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Changes in mutagenicity during crude oil degradation by fungi

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Abstract

Two fungal strains, *Cunninghamella elegans* and *Penicillium zonatum,* that grow with crude oil as a sole carbon source were exposed to three crude oils that exhibit a range of mutagenic activity. At regular time intervals following fungal incubation with the various crude oils, extracts were tested for the presence of mutagenic activity using the spiral Salmonella assay. When the most mutagenic of the oils, Pennsylvania crude oil, was degraded by *C. elegans* or by *P. zonatum,* its mutagenicity was significantly reduced; corresponding uninoculated (weathered) controls of Pennsylvania crude remained mutagenic. West Texas Sour crude oil, a moderately mutagenic oil, exhibited little change in mutagenicity when incubated with either *C. elegans* or *P. zonatum.* Swanson River Field crude oil from Cook Inlet, Alaska is a slightly mutagenic oil that became more mutagenic when incubated with *C. elegans*; weathered controls of this oil showed little change in mutagenicity. Mycelial mat weights measured during growth on crude oils increased corresponding to the biodegradation of about 25% of the crude oil.

Introduction

Microbes known to utilize toxic compounds as carbon and energy sources are logical choices for bioremediation consideration. There are also microorganisms naturally present in the soil and water that can interact with toxic pollutants introduced into the environment by human activities, such as accidental oil spills. Efficient bioremediation of environmental pollutants, such as crude oil, might be accomplished by enhancing the growth of the indigenous microbiota, which would increase their number to the point where degradation is accelerated. However, as microorganisms are promoted for environmental bioremediation efforts, the potential risk of adverse environmental or human health effects must be assessed. At issue is whether biodegradation converts toxic pollutants to nontoxic products or to intermediates that are more toxic than the original compound.

Both bacteria (Perry 1968; Jobson et al. 1972; Walker et al. 1976; Cerniglia 1992) and fungi (Perry and Cerniglia 1973; Kirk and Gordon 1988; Pothuluri et al. 1992) can degrade hydrocarbons. The enzyme systems in bacteria may convert hydrocarbons to intermediates with increased toxicity (Gibson et al. 1975; Liu et al. 1992; Middaugh et al. 1993), to nontoxic metabolites (Burback and Perry 1993), or completely to CO_2 + H_2O (Beam and Perry 1974). The mechanisms for hydrocarbon degradation by fungi have been less studied, but involve inducible oxygenases similar to those in species of *Mycobacterium* and *Nocardia* (Perry 1979). Both ligninolytic (Field et al. 1992) and nonligninolytic (Cerniglia and Yang 1984; Cerniglia 1992) fungi can oxidize various aromatics.

In recent years, mutagenicity has been studied in wastewater sludge (Ottaviani et al. 1993), rubber chemicals (Crebelli et al. 1984), napthenic and mineral oils (Granella et al. 1995), liquified petroleum gas (Yin et al. 1994), polycyclic aromatic hydrocarbons in lakewater (Chang et al. 1992), and crankcase oils (Dutcher 1986; Payne et al. 1978). However, few researchers have investigated the mutagenicity of crude oil (Claxton et al. 1991; Ellenton and Hallett 1981; Tarzwell 1971). Increasing numbers of crude oil spills into aquatic ecosystems require us to advance

Table 1. Comparison of the composition of Pennsylvania crude oil, West Texas Sour crude oil, and Cook Inlet crude oil before degradation.

Crude Oil	TCO[a] (mg/ml)	API[b] Index	C/H Ratio	%C[c]	%H	%N	%S	%O
Pennsylvania	241.3	10.24	C7H13	79.6	13.2	<0.5	0.1	1.0
West Texas Sour	220.8	5.76	C7H13	83.8	12.7	<0.5	1.9	1.2
Cook Inlet	227.1	7.4	C7H13	84.9	12.9	<0.5	0.1	1.3

[a]TCO = Chromatographic analysis of components having boiling points between 100–300 Celcius.
[b]API Index = Volatility index based upon the American Petroleum Institute ratio of light and medium weight components versus high and residual components (boiling points).
[c]Percent C,H,N,S,O = percent total mass of individual elements present in crude oil.

our knowledge about the impact of such pollutants in the environment.

