates, and to a chemical surfactant capable of enhancing biodegradation, can help devise a rationale for elucidating interactions between populations in the community and thus, how they work as a consortium.

3.3. Materials and Methods

3.3.1. Culture medium

The phosphate-buffered mineral salts medium used for growth studies and community analysis was adapted from Lal and Khanna (1996) and amended with 0.5 g/l Bacto yeast nitrogen base (Difco Laboratories, Detroit, MI). The medium contained per litre: 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4$ 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 $MnSO_4$ 2 H_2O, 0.01 g $FeSO_4$, 0.1 g $CaCl_2$ 2 H_2O, 0.1 g $ZnSO_4$ 7 H_2O, 0.01 g $CuSO_4$ 5 H_2O, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3$ 16 H_2O, 0.01 g H_3BO_4 and 0.01 g Na_2MoO_4 2 H_2O. Medium was prepared fresh for each experiment and the pH was adjusted to 7.0 with 2.5 N NaOH.

The medium used for the biodegradation of Bow River crude, referred to as PT medium, contained per litre: 1.0 g KH_2PO_4, 1.5 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7$ H_2O, 0.1 g Na_2CO_3, 0.05 g $CaCl_2 \cdot 2$ H_2O, 0.005 g $FeSO_4$, 0.02 g $MnSO_4$ and 1.0 ml of trace metals solution. The trace metals solution contained per litre: 0.0144 g $ZnCl_2 \cdot 4$ H_2O, 0.012 g $CoCl_2$, 0.012 g $Na_2MoO_4 \cdot 2$ H_2O, 1.9 g $CuSO_4 \cdot 5$ H_2O, 0.05 g H_3BO_4 and 35 ml HCl. Yeast extract (Difco Laboratories, Detroit, MI) was added at 1.0 g/l and the initial pH was 7.0. For the hydrocarbon-degrading bacteria, autoclaved Bushnell-Haas medium was used at pH 7.0 and contained per litre: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.002 g $CaCl_2 \cdot 2H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$.

3.3.2. Source and maintenance of culture

The source and maintenance of the culture is as described in Van Hamme and Ward (1999). Briefly, three cyclone fermentors were inoculated with a mixed-microbial culture from hydrocarbon-contaminated soil and maintained on either refinery sludge, motor oil or diesel fuel. After several months of twice-weekly subculturing, samples from each fermentor were combined, inoculated to 10% (v/v) and grown for one week on 10% (w/v) Bow River crude oil (Imperial Oil, Sarnia, ON) with 1.25 g/l Igepal CO-630 (Rhône-Poulenc, Cranbury, NJ) and

incubated at 30°C on an orbital shaker (Labline Instruments Inc, Melrose Park, IL) set at 175 rpm. Cultures (1.0 ml) were flash frozen on dry ice with 20% (w/v) glycerol in 1.5 ml-tubes before storing at minus 80°C. Aliquots of culture were thawed for 3 minutes in a 30°C water bath and inoculated into medium containing Bow River crude oil (2% w/v) and Igepal CO-630 (0.625 g/l) prior to each experiment. The culture was grown for seven days at 30°C and used as the source of inoculum for experiments.

3.3.3. Substrates and substrate purification

Bow River crude oil (density 0.889 g/ml) was stored in a tightly sealed container to prevent evaporation and contained 25.5% volatile material, 24.6% saturates, 39.0% aromatics, 5.4% resins and 5.5% asphaltenes. No changes in density or chemical composition were noted in the stock oil over the course of the study based on analysis of the oil fractions (Van Hamme and Ward 1999).

Bow River saturates and aromatics were purified by silica gel chromatography of artificially weathered (100°C for 24 hours), asphaltene free Bow River crude oil. Hexane (400 ml) was added to 30.0 g weathered crude and stirred for 6 hours to precipitate the asphaltenes. The asphaltenes were removed by filtering through Whatman GF/A glass microfibre filter paper (Whatman International Ltd., Maidstone, England) and the hexane soluble maltenes were concentrated under vacuum. Maltenes (~ 2.5 g) were dissolved in hexane and loaded onto a 30 cm x 3 cm glass column with a 250-ml reservoir packed with glass wool, sea sand, and 70-230 mesh silica powder (activated for 24 hours at 100°C) suspended in hexane. The column was topped with sodium sulfate and eluted with 240 ml hexane, 360 ml dichloromethane (DCM) and 360 ml chloroform-methanol (1:1). Sequential 15 ml fractions were collected and elution of the saturate and aromatic fractions was followed by gas chromatography, and both gas chromatography and absorbance at 280 nm, respectively. The saturates eluted in the first 200 ml, the next 60 ml contained very little material and was discarded, followed by 390 ml containing the aromatics, and the remainder contained the resins. The saturate and aromatic fractions were concentrated under vacuum, weighed, and stored at -20°C until needed.

Refinery sludge was obtained from Sunoco (Sarnia, ON). The sludge contained 22% DCM extractable material separated into the following fractions: 54.0% saturates, 35.8% aromatics, 5.5% resins and 4.3% asphaltenes. Igepal CO-630 (Rhône-Poulec, Cranbury, NJ), a nonionic nonylphenol ethoxylate surfactant with a hydrophilie-lipophile balance of 13, was used without further purification.

3.3.4. Experimental conditions

Cultures were inoculated with 2% (v/v) of a 7-day culture and exposed to five different conditions with samples taken at 0 hours, 18 hours, 2 days and 7 days. The conditions were as follows: 2% (w/v) Bow River crude oil, 0.625 g/l Igepal CO-630; 2% (w/v) Bow River crude oil; 5000 ppm Bow River saturates; 5000 ppm Bow River aromatics; and 12.5% (w/v) Sunoco refinery sludge. Bow River crude and refinery sludge were added directly to 250-ml flasks containing medium, while saturates and aromatics dissolved in DCM were added to empty flasks and the solvent was evaporated under nitrogen before adding medium. All flasks were incubated at 30°C on an orbital shaker set to 175 rpm. Table 3.1 shows the concentration of each oil fraction and surfactant in each flask.

3.3.5. Microbial growth and isolation

Microbial growth was determined by removing 0.5 ml of culture from well-mixed triplicate flasks and preparing serial dilutions in 50 mM phosphate buffer (pH 7.2) prior to spreading onto duplicate trypticase soy agar (TSA) plates (soybean-casein digest agar, Becton Dickinson, Cockeysville, MD). Plates were incubated at 30°C for three days, after which no significant increase in colony counts was observed, prior to counting colonies on a Quebec colony counter.

For microbial isolation, a separate flask was incubated concurrently with those used for microbial growth curves and two 0.5 ml samples removed at each time point for preparation of spread plates as described above. In each case appropriate dilutions were chosen to obtain 50 to 60 colonies on a single plate. After incubation for 3 days at 30°C, 50 colonies were purified by streaking onto fresh TSA plates. Colonies were re-streaked to ensure purity before preparing slants that were stored at 4°C prior to identification by fatty acid analysis.

3.3.6. Identification of Bacterial Isolates

Bacterial isolates were tentatively identified using Microbial Identification System (MIS; Microbial ID Inc. [MIDI], Newark, DE). The system uses gas chromatography to analyze whole-cell fatty acid profiles. All bacterial isolates were subcultured at least twice on trypticase soy broth agar (TSBA; BBL, Cockeysville, MD) plates at 28°C for 24 ± 1 h. The second subculture consisted of quadriant-steaked trypticase soy broth agar plates prepared from the first subculture. Bacterial cells (45 ± 1 mg [wet weight]) were harvested from the second and third quadrant of the trypticase soy broth agar plates. If cells were not processed within 30 min of harvesting, they were stored at - 80°C until fatty acid extraction and analysis. The harvested bacterial cells were treated chemically to extract and convert fatty acids present in the cell wall or cell membrane fractions to fatty acid methyl esters (FAME). Sample preparation consisted of four steps, namely, saponification, methylation, extraction and an alkaline wash, as described by MIDI (Microbial ID Inc. 1993). The FAMEs were injected into a Hewlett-Packard 5890 series II gas-liquid chromatography column equipped with a flame ionization detector. Calibration standards, a negative control blank, and a positive control culture preparation were run with each batch, as recommended by MIDI. The retention times of the FAMEs relative to those of the calibration standards were used by the MIS computer program to identify the fatty acids and then compared to a library containing fatty acid profiles for over 600 bacterial species, and 200 yeast and mold species. Isolate identification is based on the similarity between its fatty acid profile and those present in the database. The MIS required a minimum similarity index of 0.3 with a minimum separation of 0.1 between the first identification and any secondary identification. Identification results with similarity indices <0.3 were considered to be of insufficient match. 'No growth' indicates that insufficient biomass was produced after 24 or 48 hours of incubation at 28°C on TSBA, the standard growth conditions recommended by MIS for fatty acid analysis.

3.3.7. Enumeration of hydrocarbon-degrading microorganisms and screening assay

A "five-tube" MPN assay was modified from Hanson et al. (1993), and Brown and Braddock (1990) for the estimation of hydrocarbon-degrading microorganisms. The assay was carried

out in 24-well pre-sterilized cell culture plates (Beckton Dickinson, Lincoln Park, NJ). Cell dilutions were prepared in sterile Bushnell-Haas medium. Each test well contained 1.5 ml Bushnell-Haas medium and 100 µl of 150 µg/ml 0.2 µm filter sterilized 2,6-dichlorophenol indophenol (DCPIP; BDH Limited, Poole, England). Wells were inoculated with 167 µl of diluted culture prior to overlaying with 10 µl autoclaved Sweet Mixed crude oil (Imperial Oil, Sarnia, ON: density 0.827 g/ml). Control wells were prepared as follows: inoculum with DCPIP; crude oil and DCPIP; and DCPIP only. Plates were covered and incubated at 30°C for 48 hours after which wells were scored as positive (clear) or negative (blue) for the oxidation of crude oil. Further incubation up to 14 days did not change the results. The most probable number was calculated from the appropriate table (Greenberg et al. 1992). The dilution series prepared for the MPN was simultaneously used to prepare TSA spread plates for the evaluation of the total heterotrophic population.

3.3.8. Crude oil biodegradation

The biodegradation of Bow River crude oil with and without 0.625 g/l Igepal CO-630 was evaluated by inoculating triplicate 250-ml Erlenmeyer flasks containing 50 ml PT medium and 1.0 g/l yeast extract with 1.0 ml of inoculum. Seven-day crude oil-grown cultures containing surfactant were used as the source of inoculum. Flasks were incubated for seven and fourteen days at 30°C (175 rpm orbital shaking) prior to extracting the entire contents with 50 ml of DCM. The extraction process included centrifugation (International Equipment Co., Needham Heights, Ma) in four 30 ml Corex tubes at 12 000 *g* to break oil-in-water emulsions, followed by dehydrating the oily solvent phase over granular anhydrous sodium sulfate. Extracts were concentrated under vacuum (Haake Buchler, Saddlebrook, NJ) and transferred to pre-weighed 50-ml beakers. After drying to a constant weight in a fume hood, the total non-volatile hydrocarbon level was measured gravimetrically and compared to both sterile and uninoculated controls.

3.4. Results

3.4.1. Microbial community structure during growth on Bow River crude oil with and without Igepal CO-630

Growth curves for the community in medium containing Bow River crude oil (2% w/v) with and without the addition of a nonylphenol ethoxylate surfactant (Igepal CO-630) are shown in Figure 3.1 (from Van Hamme and Ward 1999). High inoculum levels (10^8 CFU/ml) were used as is typical in industrial fermentations. The addition of surfactant resulted in higher and more sustained levels of biomass over the 14-day incubation (10^9 CFU/ml) compared to the culture containing only crude oil (10^8 CFU/ml). The rapid initial growth is not considered to be due to the culture preferentially using surfactant as a carbon source as control cultures, containing Igepal CO-630 and no crude oil, did not show increased microbial counts in the first 24 hours. In addition, no measurable microbial growth was noted in cultures supplemented with yeast nitrogen base alone. With surfactant, the growth phase lasted 48 hours followed by a stationary phase that continued until the end of the incubation. Without surfactant, the growth phase ended after 24 hours after which there was a decline to the initial inoculum levels.

In order to justify the use of TSA plates for enumeration and community evaluation, a separate experiment was carried out to determine the proportions of hydrocarbon-degrading bacteria in the surfactant-free crude oil culture. Growth curves (Figure 3.2) of total heterotrophs using TSA plate counts and the hydrocarbon-degrading organisms by a 5-tube MPN method were almost identical. Thus, the total heterotrophic population appears to consist mainly of hydrocarbon-degrading organisms.

The community structure data are presented in Table 3.2a and b for samples with and without surfactant, respectively. Crude oil biodegradation kinetics under the same conditions have been previously reported (Van Hamme and Ward 1999). The early portion of the fermentation in crude oil containing cultures yielded primarily *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* isolates. With the addition of surfactant, the most common isolates were *P. aeruginosa* and *Flavimonas oryzihabitans*. The fatty acid profiles of *P. aeruginosa* and *F. oryzihabitans* were often indistinguishable from one another and from that

Table 3.1. Initial concentrations (ppm w/v) of oil fractions and surfactant in flasks used for community structure analysis

	Initial concentration (ppm w/v)					
	Volatiles	**Saturates**	**Aromatics**	**Resins**	**Asphaltenes**	**Igepal CO-630**
Bow River crude + Igepal CO-630	5100	4920	7800	1080	1100	625
Bow River crude alone	5100	4920	7800	1080	1100	-
Saturate fraction of Bow River crude	-	5000	-	-	-	-
Aromatic fraction of Bow River crude	-	-	5000	-	-	-
Refinery sludge*	-	15176	7252	1568	1204	-

* 6.25 g refinery sludge contained 1.4 g DCM extractable material

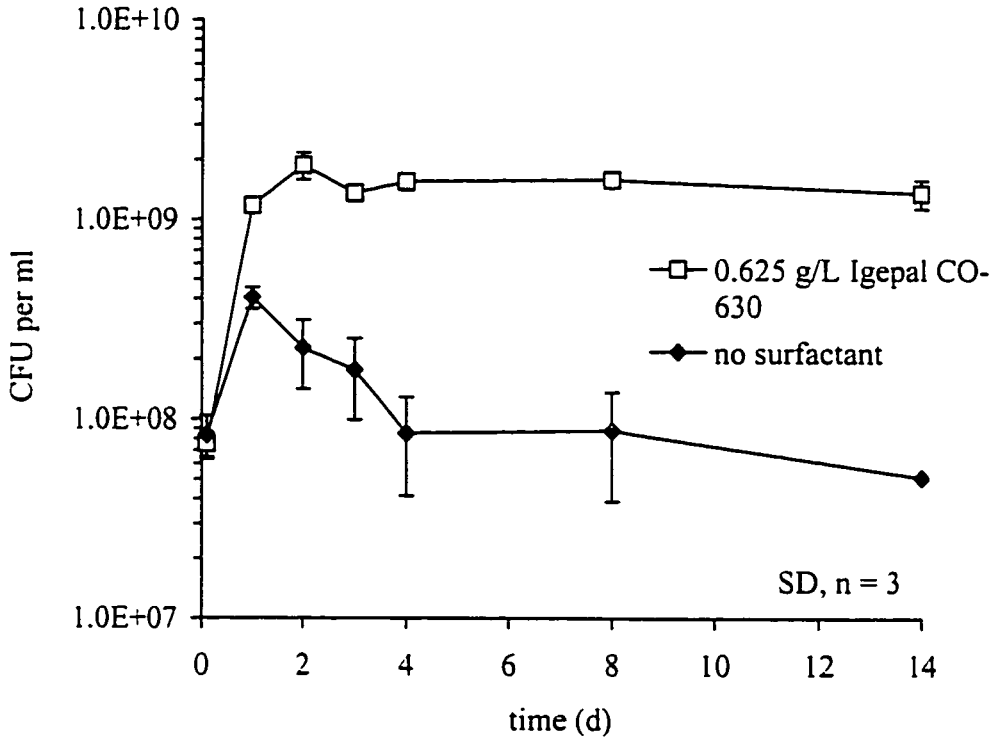

Figure 3.1 Mixed-bacterial growth with and without 0.625 g/l Igepal CO-630 on 2% Bow River crude oil (from Van Hamme and Ward 1999).

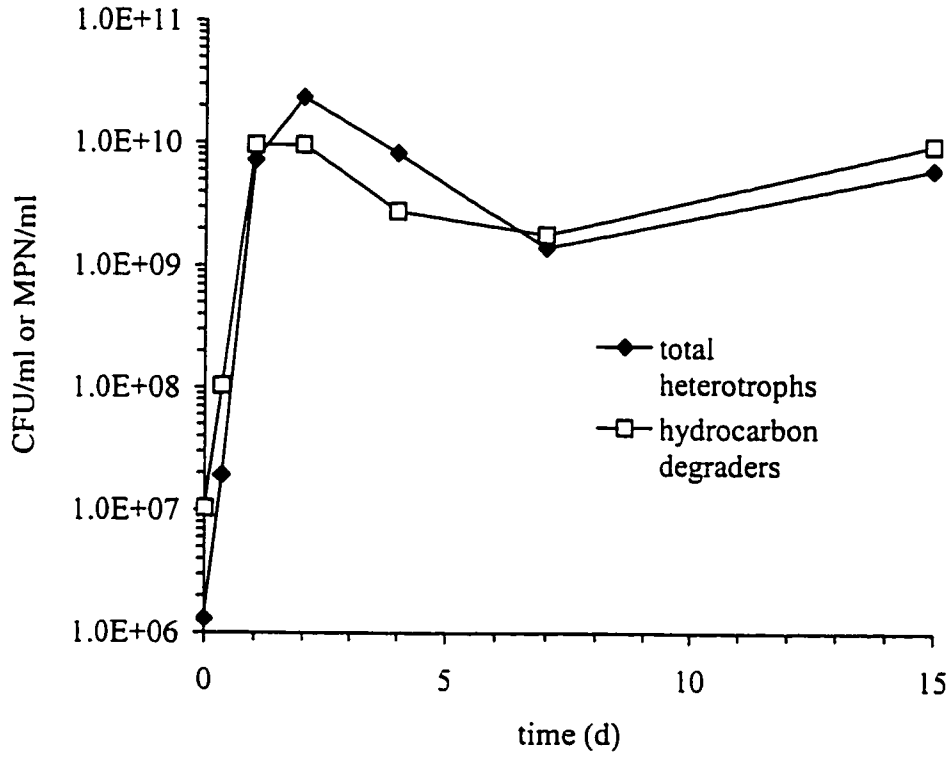

Figure 3.2 Total TSA-culturable heterotrophs and hydrocarbon-degrading bacteria over time during growth on 2% w/v Bow River crude oil without surfactant.

Table 3.2 Mixed-culture community structure during growth on 2% w/v Bow River crude oil. (a) With 0.625 g/l Igepal CO-630, and (b) without surfactant.

(a)

Time (days):	0	0.75	2	7
	Number of isolates			
Acinetobacter calcoaceticus	0	0	0	0
Alcaligenes spp.	1	0	0	4
Ochrobactrum anthropi	0	0	0	0
Pseudomonas aeruginosa and Flavimonas oryzihabitans	30	38	16	4
Stenotrophomonas maltophilia	1	0	12	6
Yersinia spp.	0	0	0	3
*Others identified	2	3	4	4
Others not identified (no match or insufficient match)	8	5	8	6
No growth	8	4	10	23
Total:	50	50	50	50
Dilution:	1.0E+06	2.0E+08	1.0E+08	1.0E+08

(b)

Time (days):	0	0.75	2	7
	Number of isolates			
Acinetobacter calcoaceticus	0	1	4	0
Alcaligenes spp.	0	0	1	0
Ochrobactrum anthropi	3	0	0	3
Pseudomonas aeruginosa and Flavimonas oryzihabitans	10	6	2	0
Stenotrophomonas maltophilia	23	24	7	2
Yersinia spp.	0	2	2	7
**Others identified	5	1	4	11
Others not identified (no match or insufficient match)	9	10	3	10
No growth	1	6	27	17
Total:	51	50	50	50
Dilution:	1.5E-06	1.0E-07	5.0E-08	2.0E-07

* other species, each less than 8% of counts at any time point: *Bacillus laterosporus, Bordetella avium, Brevundimonas diminuta, Burkholderia cepacia, Micrococcus luteus, Pantoea agglomerans, Pseudomonas syringae spp., Sphingobacterium spp., Staphylococcus epidermidis, Xanthobacter agilis.*

** other species, each less than 8% of counts at any time point: *Bacillus spp., Bordetella bronchiseptica, Brevundimonas vesicularis, Cellulomonas flavigena, Corynebacterium aquaticum, Gordona amarae, Kluyvera ascorbata, Microbacterium imperiale, Micrococcus luteus, Pseudomonas fluorescens, Sphingobacterium multivorum, Staphylococcus haemolyticus, Varivorax paradoxus, Xanthobacter agilis.*

of *Chryseomonas luteola*. Indeed, recent phylogenetic evidence indicates that these three genera are synonymous (Anzai et al. 1997). *S. maltophilia* only appeared in large numbers at the end of the growth phase in the surfactant-supplemented culture. In each case, the predominant strains declined after 2 days of growth at 30°C. For the surfactant-supplemented culture, only 4 colonies of *P. aeruginosa/F. oryzihabitans* were isolated on day 7 and, for the crude-only culture, no *P. aeruginosa/F. oryzihabitans* and only two *S. maltophilia* colonies were isolated on day 7. *Acinetobacter calcoaceticus* was isolated infrequently from the mid- to late-log phase in the sample without surfactant. *Yersinia* species (i.e. *Y. enterocolitica*, *Y. frederiksenii*, and *Y. pseudotuberculosis*), pooled in the tables for clarity, were present at low levels in both cultures, especially near the end of the time course. Overall, no dominant TSA-culturable organisms were apparent by day 7 with or without surfactant.

