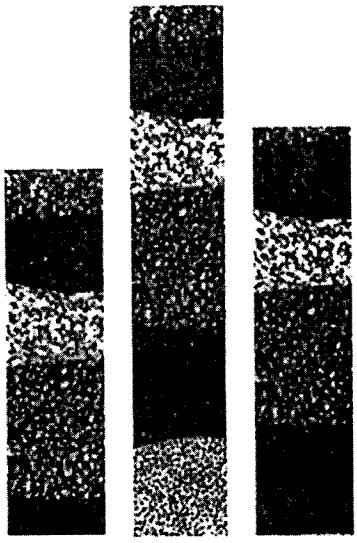
Natural Attenuation of Environmental Contaminants



Editors

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The Sixth International In Situ and On-Site Bioremediation Symposium

San Diego, California, June 4–7, 2001

BATTELLE PRESS
Columbus • Richland

NEW MARKERS FOR MONITORING THE BIODEGRADATION OF PETROLEUM FUELS IN SOIL

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ABSTRACT: We have investigated the use of per-deuterated and phenyl-labelled alkanes as indicators of the biological degradation of petroleum fuels. In laboratory scale reactors containing soil, peat moss and the target compounds, both compounds were completely degraded at a similar rate to diesel fuel within 16 days at room temperature. Degradation slowed markedly in a nitrogen purged reactor and was virtually stopped by incubation at 4 °C and by the biocide Kathon®.

Corresponding with the disappearance of the starting materials from the reactors was the appearance of fatty acids possessing the labels present in the original compounds. ^{2}H-Tetradecanoic-d_{27} acid arising from ^{2}H-tetradecane-d_{30} and 12-Phenyldodecanoic acid formed from phenyldodecane were identified. The fatty acid metabolites were found to be incorporated into complex polar lipids and were not found as free acids.

The results of this study demonstrate that ^{2}H-tetradecane and phenyldodecane in soil are both biologically degraded, converted into the corresponding fatty acids and incorporated into higher lipids. The labels in both target molecules survived the metabolic process allowing the metabolites to be traced directly to the parent molecules. The findings show that these labelled compounds can be used as sensitive monitors for the biodegradation of petroleum fuels.

INTRODUCTION

It is generally recognized that bioremediation, the biological degradation of organic pollutants, is a powerful tool for the cleanup of petroleum contaminated water and soil. Without proper verification, however, losses attributed to biodegradation may actually be due to failure to account for sampling variability, analytical variability or abiotic losses such as sorption, leaching or volatilization (Madison, 1991).

One of the most reliable ways of verifying that biodegradation has occurred is to demonstrate the appearance of metabolites that can be unequivocally traced to the pollutant (Madison 1991, Shannon and Unterman 1993, Beller *et al* 1995) . For most petroleum fuels, however, this has proven to be difficult. This is in part because the main expected products; carbon dioxide, water, acetate and fatty acids (Atlas 1981) are common in the environment and are difficult to trace to the fuel.

Aggarwal and Hinchee (1991) have explored the use of stable isotopes to link the production of carbon dioxide to the biodegradation of petroleum fuel in contaminated soil. The approach was based on the premise that the ratio of the stable isotopes ^{13}C and ^{12}C in fuels is different than that of carbon dioxide present in the air today. Others (Beller *et al* 1995) have identified products of the anaerobic biodegradation of alkyl benzenes in groundwater contaminated with gasoline.

A different way to link metabolic products to specific fuel components is to spike the fuel with parent molecules possessing recognizable labels that can be traced to the daughter products. Several reports, reviewed by Atlas (1981), describe the addition of radioactively-labelled precursors, such as ^{14}C-labelled hexadecane, to spilled fuels followed by monitoring of the ^{14}C-labelled carbon dioxide produced. This approach has also been widely used to investigate the degradation of herbicides and pesticides in soil (Fuhr 1985). These experiments provide firm evidence of biological degradation of the substrate but possess obvious containment and licensing problems when used in the field.

In the present work, we have investigated the biodegradation of phenyldodecane and fully-deuterated tetradecane, analogues of n-dodecane and n-tetradecane, typical of the straight chain alkanes present in diesel fuel and home heating oil. These analogues were chosen because they are chemically stable, biodegradable and possess readily identifiable mass spectral characteristics. As well they are expected to survive the metabolic process with labels intact and are closely similar to normal components of diesel fuel. These features make the analogues ideal for extending the technology to the field.