This study describes the interactive use of microbial culture, mutagenicity bioassays, and analytical chemistry (Walker et al. 1975) to investigate the genotoxicity that might arise during crude oil degradation. Crude oils of low, moderate, and high mutagenicity were exposed to the two fungi *Cunninghamella elegans* ATCC 36112 and *Penicillium zonatum* ATCC 24353 (Cerniglia and Perry 1973; Hodges and Perry 1973) and the presence of genotoxic agents was assessed by the spiral Salmonella assay (Houk et al. 1991). This assay has been used to test pure compounds (Houk et al. 1989), combustion samples (Houk et al. 1991), contaminated soil (DeMarini et al. 1992), and oil undergoing bioremediation in a field setting (Claxton et al. 1991). Its advantages include conservation of sample mass and enhanced assay sensitivity.

Materials and methods

Sample selection

The crude oil substrates selected for study were Pennsylvania Refining Company Pennsylvania Crude (Pennsylvania crude), Cosden Oil and Chemical Company West Texas Sour Crude (West Texas Sour crude), and ARCO Swanson River Field Cook Inlet, Alaska Crude (Cook Inlet crude) (provided by the oil companies). The three oils were selected after initial chemical analysis. Total chromatographical organics (TCO) analysis, thermogravimetric analysis (TGA), and elemental analysis (EA) indicated that the oils were of similar composition (Table 1).

The three crude oils were analyzed for mutagenicity using the spiral Salmonella assay. Pennsylvania crude exhibited high mutagenicity, West Texas Sour crude moderate mutagenicity, and Cook Inlet crude low mutagenicity. Using oils of varying mutagenicity was a logical choice to demonstrate mutagenic effects from the classes of hydrocarbons that could become environmental contaminants and candidates for bioremediation efforts. The three oils were also screened by experiments using a well plate technique (Perry and Cerniglia 1973), which indicated that the selected fungi would grow with the crude oils as sole carbon source. The three oils were ultimately selected based upon the following criteria: 1) low, moderate, and high mutagenicity values to demonstrate mutagenicity changes caused by fungal utilization, 2) ability of the oils to support the growth of *Cunninghamella elegans* and *Penicillium zonatum*, and 3) availability of sufficient oil for extensive experimentation.

Microbiology

Fernbach flasks (2.8 L) containing 5 mL crude oil and 500 mL mineral salts medium (Leadbetter and Foster 1958) were inoculated with a fungal culture (*Cunninghamella elegans* or *Penicillium zonatum* homogenized in 50 mL 0.9% saline). Flasks were incubated in stationary culture at 30 °C for 4 to 30 days; at 2 day intervals triplicate flasks were removed for mycelial mat biomass determination and crude oil extraction from the mat and the culture medium by the following modification of the method of Cerniglia (Cerniglia and Perry 1973; Cerniglia 1975). Methylene chloride was used instead of chloroform as an extractant for these experiments (Williams et al. 1988; Claxton et al. 1991) as this solvent did not interfere with the subsequent use of the spiral Salmonella assay (Houk et al. 1991). Mycelial mats were harvested using a kitchen sieve of 7 cm diameter and 2 mm pore size; the mat was rinsed by agitation with 100 ml methylene chloride for 15 minutes (3x) to extract bound oil, removed, dried overnight,

and weighed for biomass determination. The culture medium remaining in the flask was mixed with 100 ml methylene chloride to recover the small amount of oil not bound into the mat; this methylene chloride was then added to that used to extract oil from the mycelial mat. Oil residues contained in the combined mat and medium methylene chloride rinse were separated from culture medium using a 500 ml separatory funnel, dried on a 3 cm by 4 cm column of anhydrous sodium sulfate, and removed of excess methylene chloride by evaporation with a Rotovaporator apparatus (Buchi model 001). The residual oil mass was determined by weighing dried residues of 100 microliters of oil sample. All data (each time interval) are the average of triplicate flasks. Controls included one "weathered" (uninoculated) oil-and-culture-medium flask per run. Sample control flasks were examined visually and subcultured to ensure sterility; no evidence of bacterial contamination was found. These flasks, therefore, represented abiotic controls used to calculate nonbiological loss and for comparison in mutagenicity testing.

During the incubation and extraction processes, some oil was lost due to nonbiological factors (Atlas 1975). These factors include volatilization and adherence to mycelial mats, glassware, and drying agents. Therefore, it is necessary to make a correction for nonbiological loss in considering the amount of oil utilized by the fungus. Biologically linked oil degradation is calculated as biological loss. Normalization of the percentage used is based on the abiotic (weathered) control residue, which represents nonbiological loss. Averaging of values indicates that about 30 percent of each crude oil is lost by nonbiological means. Biological loss is calculated as follows: percent biological loss = 100% - (percent residue + percent nonbiological).