3.4.2. Community changes during growth on Bow River saturates and aromatics

In order to probe for organisms with different metabolic capabilities within the community, the culture was exposed to 5000 ppm Bow River saturates or aromatics. The growth patterns were similar for both substrates, although the overall microbial yield was higher in the saturate-supplemented sample (Figure 3.3). The addition of surfactant increased growth slightly on both substrates, but was omitted during community analysis. Time courses showing the community structure when grown on saturates and aromatics are shown in Table 3.3a and b, respectively. The time zero sample for both cultures contained predominantly *S. maltophilia*, *F. oryzihabitans* and *P. aeruginosa* (as mentioned above, the latter two organisms were often indistinguishable by fatty acid analysis). In the saturate sample, as in the crude oil samples, *S. maltophilia*, *F. oryzihabitans* and *P. aeruginosa* declined substantially by the end of the growth phase. In contrast, these organisms declined but were persistent in the sample supplemented with Bow River aromatics. *Alcaligenes* species (i.e. *A. xylosoxidans*, *A. denitrificans* and *A. piechaudii*), pooled in the tables for clarity, appeared in both the saturate and aromatic cultures, with the greatest proportion appearing after 7 days in the saturate culture. *Ochrobactrum anthropi* remained at low levels throughout growth on the aromatics,

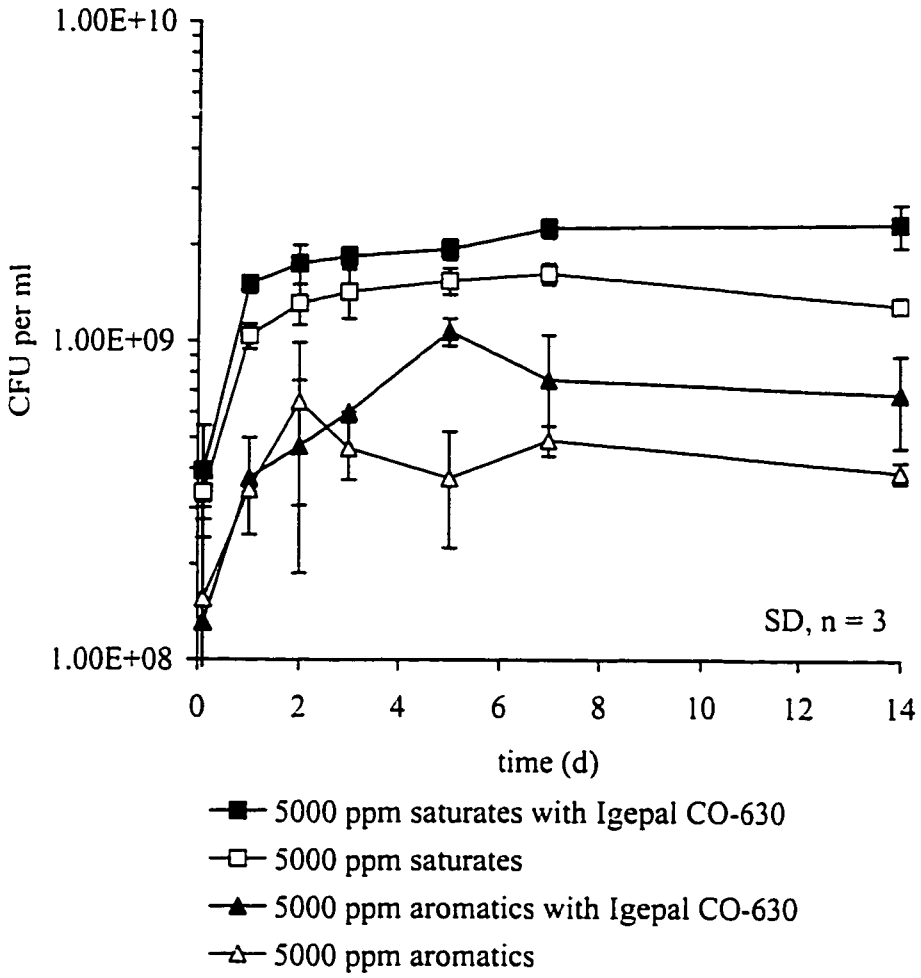

Figure 3.3 Mixed culture growth on Bow River saturates or aromatics in the presence and absence of 0.625 g/l Igepal CO-630.

Table 3.3 Mixed-culture community structure during growth on (a) 5000 ppm Bow River saturates, and (b) 5000 ppm Bow River aromatics.

(a)

Time (days):	0	0.75	2	7
	Number of isolates			
Acinetobacter calcoaceticus	0	26	17	1
Alcaligenes spp.	3	0	3	15
Ochrobactrum anthropi	1	0	1	11
Pseudomonas aeruginosa and Flavimonas oryzihabitans	21	7	0	0
Stenotrophomonas maltophilia	12	3	3	0
Yersinia spp.	0	0	3	3
*Others identified	10	2	2	7
Others not identified (no match or insufficient match)	2	12	10	12
No growth	1	0	11	1
Total:	50	50	50	50
Dilution:	2.0E+07	1.0E+07	1.0E+07	2.0E+07

(b)

Time (days):	0	0.75	2	7
	Number of isolates			
Acinetobacter calcoaceticus	0	0	0	0
Alcaligenes spp.	3	2	4	9
Ochrobactrum anthropi	1	2	2	3
Pseudomonas aeruginosa and Flavimonas oryzihabitans	15	19	10	8
Stenotrophomonas maltophilia	10	7	7	5
Yersinia spp.	1	2	2	2
**Others identified	10	4	8	10
Others not identified (no match or insufficient match)	10	12	3	10
No growth	0	2	13	3
Total:	50	50	49	50
Dilution:	2.0E+07	3.0E+07	4.0E+07	3.0E+07

* other species, each less than 8% of counts at any time point: *Arthrobacter spp., Bacillus sp., Bordetella bronchispetica, Cellulomonas flavigena, Citrobacter sp., Kluyvera sp., Pantoea agglomerans, Pseudomonas spp., Sphingobacterium multivorum, Staphylococcus haemolyticus, Variovorax paradoxus, Xanthobacter agilis.*

** other species, each less than 8% of counts at any time point: *Arthrobacter spp., Bordetella spp., Brevundimonas diminuta, Cedecea davisae, Chryseomonas luteola, Citrobacter sp., Comamonas acidovorans, Corynebacterium aquaticum, Gordona terrae, Kluyvera ascorbata, Methylobacterium mesophilicum, Pseudomonas fluorescens, Pseudomonas syringae sp., Rhodococcus globerulus, Salmonella sp., Xanthobacter agilis*

and was frequently isolated in the saturate sample by day 7. Unlike all other experimental conditions, the saturate-grown culture yielded predominantly *A. calcoaceticus* during the mid- to late-log phase. *Yersinia* species were also present in low numbers throughout the experiment. Once again, no dominant isolates were apparent by the end of the time course.

3.4.3. Community changes during growth on refinery sludge

A two-day growth phase followed by a slow decline to the stationary phase at day 5 was noted when refinery sludge (12.5% w/v) was used as a carbon source (Figure 3.4). The viable count peaked at 4 x 10^9 CFU/ml, which was slightly higher than the surfactant-supplemented crude oil grown sample (1.9 x 10^9 CFU/ml). As for the saturate and aromatic-grown cultures, only a slight increase in microbial numbers was seen when surfactant was added to the sludge, and was not included during community analysis. *P. aeruginosa/F. oryzihabitans* and *S. maltophilia* were the dominant isolates initially and declined over the course of the experiment (Table 3.4). *O. anthropi* and *Yersinia* species were present in low numbers throughout the experiment, and *Alcaligenes* species were present in high numbers at 2 and 7 days. The organisms were divided into a variety of genera by the end of the time course.

3.4.4. Crude oil biodegradation

The original mixed culture, species of the most frequently isolated genera, and *Rhodococcus globerulus* were evaluated with respect to crude oil biodegradation in the presence and absence of Igepal CO-630. *R. globerulus* was selected due superior emulsification ability observed in pure culture. Residual oil was quantified gravimetrically after 7 and 14 days (Figure 3.5) although incubation beyond 7 days had no effect in most cases. In the absence of surfactant, all of the isolates showed little biodegradation potential, while the original mixed culture degraded at least 18% of the non-volatile TPH. After 24 hours, oil was emulsified in the mixed culture system, a condition that remained stable throughout the remainder of the incubation. *R. globerulus* was the only isolate tested with strong emulsification ability in this system, but the

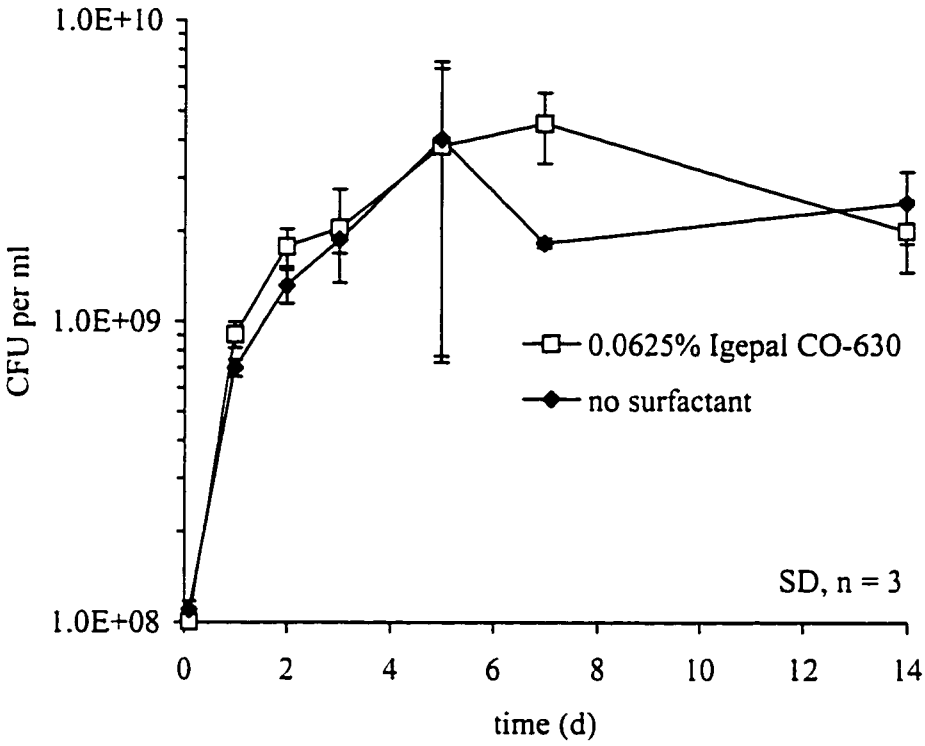

Figure 3.4 Mixed culture growth on 12.5% w/v Sunoco refinery sludge with and without 0.625 g/l Igepal CO-630.

Table 3.4 Mixed-culture community structure during growth on 12.5% w/v refinery sludge.

Time (days):	0	0.75	2	7
	Number of isolates			
Acinetobacter calcoaceticus	0	1	2	0
Alcaligenes spp.	1	2	9	13
Ochrobactrum anthropi	1	2	2	0
Pseudomonas aeruginosa and Flavimonas oryzihabitans	13	10	7	1
Stenotrophomonas maltophilia	14	12	14	5
Yersinia spp.	2	5	4	5
*Others identified	6	2	5	5
Others not identified (no match or insufficient match)	9	16	7	16
No growth	4	0	0	5
Total:	50	50	50	50
Dilution:	5.0E+07	1.0E+07	5.0E+08	3.0E+08

* other species, each less than 8% of counts at any time point: *Bacillus laterosporus, Bordetella bronchiseptica, Cedecea davisae, Chryseobacterium indologenes, Escherichia coli, Flavobacterium odoratum, Flavobacterium resinovorum, Kluyvera cryocrescens, Micrococcus lylae, Micrococcus roseus, Salmonella sp., Sphingobacterium spiritovorum, Varivorax paradoxus, Xanthobacter agilis.*

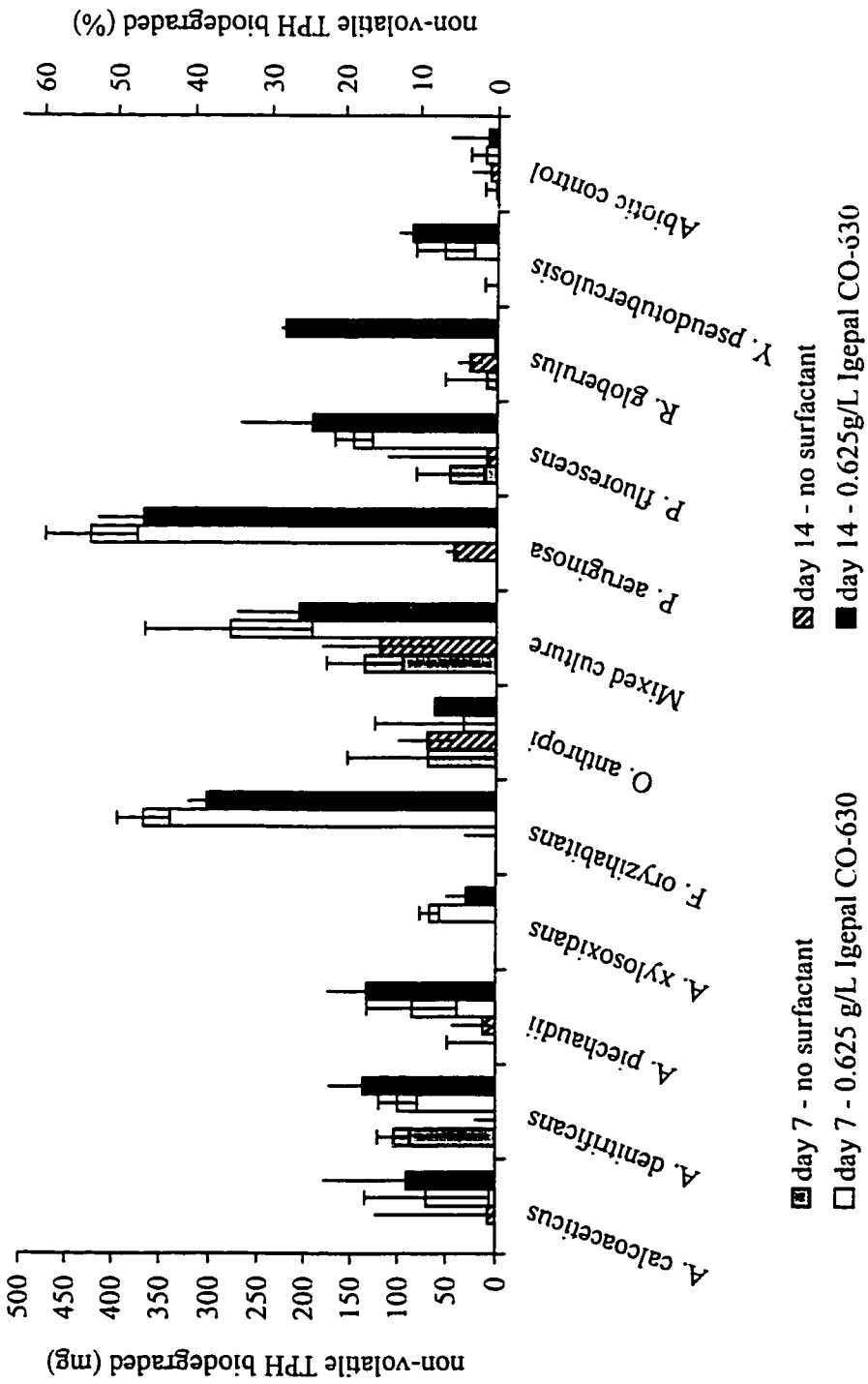

Figure 3.5 Bow River crude oil (2% w/v) biodegradation by pure isolates and the original mixed-bacterial culture with and without 0.625 g/l Igepal CO-630 after 7 and 14 days.

emulsion formed was not long lasting. Specifically, large (1-5 mm) irregular oil droplets would float to the culture surface when agitation ceased. The culture fluid appeared quite viscous with many smaller oil droplets suspended in the liquid medium.

With surfactant addition, the mixed culture was able to degrade 30% of the oil. For the pure cultures clear evidence for biodegradation by *A. calcoaceticus* and *Alcaligenes xylosoxidans* was not evident. *A. denitrificans*, *A. piechaudii*, *O. anthropi* and *Y. pseudotuberculosis* did display low levels of crude oil biodegradation (approximately 10%) in pure culture. Degradation by *P. fluorescens* increased significantly, approaching 25% removal, which was similar to that of the mixed culture. A comparable result was noted for *R. globerulus* after a 14-day incubation. The greatest surfactant-enhancement was noted for *F. oryzihabitans* and *P. aeruginosa*. Degradation by *P. aeruginosa* exceeded that observed with the mixed culture almost two-fold, reaching 50%, corresponding to a 10-fold increase compared to *P. aeruginosa* without surfactant supplementation.

3.5. Discussion

3.5.1. Dominant genera

A group of six identifiable genera in the mixed-bacterial culture, *Acinetobacter*, *Alcaligenes*, *Ochrobactrum*, *Pseudomonas/Flavimonas*, *Stenotrophomonas*, and *Yersinia*, were the dominant TSA-culturable isolates regardless of the carbon source. All six genera are Gram negative rods and have been isolated from water, soil and clinical materials. In this study, evidence of hydrocarbon degradation by *Alcaligenes*, *Pseudomonas/Flavimonas*, *Ochrobactrum* and *Yersinia* was provided. *Pseudomonas/Flavimonas* were the strongest hydrocarbon degrading organisms in the system when an emulsion was formed with chemical surfactant. With the exception of *Yersinia*, the above genera have been previously reported to metabolize hydrocarbons (Bajpai et al. 1998; Boonchan et al. 1998; Bury and Miller 1993; Foght et al. 1996; Guerin and Boyd 1995; Bottane 1992), and have been simultaneously isolated from hydrocarbon contaminated environments (Kämpfer et al. 1993; Wünsche et al. 1997). *Acinetobacter* spp. have been shown to degrade a variety of substrates including alkanes from C_{13} to C_{44} (Sakai et al. 1994), intermediates of alkane metabolism (Bajpai et al.

1998; Lal and Khanna 1996), and benzoate (Collier et al. 1998; Bundy et al. 1998). Naphthalene (Guerin and Boyd 1995), phenanthrene (Moller and Ingvorsen 1993), phenol, *p*-cresol, benzoate and benzaldehyde (Ampe et al. 1996) have been reported as substrates for *Alcaligenes* species. *Ochrobactrum anthropi* can metabolize decane (Bury and Miller 1993) and has been isolated from contaminated fuel (Wasko and Bratt 1990). *Pseudomonas* species can grow on C_{11} to C_{36} straight chain alkanes (Jerábková et al. 1997; Miller and Bartha 1989), branched alkanes (Gough et al. 1992), phenanthrene, anthracene and naphthalene (Foght and Westlake 1988), benzothiophenes and dibenzothiophenes (Kropp et al. 1997). A *Pseudomonas* sp. also degrades the high-molecular weight resin fraction of crude oil (Venkateswaran et al. 1995). *Stenotrophomonas maltophilia* efficiently degrades phenanthrene, fluorene, pyrene, chrysene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and benz[*a*]anthracene (Boonchan et al. 1998).

The apparent lack of other commonly reported hydrocarbon-utilizing microorganisms might be due to the extensive selection process used and the specific culture conditions. Most importantly, the culture was aggressively subcultured during initial isolation to promote rapid growth and degradation. It is germane to note that only one *Rhodococcus*, and very few *Gordona* and *Norcardia*, isolates were obtained. *Rhodococcus* species have been reported to grow on and emulsify hydrocarbons (Bredholt et al. 1998) and the organism isolated in this study produces very small colonies on TSA plates, even after extended incubation. This may have led to under representation in the community data and to the number of unidentified isolates. In addition, when grown in pure culture cells adhered directly to oil droplets and, when grown on water-soluble substrates, cells clumped as macroscopic aggregates.

Three of the most frequently isolated organisms in the present study, *A. calcoaceticus*, *O. anthropi*, and *P. aeruginosa*, are reported biosurfactant producers (Arino et al. 1996; Wasko and Bratt 1990; Desai and Banat 1997). A crude extract from *O. anthropi* emulsified hexane, pentane, octane, hexadecane, and petroleum ether (Wasko and Bratt 1990). A rhamnolipid from *P. aeruginosa* ATCC 9027 increased dispersion and enhanced mineralization of octadecane from 5% to 20% in 84 hours when added to 300 mg/l (Zhang and Miller 1989). A biosurfactant from *P. aeruginosa* USB-CS1 emulsified oily sludge and

enhanced biodegradation by native sludge microflora (Rocha and Infante 1997). Of the genera in the present study, *A. calcoaceticus*, *Alcaligenes xylosoxidans*, and *F. oryzihabitans/P. aeurginosa* displayed oil emulsification. However, the most efficient oil emulsification was observed in *R. globerulus* cultures, a less frequently isolated organism. The observed and reported metabolic capabilities of the key genera identified in this study agree with the overall nature of the mixed culture and the evaluation of total hydrocarbon-degrading microorganisms.

3.5.2. Effect of substrate and chemical surfactant

The refinery sludge-containing culture was enriched in saturates compared to the crude oil-containing cultures. The aromatic, resin and asphaltene concentrations were similar (Table 3.1) and, in the absence of surfactant, the microbial community responded similarly to crude oil-containing cultures. The same general trends in community structure were also observed for the culture supplemented with the aromatic fraction of Bow River crude. The pattern was altered with the saturate fraction in that *A. calcoaceticus* dominated the culture during the initial incubation period. While *A. calcoaceticus* is often isolated from hydrocarbon-contaminated sites (Kämpfer et al. 1993; Wünsche et al. 1997), it was only the dominant isolate in this study under conditions where no aromatic compounds were present. In a previous study (Van Hamme and Ward 1999), the mixed culture degraded Bow River crude oil more effectively in the presence of a nonylphenol ethoxylate surfactant. A striking observation in the present study was the two-day lag in growth for *S. maltophilia* when surfactant was included in the crude oil culture. *P. aeruginosa/F. oryzihabitans*, the organisms that were co-dominant with *S. maltophilia* in all other cultures, were not so affected by the surfactant. While it is surprising that *S. maltophilia* was not detected in the time zero sample, this organism has been reported to be sensitive to alkylphenol ethoxylate. Specifically, Boonchan et al. (1998) recently reported that growth on glucose and the degradation of pyrene by *S. maltophilia* was negatively affected by an octylphenol ethoxylate that is almost identical in structure to the nonylphenol ethoxylate used in our work.

