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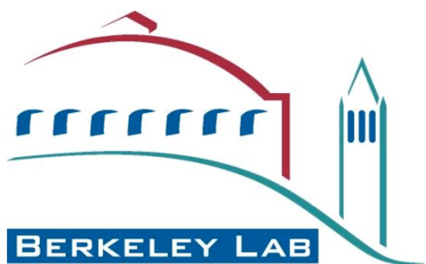

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Microbial Hydrocarbon and Toxic Pollutant Degradation Method

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Microbial Hydrocarbon and Toxic Pollutant Degradation Method

Purpose: The goal of this project is to determine optimum conditions for bacterial oxidation of hydrocarbons and long-chain alkanes that are representative of petroleum contamination of the environment. Polycyclic Aromatic Hydrocarbons (PAHs) are of concern because of their toxicity, low volatility, and resistance to microbial degradation, especially under anaerobic conditions. The uniqueness of our approach is to use carbon-11 in lieu of the traditional use of carbon-14.

Background: Petroleum hydrocarbon contamination of marshland sediments is a grave concern following oil spills from ships and leaks from oil refineries. Marshlands are environmentally sensitive habitats because of the varied wildlife they support and the anoxic conditions they present (Teal and Kanwisher, 1961 and 1966). Because of the relatively low level of water circulation found in marshes, conditions tend to be anaerobic and thus hydrocarbons and other pollutants are oxidized slowly (Blumer and Sass, 1972). In these environments, the sulfate-reducing bacteria (SRB) are able to mineralize oil hydrocarbons by using sulfate as the terminal electron acceptor in cellular respiration instead of oxygen (Klepac-Ceraj et al. 2004, Shin et al. 2000). Even in oxic environments, degradation of pollutants can be made more effective by additives such as phosphates and nitrates (Fedorak and Westlake 1981, Olivieri et al. 1978, Röling et al. 2002).

The ability of microorganisms to degrade hydrocarbons is now well known since ZoBell's review on the fact that bacteria can utilize hydrocarbons as a sole source of energy and carbon (Zobell 1946). Not only bacteria but fungi can mineralize (reduce to CO_2) crude oil in the aliphatic fraction range, n-C_{12} to n-C_{26} (April et al. 2000). The most sensitive method for assay of the rate of degradation of hydrocarbons has been through the use of radiocarbon assays, usually of the evolved $^{14}CO_2$ gas from oxidation of a carbon-14 labeled hydrocarbon added to water or sediment. Methods used for both hydrocarbons and labeled pesticides generally involve a single growth flask wherein the evolved $^{14}CO_2$ is flushed from the headspace by air and then passed through a trap containing scintillation counting cocktail (e.g. Kazano et al. 1972). To obtain the rate of oxidation, a series of flasks were needed and this apparatus along with the sterile control flasks presented a cumbersome method particularly for evaluation of contaminants that are slowly mineralized by aquatic microorganisms. A single flask method using aliquot sampling was devised to avoid this problem (Fedorak et al. 1982).

A major deterrent to the use of these carbon-14 based methods is the potential exposure of laboratory equipment and personnel to radioactive contamination from the 5080-year half-life of carbon-14 even though only 0.2 to 10 microCi are used per experiment. Because the biological half-life of most carbon compounds is only 10 days, the absorbed radioactive dose in personnel is not significant, but the contamination of laboratory equipment including assay instruments requires special care. Two alternatives to tracer studies with carbon isotopes are stable carbon-13 and the short half-life carbon-11 ($t_{1/2}$ = 20.38 min). Carbon-13 is difficult to use in kinetic studies as it requires mass spectroscopy for analysis and the natural background (1.1%) is many times higher than the range of tracer doses.

Materials and Methods:

Reaction Vessel Design: A reaction vessel was designed and constructed as depicted in Figure 1. The 150 mL pyrex glass vessel is equipped with 7 openings. A gas input port allows N_2 sweep gas to enter the reaction vessel through a frit near the bottom of the vessel. The frit maintains the sediment in the slurry and prevents it from leaving the main body of the vessel. The sweep gas bubbles through the slurry from the bottom and exits the outlet port. On the sides of the reaction vessel are four probe access ports, for the insertion of electrode probes (Thermo Orion) for real-time measurements of pH, dissolved oxygen, sulfides, and temperature during the experiments. Finally a large diameter septa cap on the top of the vessel allows for the addition of reagents into the reaction vessel.