Mutagenicity

Mutagenicity was determined by spiral Salmonella assay analysis of the extracted oil samples. To conduct the spiral assay, the methylene chloride extract (stock concentration of approx. 200–250 mg/ml) was dispensed by a spiral plater onto the surface of a rotating agar plate, forming an Archimedes spiral (Houk et al. 1989). The extract was delivered at a logarithmically decreasing rate, generating a concentration gradient along the spiral track of about 12:1. Doses are defined by each revolution of the spiral and are extrapolated by computer to equivalent pour-plate values; given the above-mentioned stock concentration for this sample set, the range of doses on a spiral plate was equivalent to between 3 and 45 mg per standard pour plate. The vehicle (methylene chloride) evaporated rapidly and did not affect the bacterial tester strain. *Salmonella typhimurium* Strain TA98 was selected for this study as it was the most sensitive strain in initial studies. Overnight cultures of TA98 were concentrated 20-fold in 0.015M phosphate buffer as described previously (Houk et al. 1992) and delivered at a constant rate by the spiral plater onto the original sample spiral track. In this way, a uniform number of bacteria would be exposed to a concentration gradient of the test material on a single agar plate. Plates were inverted and incubated at 37 degrees C for 72 hours. Mutant (revertant) colonies were quantified with a laser scanner (Spiral Biotech, Inc.) interfaced with a personal computer equipped with software capabilities (SALS 3.0 Spiral Biotech) for dose-response analysis. Tested extracts included a control ('weathered') oil sample and three experimental ('treated') samples collected on each sampling day. Spontaneous revertant values were obtained by using methylene chloride as the negative control. Extracts were tested in triplicate, and experiments were conducted at least twice.

Chemistry

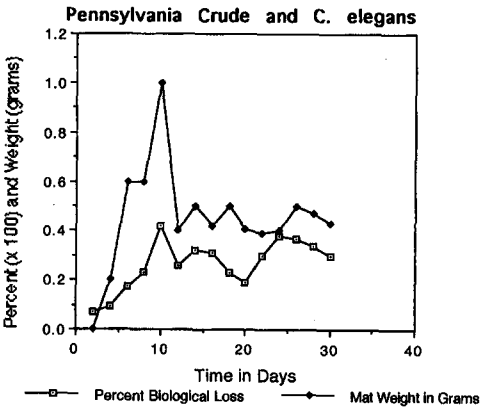
The three crude oils were analyzed for their chemical composition. Thermogravimetric analysis (TGA), Total Chromatographic Organics (TCO), and Elemental Analysis (EA) were performed on each sample. TGA analyses used a PL Thermal Sciences (Omnitherm) 1500 TGA. A mass ranging from 8 to 11 mg of each sample was heated (under nitrogen) from ambient to 700 degrees C at 20 degrees C/minute. Weight versus temperatures profiles were collected for each sample. Data collected from this procedure indicated the relative volatility of each crude oil, which allowed us to extrapolate the relative abundance of components such as paraffins, lubricating oils, or asphaltenes.

TCO was performed using a gas chromatographic-flame ionization detection system (GC-FID), which quantitated the hydrocarbons having boiling points between 100–300 degrees C; this analysis was useful in determining the relative abundance of volatile, semi-volatile, or gravimetric components present in the crude oils prior to experimentation. The range of these components was determined from the elution time of C8 and C17 straight chain HCs. Quantitation was by comparison of peak area response of the unknowns eluting within this time frame versus cali-

bration response using an EPA established procedure (Lenzen et al. 1978).

The EA was performed by Galbraith Laboratories (Knoxville, TN). The percent of molecular C,H,N,S, and O making up the sample mass was obtained. These data were used to calculate a molar C/H ratio, and a mean hydrocarbon (HC) formula was determined. This HC formula would suggest an average length for the HC chains present in the crude oil. The mean HC formula provides an estimate of the general presence of straight, substituted, or aromatic HCs in a mixture.