3.5.3. Temporal community changes

Despite differences in overall community structure and initial substrate composition, the frequent isolation of *P. aeruginosa/F. oryzihabitans* and *S. maltophilia*, or *A. calcoaceticus* in the saturate-containing culture, was consistently reduced after one week of incubation. As substrates are oxidized by competent organisms, a variety of metabolites, such as aliphatic organic acids, diacids and aromatic ketones, accumulate (Langbehn and Steinhart 1995) and the turnover of cellular materials will occur due to cell lysis. The required interaction of two or more populations for the complete mineralization of a specific compound has been illustrated in a number of cases. For example, Casellas et al. (1998) showed that a co-culture of *Arthrobacter* sp. F101 and *Pseudomonas mendocina* MC2 was able to completely degrade fluorene. Surprisingly, pure cultures of both *P. aeruginosa* and *F. oryzihabitans* were capable of eliminating a greater amount of TPH than the original mixed culture in chemically emulsified systems. In this study, *Alcaligenes* spp. and *Ochrobactrum anthropi* were among the frequently isolated organisms after 7 days, especially with the saturate fraction, aromatic fraction and refinery sludge. Both of these organisms have been reported to metabolize hydrocarbons (Guerin and Boyd 1995; Bury and Miller 1993) and various metabolic intermediates (Busse and Auling 1992; Swings et al. 1992). Interestingly, Guerin and Boyd (1995) found that *Alcaligenes* sp. NP-alk required a longer induction period than *P. putida* (ATCC 17484) during growth on naphthalene, which agrees with the lag noted in the appearance of *Alcaligenes* sp. compared to *Pseudomonas* spp. in this study.

The MIS proved to be useful for the identification of environmental isolates. Indeed, the rate of identification (68.6% of 1000 isolates) agrees with Wünshe et al. (1997) who identified 75% of hydrocarbon-degrading soil isolates using a combination of fatty acid analysis and the BIOLOG system. Analogously, Tang et al. (1998) were able to identify 77.8% of 72 aerobic pathogenic Gram-negative bacilli from clinical specimens. Haack et al. (1994) found that fatty acid methyl ester analysis was a reproducible and reliable tool for the differentiation of a model microbial community consisting of 10 known soil bacteria under a variety of conditions.

Despite the complexity of the system under study, some useful relationships between substrate, culture conditions, and community structure were revealed. Future work will focus on characterizing interactions of populations in simplified artificial communities.

3.5.4. Acknowledgements

The technical expertise of Jenny Belvedere in identifying the strains by MIDI fatty acid analysis at the University of Guelph Laboratory Services Division is greatly appreciated. A special thanks is extended to Candace Rutka for her assistance with the crude oil biodegradation studies.

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

4.1. Abstract

A quantitative SPME-GC-FID (solid phase microextraction) method for low molecular weight hydrocarbons from crude oil was developed and repeatedly applied to biodegradation samples during continuous incubation. Repeated sampling was achieved through headspace extractions at 30°C for 45 minutes from flasks sealed with Teflon Mininert caps. 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} 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 3 fold higher for the mixed culture, reaching 45% degradation after 48 hours. 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.

4.2. Introduction

Studies related to the 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 (Coppock and Monstrom 1995; Field et al. 1992; Moseley and Meyer 1992). Indeed, many laboratory biodegradation studies examine only

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artificially weathered crude oil (Thouand et al. 1999), or do not quantify the fate of volatile hydrocarbons (Van Hamme and Ward 1999). Cost-effective remedial technologies, including biofiltration, are being developed to comply with regulated emission levels during all stages of petroleum production and use (Leson and Smith 1997). Minimizing volatile hydrocarbon losses during waste remediation is important and schemes, such as the addition of sorbing materials, have been examined (Rhykerd et al. 1998). However, chemical analysis of petroleum hydrocarbons during biological treatment is challenging and volatile hydrocarbon analysis can be difficult and expensive.

Solid phase microextraction is a relatively new solventless-extraction technique developed for rapid, accurate sample analysis (Pawliszyn 1997). Briefly, the sample is sealed in a vial and incubated at constant temperature under a standard set of conditions (i.e. sample volume, mixing, pH and salt concentration). A needle containing a protractible 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 (1997) 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 (Langenfeld et al. 1996). Following equilibration, the fibre is retracted and introduced directly into the analytical instrument of choice for quantitative analysis. Examples include HPLC (Boyd-Boland and Pawliszyn 1996), infrared spectroscopy (Stahl and Tilotta 1999) and GC (Agozzino et al. 1999; Chai and Pawliszyn 1995; Llompart et al. 1999). No intermediate steps are required increasing the precision and accuracy of determinations compared to conventional extraction (Pawliszyn 1997).

SPME fibres can be used to extract polar and non-polar analytes from any type of sample matrix (Pawliszyn 1997). Methods are rapidly being developed to exploit SPME in environmental analysis. In a recent review (Clement and Yang 1999), 24 papers were cited on the use of SPME. Aqueous samples can be analyzed by direct introduction of the fibre into the

water phase (Doll et al. 1999; Langenfeld et al. 1996; Paschke et al. 1999) or via headspace sampling (Djozan and Assadi 1999). Complex aqueous samples can also be analyzed directly using hollow fibre membrane protection systems (Zhang et al. 1996). Analyte in solid samples must be extracted from the headspace (Havenga and Rohwer 1999) or by recovering analyte from aqueous extracts (Dermietzel and Strenge 1999).

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 throughout biodegradation time course studies were two desired methodological characteristics.

4.3. Materials and Methods

4.3.1. 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 $MgSO_4 \cdot 7\ H_2O$, 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 $MnSO_4 \cdot 2\ H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $ZnSO_4 \cdot 7\ H_2O$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3 \cdot 16\ H_2O$, 0.01 g H_3BO_4 and 0.01 g $Na_2MoO_4 \cdot 2\ H_2O$. 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 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; 21% 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.

4.3.2. Source and maintenance of culture

The source and maintenance of the mixed culture has been previously described (Van Hamme and Ward 1999). The isolates (*Pseudomonas aeruginosa*, *P. fluorescens* and *Rhodococcus globerulus*), identified by fatty acid analysis, were obtained from the original mixed culture (Van Hamme et al. 2000). All cultures were pre-grown as a source of inoculum for 3 days in flasks stopped 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 surfactant).

4.3.3. 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 minutes on an orbital shaker) to extract volatile hydrocarbons. Equilibration was carried out for 45 minutes 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 ID) injector sleeve (Supelco, Oakville, ON) and a fused silica column (Restek Rtx-5MS 5% diphenyl – 95% dimethyl polysiloxane, 30 m x 0.32 mm, 0.25 µm film thickness; Restek Corp., Bellefonte, PA) connected to a flame ionization detector (FID). A three minute 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 minutes, 7.5°C/minute to 225°C and 1.0 minutes at 225°C for a total run time of 31 minutes. 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 a 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 a HP 6890 gas chromatograph using helium as the carrier gas. SPME and GC temperature conditions are as described above and a HP-5 5% phenylmethylsiloxane column was used for separations (30.0 m x 250 μm x 0.25 μm nominal). Compounds were identified by comparison spectra to a standard library (NIST Mass Spectral Search Program, Version 1.1a).

4.3.4. 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., Kyoto, Japan) was used to inject 1 μl samples into a wide bore inlet liner using the following conditions: column set to 35°C for 5 minutes, 7.5°C/minute to 225°C, hold for 1 minute, 10°C/minute to 300°C, hold for 5 minutes; injector set to 225°C; and detector set to 275°C. A 5-point toluene standard curve (R^2 0.998, 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 to 500 ppm) were prepared in carbon disulfide and liquid injections were made. The column was held at 35°C for 5 minutes, increased by 1°C/minute to 40°C and then by 10°C/minute to 50°C. The analyte masses were summed in 2.5 minute retention-time windows. Twenty-five microlitres of crude oil contained the following masses of hydrocarbons in milligrams (mean +/- standard

deviation, n = 4): 0 to 2.5 minutes, 1.50 +/- 0.12; 2.5 to 5.0 minutes, 0.50 +/- 0.05; 5.0 to 7.5 minutes, 0.26 +/- 0.04; 7.5 to 10.0 minutes, 0.22 +/- 0.02; 10.0 to 12.5 minutes, 0.32 +/- 0.07; and 12.5 to 15.0 minutes, 0.40 +/- 0.04.

Known volumes of Bow River crude oil (2.5 to 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-minute 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.

4.4. Results

4.4.1. 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 seconds removed >99% of the analyte. The equilibration, or sampling, time was optimized for maximum analyte recovery in the minimum time. Compounds eluting before 10 minutes (72.5°C, *n*-C_{10}) reached equilibrium in three minutes (Figure 4.1). Compounds eluting between 10 and 15 minutes (72.5-110°C, *n*-C_{10} to *n*-C_{12}) were extracted slowly, showing linear kinetics after three minutes of rapid accumulation. Overall, the initial period of rapid analyte accumulation was complete after 10 minutes of exposure with slower change between 30 and 60 minutes. Thus, a 45-minute equilibration time was chosen corresponding to the total GC run time.

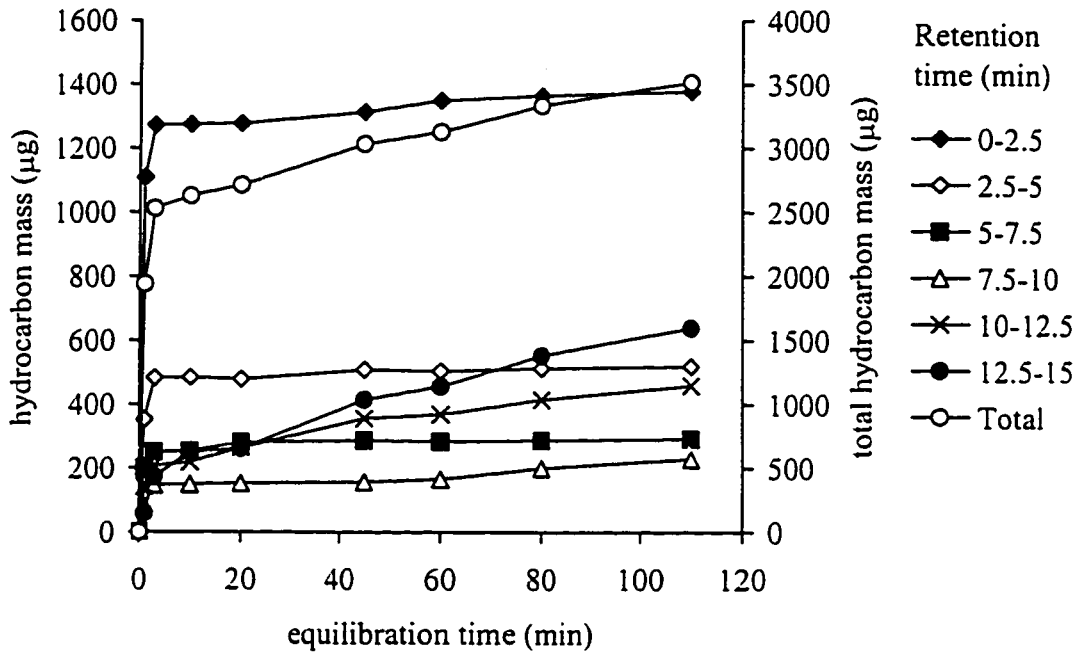


Figure 4.1. Effect of equilibration time on recovery of volatile compounds from headspace of Bow River crude oil samples by SPME (30°C waterbath, 145-ml total volume, 25 ml medium, 25 µl crude oil).

4.4.2. SPME response curves, quantification and reproducibility

Standard curves of total peak area versus total hydrocarbon mass were prepared for calibration. Six standard curves (Table 4.1) for 2.5-minute retention time windows from 0 to 15 minutes were required as curves shifted from linear to logarithmic for increasing retention time ranges. Beyond 15 minutes (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 4.2a). Note the increasing sensitivity for higher molecular weight compounds based on retention time. Figure 4.2b 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 was evident. Decreased ratios were observed beyond 7.5 minutes for 35 µl oil.

4.4.3. 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 was pierced upwards of 80% of the compounds were lost from acidified flasks repeatedly sampled over 120 hours (Figure 4.3a). Losses were negligible in flasks sealed with Teflon Mininert caps (Figure 4.3b). Killed and sterile controls were stable for longer than 20 days during biodegradation experiments.

Table 4.1 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 (degrees C)	Concentration in oil (μg/μl)	Analyte range (volume oil, μl)	Response curve (y = response; x = μg)	R-squared value
0.0 to 2.5	35	66.4	2.5 to 35	y = 30.74 x - 1522.1	0.999
2.5 to 5.0	35	23.0	2.5 to 35	y = 167.4 x + 4020	0.993
5.0 to 7.5	35 to 53.75	12.8	2.5 to 35	y = 315.86 x + 11810	0.983
7.5 to 10.0	53.75 to 72.5	9.7	2.5 to 35	y = 52942Ln(x) - 141924	0.984
10.0 to 12.5	72.5 to 91.25	16.4	2.5 to 35	y = 56836Ln(x) - 133563	0.999
12.5 to 15.0	91.25 to 110	18.1	2.5 to 35	y = 38908Ln(x) - 29091	0.971
	Total:	146.3			

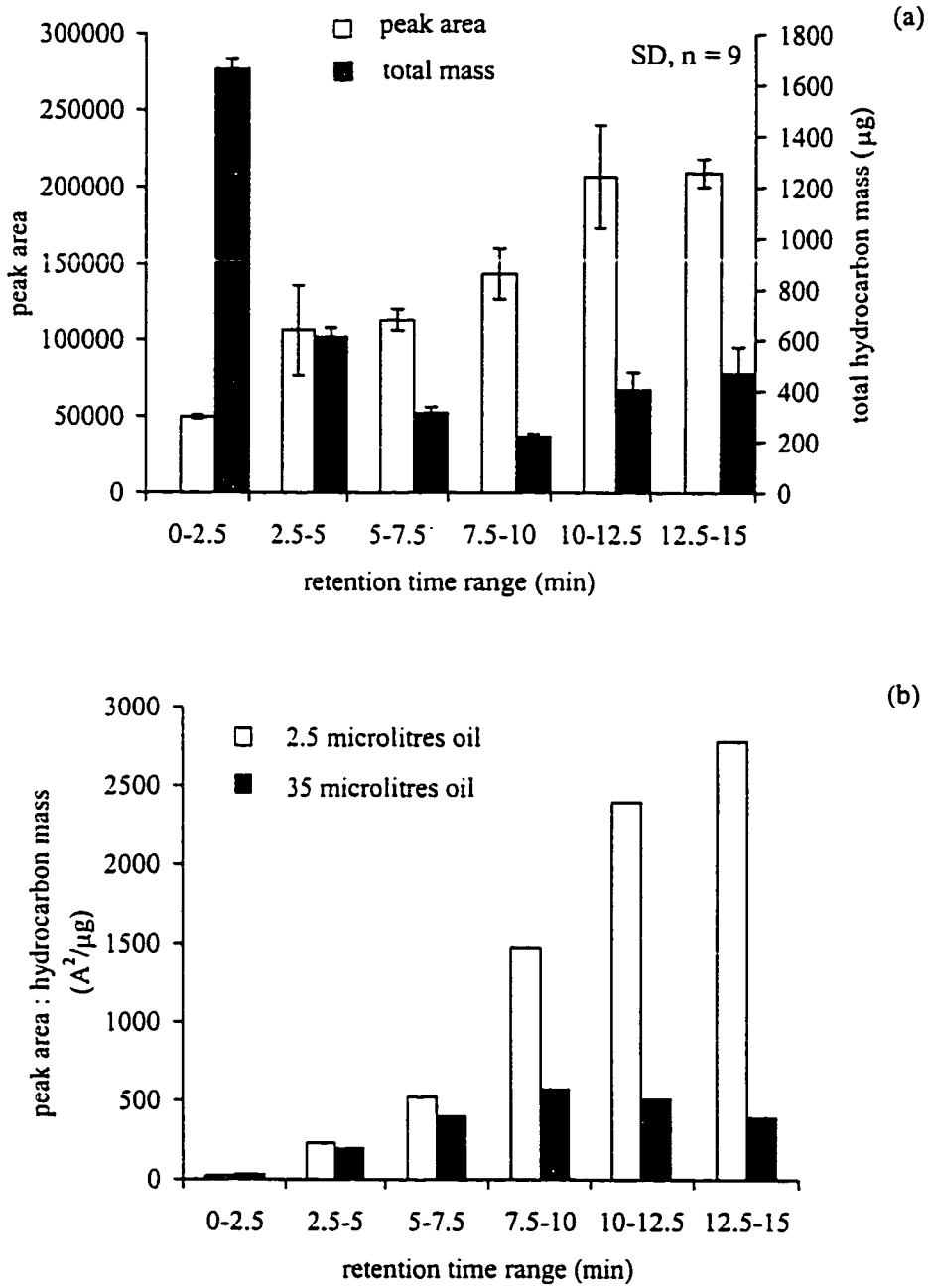


Figure 4.2. (a) Reproducibility and comparison of FID response compared to total analyte mass for increasing retention time windows. (b) Comparison of total peak areas to hydrocarbon mass for increasing retention time ranges.

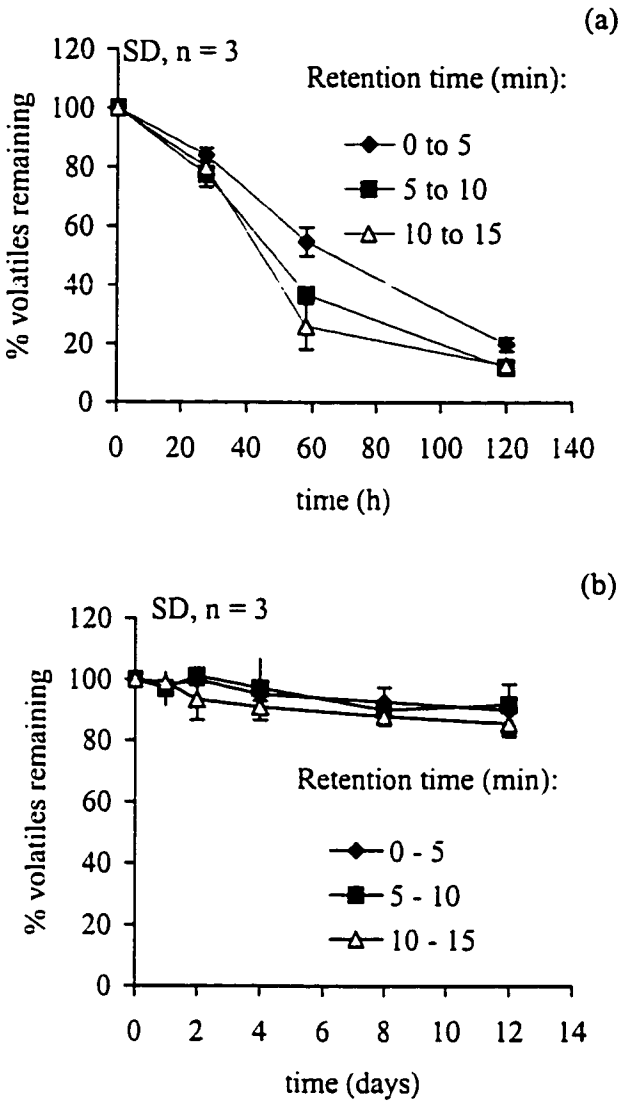


Figure 4.3. Comparison of capping systems for retention of volatile compounds above Bow River crude oil cultures in acidified controls for various retention time ranges. (a) Teflon-lined silicone septum, repeatedly pierced. (b) Re-sealing Teflon Mini-nert cap.

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 4.2a). Oil could be autoclaved and stored in a Mininert-capped vial without significant volatile hydrocarbons losses (Table 4.2b).

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*) were 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 4.4). 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 four. The mixed culture from which the isolates were originally obtained displayed a faster degradation rate and eliminated 55% of the low molecular weight hydrocarbons in four days.

Representative chromatograms for a control and biodegradation flasks containing the mixed culture, *P. aeruginosa*, *R. globerulus*, and the co-culture are shown in Figure 4.5 a-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 mass spectrometry as methylcylcohexane. While degradation of compounds eluting after 15 minutes 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 4.3 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*-C6 and *n*-C7 while *n*-C9 to *n*-C11 were eliminated. *R. globerulus* and the co-culture removed 50% of the *n*-C8, compared to *P. aeruginosa* alone which completely removed this compound. The original mixed culture exhibited higher maximum

Table 4.2. Effect of (a) perchloric acid and (b) autoclaving on volatile hydrocarbon sampling efficiency from biodegradation flasks using headspace SPME.

(a)

% acid (v/v)	pH	% recovery*
0	7.00	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

* compared to pH 7.00 sample.