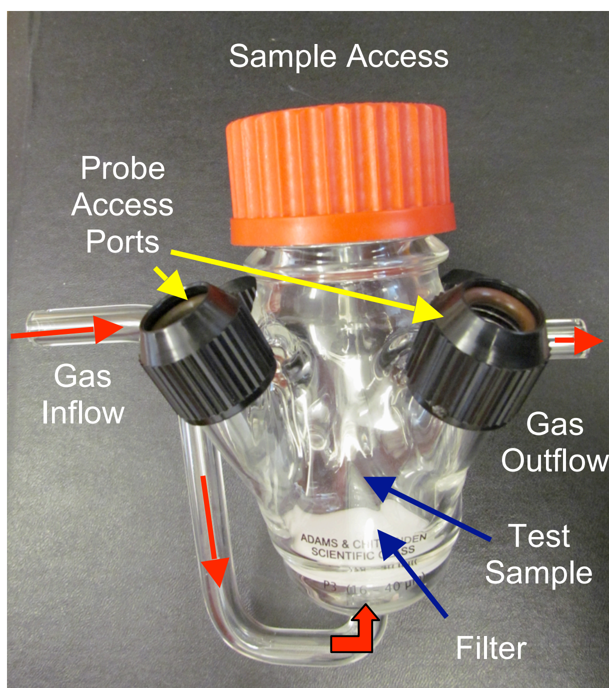

Figure 1. Reaction Vessel for Sample Incubation and Reaction Monitoring

Marshland Slurry Preparation: Marshland sediment and marsh water will be collected from non-radioactive sites (e.g. Gulf of Mexico Region, Bay Area, etc.). The marsh water will be mixed with the sediment samples (50:50, vol/vol) to provide a slurry, 100 ml of which will be added to the reaction vessel. Experiments will be conducted in both aerobic and anaerobic conditions.

Initial Enrichment Conditions: Sulfate-reducing conditions will be established by the addition of NH_4Cl, KH_2PO_4, Na_2SO_4, $Na_2S{\cdot}9H_2O$ and vitamin B-12 in varying amounts. Initial quantities of these nutrients and hydrocarbons will be, per L of marsh water: 380 mg NH_4Cl, 570 mg KH_2PO_4, 550 mg hydrocarbons (Olivieri et al. 1978), 2.84 g Na_2SO_4, 0.35 g $Na_2S{\cdot}9H_2O$ and 1 μg vitamin B-12 (So et al. 2001).

Mineralization of Hydrocarbons: Radiolabeled hydrocarbons including Me-[^{11}C]Toluene, 1-[^{11}C]n-Dodecane, 1-[^{11}C]n-Hexadecane and Me-[^{11}C]1-Methylnaphthalene will be radiolabeled using the corresponding Grignard or Lithium reagent (Figure 2,Kihlberg et al. 1994, Langstrom et al. 1980, Rahman et al. 1987) These substrates will be added to the chamber by microliter syringe through the septa on the top of the vessel.