During experimentation, GC-FID analysis was performed on aliquots of each crude oil at three timepoints: at initial treatment (T=0), at the time of a noticeable change in mutagenicity, and at the final treatment timepoint to monitor any change in HC composition as degradation proceeded. Stocks of each crude oil were prepared at 200 mg/ml (in methylene chloride). Analysis was performed using automated 2 microliter splitless injections upon a J&W PTE–5 fused silica capillary (30M, 0.25 mm i.d., 0.25 micrometer film thickness) installed in a Waters Associates, Dimension 1 GC. Helium was utilized as the carrier gas with a linear velocity of 18.7 cm/sec. The injector temperature was maintained at 200 degrees C during the run with a timed column program (4 degrees for 4 min, followed by a linear 2 degree C/minute ramp to 300 degrees C). The final temperature was maintained for 30 minutes to complete the run. Data were captured using a Varian DS–604 data station. A select hydrocarbon standard mix (C7, C10, C12, C14, C16, and C17) was utilized throughout the run sequence to calibrate peak retention indices.



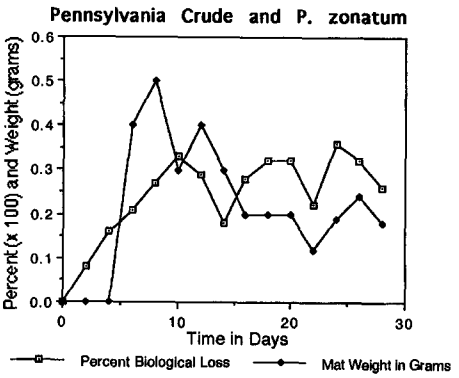


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Figure 1A.

Results and discussion

Both *C. elegans* and *P. zonatum* grew well on the crude oils in the experimental system with the formation of either a sturdy or fragile mycelial mat. Character and relative weight (Figure 1) of the mat was apparently determined by the nature of the oil. With sturdy mats, oil was firmly bound up into the mat, which was 1 to 2 cm thick and rubbery. The oil was readily extracted from these mats with methylene chloride. An average of 44% of oil ('residue') was recovered from the sturdy mats. Sturdy mats were typically formed during growth on Pennsylvania crude oil. The fragile mats appeared as a thin, carbon-paper-like film on the medium. Extraction of fragile mats was more difficult; nevertheless an average of 44% of oil ('residue') was recovered. Fragile mats occurred with West Texas Sour and Cook Inlet crudes.

The relationship between biological loss and dry mat weight is presented for Pennsylvania crude, West Texas Sour crude, and Cook Inlet crude (Figure 1). When *C. elegans* grew on Pennsylvania crude the mat weight increased to a maximum of 1 gram on day 10, paralleling the increase in biological loss that peaked at 42% on that day. *P. zonatum* growing on Pennsylvania crude reached a maximum weight of 0.5 grams on day 8; the initial peak in biological loss occurred at day 10. No correlation between mycelial mat weight and biological loss is apparent with West Texas Sour crude. With Cook Inlet crude as substrate, a small, fragile mycelial mat is obtained. This mat weight correlates with biological loss. Growth of *C. elegans* on Cook Inlet crude resulted in a utilization of 23% on day 22 with a corresponding elevated mat weight of 0.2175 grams. *P. zonatum* grown on Cook Inlet crude