(b)

Retention time range (minutes)	Elution temperature (degrees C)	% recovery**
0-2.5	35	90.1 (2.1)
2.5-5	35	95.3 (0.9)
5-7.5	35 to 53.8	98.9 (3.7)
7.5-10	53.8 to 72.5	96.5 (4.6)
10-12.5	72.5 to 91.3	98.8 (8.5)
12.5-15	91.3 to 110	98.7 (1.5)
total	35 to 110	93.4 (0.4)

** compared to unautoclaved controls (mean +/- SD, n = 3)

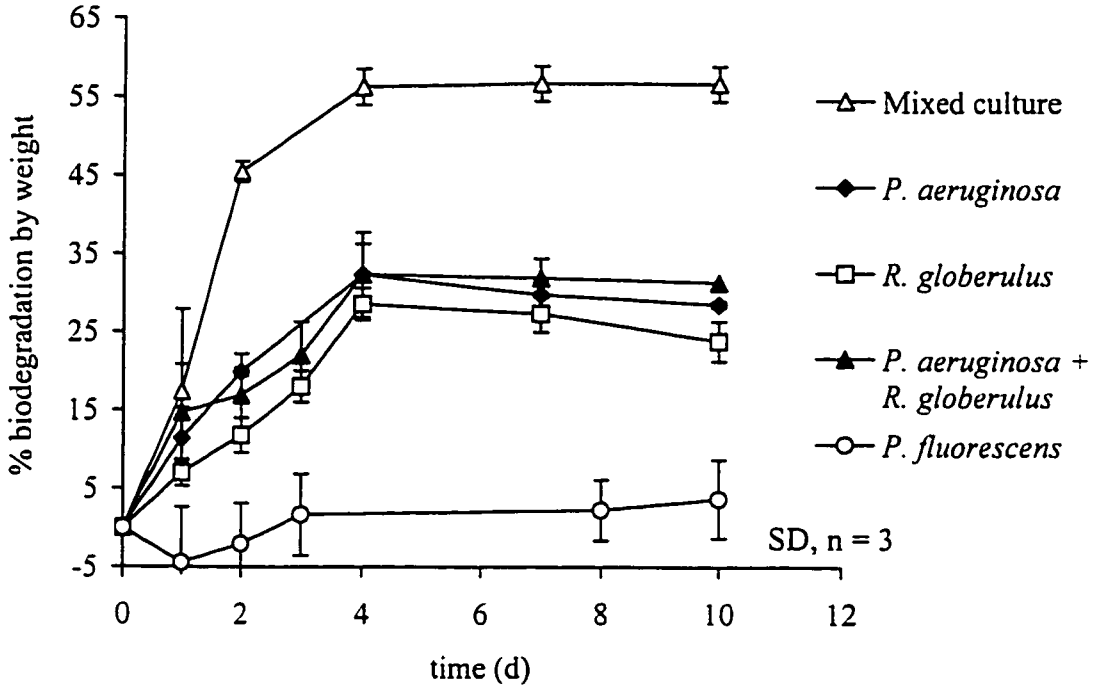


Figure 4.4. Volatile hydrocarbon biodegradation kinetics (C_5 to C_{11}) during growth on Bow River crude oil.

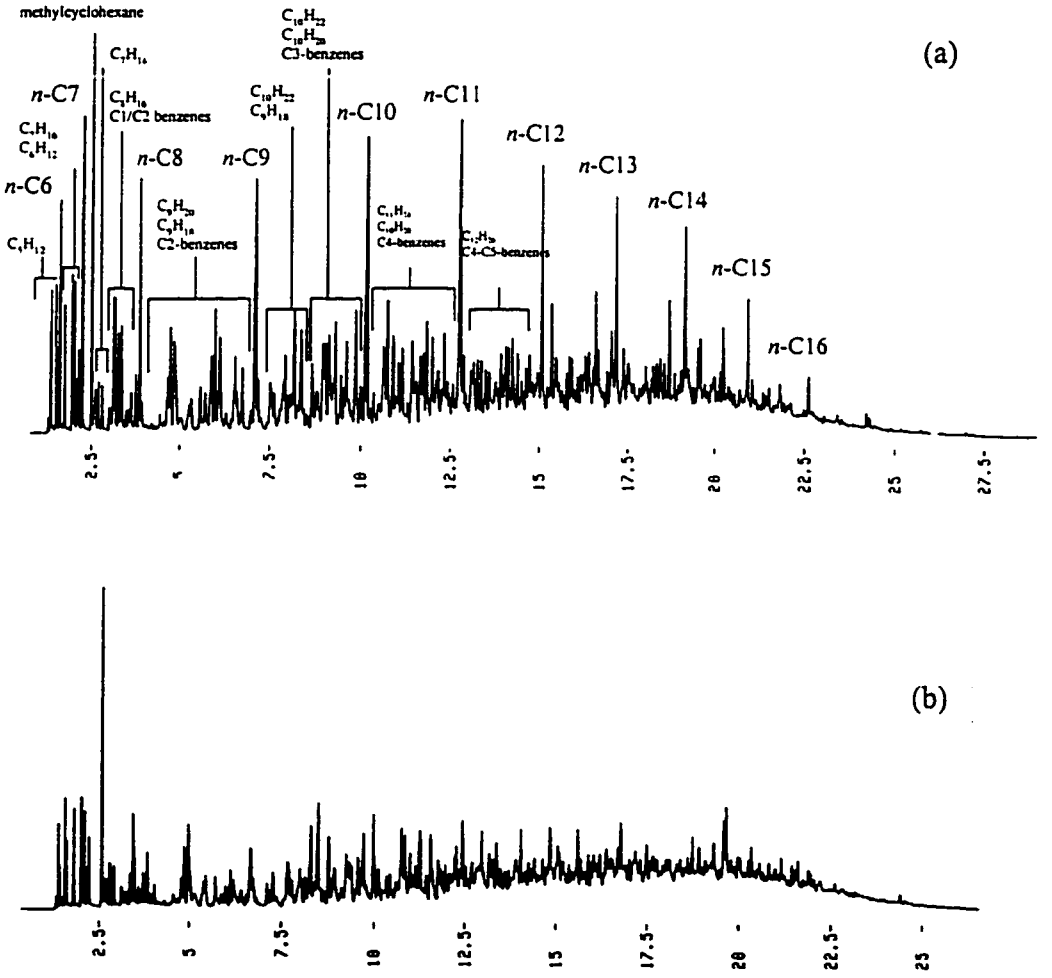
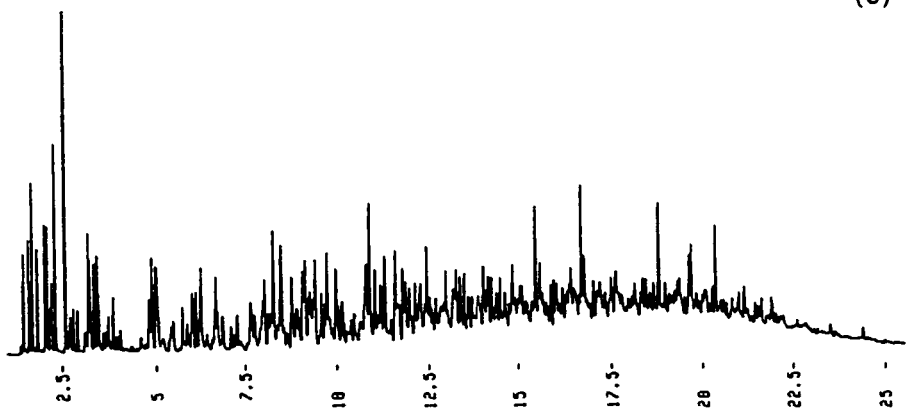
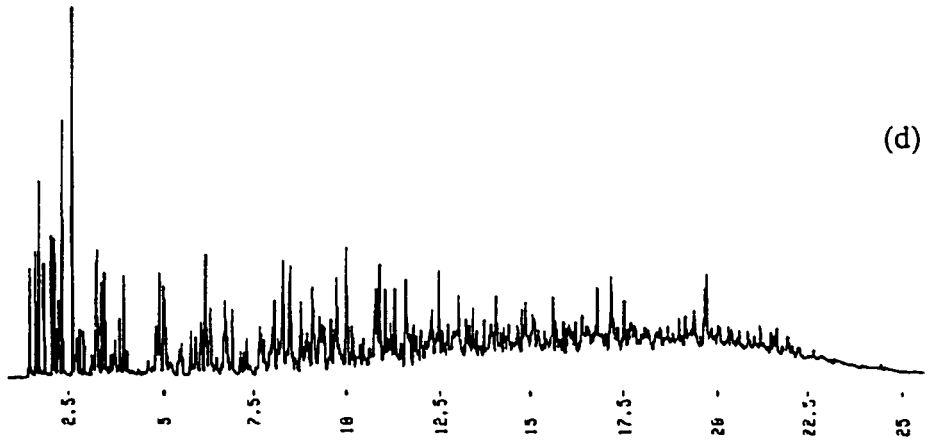


Figure 4.5. Representative chromatograms from SPME-GC-FID analysis of volatile hydrocarbons in the headspace of biodegradation flasks containing Bow River crude oil after a 10 day incubation. (a) Control, (b) Mixed culture, (c) *P. aeruginosa*, (d) *R. globerulus*, (e) Co-culture.

(c)



(d)



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(e)

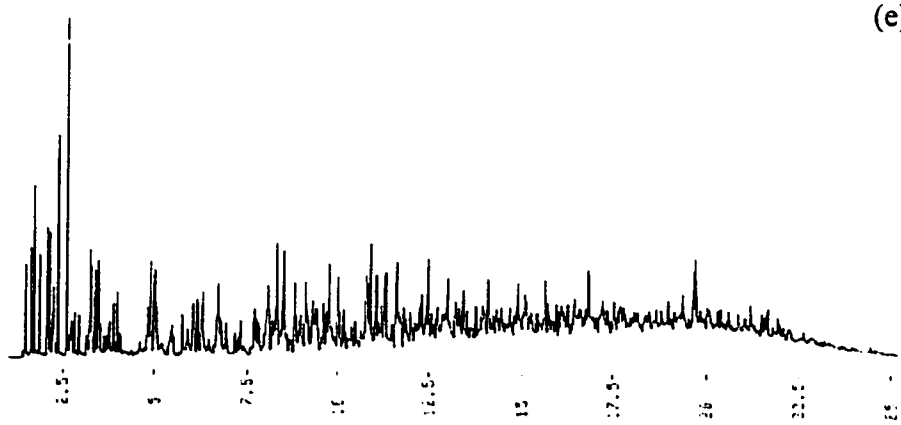

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Table 4.3 Extent and linear alkane (hexane to undecane) biodegradation rate for the mixed and pure cultures.

	Mass remaining (micrograms)[a]					
Culture	*n*-C6	*n*-C7	*n*-C8	*n*-C9	*n*-C10	*n*-C11
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)
Pseudomonas 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)
Pseudomonas 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)
Rhodococcus 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 (micrograms/h)[b]					
Culture	*n*-C6	*n*-C7	*n*-C8	*n*-C9	*n*-C10	*n*-C11
Control	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	0.83	0.54	0.22	0.06
Pseudomonas fluorescens	-	-	-	-	-	-
Rhodococcus 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 hours

removal rates for *n*-alkanes although a 24-hour lag period was observed that was not evident for pure cultures. 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.

4.5. 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 the 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, 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. (1996) 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-minute extraction was chosen in this study as the period of rapid analyte accumulation, during which 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 (Havenga and Rohwer 1999) 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 (Pawliszyn 1997) 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 (1999) 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 the biodegradation of diesel fuel and components (C_{14} to C_{17} *n*-alkanes, *p*-xylene and naphthalene) in water and soil have appeared (Ericksson et al. 1998; Ericksson et al. 1999). However, Ericksson et al. (1998) 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 demand greater SPME sensitivity with increasing carbon number (Martos et al. 1997).

Fortunately, the substrate in this study could be well-characterized and external calibrations 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 (Martos et al. 1997). 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 (Pawliszyn 1997).

Since repeated analysis of live samples was a requirement of the developed method internal standard addition was inappropriate due to the potential for biodegradation. 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 (Schwarzenbach et al. 1993). 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 rather than biodegradation. Ericksson et al. (1998) used open flasks loosely sealed with aluminum foil to monitor diesel fuel biodegradation and only detected 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 were 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 the biodegradation of mixed substrates due to the potential for greater metabolic diversity (Komukai-Nakamura et al. 1996; Sugiura et al. 1997). While the biodegradation data shown is for all of the material 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 or *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 (Martos and Pawliszyn 1997). 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. (1996) 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 75μm polyacrylate fibre (Whyte et al. 1998) which is more suitable for polar analytes (Pawliszyn 1997). 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 the 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 gas chromatography 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 the 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.

4.5.1. Acknowledgements

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

5.1. Abstract

Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on Bow River crude oil was investigated using solid phase microextraction (SPME). Substrate and inoculum treatments were examined in relation to C_5-C_{11} hydrocarbon degradation. In the substrate range tested, sealed batch cultures could be repeatedly analyzed over 14-day time course experiments. Up to 1600 mg/l biomass was tested without achieving high levels of volatile hydrocarbon partitioning and affecting analysis. Inoculum age rather than concentration had the most profound impact on biodegradation. Increasing the inoculum concentration from 0.63 to 63 mg/l doubled the degradation observed in the first 24-hours. When crude oil-grown inocula were in late log biodegradation reached 55-60%, and methylcyclohexane and other branched compounds eluting before *n*-C_8 were recalcitrant. Methylcyclohexane recalcitrance was correlated with reduced levels of hydrocarbon-degrading bacteria and physical loss of volatile hydrocarbons from inoculum flasks. Inoculum from early stationary phase cultures displayed a decreased lag phase prior to degradation, achieved 90% removal and eliminated most compounds up to C_{11} including methylcyclohexane. Cultures retained activity if grown in sealed flasks or in crude oil medium amended with yeast extract in lieu of yeast nitrogen base.

5.2. Introduction

The petroleum industry and the use of its derived products contribute significantly to volatile hydrocarbon release (Field et al. 1992). Emissions reduction has achieved international interest due to direct and indirect impacts on humans, plants and animals (Thijsse et al. 1999; Yang and Lo 1998). Biological methods to eliminate hazardous products and to achieve odour control (Leson and Smith 1997; Stehmeier et al. 1996) are an attractive alternative to phase transfer techniques such as activated carbon (Voice et al. 1992).

Novel biofiltration technologies will have application in systems currently being used

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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. (1999) 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. (1999) showed toluene removed from wastewater with a stripping tank could be biodegraded in series with a liquid bioreactor.

For mixed hydrocarbon streams, 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 co-substrate, while toluene degradation is reduced in the presence of styrene (Burack and Perry 1993). These interactions are further complicated in mixed culture systems. However, mixed cultures have the potential for a broader range of metabolic activity, and the interaction of two or more strains is often a prerequisite for growth and biodegradation (Lloyd-Jones and Trudgill 1989).

A rapid and accurate method for monitoring low molecular weight hydrocarbon biodegradation using solid phase microextraction (SPME) has been described (Van Hamme and Ward 2000) simplifying mixed-hydrocarbon substrate analyses. Here we examined factors affecting volatile hydrocarbon biodegradation by a mixed-culture in closed, batch systems. Specifically, substrate concentration and inoculum treatment effects were of interest.

5.3. Materials and Methods

5.3.1. Culture source and preparation

A mixed-bacterial culture isolated from petroleum-contaminated soil and maintained in cyclone fermentors on various hydrocarbon substrates was used (Van Hamme and Ward 1999). The culture was stored at -80°C in 20% w/v glycerol and pre-grown at 30°C (175 rpm orbital shaking) in 50 ml batch cultures on 2% w/v Bow River crude oil (density 0.905 g/ml; Imperial Oil, Sarnia, ON) with 0.625 g/l Igepal CO-630 (Rhône-Poulenc, Cranbury, NJ) and 1.0 g/l yeast nitrogen base (Difco Laboratories, Detroit, MI). In several experiments, the yeast nitrogen base was replaced with yeast extract (Difco Laboratories, Detroit, MI). The bacterial community has been described (Van Hamme et al. 2000).

5.3.2. Culture media, 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 $MnSO_4 \cdot 2\ H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $ZnSO_4 \cdot 7\ H_2O$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3 \cdot 16\ H_2O$, 0.01 g H_3BO_4 and 0.01 g $Na_2MoO_4 \cdot 2\ H_2O$.

Bow River crude oil was stored in Mininert capped (VICI Precision Sampling Inc., Baton Rouge, LA) bottles at 4°C. Unwashed culture (25 µl) was added to 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 Co., Reno, NV) immediately before sealing flasks with Teflon Mininert caps. Flasks were incubated at 30°C on an orbital shaker (175 rpm). Controls were prepared by pre-autoclaving oil or by acidification with 0.2% v/v perchloric acid (Van Hamme and Ward 2000). Triplicate flasks were repeatedly analyzed over time.

5.3.3. SPME-GC-FID and SPME-GC-MS analysis

As previously described (Van Hamme and Ward 2000), a 30 µm polydimethysiloxane (PDMS) fibre (Supelco, Oakville, ON) 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-minute equilibration time was used. A narrow bore (0.75 mm ID) injector sleeve (Supelco, Oakville, ON) and a fused silica column (Restek Rtx-5MS 5% diphenyl – 95% dimethyl polysiloxane, 30 m x 0.32 mm, 0.25 µm film thickness; Restek Corp., Bellefonte, PA) were used with a Shimadzu GC-14A (Shimadzu Corp., Kyoto, Japan) for FID analysis. For sample injections, the split valve was closed and the fibre held in place for three minutes (injector 225°C, detector 275°C). The column temperature program was as follows: 5 min at 35°C, 1°C/min to 40°C, 10°C/min to 225°C and hold for 1.5 minutes. 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 a HP 6890 gas chromatograph using helium as the carrier gas. SPME and GC temperature conditions were as described above and a HP-5 5% phenylmethylsiloxane column was used (30.0 m x 250 µm x

0.25 μm nominal). Compounds were identified by comparing spectra to a standard library (NIST Mass Spectral Search Program, Version 1.1a).

5.3.4. Calibration curves

Hydrocarbons in Bow River crude oil were quantified by GC-FID analysis of standards prepared in carbon disulfide (99.9%; BDH Inc., Toronto, ON) using the above temperature program extended to 300°C. FID response was standardized with a 5-point toluene calibration curve. Mass values for short retention time ranges were calculated and used to prepare SPME standard curves. For biodegradation samples analyzed with SPME, mass values were calculated from these curves and summed to yield a total mass.

Methylcyclohexane (99%+ anhydrous; Aldrich Chemical Co., Milwakee, 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 from 50 μg to 400 μg methylcyclohexane was prepared ($R^2 = 0.998$) and used for quantification.

5.3.5. 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 three days. Further incubation was not required prior to counting total heterotrophs as CFU/ml. Oil-degrading bacteria were enumerated using a 5-tube most probable number (MPN) approach with crude oil as the sole carbon and energy source (Van Hamme et al. 2000).

5.4. Results

Volatile hydrocarbon (C_5 to C_{11}) biodegradation by a mixed-bacterial culture during growth on crude oil was monitored using SPME. Compounds eluting above dodecane did not show a concentration response and compounds above hexadecane were not extracted (Table 5.1). Figure 5.1a is a chromatogram showing volatile hydrocarbons extracted from biodegradation flasks and a typical chromatogram following biodegradation by the mixed-bacterial culture is

shown in Figure 5.1b. Note that methylcyclohexane and other low molecular weight branched alkanes, cycloalkanes and aromatics were recalcitrant.

5.4.1. 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 5.2 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 early stages when biomass concentrations were low.

Volatile hydrocarbon removal kinetics were examined to verify that sufficient oxygen was available for aerobic biodegradation. With 9, 18 or 36 mg oil, biodegradation reached 60% after 5 days (Figure 5.3a). During the initial 24 hours, biodegradation rates were similar for all three concentrations at 10-14 μg/hour. The maximum degradation rates for the period between 24 and 48 hours were correlated to initial oil mass (Figure 5.3b). The total mass degraded was also linearly related to the initial substrate concentration.

The mixed-bacterial culture grown on crude oil was concentrated and inoculated at 0.63, 6.3, 63 mg/l, corresponding to 0.1, 1.0 and 10.0% (v/v) inoculum, respectively. In other experiments 0.1% v/v inoculum was used. Figure 5.4 shows that a 100-fold inoculum concentration increase doubled the degradation observed in the initial 24-hours. Degradation after 7 days was similar for all three cultures at 55-60%, and low molecular weight compounds were not removed.