EXPERIMENTAL SECTION

Reactor Design. Laboratory scale reactors were comprised of 0.5 litre Mason jars containing 100-200g Guelph sandy loam soil, 1 g commercially available peat moss and the target compounds. The target compounds ^{2}H-tetradecane and phenyldodecane were added to the soil dissolved in 1-2 ml of acetone giving final concentrations of 200-1300 μg/g. Where diesel fuel was employed, it was added directly to the soil and mixed well. Except in the case of nitrogen-purged controls, the Mason jar lid inserts were replaced with circles of filter paper to allow the passage of air into the jar. Nitrogen- control reactors were purged with nitrogen and sealed with a standard Mason jar lid. Kathon® controlled reactors were treated with 46 μg/g biocide. The reactors were kept hydrated and incubated at either room temperature (25 °C) or refrigerated (4 °C) depending on the regimen required. Soil samples (2 grams) removed from the reactors were flushed with nitrogen and stored at -20 °C prior to extraction and analysis.

Extraction and Analysis of Soil. Extraction of the non-polar target compounds and diesel fuel from soil was done by sonication using dichloromethane as a solvent (USEPA SW-846). Polar lipids were extracted from the soil using a Bligh & Dyer (chloroform/methanol/water) extraction (Kates 1972).

The target compounds in dichloromethane extracts of soil were determined by combined gas chromatography-mass spectrometry (GCMS). Quantification was done using ^{2}H-dodecane-d_{26} as an internal standard. Under these conditions fatty alcohols and aldehydes are also extracted and are detectable if present. Fatty acids present in chloroform/methanol/water extracts of soil were analyzed as the methyl esters by GCMS employing the same chromatographic conditions as above.

Identification of Neutral and Polar Lipids. Complex lipids present in chloroform/methanol/water extracts of soil were fractionated by thin layer chromatography using silica gel plates eluted with polar lipid and neutral lipid solvent systems as described (Kates 1972). The compounds were visualized by ultraviolet light or by charring with sulphuric acid. For analysis, the thin layer bands were scraped and the lipid material converted into fatty acid methyl esters.

RESULTS AND DISCUSSION

Evidence of Target Compound Depletion. In initial experiments, reactors were spiked with 400 µg/g each ^{2}H-tetradecane and phenyldodecane. The soil had a moisture content of about 20%. The reactors were incubated under aerobic conditions at 4 °C and 25 °C. One set of reactors was flushed with nitrogen, sealed and incubated at 25 °C. The soil was periodically sampled over a three week period, extracted and analyzed by GCMS.

The results, summarized in Figure 1, show that under aerobic conditions at room temperature, both target compounds are completely degraded within about 16 days. The initial rate of degradation of deuterated tetradecane displayed apparent first order kinetics with a half life of about 87 hours. There was a reproducible time lag of about 48 hours in the degradation of phenyldodecane during which time the rate was negligible. The period of maximum degradation for this compound occurred during 92 and 192 hours.