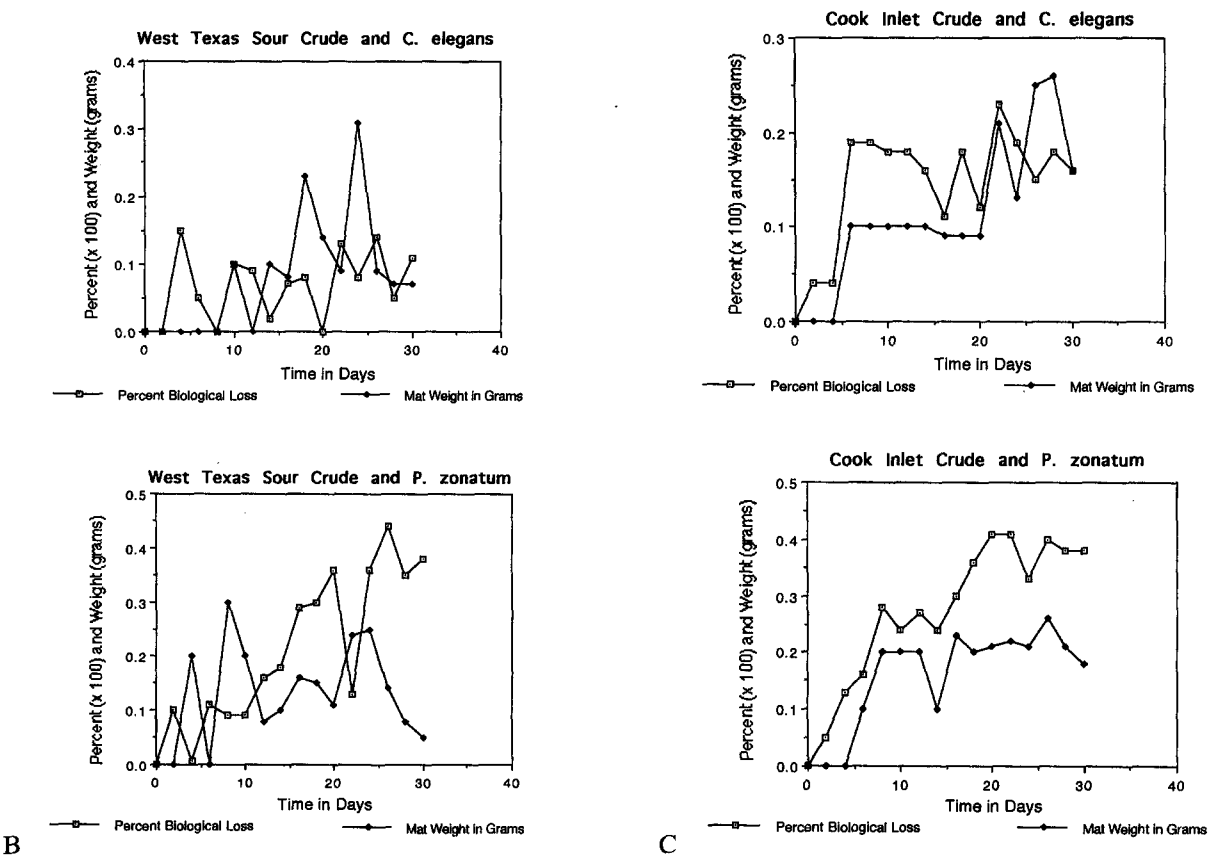

Figure 1. Comparison of percent biological loss and mycelial mat weight of *Cunninghamella elegans* and *Penicillium zonatum* grown on (*A*) Pennsylvania crude, (*B*) West Texas sour crude, and (*C*) Cook Inlet crude.

resulted in biodegradation of 35% over 22 days, with a corresponding mat weight gain to a maximum of 0.26 grams at day 26.

The changes in mutagenicity detected during the experimentation are presented in Table 2. Quantitation of mutagenic activity is based on a somewhat subjective comparative rating system explained in Table 2 (- = nonmutagenic to +++++ = most highly mutagenic). Under normal experimental conditions, some variation in mutagenicity occurs, due in part to the natural complexity of crude oil. Therefore, we consider this minor irregular fluctuation pattern a normal component of the spiral Salmonella assay when testing crude oils. In interpreting our data we considered a mutagenic response significant when the pattern switched from positive to negative or negative to positive and did not revert back to its original mutagenicity value over time (as with *P. zonatum* incubated with Pennsylvania crude). Weathered controls of the three oils exhibit slight but insignificant variations in mutagenic activity over time. The mutagenicity of the most potent oil, Pennsylvania crude, was reduced following degradation by either fungus; reductions in activity were more pronounced with *P. zonatum*. The activity of the moderately mutagenic West Texas Sour crude exhibited little change during degradation, although a slight transient increase was noted (Table 2). The weakly mutagenic Cook Inlet crude became more mutagenic when incubated with *C. elegans*; a transient increase in mutagenicity (around days 4 - 8) occurs with *P. zonatum*.

Chemical analyses were performed to characterize the oils (Table 1), to determine the pattern of changes in hydrocarbons of various molecular weights during degradation, and to try to elucidate the reason for the changes in mutagenicity. Representative chromatograms of the GC-FID analysis of West Texas Sour crude samples undergoing degradation are pre-

Table 2. Mutagenic activity of control (uninoculated) and treated oils.[a]