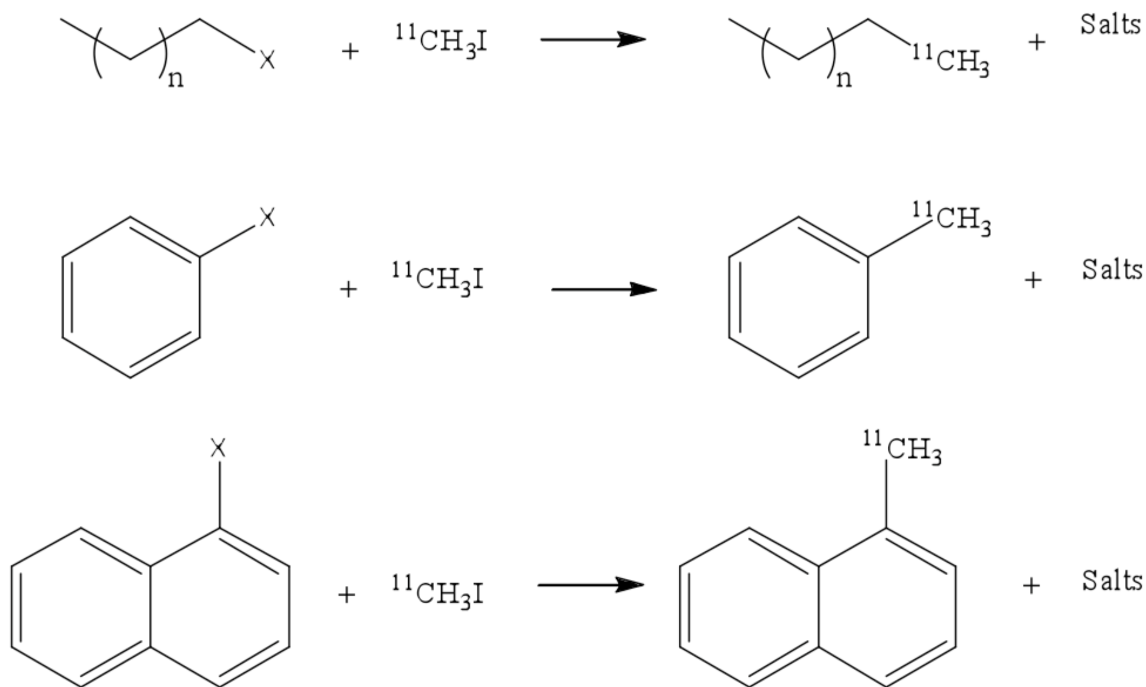

Figure 2 Radiolabelling of Hydrocarbons

The reaction vessel will be connected to a closed aeration system (Figure 4). A stream of N_2 gas bubbles through the slurry and carries evolved gases such as $^{11}CO_2$ created during the oxidation of the ^{11}C-tagged hydrocarbons into scintillation vial traps. Ethylene glycol methyl ether will trap the volatilized hydrocarbons and KOH will trap the $^{11}CO_2$. Radioactivity will be measured by gamma scintillation counting using a gamma counter (PerkinElmer, Wizard 3 model 1480) and gas chromatography (GC). Prior to flushing N_2 gas through the aeration system, for each sample a small volume of gaseous headspace from the reaction vessel (250µl) can also be analyzed by GC .

An SRI model 8610C model gas chromatograph will be utilized, equipped with a Methanizer, Thermal Conductivity Detector, Flame Ionization Detector, and Gamma Detector (Carroll & Ramsey Associates, model 105S-1). The GC contains a Zebron ZB5 column, which is capable of separating alkanes (dodecane, hexadecane), aromatic hydrocarbons (toluene, naphthalene) and their metabolites from carbon dioxide. The FID will be able to detect organic molecules and,

with the Methanizer, CO_2 with a high sensitivity (lower than 100ppm). The Gamma Detector will be able to detect ^{11}C-labeled compounds. PeakSimple Software will be used to collect the data from the gas chromatograph.