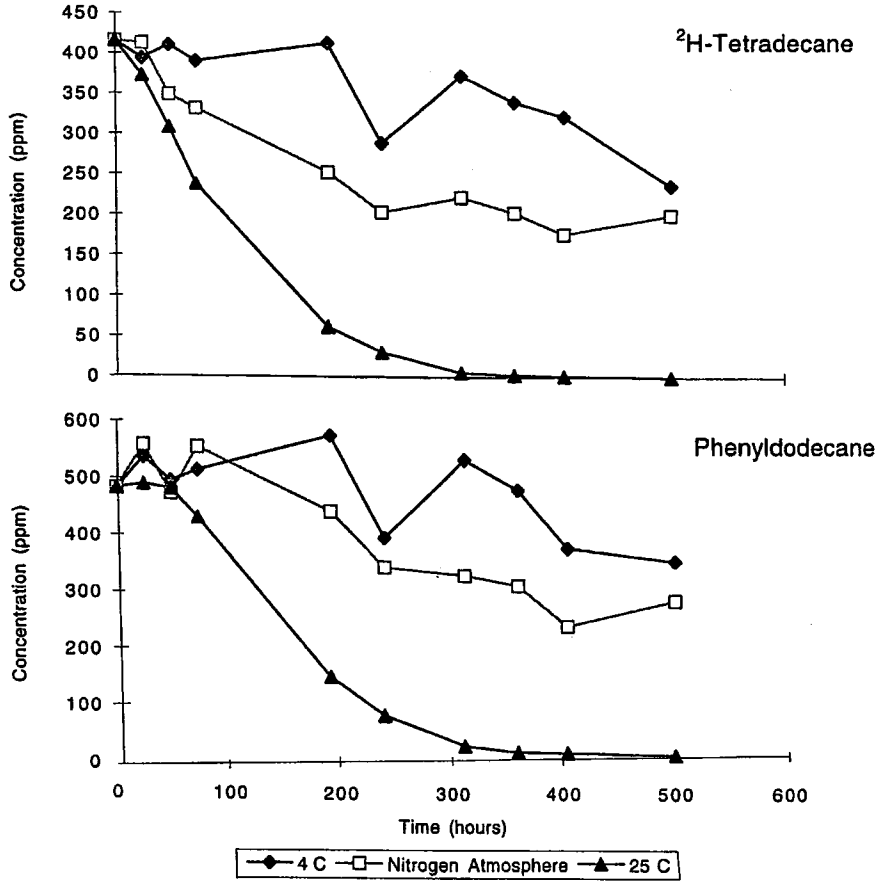

FIGURE 1: Degradation of ^{2}H-Tetradecane and Phenyldodecane in Laboratory Scale Reactors

Evidence for Biological Degradation. Evidence that the loss of target molecules from soil containing reactors was the result of biological action was collected in two ways. First, experiments were conducted to examine the effect of metabolic inhibitors on the reactor. Second, the reactors were examined for metabolic products.

As illustrated in Figure 1, the degradation of ^{2}H-tetradecane and phenyldodecane was markedly slower in soil reactors under a nitrogen atmosphere. About 50% of the material was degraded during the three week test period. No loss of either target compound was detected in the 4°C reactors before 200 hours of incubation. During the subsequent 300 hour period, the concentrations of both compounds decreased to 60-70% of their starting values.

The results of incubation of ^{2}H-tetradecane with soil in the presence of the biocide, Kathon®, is shown in Figure 2. The mode of action of Kathon® is to inhibit respiration causing bacterial, fungal, and yeast cell death (Supelco, 1997). The biocide essentially stops the reaction confirming that the observed loss of the target compounds is the result of biological activity.

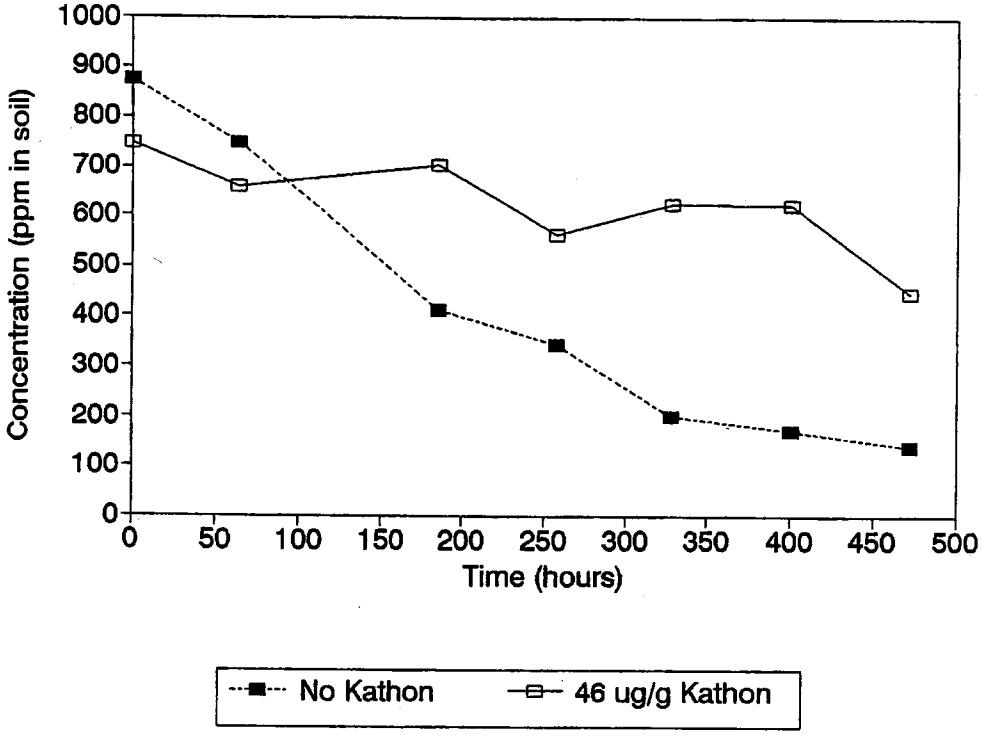

FIGURE 2. Degradation of ^{2}H-tetradecane in the presence of local garden soil and the biocide Kathon®.