	Incubation (Days)	Pennsylvania Mutagenic Response		West Texas Sour Mutagenic Response		Cook Inlet, Alaska Mutagenic Response	
		Control	Treated	Control[b]	Treated	Control	Treated
C. elegans	1	+++++	+++	-	+	-	-
	2	+++++	+++	+/-	-	-	-
	3	nd[c]	nd	-	-	-	-
	4	+++++	+++	-	-	-	-
	6	+++++	+++	+++	++++	-	-
	7	+++++	+	+++	++	nd	nd
	8	+++++	+	+++	+++	+/-	+
	9	+++++	+++	nd	nd	nd	nd
	10	+++++	+++	-	++	-	++
	11	+++++	+++	nd	nd	nd	nd
	12	+++++	++	++	++++	+/-	++
	13	+++++	-	nd	nd	nd	nd
	14	+++++	++++	++	+	-	+
	16	nd	nd	-	++	+/-	++
	18	nd	nd	-	+	nd	nd
P. zonatum	1	+++++	+++	-	-	-	-
	2	+++++	+++	-	-	-	-
	3	+++++	++++	-	-	-	-
	4	+++++	++++	-	-	-	+
	6	+++++	-	-	-	+/-	++
	8	+++++	-	-	-	-	+
	10	+++++	-	-	-	-	-
	12	+++++	-	-	-	-	-
	14	+++++	-	-	-	+/-	-
	16	+++++	+	-	-	+	-
	18	nd	nd	-	-	nd	nd
	20	nd	nd	-	-	nd	nd

[a]Qualitative expressions of mutagenic activity are based on calculations of slope values (revertants per mg, rev/mg) derived from the linear portion of the dose-response curves. Three treated flasks and one control (uninoculated) flask were included for each incubation period. Contents (extracts) were tested on at least 2 separate occasions, and triplicate plates were included for each test. The slope values generated from the separate tests were averaged to obtain the levels of activity given in the table.
+++++: 25 rev/mg; ++++: $15 \leq 25$ rev/mg; +++: $> 8 \leq 15$ rev/mg
++: $> 4 \leq 8$ rev/mg; +: $> 1 \leq 4$ rev/mg; -: ≤ 1 rev/mg; +/-: inconclusive
[b]The discrepancy between weathered controls of West Texas sour crude for *C. elegans* and *P. zonatum* could not be explained by our experimental system.
[c]not done.

sented in Figure 2. At day 0 a uniform hydrocarbon pattern is evident (dominant peaks eluting in a consistent time differential). Very low molecular weight HC (eg., hexanes, heptanes, octanes) are seen as sharp peaks eluting during the first 15 minutes of the analysis. Day 4 analysis indicates that a majority of the very low molecular weight components are not present at the same concentration, presumably due to fungal degradation and natural evaporation. Also by day 4 (a mutagenic inflection point) the concentration of the straight chain hydrocarbons (eg., C10, C12, C14, C16, C17) had begun to change. These components are now present in lower concentrations (as observed in peak height comparisons to non-straight chain hydrocarbons eluting nearby). Analysis of the day 12 treatment sample reveals a near total breakdown relative to the hydrocarbon pattern observed under day 0 treatment conditions. This hydrocarbon breakdown pattern was consistent in the three crude oils. The data from the GC-FID analysis are reported because the reduction