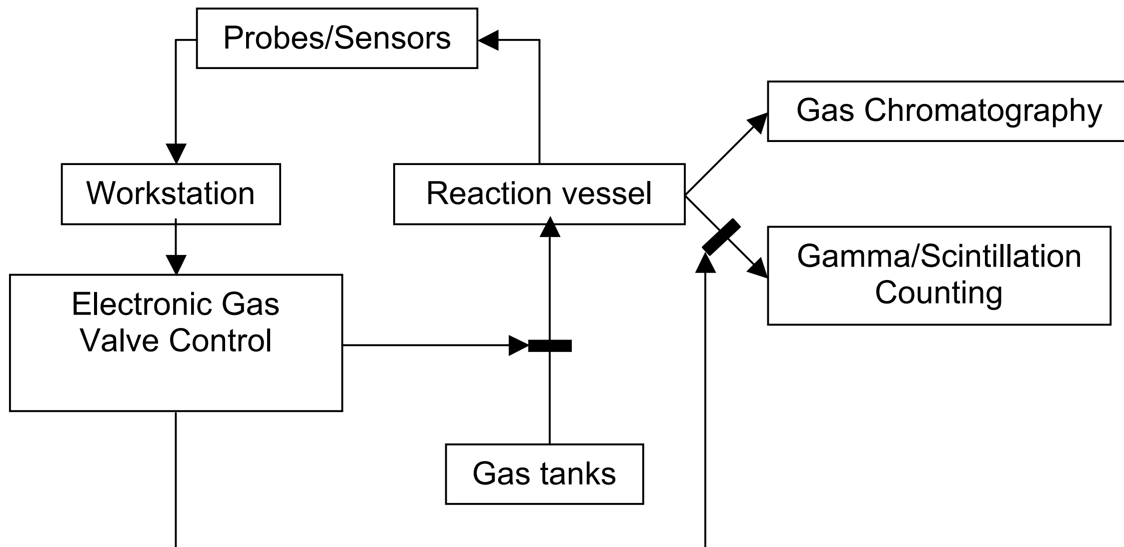

Figure 3: Experimental Schematic

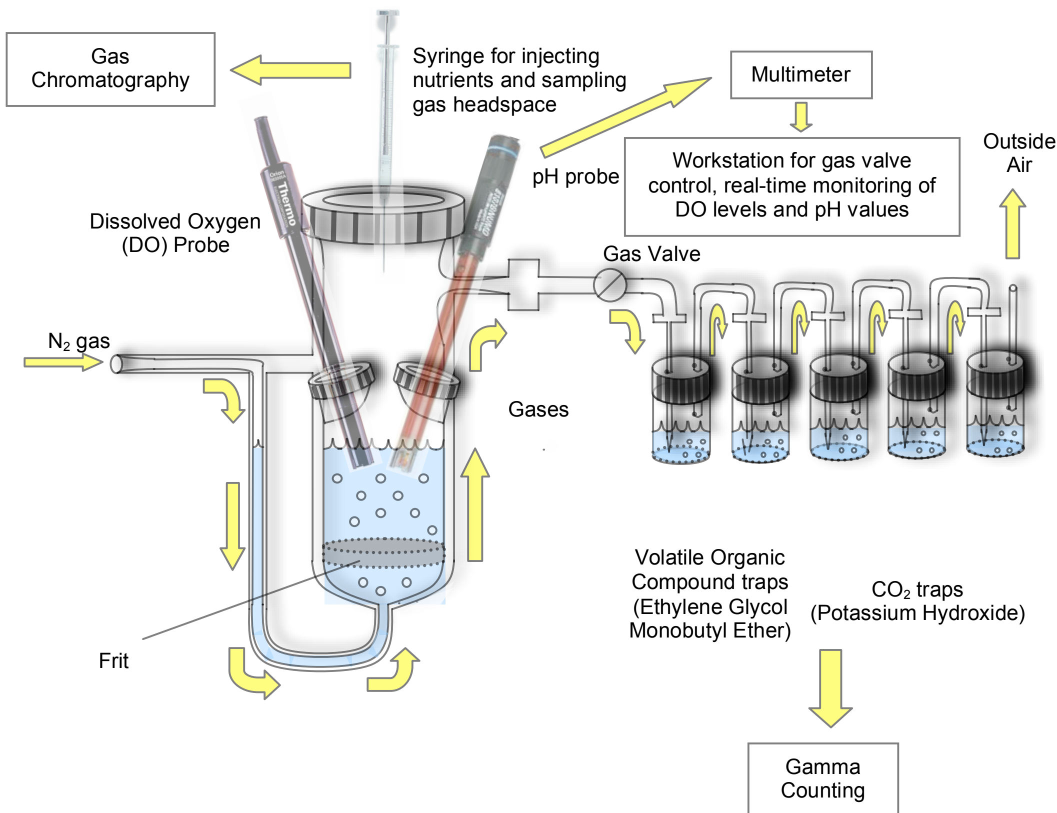

Figure 4 Gas Chromatography and Scintillation Counting

Theoretical projections for expected sensitivity

Available $^{11}CO_2$ at optimum:

The expected yield of $^{11}CO_2$ is determined from a model that assumes a linear increase in degradation product from aerobic or anaerobic oxidation of the labeled contaminant tracer.(Karl 1992) The amount available to assay is the product of the accumulation and the radioactive decay. Thus,

$$f(t) = \mu \cdot t \cdot A \cdot e^{-\lambda t} \qquad (1)$$

Where A is the activity added initially, μ is the rate of degradation (oxidation), λ is the physical decay constant and t is time. This model is based on the assumption that the fraction of A that is degraded per time interval is constant but A is decaying while the degradation product in being produced. After taking the first derivative of (1) and setting it equal to zero, the time of maximum accumulation is given by:

$$t_{max} = 1/\lambda,$$

given that $\lambda = \ln(2) / t_{1/2}$ and carbon-11 has a half-life of 20.38 minutes:

$$t_{max} = (\ln(2) / 20.38 \text{ min})^{-1} = 29.4 \text{ min} \qquad (2)$$

We can now estimate the measurable yield after inoculating soil with a tracer hydrocarbon such as hexadecane. From the literature on oil spills in cold water(Karl 1992), the rate constant μ for oxidation at 0°C is calculated to be 1.39×10^{-7} min^{-1}, using known values for the concentration of hydrocarbons in sediment and hydrocarbon degradation rate and assuming first-order kinetics.[1] Using equation 1 and inserting values for the 0°C rate constant and an injected tracer amount of 1 mCi one calculates:

$$f(29.4 \text{ min}) = 1.5 \times 10^{-3} \mu\text{Ci} = 55.5 \text{ dps}. \qquad (3)$$