Investigation of Metabolic Products. To look for metabolic products, soil samples subjected to 16 days of incubation were extracted, resolved into polar lipid fractions by thin layer chromatography and reacted to form methyl esters of any fatty acids present. The methylation reaction used converts both free fatty acids and those found in complex lipids, such as glycerides or waxes, into their methyl esters.

Analysis of the polar lipid fractions showed a range of normal fatty acid methyl esters from about 12 to 20 carbons in length in samples for both control and test reactors. These most likely represent the fatty acid component of the polar lipids arising from the microbial population.

In three of the polar lipid fractions from the reactors spiked with the target compounds and exposed to air at room temperature, compounds identified as ^{2}H-tetradecanoic acid-d_{27} methyl ester and phenyldodecanoic acid methyl ester were found. The mass spectrum and gas chromatographic retention time of the deuterated metabolite matched that of authentic ^{2}H-tetradecanoic acid-d_{27} methyl ester available commercially. The spectra of the metabolite and the authentic material are shown in Figure 3.

Authentic phenyl dodecanoic acid was not available commercially, but the mass spectrum of the phenyl-labelled metabolite possessed all of the expected elements including ions at 91 and 105 mass units characteristic of alkyl benzenes, m/z 74 typical of methyl esters, m/z 290 corresponding to the molecular weight of the compound and m/z 258 corresponding to the loss of methanol form the molecular ion. The mass spectrum was also closely similar to authentic purchased samples of the related homologues phenyldecanoic acid methyl ester and phenyl hexanoic methyl ester.

These metabolites were not detected in the control reactor which contained the same soil and peat mix without the target compounds. No fatty alcohols, fatty aldehydes or water soluble metabolites that could be traced to the starting material were detected in any of the reactors.

The nature of the polar lipids in which the metabolites were found was not firmly established, but the mobility of the material on the thin layer plates would be consistent with phosphatidyl ethanolamine or phosphatidyl glycerol. Fatty acids produced from n-alkanes by bacteria in culture have been shown to be incorporated into cellular glycerolipids including phosphatidyl ethanolamine and phosphatidyl glycerol (Makula and Finnerty 1972).

The detection of metabolic products possessing the labels present in the starting material, and their incorporation into higher glyceride structures confirms that the removal of the starting material from the reactors is the result of biological action. This is also supported by our ability to stop the reaction using a commercially available biocide.