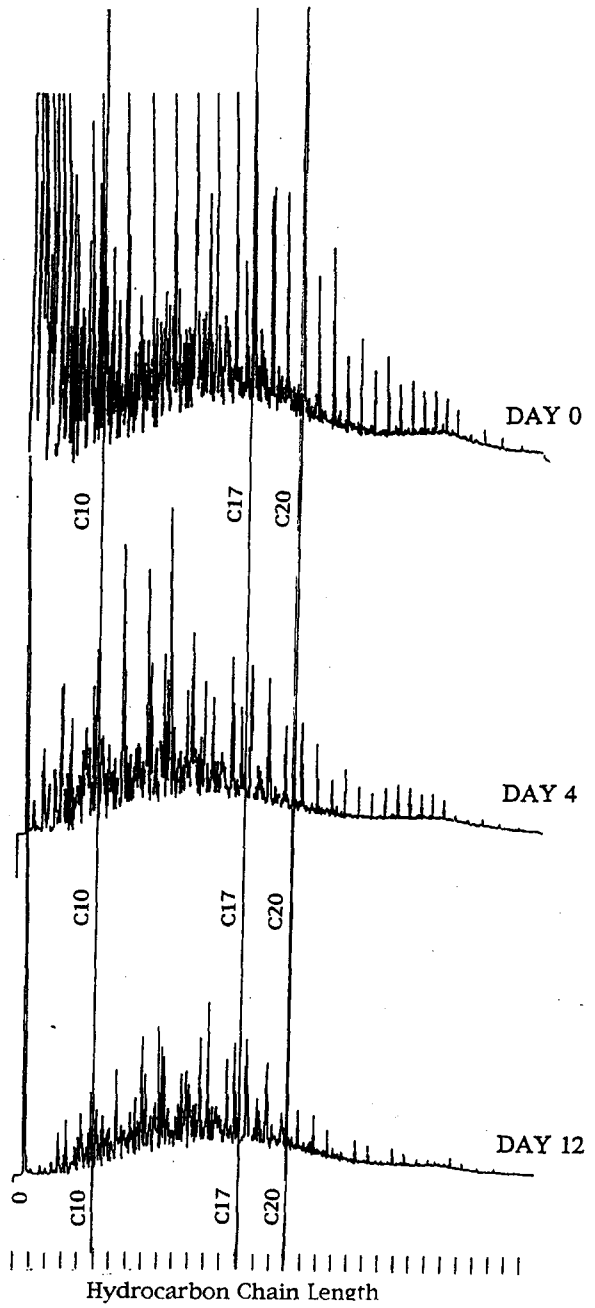

Figure 2. GC-FID Chromatograms for West Texas Sour crude representative of the relative change in composition of crude oil undergoing degradation. Comparisons among chromatograms reveal a decrease in numbers of low molecular weight components as degradation progresses.

in C10 and above components is evidence of degradation during incubation. Components of crude oils undergoing nonbiological breakdown would decrease proportionately at the same rate, while biologically-induced degradation causes uneven rates of decrease, especially on the low end of the molecular weight scale (Atlas 1975) where fungi degrade hydrocarbons more quickly (Cerniglia 1992).

Representative samples from each degradation treatment were characterized by GC-FID to estimate alterations in composition that might account for the observed changes in mutagenicity. Three data points were examined to mark the test initiation (day 0), first mutagenicity inflection point (either day 2 or 4), and test completion where a mutagenic plateau was established (either day 12 or 16). A very slight increase in low and medium weight hydrocarbons was observed in degradation analysis for the Pennsylvania crude oil (91.4% to 92.6%), the oil that reflected the most consistent decrease in mutagenicity over time. Low and medium weight hydrocarbons decreased from 90.3% to 84.6% (5.7% change) in West Texas Sour crude; this oil exhibited little change in mutagenicity during incubation. The Cook Inlet sample lost approximately 4% (86% to 82%) of the low and medium weight hydrocarbons by day 16; mutagenicity of this oil increased during the incubation period. Comparing these data with mutagenicity results does not yield a useful pattern that would explain the observed changes in mutagenicity.

Conclusions

Many species of fungi can catabolize hydrocarbons (Cerniglia and Perry 1973; Perry and Cerniglia 1973; Kirk and Gordon 1988; Pothuluri 1992), and this study indicates that these catabolic reactions may convert crude oil to intermediates that are either more or less mutagenic than the original oil. Growth of *Penicillium zonatum* on Pennsylvania crude produced a consistent decrease in mutagenicity. The results with the other oils were less pronounced and somewhat inconsistent, although a slight increase in mutagenicity occurred with *Cunninghamella elegans* during biodegradation of Cook Inlet crude.

In this study conventional chemical analyses (typical of those done to monitor crude oil bioremediation efforts) gave no indication of the level of mutagens present before, during, and after degradation by the fungi. As Rhodes pointed out (Rhodes et al. 1995), the limitations of conventional chemical methods must

be clearly understood when used to monitor or evaluate petroleum hydrocarbon contaminations. The results presented here also indicate that a mutagenicity bioassay can be employed to monitor toxicity (i.e. mutagenicity) during the biodegradative process. Despite the application of both chemical and biological analyses, the mechanisms whereby crude oil is rendered mutagenic or nonmutagenic could not be determined in this study. Because an inducible oxygenase has been postulated as a significant factor in this conversion (Perry 1979; Cerniglia and Yang 1984; Field 1992), enzymatic studies might provide more information about the conversion mechanism.

This study provides information that contributes some understanding of the nature of microbe/pollutant interactions in the environment. *Cunninghamella elegans* and *Penicillium zonatum* are naturally present in the ecosystem (Hodges and Perry 1973; Perry and Cerniglia 1973) or might be introduced in large numbers as a part of bioremediation efforts. Bioremediation researchers and practitioners should note that such organisms in ecosystems may cause increased as well as decreased mutagenicity in contaminant petroleum hydrocarbons.

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