5.4.2. Inoculum age

Biodegradation in cultures initiated with inocula from different stages of growth on 20 g/l Bow River crude oil in open flasks was examined. A growth curve has been published (Van Hamme

Table 5.1 Summary of gas chromatography conditions and SPME response curves for volatile hydrocarbons from Bow River crude oil

Retention time (min)	Elution temperature (degrees Celcius)	Mass in 25 ml oil (μg)	Analyte range (volume oil, μl)	Response curve (y = response; x = μg)	R-squared value
0.0 to 2.5	35	856	2.5 to 35	y = 29.74 x + 3195.4	0.970
2.5 to 5.0	35	587	2.5 to 35	y = 182.2 x - 440.6	0.994
5.0 to 7.5	35 to 37.5	125	2.5 to 35	y = 515.9 x + 4554.4	0.995
7.5 to 10.0	37.5 to 40	117	2.5 to 35	y = 552.75 x + 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			

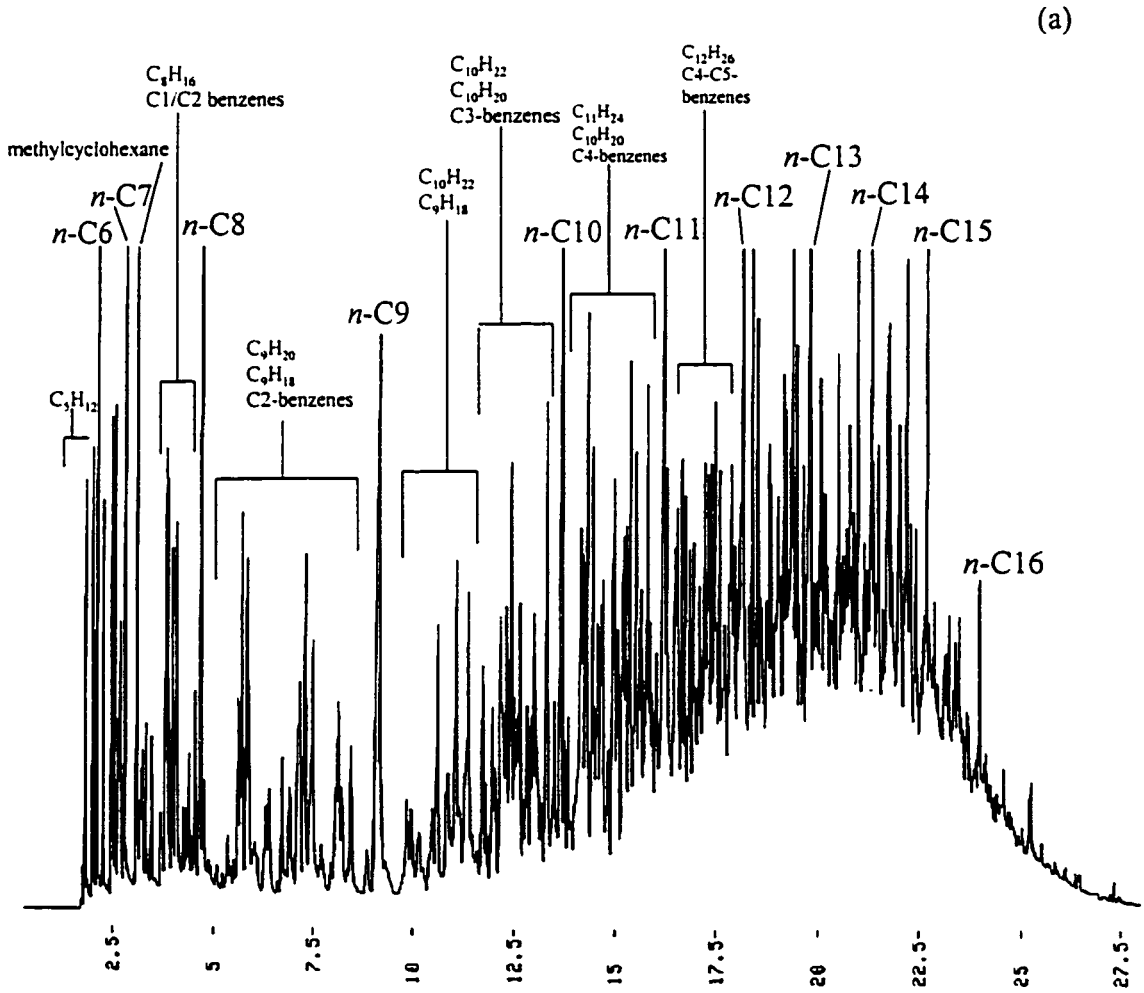
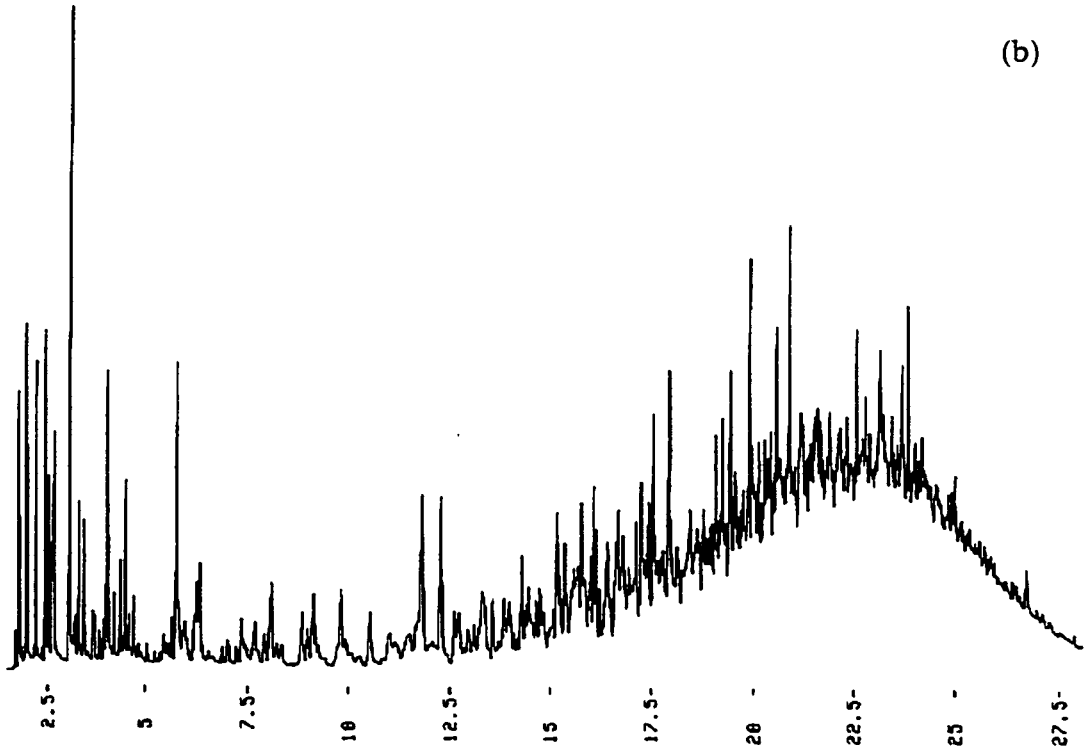


Figure 5.1 (a) Headspace SPME-FID chromatogram over a biodegradation flask containing Bow River crude oil. (b) Representative chromatogram following biodegradation by 7-day old mixed-bacterial inoculum.

(b)



Cont.

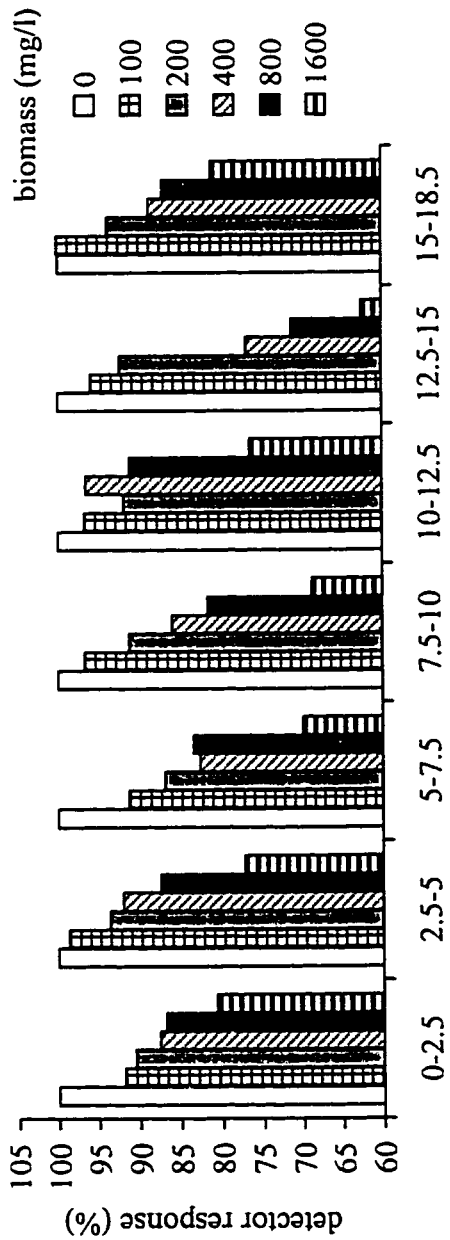


Figure 5.2 Volatile hydrocarbon partitioning by mixed culture biomass.

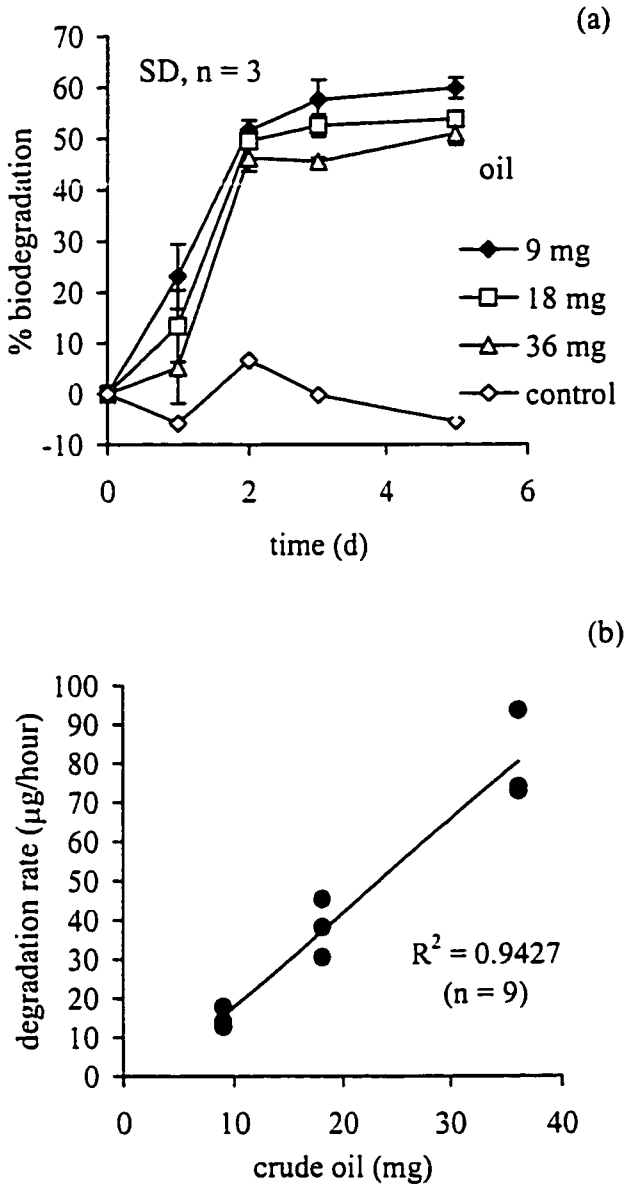


Figure 5.3 (a) Crude oil concentration effect on volatile hydrocarbon biodegradation kinetics. (b) Relationship between oil concentration and degradation rate between 24 and 48 hours.

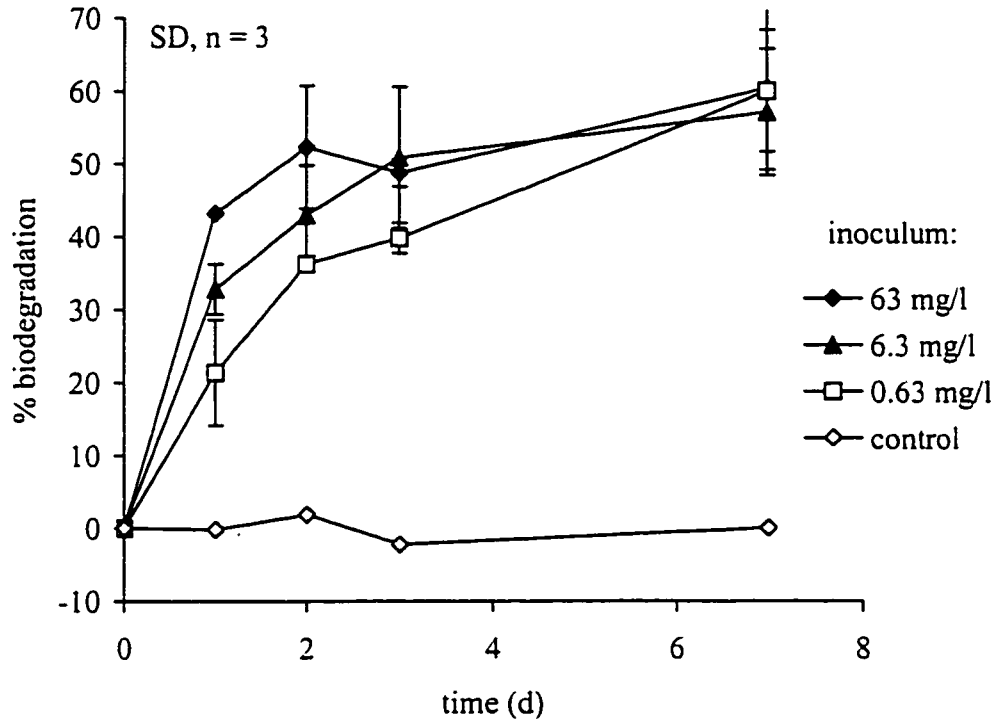


Figure 5.4 Inoculum concentration effect on volatile hydrocarbon biodegradation kinetics during growth on crude oil.

and Ward 1999) and was characterized by 24 hours of rapid growth (from 10^8 to 10^9 CFU/ml) followed by a decline to stationary phase between 24 and 48 hours. 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. Degradation kinetics are shown in Figure 5.5a. Flasks initiated with early stationary or stationary phase inocula displayed a 24-hour lag period followed by 24 hours of rapid degradation, ultimately removing 60% of the volatile hydrocarbons. The lag period prior to degradation was eliminated for both lag and late log inocula with 30% degradation after 24 hours. After 4 days, activity in flasks initiated with late log inoculum slowed and ultimately reached 70% degradation. Conversely, flasks initiated with lag phase inoculum were active until day 7 with greater than 90% removal. The lag phase inoculum was diluted 50 and 500 times without loss of activity. A sample chromatogram (Figure 5.5b) shows that the lag phase inoculum removed all compounds eluting below 37.5°C.

Volatile hydrocarbon removal kinetics for 2.5 minute retention time slices were plotted and used to calculate the maximum degradation rate and final extent of degradation. For lag phase inoculum, degradation rates between 0 and 24 hours were calculated while for stationary phase (7-day) inoculum the region between 24 and 48 hours was used. When normalized for initial concentration, the maximum degradation rate increased with increasing retention time and, hence, molecular weight. This relationship was stronger for lag phase inoculum (Figure 5.6a). Conversely, an inverse relationship between the fraction degraded and retention time was observed for lag-phase inoculum (Figure 5.6b). Degradation by lag phase inoculum was twice that of stationary phase inoculum for compounds eluting below 37.5°C (7.5 minutes). Methylcyclohexane, recalcitrant with inocula beyond lag phase, was removed after a 4-day acclimation period (Figure 5.7a).

Growth curves of total heterotrophs and hydrocarbon-degrading bacteria were prepared for cultures initiated with lag phase and stationary phase (7-day) inocula. Figure 5.7b shows that total heterotrophic growth was similar in both culture systems. Conversely, the initial level

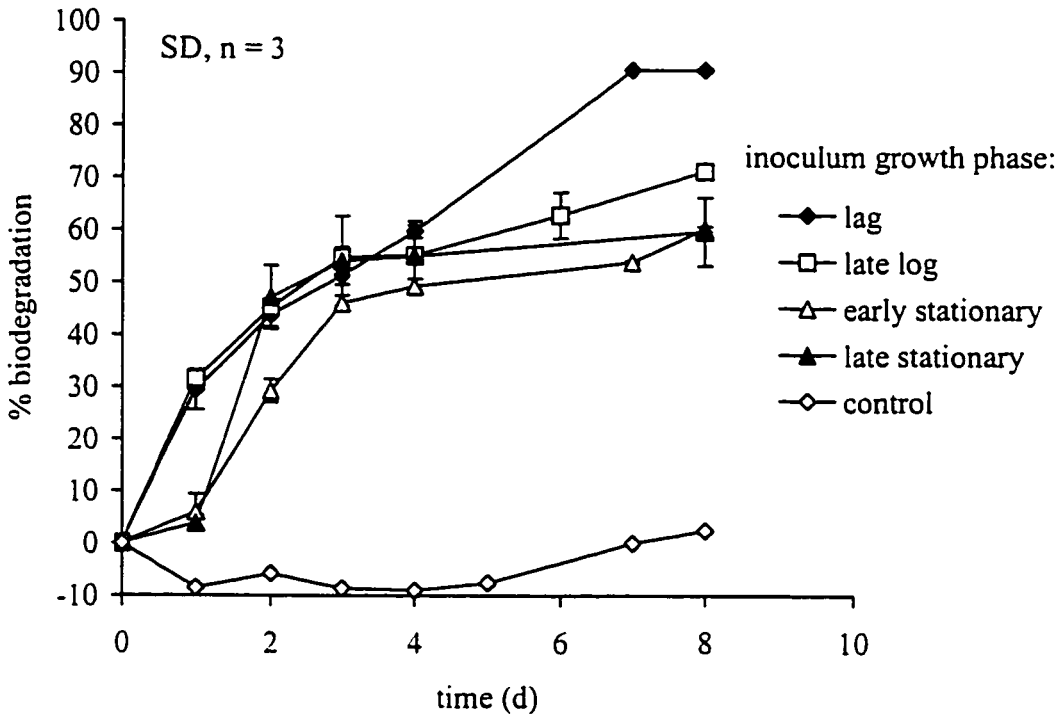
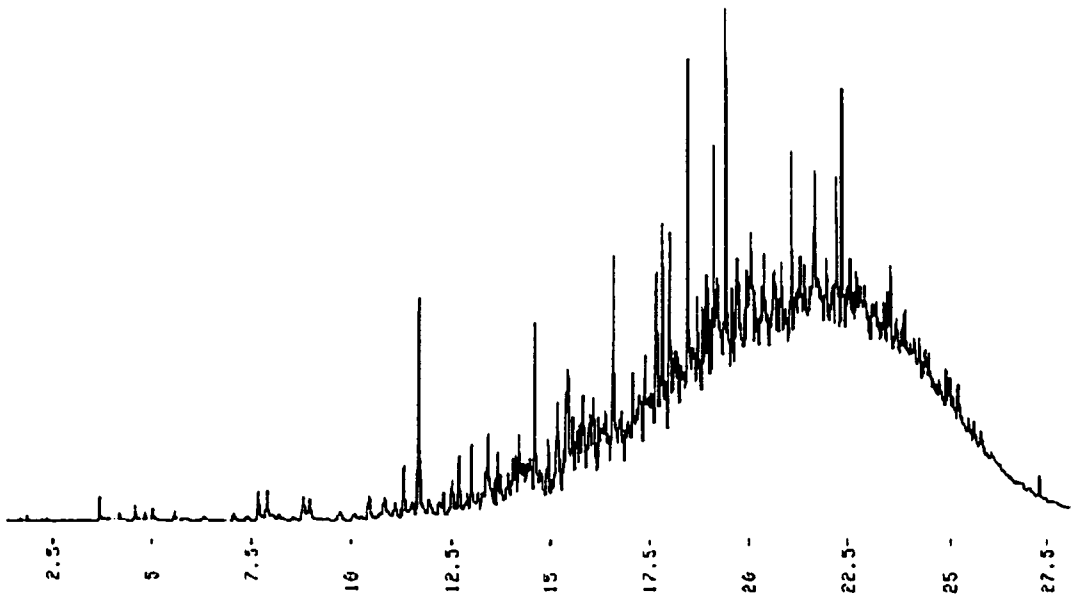


Figure 5.5 (a) Inoculum age (0.1% v/v) effect on volatile hydrocarbon biodegradation kinetics during growth on crude oil. (b) Chromatogram following biodegradation by lag phase inoculum.

(b)



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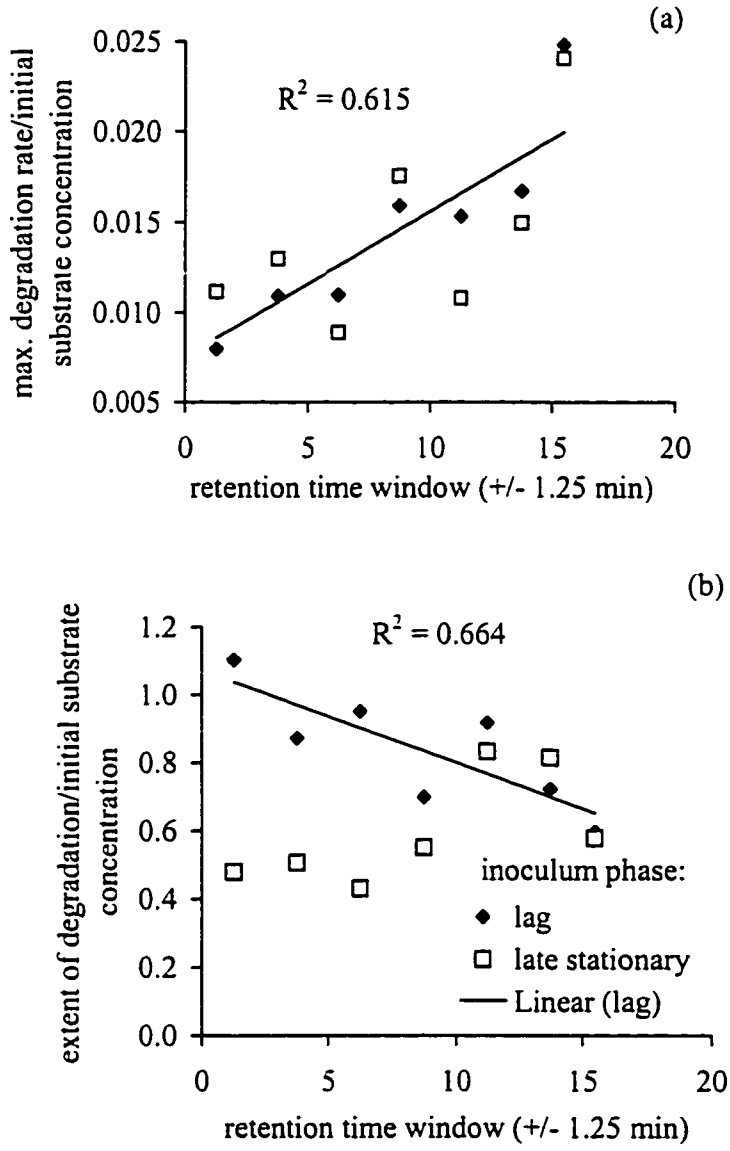

Figure 5.6 (a) Relationship between retention time and maximum degradation rate for lag phase (0-hour) and stationary phase (7-day) inoculum. (b) Relationship between retention time and biodegradation for lag phase (0-hour), and stationary phase (7-day) inoculum.