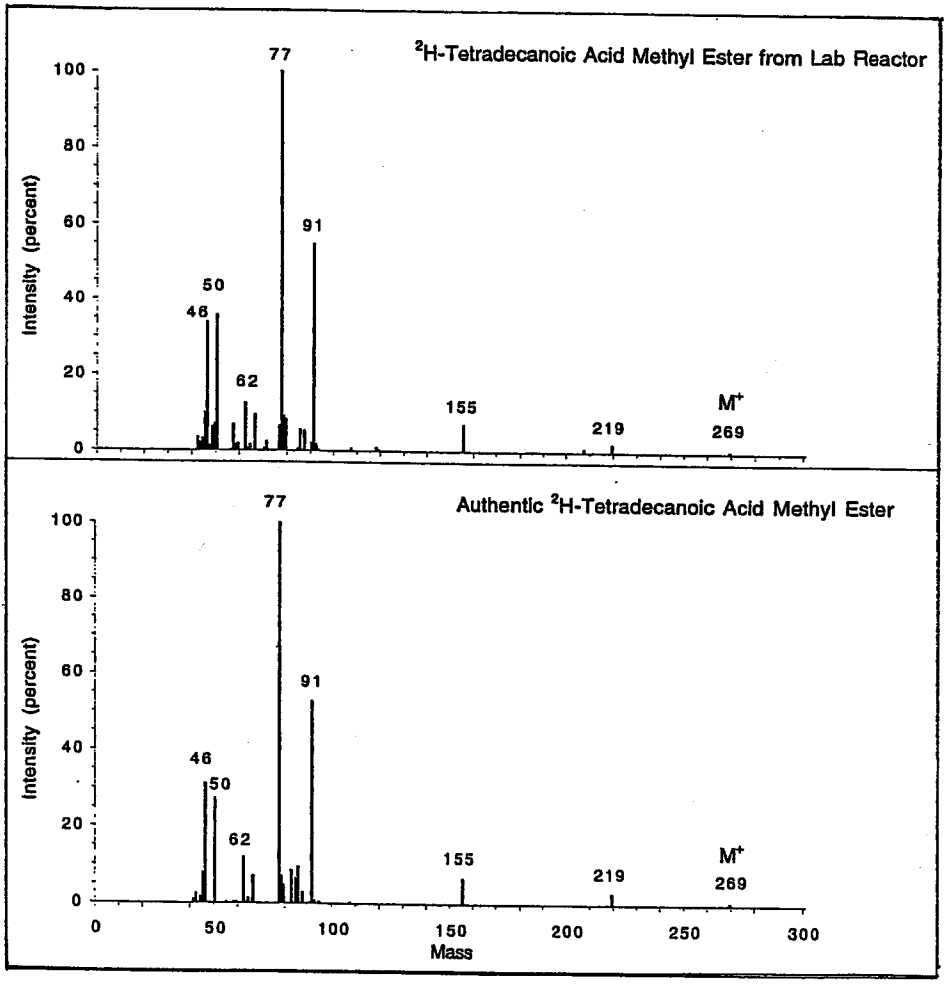

FIGURE 3. Mass spectra of ^{2}H-tetradecanoic acid-d_{27} methyl ester derived from ^{2}H-tetradecane in the lab scale reactors and authentic ^{2}H-tetradecanoic acid-d_{27} methyl ester.

Relationship Between Compounds and Metabolites. The results of the initial reactor experiments establish that both phenyl-labelled and fully deuterated alkane analogues are oxidized to the corresponding fatty acids and incorporated into higher lipids by micro-organisms in soil. In the following experiments, the kinetics of the appearance of these metabolites were investigated.

To track the appearance of the metabolites over time, samples of soil were taken over a three week period from the aerated reactors incubated at 4 °C and 25 °C and nitrogen-flushed reactor incubated at 25 °C. The total lipid extract of each was methylated and analyzed by GCMS. The results, summarized in Figure 4, show that under aerobic conditions at room temperature, the metabolite ^{2}H-tetradecanoic acid-d_{27}, formed from the degradation of ^{2}H-tetradecane-d_{30}, increases in concentration to a maximum of about 3 ppm after 150 hours then decreases. This trend has been observed in other studies of the rate of appearance of metabolites (Beller *et al* 1995) and may indicate further conversion of the metabolite. It is commonly accepted that fatty acids undergo beta-oxidation in bacteria, yeasts and fungi expected to be present in soil (Atlas 1981). We found no trace of chain-shortened fatty acids possessing deuterium labels in any of the samples from this study.

Much lower amounts of ^{2}H-tetradecanoic acid-d_{27} were formed in the nitrogen-flushed reactor in the first 400 hours. This is consistent with the assumption that oxygen is required for the conversion of alkanes to fatty acids by microorganisms in soil (Bartha 1986). There are reports of the biological degradation of petroleum hydrocarbons occurring under anaerobic conditions (Caldwell et al, 1999, Fuhr, 1985) but, in this case we cannot rule out that t6he metabolism that occurred under a nitrogen atmosphere may also have been due to incomplete purging or to trace amounts of oxygen leaking into the reactor.

Neither metabolite was detected in the soil from the 4 °C reactor. This is consistent with reports that metabolism of alkanes is greatly slowed at low temperatures (Bartha 1986, Pollard *et al* 1995). This result is also of interest because it implies that preservation of field samples at refrigerator temperatures may prevent biological degradation for three weeks or more, assuming that the target molecules behave in a similar manner to diesel fuel.

The overall yield of the metabolites, taken at the time point at which the maximum concentrations occurred, did not exceed 1% of the total amount of starting material on a molar basis. The total accumulation of the deuterated fatty acid reached about 2.5% of the total fatty acid content of the samples at this point. These levels of incorporation are low compared to reports of the behaviour of bacteria in culture. It has been reported (Makula and Finnerty 1968) that *Micrococcus cerificans* grown on n-tetradecane in culture produced tetradecanoic acid that accumulated in the biomass until it represented the main fatty acid. These cultures also produced the corresponding alcohol. This was not observed in our experiments. The rates of fatty acid production found in our experiments may underestimate the true rate if subsequent metabolism is occurring, however, we take the generally low yield to indicate that the bulk of the starting material is mineralized to carbon dioxide.

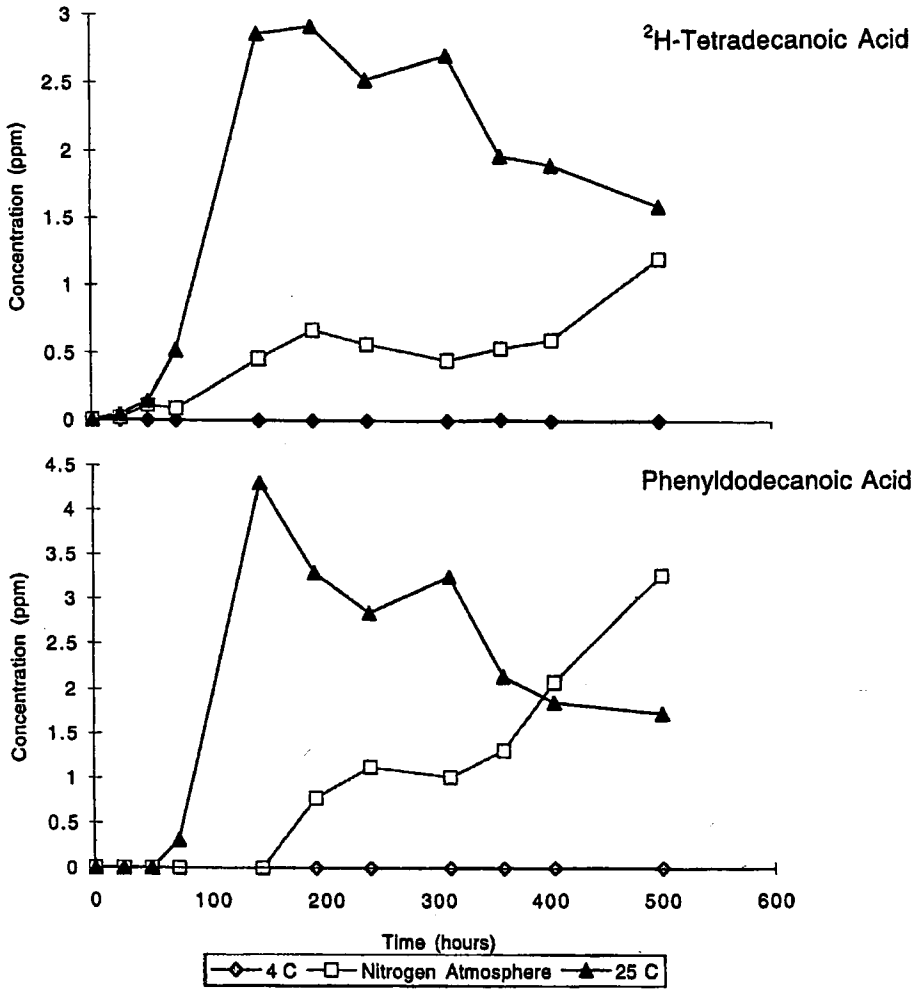

FIGURE 4: The Rates of Formation of 2H-Tetradecanoic and Phenyldodecanoic Acids at 4 °C, 25 °C, and Under Nitrogen

Degradation in the Presence of Diesel Fuel. To assess the suitability of ^{2}H-tetradecane and phenyldodecane as markers for the degradation of diesel fuel, reactors containing soil (200g) were spiked with the two target compounds at concentrations of 1300 µg/g and with diesel fuel at a concentration of 13, 000 µg/g. This level of fuel was chosen as it is typical of moderately contaminated sites and allows adequate distribution of the fuel in the soil reactor without separation of free product. The reactors (in triplicate) were incubated at 25°C and lost moisture replenished as before. Duplicate samples (2 grams each) were collected daily for 4 weeks and stored frozen until analysis.