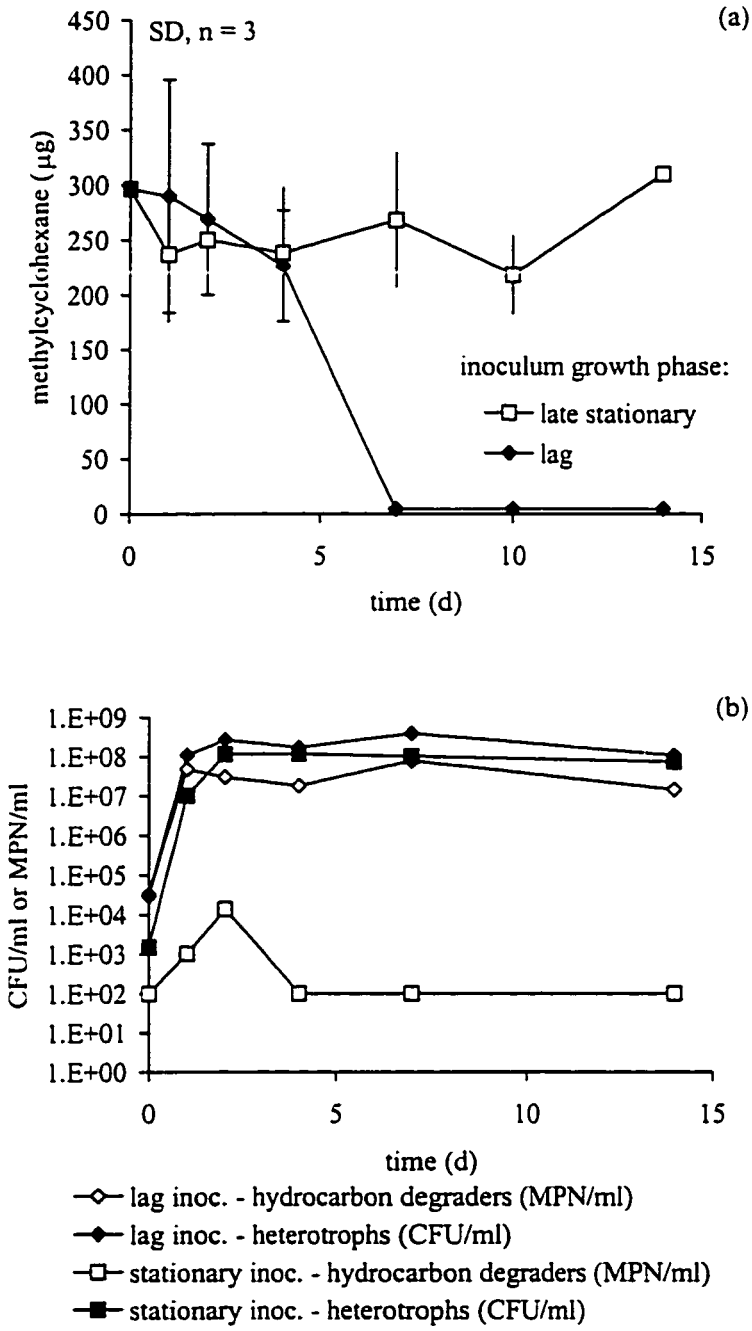

Figure 5.7 (a) Methylcyclohexane removal by lag phase (0-hour), and stationary phase (7-day) inoculum. (b) Inoculum age effect on oil-degrading bacteria.

of hydrocarbon-degraders was less than 50% for the stationary phase inoculum and declined to low levels after reaching 10^4 MPN/ml in 2 days. The percentage of hydrocarbon-degraders was higher with lag phase inoculum and the growth pattern followed that of the total heterotrophs with stable numbers (10^7 MPN/ml) throughout the 14-day time course.

5.4.3. Preservation of enhanced activity

Enhanced biodegradation, characterized by a short lag phase prior to degradation and methylcyclohexane removal, was not recovered when the original inoculum flask was subcultured after 7 days to produce a second inoculum culture. A 48-hour lag period prior to degradation was evident for cultures initiated with lag phase inoculum while the period preceding degradation decreased and was eliminated with late log (24-hour) and early stationary (48-hour) inocula, respectively (Table 5.2, row A). In each case, degradation reached approximately 60%. Freezing the stationary phase (7-day) inoculum culture with 20% glycerol did not result in recovery of enhanced biodegradation activity.

Sealed biodegradation cultures initiated with lag phase inoculum fed with additional crude oil or subcultured after 14 days retained enhanced activity (Table 5.2, row B). Again, culture aliquots stored at -80°C in 20% w/v glycerol and grown in open flasks lost enhanced activity after prolonged incubation (Table 5.2, row C). Boosting the crude oil concentration in open inoculum flasks from 20 g/l to 100 g/l was not effective for retaining enhanced activity (Table 5.2, 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 hours (Figure 5.8). Enhanced activity was retained in open inoculum cultures if yeast nitrogen base was replaced with yeast extract (Table 5.2, row E). Adding either of these to biodegradation flasks had no effect on biodegradation kinetics.

Table 5.2 Inoculum preparation effect on the preservation of enhanced volatile hydrocarbon degradation capabilities

Inoculum	Inoculum age (d)	Degradation lag (d)	% 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	fedbatch	0	90.0 (0.4)
initiated with lag phase inoculum	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	lag (0)	0	89.6 (0.0)
inoculum flask	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)

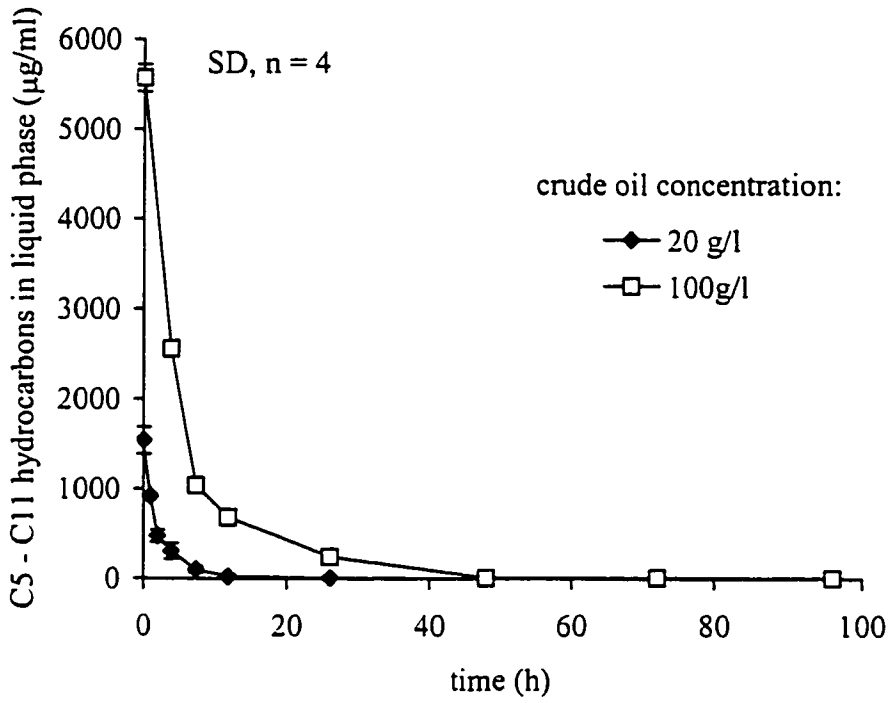

Figure 5.8 Volatilization of C_5 to C_{11} hydrocarbons from open inoculum flasks containing 20 g/l or 100 g/l Bow River crude.

5.5. Discussion

The SPME methodology used for volatile hydrocarbon quantification allowed for rapid and repeated analysis of live cultures growing on Bow River crude oil. As previously observed (Van Hamme and Ward 2000), 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. Theoretical oxygen demand calculations (page 84), the observation that the degradation rate and extent increased linearly over the substrate range used, and results from the fed-batch experiment verified system validity. Increasing substrate concentrations did reduce the fraction degraded in the first 24 hours, potentially a toxicity effect as volatile hydrocarbons may negatively affect microbial activity by damaging membrane integrity or altering enzyme structure and function (Sikkema et al. 1995).

High biomass levels were required to affect low molecular weight hydrocarbon partitioning. Had alterations in analyte partitioning occurred, quantification with the prepared SPME standard curves would have been inaccurate (Martos and Pawliszyn 1997; Pawliszyn 1997). On the other hand, to reduce volatilization, 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 (Stratton et al. 1999) have been shown to have high sorptive capacity for phenanthrene, regardless of biodegradative capabilities (Stringfellow and Alvarez-Cohen 1999). Binding would increase with molecular weight (Schwartzenbach 1993) and may reduce bioavailability of high molecular weight substrates. However, adding recalcitrant sorbing agents (Laor et al. 1999; Ortego-Calvo and Saiz-Jimenez 1998) or sorbing agents acting as co-substrates (Rhykerd et al. 1999) can enhance crude oil biodegradation. Further work in this area is warranted and SPME would be a suitable methodology (Doll et al. 1999).

The mixed culture used in this study was previously observed to be inactive against methylcyclohexane and other branched hydrocarbons eluting before *n*-C_8 (Van Hamme and Ward 2000). Increasing the inoculum concentration did increase degradation rates high at

levels, 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 mid- to late-log inocula, increasing lag times and methylcyclohexane recalcitrance were observed. The expected decrease in lag time prior to degradation with increasing inoculum age was observed in subcultures of the original inoculum flask but methylcyclohexane remained recalcitrant. This indicates that the metabolic capabilities or community makeup of the culture had changed. Indeed, the proportion of hydrocarbon-degrading bacteria declined in inoculum cultures prepared in open systems translating to unstable populations in biodegradation flasks.

Apparently, selection for heterotrophs capable of growth on metabolites of hydrocarbon-degrading organisms took place in open inoculum flasks. Methylcyclohexane degradation was not induced by freezing or the presence of glycerol. Cultures grown in closed flasks retained biodegradation capabilities indicating that stable selection depended on low molecular weight compounds being present and available for uptake. Lloyd-Jones and Trudgill (1989) showed that a *Rhodococcus* sp., *Flavobacterium* sp. and a *Pseudomonas* sp. grow on branched cyclohexanes, alcohols, ketones and acids only when inoculated as a co-culture. They also noted that, if pre-cultured in nutrient broth, growth on methylcyclohexane as the sole carbon and energy source continually declines over time and is eliminated after 72-hours. This phenomenon correlated to the loss of two unidentified plasmids from the consortium (Lloyd-Jones and Tudgill 1989). 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. Positive effects of yeast extract on the degradation of C_{10}-C_{21} alkanes, branched alkanes and a substituted cyclohexane in diesel fuel by a psychrotrophic *Rhodococcus* sp. has been observed (Whyte et al. 1998).

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 described for biofilters treating mixtures of toluene, ethylbenzene and xylene (Veiga et al. 1999). Considering the treatment endpoint and residence time of the most recalcitrant compound is necessary for good process design. Biofilters (Yeom and Yoo 1999) or design of

batch systems with volatile hydrocarbon recycling may allow for more effective biodegradation during heterogeneous oily waste treatment. Berthe-Corti et al. (1998) 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 long-term inoculum preparation and maintenance effects on volatile hydrocarbon biodegradation by a mixed-bacterial culture were illustrated. To retain methylcyclohexane-degrading capabilities, cultures must be maintained in a closed system or supplemented with yeast extract. The exact mechanism for this phenomenon has yet to be elucidated. Future work will focus on developing methods for retaining volatile compounds in the aqueous phase of biodegradation systems using cultures or partitioning agents with high sorption coefficients.

5.5.1. Acknowledgements

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6. Physical and metabolic interactions of *Pseudomonas* sp. JA5-B45 and *Rhodococcus* sp. F9-D79 during growth on crude oil and the effect of a chemical surfactant thereon

6.1. Abstract

Physical and metabolic interactions between *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 were examined during growth on Bow River crude oil. The effects of a non-ionic chemical surfactant were also evaluated. Phase contrast microscopy and staining revealed that strain F9-D79 grew attached to the oil-water interface and produced a mycolic acid-containing capsule. Crude oil emulsification was observed and both emulsification and surface activity were associated with the cellular fraction. Strain JA5-B45 grew in the aqueous phase, and was unable to emulsify oil but did excrete an emulsifier capable of forming kerosene-water emulsions. In co-culture a more stable emulsion was formed and strain JA5-B45 had an affinity for the capsule produced by strain F9-D79. Total petroleum hydrocarbon (TPH) biodegradation was slightly enhanced. Chemical surfactant removed strain F9-D79 from the oil-water interface resulting in a linear alkane degradation rate, and cells grew dispersed in the aqueous phase as 0.5-μm cocci rather than 2.5-μm rods. Surfactant increased TPH degradation by strain JA5-B45 from 4 to 22% and included both saturates and aromatics. In co-culture, TPH degradation increased from 13 to 40% upon surfactant addition. Culture pH normally increased from 7.0 to between 7.5-8.5 although surfactant addition to strain F9-D79 cultures resulted in a pH drop to 5.5. A dual role for chemical surfactant in the co-culture is suggested: 1.) To directly improve hydrocarbon-uptake by *Pseudomonas* sp. JA5-B45 through emulsification, and 2.) to prevent *Rhodococcus* sp. F9-D79 from adhering to the oil-water interface, indirectly increasing hydrocarbon availability.

Van Hamme, J.D., and Ward, O.P. Submitted to Appl. Environ. Microbiol.

6.2. Introduction

Biodegradation of heterogeneous petroleum waste streams typically relies on mixed-microbial cultures. Physical, metabolic and community interactions contribute to the dynamic nature of these systems. For example, populations employing different hydrocarbon-uptake modes would be represented in a community. The uptake modes include: uptake of water-soluble hydrocarbons, direct adherence to hydrocarbon-water interfaces, and biosurfactant-mediated micellar transfer (Bouchez-Naïtali et al. 1999; Hommel 1990). As a result, methods to enhance hydrocarbon-solubility, such as addition of chemical or biological surfactant, will have differential effects on members of a given mixed culture (Bruheim et al. 1999; Van Hamme et al. 2000).

Pseudomonas spp. and *Rhodococcus* spp. are often isolated from hydrocarbon-contaminated sites and hydrocarbon-degrading cultures (Bouchez- Naïtali et al. 1999; Lloyd-Jones and Trudgill 1989). These two genera have a broad affinity for hydrocarbons and are able to degrade alkanes, alicyclics, thiophenes and aromatics (Bock et al. 1996; Folsom et al. 1999; Koike et al. 1999; Oldfield et al. 1998; Paje et al. 1998; Shumacher and Fakoussa 1999). In addition, both produce a range of biosurfactants (Desai and Banat 1997) such as rhamnolipids by *Pseudomonas* species (Sim et al. 1997) and trehaloselipids by *Rhodococcus* species (Bredholt and Eimhjellen 1999; Kretschmer et al. 1982; Ramsay et al 1988; Vogt Singer and Finnerty 1990). Typically, *Rhodococcus* spp. are more hydrophobic and display a higher affinity for hydrocarbon-water interfaces than *Pseudomonas* spp. (Stringfellow and Alvarez-Cohen 1999), revealing different modes of hydrocarbon uptake. Co-cultures of *Pseudomonas* sp. and *Rhodococcus* sp. have been reported to mineralize chloronitrobenzenes (Park et al. 1999). Ko and Lebault (1999) illustrated that *R. equi* P1 competitively used hexadecane in a hydrocarbon mixture, reducing hexadecane-dependent co-oxidation of decalin by *P. aeruginosa* K1.

Previously, strains isolated form a mixed-bacterial culture growing on crude oil and crude oil fractions (Van Hamme et al. 2000) were screened for crude oil biodegradation and emulsification abilities. Two strains identified by fatty acid analysis were particularly

interesting. *Rhodococcus* sp. strain F9-D79 forms excellent, though transient, crude oil-water emulsions between 24 and 48 hours of incubation. *Pseudomonas* sp. strain JA5-B45 does not emulsify oil but efficiently degrades crude oil in the presence of chemical surfactant.

Given these observations, it was hypothesized that a co-culture of the two organisms may enhance biodegradation through the combination of superior emulsification and degradation capabilities. In this investigation, the co-culture was used as a surrogate to study metabolic and physiological interactions that may occur in a mixed culture system, and to determine how a chemical surfactant may change those interactions.

6.3. Materials and Methods

6.3.1. Culture media and substrates

The medium used for culture selection and biodegradation of Bow River crude oil, contained per litre: 1.0 g KH_2PO_4, 1.5 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g Na_2CO_3, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 0.005 g $FeSO_4$, 0.02 g $MnSO_4$ and 1.0 ml of trace metals solution. The trace metals solution contained per litre: 0.0144 g $ZnCl_2 \cdot 4\ H_2O$, 0.012 g $CoCl_2$, 0.012 g $Na_2MoO_4 \cdot 2\ H_2O$, 1.9 g $CuSO_4 \cdot 5\ H_2O$, 0.05 g H_3BO_4 and 35 ml HCl. Yeast extract (Difco Laboratories, Detroit, MI) was added at 1.0 g/l unless otherwise noted and the initial pH was 7.0.

6.3.2. Substrates and purification

Bow River crude oil (density 0.905 g/ml; 21.7% volatiles, 24.5% saturates, 42.2% aromatics, 5.8% resins, 5.5% asphaltenes) was used (Imperial Oil, Sarnia, ON) for biodegradation experiments. The oil was stored at 4°C in a glass bottle sealed with a Mininert cap. No changes in oil composition were noted by SARA and solid phase microextraction analysis (Van Hamme and Ward 1999; Van Hamme and Ward 2000) over the course of the study. The nonlyphenol ethoxylate surfactant, Igepal CO-630 (Rhône-Poulenc, Cranbury, NJ), was used without further purification. Hexadecane (99% Sigma, St. Louis, MO), D-glucose and sodium acetate (BDH Ltd., Toronto, ON), trypticase soy broth (30 g/l, Beckton-Dickinson, Cockysville, MD) and diesel fuel from a local filling station were also used as growth substrates.

6.3.3. Culture source and maintenance

Isolates were obtained during a study of the community structure (Van Hamme et al. 2000) of a mixed-bacterial culture obtained from petroleum-contaminated soil (Van Hamme and Ward 1999). Pure cultures were streaked onto trypticase soy agar (40 g/l soybean-casein digest agar, Beckton-Dickinson, Cockysville, Md.) plates and purity verified by Gram stain and colony morphology. After identification by MIDI fatty acid analysis (Van Hamme et al. 2000), isolates were stored on beads at -80°C using the Microbank bacterial preservation system (Pro-Lab Diagnostics, Richmond Hill, ON). The isolates were identified as *P. aeruginosa* and *R. globerulus* and are referred to here as *Pseudomonas* sp. strain JA5-B45 and *Rhodococcus* sp. strain F9-D79. Prior to initiating a degradation experiment, a single frozen bead was streaked onto a TSA plate and grown for 3 days at 30°C. A single colony was used to inoculate each flask as *Rhodococcus* sp. strain F9-D79 could not be resuspended precluding optical density measurements.

6.3.4. Crude oil biodegradation

Bow River crude oil biodegradation with and without 0.625 g/l Igepal CO-630 was carried out in triplicate 250-ml Erlenmeyer flasks containing 50 ml medium and 1.0 g/l yeast extract. Flasks were incubated at 30°C (175 rpm orbital shaking) prior to extracting the entire contents with 50 ml of dichloromethane (DCM). The extraction process included centrifugation (International Equipment Co., Needham Heights, MA) in four 30-ml Corex tubes at 12 000 *g* to break oil-in-water emulsions followed by dehydrating the oily solvent phase over granular anhydrous sodium sulfate. Extracts were concentrated under vacuum (Haake Buchler, Saddlebrook, NJ) and transferred to pre-weighed 50-ml beakers. After drying to constant weight in a fume hood, the total non-volatile hydrocarbon level was measured gravimetrically and compared to both uninoculated and killed (0.1% v/v perchloric acid) controls. Following extraction, supernatant pH values were measured with an Orion SA 520 pH meter (Orion Research Inc., Boston, MA).

6.3.5. SARA analysis and GC-FID analysis of the saturate fraction

Saturate, aromatic, resin and asphaltene (SARA) fractionation from crude oil is described in Chapter 2 and Van Hamme and Ward (1999). Prior to crude oil extraction from biodegradation flasks, 50 µl of 28 µl/ml squalane and 10 µg/ml fluoranthene (Sigma, St. Louis, MO) in DCM was added as internal standards of the saturate and aromatic fractions.

Biodegradation of Bow River crude saturates was evaluated by GC-FID (Shimadzu GC 14A, Shimadzu Corp., Kyoto, Japan). Following SARA analysis, the saturate fraction was dissolved in 5 ml HPLC grade hexane (EM Science, Gibbstown, NJ) in a volumetric flask. Samples were then injected with an autoinjector (Shimadzu AOC-17, Shimadzu Corp., Kyoto, Japan) onto a fused silica column (Restek Rtx-5MS, 5% diphenyl – 95% dimethyl polysiloxane, 30 m x 0.32 mm, 0.25 µm film thickness; Restek Corp., Bellefonte, PA). The GC was operated with a split of 60 ml/min and purge of 5.5 ml/min. Helium was used as a carrier gas (10 ml/min) with nitrogen as makeup (40 ml/min). The injector and detector temperatures were 280°C and 310°C, respectively. The oven temperature program was as follows: 80°C for 3 minutes, 5°C/min to 315°C, final hold for 2 minutes. A Shimadzu CR601 Chromatopac (Shimadzu Corp., Kyoto, Japan) was used for peak area measurements. Standards were prepared from known concentrations of purified Bow River saturates. Standard mixtures (Restek Corp., Bellefonte, PA) of known saturated (Diesel Range Organics) hydrocarbons were analyzed to judge elution times. Total saturates from C_{16} to C_{30} could be readily detected in linear range from 1.3 to 21.0 mg/ml.

6.3.6. Surface tension

Surface tension measurements were made with a CSC-du Noüy ring tensiometer (Central Scientific Company, Fairfax, VA) equipped with a platinum-iridium ring. Whole broth surface tensions were measured prior to centrifuging cultures at 12 000 *g* for 10 minutes. Surface tensions of cell-free supernatants and resuspended cell pellets (50 mM phosphate buffer, pH 7.0) were measured. Five readings per sample, and of fresh medium with carbon source, were made.

6.3.7. Staining and microscopy

A Nikon Optiphot Phase Contrast Microscope (Nippon Kogaku K. K., Japan) was for examination of live cultures. India Ink was used as a negative stain for capsule visualization, and mycolic acids detected with the Ziehl-Neilson stain (Barrow and Feltham 1993). Live cultures were stained with a LIVE *Bac*Light Bacterial Gram Stain Kit (Molecular Probes, Eugene, OR) and visualized with a fluorescence microscope.

6.3.8. Surfactant toxicity – biomass measurement

Surfactant toxicity to *Rhodococcus* sp. F9-D79 was evaluated by quantifying biomass production following 5 days of growth at 30°C on TSB supplemented with a range of Igepal CO-630 concentrations. Cells recovered by centrifugation were washed three times with deionized water prior to drying at 100°C for 24 hours.

6.3.9. Emulsification assay

Five millilitres of culture supernatants or resuspended cells (50 mM phosphate buffer, pH 7.0) were vortexed (S/P Vortex Mixter, American Scientific Products, McGaw Park, IL) with 5 ml of kerosene (Trileaf Distribution, Toronto, ON) at high speed for 60 seconds in 10 ml-graduated cylinders. Kerosene was added dropwise and measurements were recorded after 1 hour and all emulsions were stable for at least 24 hours. The volume of the middle phase was normalized to the total volume to calculate percent emulsification. Cultures were grown on 2% w/v Bow River crude oil for the assay and cells were recovered from the solvent-water interface of DCM extracted samples.