The results, illustrated in Figure 5, show that the degradation of the diesel fuel occurred at a uniform rate for the first 2 weeks, then stopped after about three quarters of the fuel had been consumed. The time lag noted in earlier experiments during the initial 24-48 hours was also seen in these experiments. The target compounds, ^{2}H-tetradecane and phenyldodecane degraded at a uniform rate in the presence of diesel fuel in our reactors.

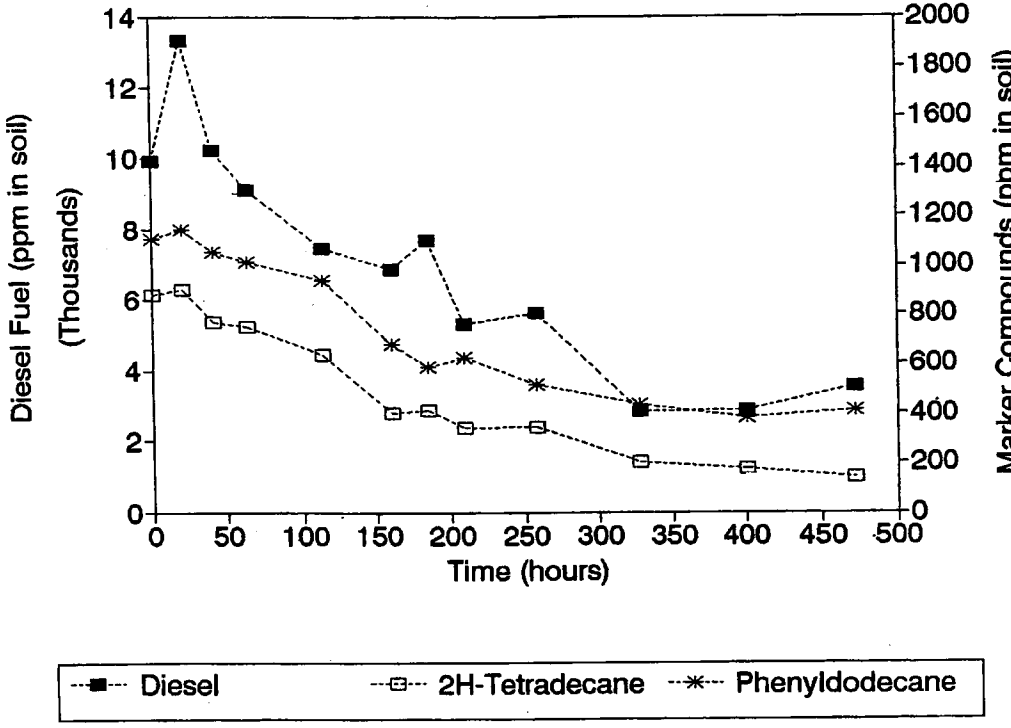

FIGURE 5: The Rates of Degradation for Diesel Fuel, 2H-Tetradecanoic and Phenyldodecanoic Acids at room Temperature

CONCLUSIONS

The results of this study demonstrate that in lab reactors containing soil, the target compounds ^{2}H-tetradecane and phenyldodecane are biologically degraded, converted into their corresponding fatty acids and incorporated into higher lipids. The labels in both target compounds survived the metabolic process and gave identifiable daughter compounds traceable directly to the parent molecules. To our knowledge this is the first demonstration of the formation of metabolic products of stable isotope and phenyl labelled alkanes during biological degradation in soils

The findings show that both phenyl and deuterium labelled alkanes can be used as sensitive tools for the confirmation of biodegradation. The rates of degradation of ^{2}H-tetradecane and phenyldodecane are similar to that of diesel fuel and their unlabelled analogues. We predict that longer or shorter chain length labelled compounds offer the opportunity for easy manipulation of the rate of degradation to suit needs in the field. Further, the metabolic products are easy to detect in soil because they appear against a blank background, unlike native metabolites. Under optimal conditions of temperature and nutrients, the metabolites can be seen within a few days. The results also indicate that the metabolites are themselves further degraded. This is an important prerequisite for the use of these labelled-compounds as markers for biodegradation at contaminated sites.

Potential application of these markers include assessment of biodegradation potential of soils in lab scale experiments or under actual field conditions and for verification of biodegradation during the bioremediation of contaminated sites. We are currently investigating these applications.

ACKNOWLEDGEMENT

The project was funded jointly by National Science and Engineering Research Council of Canada, Bell Canada and Investigative Science Incorporated.

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