6.4. Results

6.4.1. Co-culture effects on crude oil emulsification

The physical state of Bow River crude oil was observed in shake flasks containing *Rhodococcus* sp. F9-D79, *Pseudomonas* sp. JA5-B45 or a co-culture of the two (Figure 6.1a).

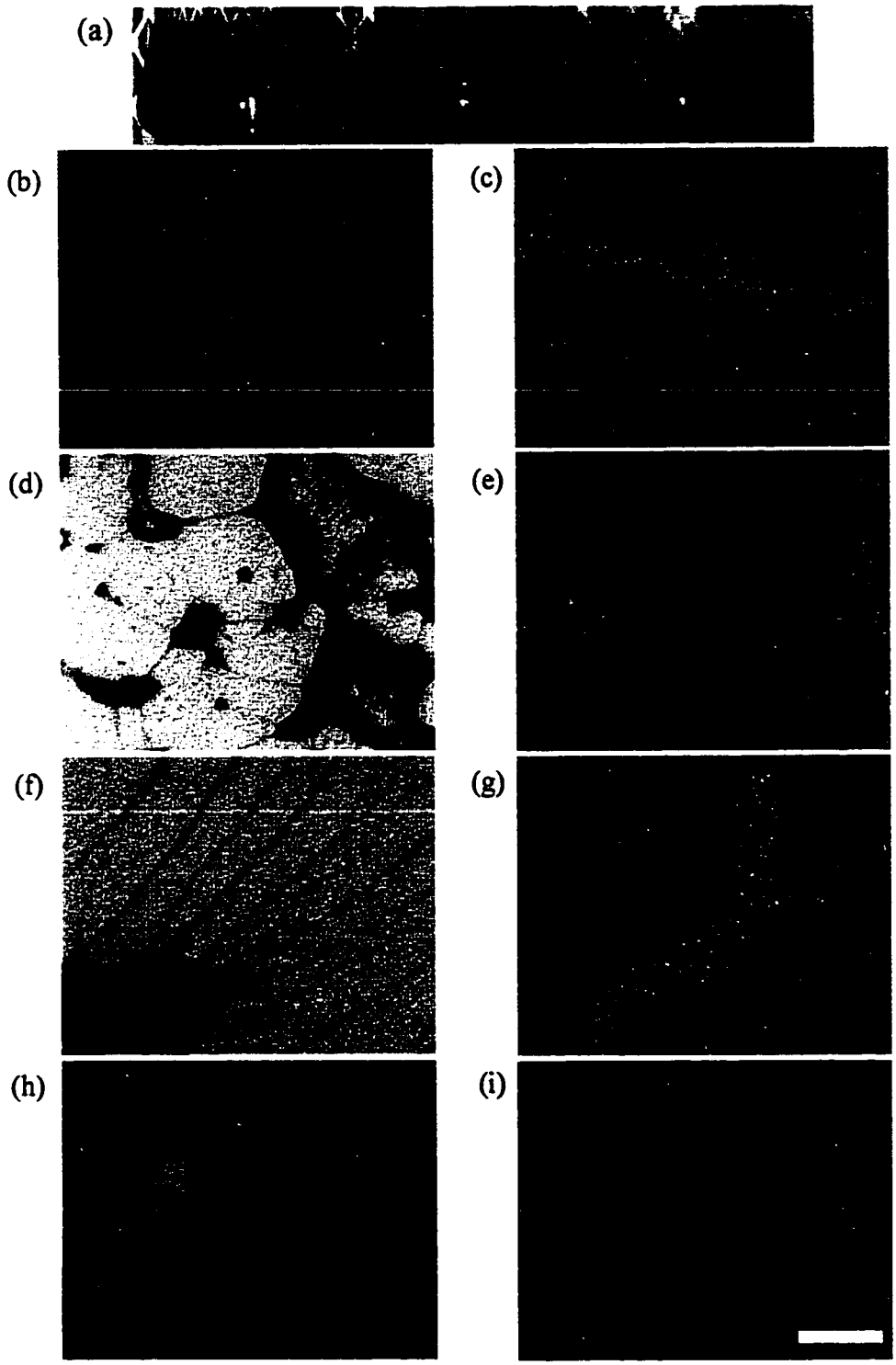


Figure 6.1

(a) Photograph of 48-hour crude oil cultures inoculated with, from left to right: *Rhodococcus* sp. F9-D79; co-culture; and *P. aeruginosa*. (b-i) Photomicrographs: (b,d,f) – Gram stains; (c,e,g) – phase contrast; (h,i) – negative stain; (b,c) – *P. aeruginosa*; (d,e,h,i) – *Rhodococcus* sp. F9-D79; (f,g) – co-culture.

The F9-D79 culture passed through a stage (24-48 hours) where emulsification occurred but the co-culture produced a more stable emulsion. The lack of turbidity in the F9-D79 culture sharply contrasts the turbid, non-emulsified, JA5-B45 culture.

6.4.2. Microscopic characterization of pure and co-cultures

Rhodococcus sp. strain F9-D79 grew adhered to oil droplets while *Pseudomonas* sp. strain JA5-B45 grew in the aqueous phase of crude oil cultures. Figure 6.1b shows Gram negative rods of strain JA5-B45 and Figure 6.1d shows clumps of oil associated Gram positive F9-D79 rods. Strands of fibrous material extended between strain F9-D79 clumps and, in co-culture, strain JA5-B45 aligned with this material (Figure 6.1f). Phase contrast microscopy revealed opaque JA5-B45 rods, motile and non-motile, in the aqueous phase of live, crude oil-grown cultures (Figure 6.1c). Non-motile F9-D79 cells growing in clumps appeared white under phase contrast and accumulated solely on oil droplets (Figure 6.1e). In co-culture, strands of strain JA5-B45 cells remained in the aqueous phase adjacent to adherent F9-D79 cells (Figure 6.1g). Live fluorescent Gram stain probes confirmed the identity of the two organisms. Probe binding was inefficient although Gram negative rods and Gram positive rods were observed in only the aqueous and hydrocarbon phases, respectively.

The cell-associated material produced by strain F9-D79 was examined in more detail. Figure 6.1h and i show a negative stain of strain F9-D79 growing on crude oil. Cells are visible on oil droplets with a bright halo extending into the aqueous phase. Mycolic acids were detected with an acid-fast stain. Strain F9-D79 grown on soluble substrates yielded neither a capsule nor a positive acid-fast reaction despite their tendency to grow in clumps (Table 6.1). Strain JA5-B45 was acid-fast negative and capsule negative on all substrates.

6.4.3. Alteration of Rhodococcus *sp. strain F9-D79 morphology by chemical surfactant*

Since cultures treated with surfactant were consistently emulsified, surfactant effects on strain F9-D79 were examined. Strain F9-D79 cells grew dispersed in the aqueous phase on soluble and insoluble substrates only in the presence of surfactant. Adherent cells could be washed

Table 6.1 Summary of microscopic observations of 48-hour *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 grown on soluble and insoluble carbon sources

Organism	Carbon source*	Characteristic				
		Capsule	Acid-fast reaction	Growth	Cellular morphology	Size (μm)
Rhodococcus sp. F9-D79	Soluble	–	–	particulate	palisade, non-motile	2.5 - 3.5 x 0.7
	Insoluble/hydrophobic	+	+	oil phase	palisade, non-motile	2.5 - 3.5 x 0.7
	Insoluble + surfactant	–	+	dispersed/aqueous phase	single, non-motile	0.5 x 0.5
Pseudomonas sp. JA5-B45	Soluble	–	–	dispersed	chains, single, motile, non-motile	1.8 - 2.7 x 0.7
	Insoluble/hydrophobic	–	–	aqueous phase	chains, single, motile, non-motile	1.8 - 2.7 x 0.7
	Insoluble + surfactant	–	–	aqueous phase	chains, single, motile, non-motile	1.8 - 2.7 x 0.7

* carbon sources tested same as those in Figure 6.3 plus 0.2% and 2.0% (w/v) Bow River crude oil

from crude oil droplets with surfactant treatment. A capsule was not produced and cells were single, non-motile cocci 0.5 μm in diameter rather than rods 2.5 to 3.5 μm long (Table 6.1). Surfactant was not acutely toxic to strain F9-D79 with biomass yields ranging from 70 to 80% of a no-surfactant control in TSB broth containing 0.312 to 2.5 g/L Igepal CO-630 (Figure 6.2). No surfactant effect on JA5-B45 morphology was evident.

6.4.4. Surface tension and emulsification abilities

Surface-active agents were not detected in supernatants of crude oil-grown cultures. Measurements were confounded by crude oil and the highly viscous nature of the bioemulsified cultures so a variety of other soluble and insoluble substrates were tested. No surface tension reductions were observed for strain F9-D79 or strain JA5-B45 grown on TSB or glucose-acetate (Figure 6.3a and b). No surface tension reductions were observed for strain JA5-B45 cultures grown on diesel fuel or hexadecane, and the reduction observed in the whole broth of 2% diesel cultures was not recovered in the pellet or supernatant. Conversely, significant reductions in surface tension were associated with strain F9-D79 cells grown on 2% diesel and 0.2% hexadecane.

Bioemulsifiers were detected with a kerosene-water emulsification assay (Figure 6.4a). Strain F9-D79 cultures grown on crude oil had an emulsification value of 20% associated with the cellular fraction. The supernatant of strain JA5-B45 cultures had an emulsification value of 50% although no emulsification of crude oil was observed. The supernatant and cellular fraction from the co-culture displayed 50 and 20% emulsification, respectively. The extracellular nature of strain JA5-B45 emulsifier was confirmed by assaying the supernatants of repeated cell washes (Figure 6.4b). Washes of both strain JA5-B45 and the co-culture retained emulsification activity, albeit reduced, after two washes. Strain F9-D79 cells yielded no emulsifiers after three washes.

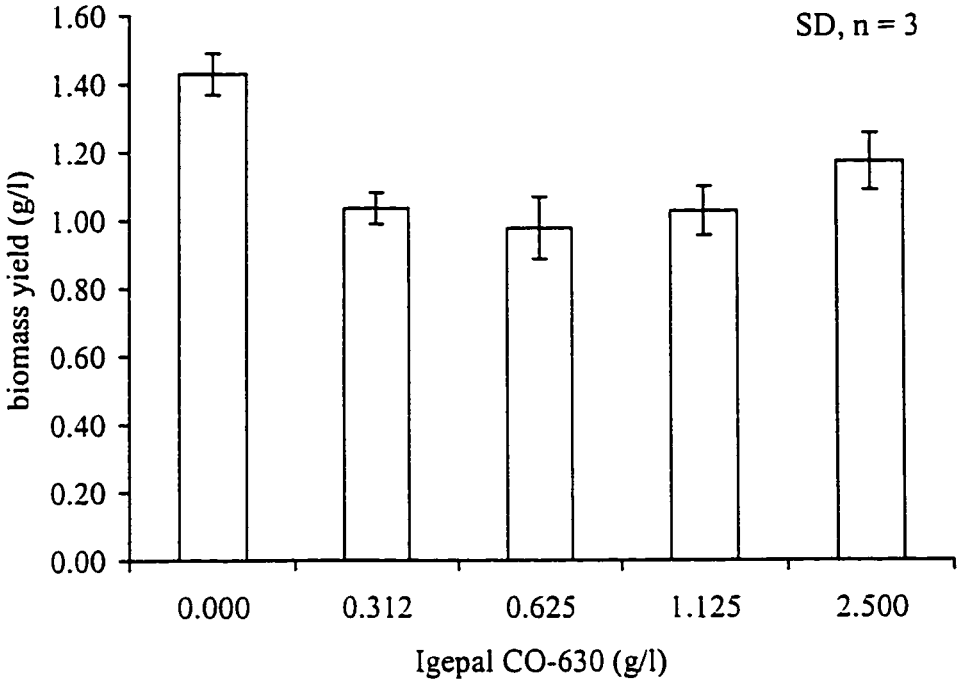


Figure 6.2. *Rhodococcus* sp. strain F9-D79 biomass yields following growth for 5 days at 30°C in TSB medium supplemented with chemical surfactant.

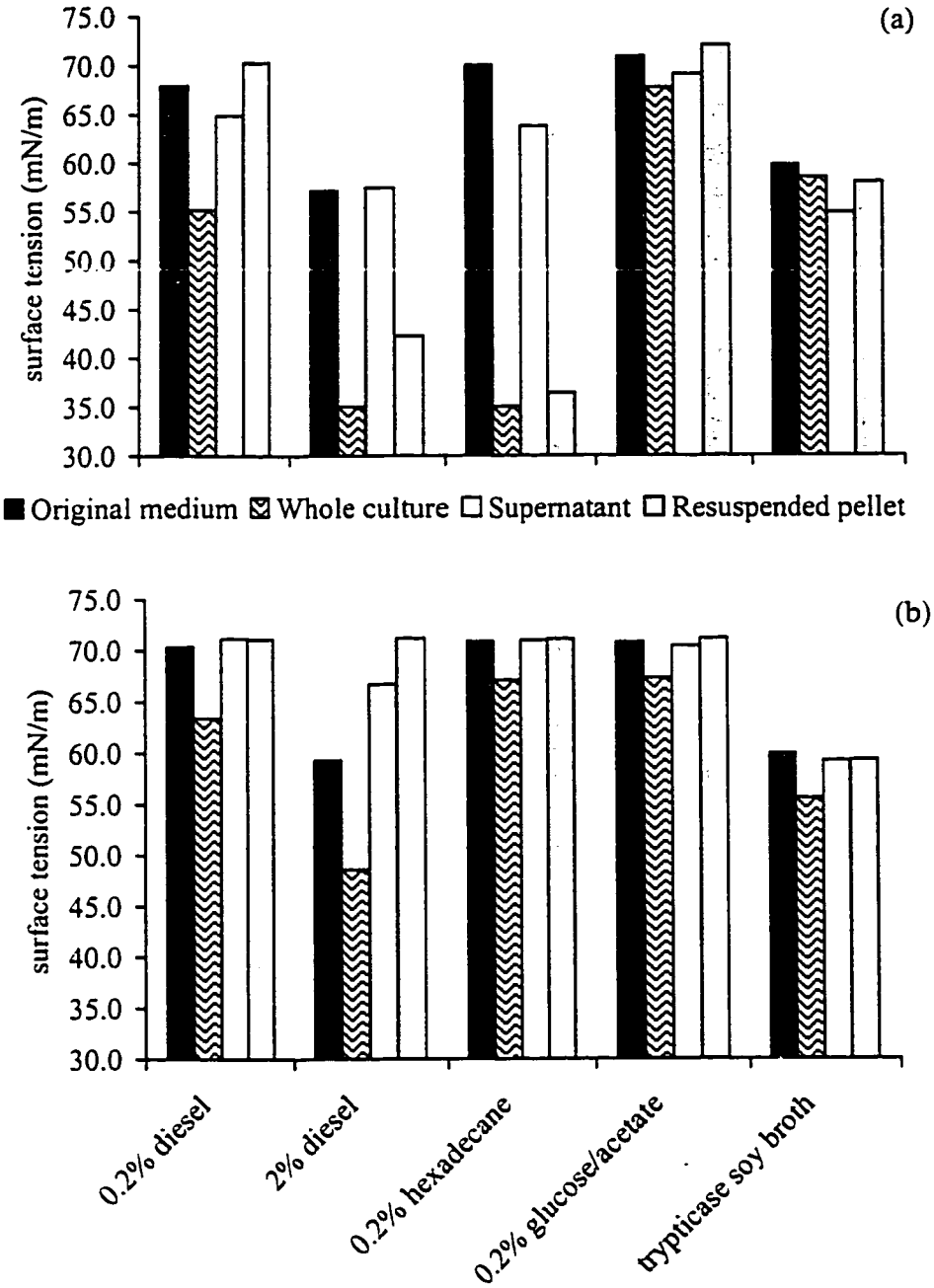


Figure 6.3. Surface tension of (a) *Rhodococcus* sp. F9-D79, and (b) *P. aeruginosa* cultures following 48-hour incubation on soluble and insoluble carbon sources.

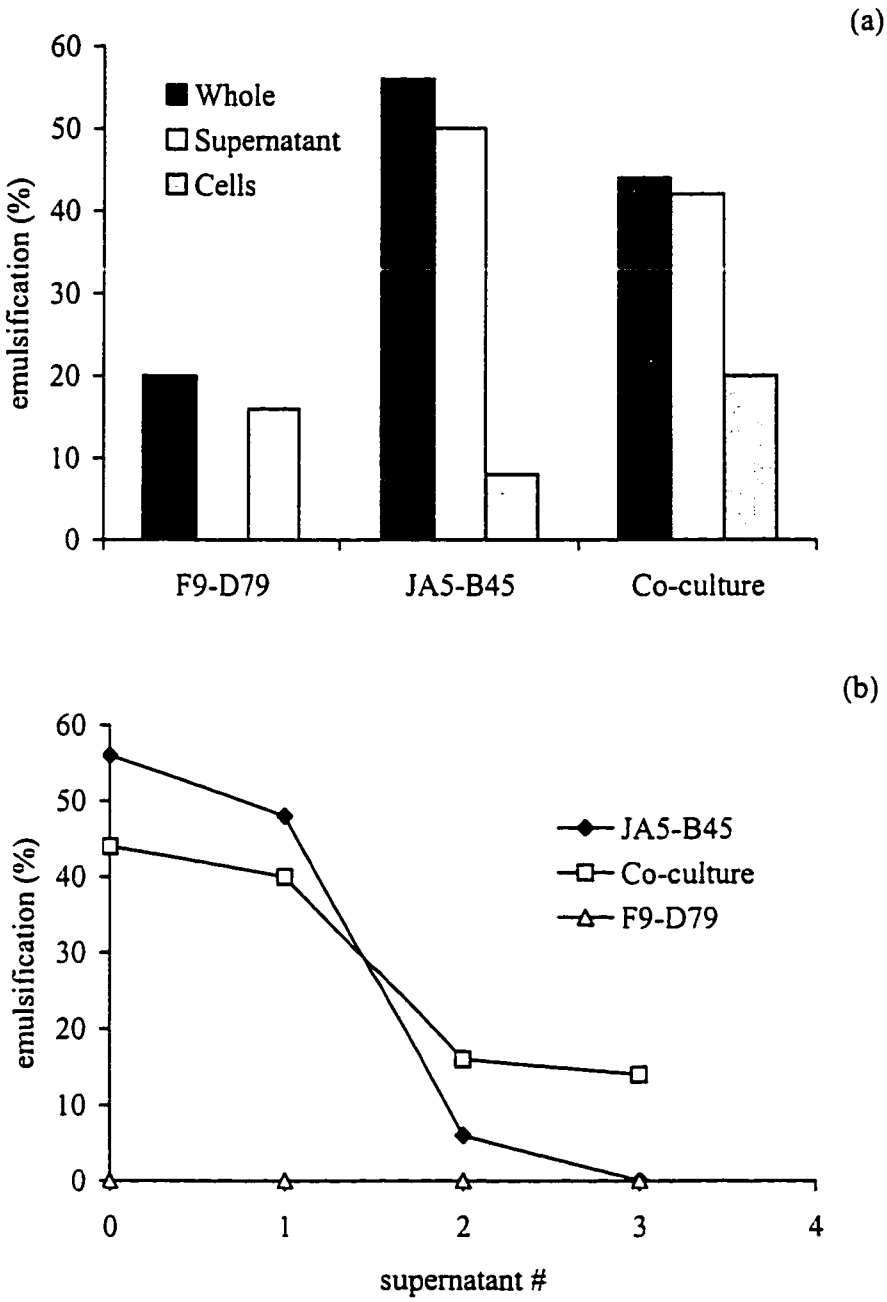


Figure 6.4. (a) Emulsification activity in 48-hour *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 cultures. (b) Emulsification activity in cell wash supernatants.

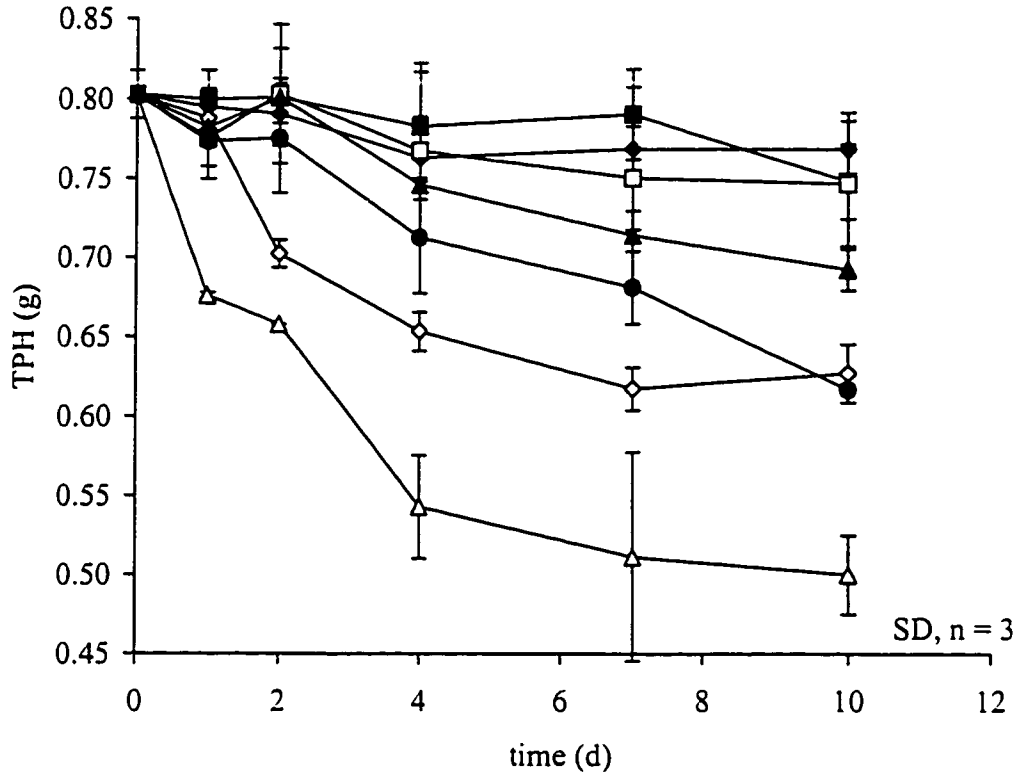
6.4.5. Biodegradation of crude oil – co-culture, chemical surfactant and pH

Bow River crude oil degradation time course studies for the two pure cultures and the co-culture were carried out (Figure 6.5). By weight, TPH removal by the pure cultures was minimal although strain F9-D79 degraded 15-20% of the saturate fraction (Figure 6.6a), corresponding to 55-60% of the GC resolvable compounds (data not shown). Strain JA5-B45 removed <10% of the saturate fraction by weight and insignificant quantities of the aromatic fraction were removed in both cases (Figure 6.6b). A slight enhancement was noted in TPH removal by the co-culture. The saturate fraction was degraded at the same rate as the strain F9-D79 culture and the aromatics were not affected.

TPH biodegradation by strain F9-D79 was not affected by chemical surfactant addition although the degradation rate of the saturate fraction was reduced, remaining linear over the 10-day incubation. In the presence of surfactant, TPH biodegradation by strain JA5-B45 increased from 4 to 22% and included 30% of the saturate fraction and 22% of the aromatic fraction by weight. TPH degradation by the co-culture increased from 13 to 40%, with 40% of the saturate and 35% of the aromatic fractions being removed (Figure 6.6a and b).

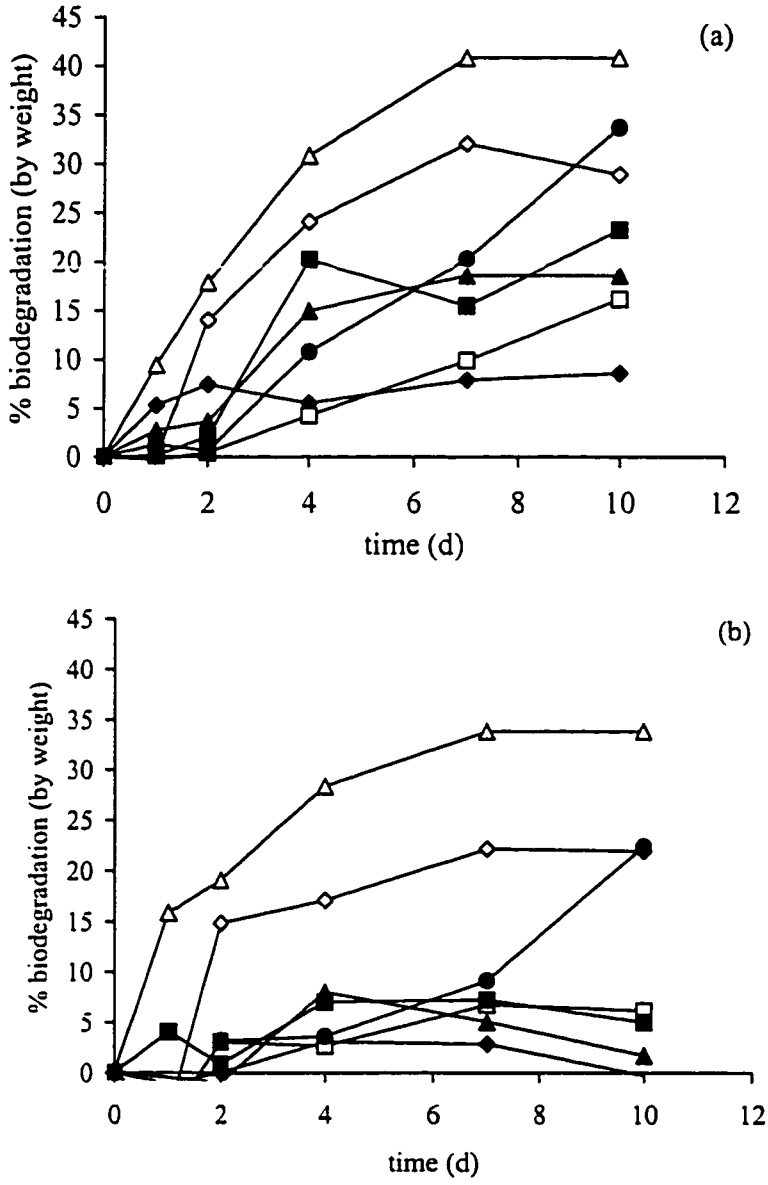
Surfactant was added to a bioemulsified co-culture after 4 days to determine if a boost in degradation would occur. When surfactant was added, strain F9-D79 cells were removed from the interface and a fine emulsion was formed. An increase in TPH degradation was noted, evident in both the saturate and aromatic fractions.

The pH of media from crude oil biodegradation flasks was monitored (Figure 6.7). Without surfactant the pH of all three cultures increased from 7.0 to between 7.5 and 8.5 after 4 days. The pH of strain F9-D79 and co-cultures continued to increase after day 4, reaching 9.1 by the end of the fermentation. With surfactant, the final pH of media from the co-culture and the JA5-B45 culture was 1.4 and 1.0 units lower than without, reaching 8.2 and 7.2, respectively. The pH in surfactant-amended strain F9-D79 cultures dropped to 5.5 after 2 days. Similarly, delayed surfactant addition to the co-culture stabilized the pH at 7.9, 1.2 units lower than the surfactant-free co-culture after 10 days.



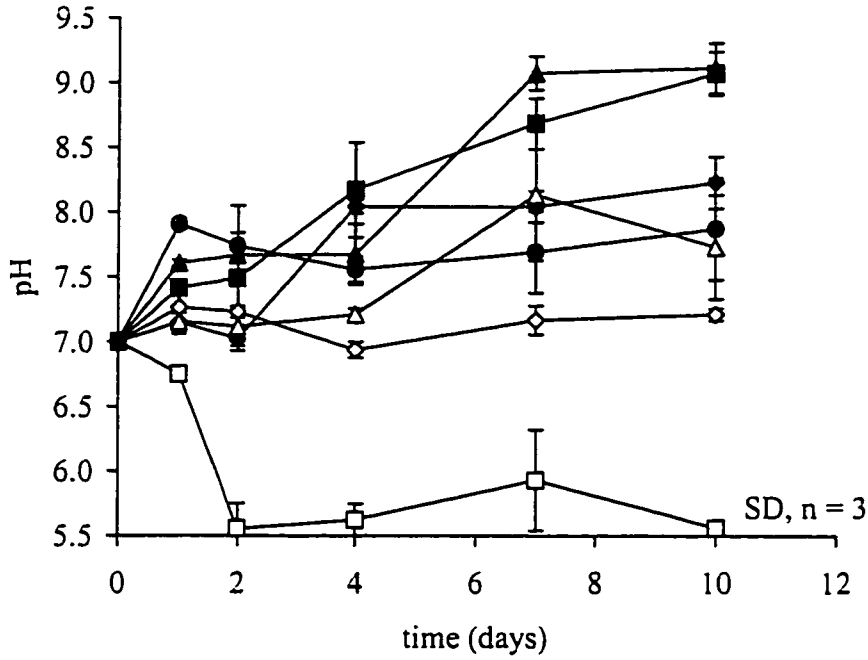
Closed symbols – no surfactant, Open symbols – 0.625 g/l Igepal CO-630:
◆, ◇ *Pseudomonas* sp. strain JA5-B45; ■, □ *Rhodococcus* sp. strain F9-D79;
▲, △ Co-culture; ● Co-culture + surfactant at day 4.

Figure 6.5. Bow River crude oil biodegradation time course curves for pure and co-cultures of *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 with and without 0.625 g/l Igepal CO-630.



Closed symbols – no surfactant, Open symbols – 0.625 g/l Igepal CO-630:
◆, ◇ *Pseudomonas* sp. strain JA5-B45; ■, □ *Rhodococcus* sp. strain F9-D79;
▲, △ Co-culture; ● Co-culture + surfactant at day 4.

Figure 6.6. Biodegradation of (a) saturates and (b) aromatics by pure and co-cultures of *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 with and without 0.625 g/l Igepal CO-630 on crude oil.



Closed symbols – no surfactant, Open symbols – 0.625 g/l Igepal CO-630:
◆, ◇ *Pseudomonas* sp. strain JA5-B45; ■, □ *Rhodococcus* sp. strain F9-D79;
▲, △ Co-culture; ● Co-culture + surfactant at day 4.

Figure 6.7. Supernatant pH values from crude oil biodegradation samples of pure and co-cultures of *Rhodococcus* sp. F9-D79 and *Pseudomonas* sp. JA5-B45 with and without 0.625 g/l Igepal CO-630

6.5. Discussion

The physical and metabolic properties of two distinct hydrocarbon-utilizing bacteria were characterized during crude oil fermentations. Chemical surfactant effects on their interactions in co-culture was also assessed. Visual observations of culture flasks indicated that: (1) *Rhodococcus* sp. strain F9-D79 grew adhered to the oil phase, produced a capsule, formed transient oil-in-water emulsions, and could be removed from oil-water interfaces with chemical surfactant; (2) *Pseudomonas* sp. strain JA5-B45 grew in the aqueous phase and was unable to emulsify crude oil; and (3) a co-culture of the two resulted in a more stable, prolonged state of emulsification.

Microscopic observations confirmed that strain F9-D79 attached directly to oil droplets in order to access hydrocarbons. *Acinetobacter* spp. (Baldi et al. 1999; Marin et al. 1996) and *Rhodococcus* spp. (Bredholt et al. 1998; Whyte et al. 1999) have been observed growing on hydrocarbon droplets and direct adherence to hydrocarbons is a common uptake mechanism for *Rhodococcus* spp. (Lang and Philp 1998). In this case, a capsule was observed extending into the aqueous phase which, based on the positive acid-fast reaction, consisted of mycolic acids. Actinomycetes (family Nocardiaceae) are known to produce mycolic acids and *Rhodococcus* spp. generally have 34-52 carbon atoms in the alkyl branches (Goodfellow 1992). The mycolic acids may be covalently bound or exist as readily extractable trehalose and glycerol mycolates (Sutcliffe 1998). While difficult to recover from hydrocarbon substrates due to their adherent nature, hydrocarbon-grown strain F9-D79 cells did have surface-active and emulsification properties. The majority of surface-active agents in *Rhodococcus* spp. are cell-bound glycolipids (Lang and Philp 1998) and a glycoprotein with emulsifying abilities was found to be cell-associated in *Rhodococcus erythropolis* ST-2 (Bailey and Ward 1997).

Chemical surfactant washed strain F9-D79 cells from the oil phase, presumably through disruption of cell-oil hydrophobic interactions upon emulsification. Alternately, the hydrophobic nonylphenol moiety may have intercalated with the distal moiety of bound and free mycolic acids in the outer layer of the cell envelope. This would have rendered the cells

themselves more uniformly hydrophilic. It has been hypothesized that surface amphiphiles orient themselves with their hydrophobic heads pointing away from the cell in the lipid layer of mycolata (Sutcliffe 1998). Surfactant altered the cellular morphology of strain F9-D79 from a rod to coccal form and eliminated growth on the oil phase. Rhodococci typically form rods or branched mycelia that fragment to irregular rods and cocci (Goodfellow 1992). On soluble substrates amended with surfactant, F9-D79 cells no longer clumped and the overall biomass yield was reduced by 20%. Thus, chemical surfactant effects a change in morphology rather than causing acute toxicity. Chemical surfactants may be toxic to cells through membrane disruption or by reacting with essential proteins (Volkering et al. 1998). Alternately, an increase in membrane fluidity without cell death may occur (Glover et al. 1999). Surfactants have also been shown to inhibit attachment of microorganisms to oil-water interfaces thus reducing biodegradation (Neu 1996; Volkering et al. 1998). For example, Stelmack et al. (1999) showed that two non-toxic surfactants inhibited both adhesion to non-aqueous phase liquid and growth on anthracene for a *Mycobacterium* strain and a *Pseudomonas* strain

Metabolically, the adherent strain F9-D79 degraded both linear and branched alkanes in crude oil. These compounds may have been used both as a carbon and energy source and for incorporation into cell envelope structures. No aromatic compounds were degraded although the trait does exist in this genus (Allen et al. 1997). Chemical surfactant addition reduced the alkane degradation rate indicating that the normal hydrocarbon uptake mode was damaged. Surfactant-supplemented strain F9-D79 cultures exhibited pH values 3.5 units lower than surfactant-free cultures. It appears as though chemical surfactant caused strain F9-D79 cells to release non-covalently bound fatty or mycolic acids as adherence to hydrophilic oil droplets was unnecessary or impossible.

In contrast to strain F9-D79, strain JA5-B45 was observed growing solely in the aqueous phase of crude oil and hydrocarbon cultures. Thus, contact with large oil droplets is evidently not a mode of hydrocarbon-uptake for this organism and, as expected, the cells did not produce a capsule or mycolic acids. Surface active agents were not detected. Despite the fact that strain JA5-B45 did not emulsify crude oil, an extracellular bioemulsifier was detected in the supernatant of hydrocarbon-grown cultures.

Upon chemical surfactant addition to strain JA5-B45 cultures it became apparent that this strain was indeed limited by an inability to access water insoluble substrates. The organism was active against both saturated and aromatic compounds in the crude oil when surfactant was added. The presence of both alkane and aromatic degradation pathways in a single strain has been reported (Whyte et al. 1997). Surfactant-amended cultures remained neutral, possibly the result of acidic metabolites offsetting the normal pH increase due to urea utilization.

In co-culture, the two organisms retained their positions with respect to the oil-water interface. The strains appeared different under phase contrast microscopy and a fluorescent Gram stain procedure confirmed their identities. Strain JA5-B45 growing in the aqueous phase appeared to have a strong affinity for the capsule surrounding strain F9-D79 cells. Whyte et al. (1999) observed, using transmission electron microscopy, similar strands of extracellular material between cells of *Rhodococcus* sp. Q15.

The combination of superior emulsification and metabolic capabilities of strain F9-D79 and strain JA5-B45 slightly enhanced crude oil biodegradation. Biosurfactants from one organism may positively or negatively affect biodegradation in pure or mixed cultures of other organisms (Barkay et al. 1999; Bruheim et al. 1997; Foght et al. 1989, Zhang and Miller 1994). In this case, it appears that the emulsion formed primarily by strain F9-D79 does not allow strain JA5-B45 to access aromatic hydrocarbons. Adding chemical surfactant to an emulsified co-culture boosted degradation of saturate and aromatic compounds by washing strain F9-D79 cells from the interface and forming a more dynamic micellar solution. Since the strain F9-D79 cells coat oil droplets and do not appear to be easily removed, less water soluble compounds will tend to remain partitioned in the organic interior. Conversely, micellar solutions of chemical surfactant and oil will be in a constant state of flux, breaking apart and reforming. When added at the beginning of fermentations, chemical surfactant inhibited strain F9-D79 attachment to the oil phase and enhanced degradation.

In this hydrocarbon-degrading co-culture system, a dual role for chemical surfactant can be suggested. First, the chemical surfactant directly increases the availability of both the saturate and aromatic fractions for strain JA5-B45. Second, the surfactant inhibits attachment

to, or removes strain F9-D79 from, the oil-water interface with the formation of a more dynamic emulsion indirectly increasing crude oil availability for strain JA5-B45. From a practical standpoint, it will be difficult to predict the effect of a given surfactant, be it chemical or biological, on biodegradation of hydrocarbons when using mixed-culture systems. The mixed-culture from which the two strains under study were isolated did produced oil-water emulsions and degradation was greatly enhanced by surfactant addition (Van Hamme and Ward 1999). However, in the original system, the addition of chemical surfactant to a bioemulsified culture did not enhance degradation as seen here.

6.5.1. Acknowledgements

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7. Conclusions

The physical, chemical and microbiological interactions within a mixed-bacterial culture during crude oil biodegradation were the central themes of this thesis. A mixed-bacterial culture isolated from a petroleum-impacted site was able to biologically treat mixed-hydrocarbon substrates in fermentor-based systems. Linear, branched and cyclic alkanes, and aromatics (benzene homologues and PAHs) were easily degraded, while resins and asphaltenes tended to be recalcitrant. Combinations of the above substrates altered the bacterial community structure, and conversely, the community structure determined substrate specificity. In addition, water-soluble nutrients such as yeast extract were essential for maintaining broad metabolic capacities, especially with respect to volatile components. The study of volatile hydrocarbon biodegradation was greatly enhanced by the developed SPME methodology. Controlling treatment outcomes during fermentations will depend on understanding the inoculum and target substrate. Of particular importance is the predominant hydrocarbon-accession mode used by the microorganisms with the greatest biodegradative potential.

Assuming that uptake of water-soluble hydrocarbons is negligible for non-volatile substrates, the simultaneous occurrence of both attachment to hydrocarbon droplets and uptake of pseudosolubilized hydrocarbons should theoretically not occur in homogeneous fermentor-based systems. Multiple accession modes can be envisioned in natural environments and the mode may change during a batch fermentation time course. In this thesis, chemical surfactants were shown to enhance biodegradation not only through oil micellization, but also by limiting attachment of hydrophobic strains to oil-water interfaces. The emulsion type formed by the selected adherent strain (*Rhodococcus* sp. strain F9-D79) limited biodegradation by strains with superior metabolic capacities depending on micellar-mediated accession (particularly *Pseudomonas* sp. strain JA5-B45). This appears to result from oil droplet stability once coated with a cell-associated capsule. In addition, chemical surfactant effects depend on HLB, cmc, and the oil to surfactant concentration ratio.

Overall, examining the physical, chemical and biological interactions in the mixed culture, and pure and co-cultures derived therefrom, was useful. Specific conclusions drawn from the work follow.

1. Mid-range HLB chemical surfactants are most effective for crude oil biodegradation enhancement by the mixed culture.
2. Sufficient surfactant must be added to enhance hydrocarbon availability without diluting the substrate within excess micelles.
3. HLB, cmc and surfactant structure must be considered during surfactant selection, and screening benefits from the inclusion of structural homologues across a wide, cmc spanning, concentration range.
4. Chemical surfactants are inadequate for effective volatile-hydrocarbon partitioning in bioreactors due to the high concentrations required.
5. Chemical surfactant may enhance biodegradation through micellar-mediated uptake or pseudosolubilization. It was shown that surfactant can also remove or prevent attachment of an adherent, capsule producing *Rhodococcus* sp. strain F9-D79. This allows for enhanced biodegradation by a *Pseudomonas* sp. strain JA5-B45.
6. Chemical surfactant can have profound impacts on microbial physiology, exemplified by the *Rhodococcus* sp. mentioned above. Incubation in the presence of chemical surfactant results in a morphological change from rod to coccal form and prevents capsule formation.
7. A few capable strains dominate fermentations at early stages (e.g. *Pseudomonas* and *Stenotrophomonas* spp.) while, regardless of substrate, non-hydrocarbon-degrading bacteria increase in proportion over time.
8. Specific substrates result in capable strains becoming dominant as highlighted by *A. calcoaceticus* during growth on Bow River saturates.
9. SPME can be applied to live microbial systems to evaluate C_5 to C_{11} volatile-hydrocarbon biodegradation during growth on crude oil.

10. Volatile hydrocarbon degrading capabilities are present in the mixed culture maintained on crude oil in open systems, and this activity can be maintained in closed systems or in yeast extract amended cultures.
11. Individual populations in the mixed culture may enhance hydrocarbon degradation through combinations of emulsification and degradative abilities. In this case, *Rhodococcus* sp. strain F9-D79 enhanced biodegradation by *Pseudomonas* sp. strain JA5-B45.
12. The emulsion quality produced by adherent microorganisms such as *Rhodococcus* sp. strain F9-D79 may limit biodegradation due to the static nature of enclosed oil droplets, and reduced mass transfer, as compared to chemical surfactant.

As with any work, there remains room for extension through future study. Specific areas that deserve more attention follow.

1. Applying more detailed physiological, metabolic and molecular methods such as 16S rRNA analysis to characterize mixed-cultures in selective, fermentor-based hydrocarbon treatment systems.
2. Developing bioreactors with closed-loop capabilities or biofilter/bioreactor systems for dual volatile and non-volatile hydrocarbon treatment.
3. Fully characterizing *Rhodococcus* sp. strain F9-D79 capsular material, and quantifying hydrocarbon partitioning in relation to biodegradation for capsule-enclosed oil droplets. Comparisons to chemical and extracellular biosurfactants would be useful.
4. Examining volatile hydrocarbon partitioning by biological and non-biological materials and the effect on biodegradation. SPME analysis would be a useful tool for these types of study.
5. Determining the mechanism resulting in methylcyclohexane recalcitrance after prolonged culture incubation in open systems lacking yeast extract.
6. Expanding the surfactant-enhanced crude oil biodegradation database to other structural forms and biosurfactants. Nonylphenol ethoxylates

are currently being banned in Europe (Renner 1997) and may be phased out in North America due to nonylphenol, the primary biodegradation product, being an estrogen-mimicking compound (John and White 1998; Fujita and Reinhard 1997).

7. Developing analytical techniques for use in conjunction with advanced statistical computing (Almeida et al. 1998; Smith et al. 1998) to examine substrate-metabolite impacts on mixed culture structure and function.

From a practical standpoint, biological methods have the potential to remediate hydrocarbon spills (Freijer et al. 1996), refinery wastes (Yamaguchi et al. 1999), hydrocarbon-contaminated bituminous sand tailings (Herman et al. 1993; MacKinnon and Boerger 1986), and volatile hydrocarbons from petroleum (Aelion and Kirtland 2000). In addition, fuel upgrading through desulfurization and denitrogenation, especially with heavy Canadian crude oils (Vazquez-Duhalt et al. 1993), is currently an important research area. Fine chemical synthesis and replacing petroleum-based products such as plastics (Groethe et al. 1999) will remain a lucrative area for microbiological applications. These areas will become increasingly important as world nations focus their attentions on global carbon budgets (Telesetsky 1999).

As petroleum is currently the central fuel source for developed and developing nations, applications for petroleum microbiology are strong. However, microbiology also has a place in the transition to sustainable, low-impact fuel systems. Biological methanol, ethanol and hydrogen production (Asada and Miyake 1999; Woodward et al. 2000) for hydrogen-fuel cells is a prime example.

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