The function f(t) for the 0°C rate constant, twice that for 20°C as was measured, and 10 times the 0°C constant, we calculated Figure 5.

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[1] From Karl 1992, degradation rate of hexadecane at O°C = 10^{-12} moles n-C_{16} g^{-1} sediment dry weight day^{-1} and [total alkanes] = 5×10^{-9} g^{-1} Antarctic sediment dry weight.

Sesnitivity of HPLC assay:

The conventional HPLC columns and radiation detectors require injected amounts of microcuries for adequate signal to noise and quantitative analysis As the samples taken from this system will most likely be of lower activity, unless larger quantities of labeled hydrocarbons are initially added which would lead to increased radiation exposure or the complication of highly shielded setup, a more sensitive HPLC radiation detector is needed. A recently published development in HPLC radioactivity detector system (Huber et al, 2011) may most likely solve this issue. For very low rate constants the expected yield is very low as shown in equation (3). Can 1-2 nanoCi be detected by HPLC and peaks of chemical components be shown with an acceptable signal to noise? The recent work of Huber, et al (2011) shows the feasibility of this approach.

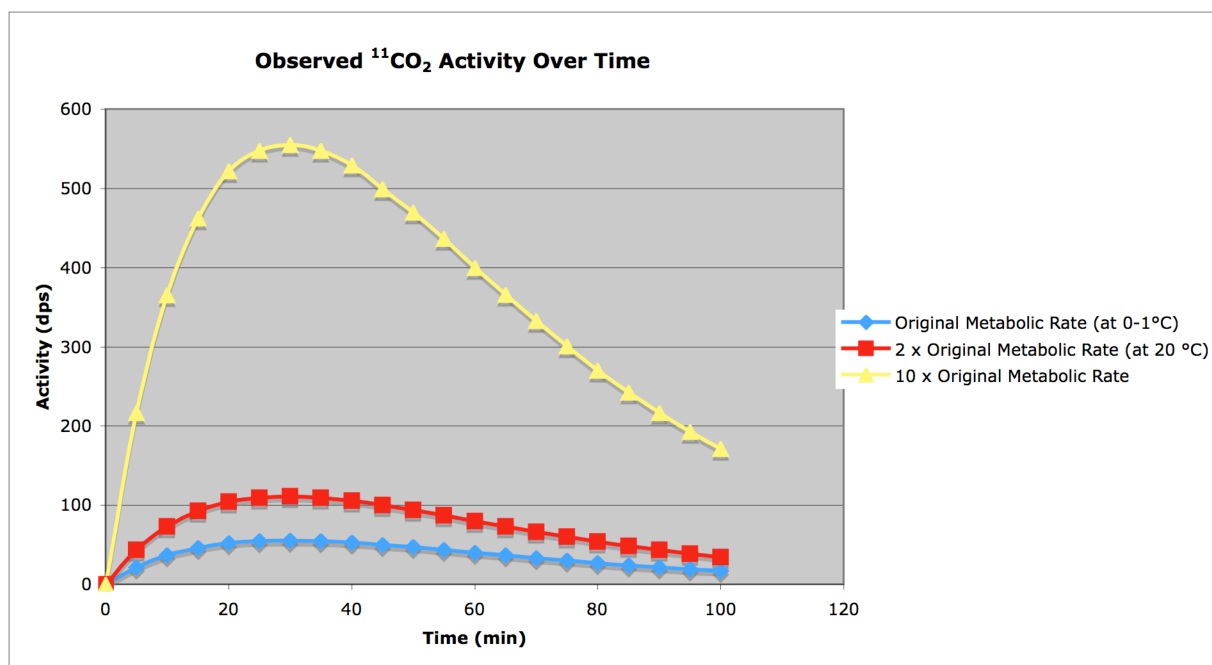

Figure 5 Production of $^{11}CO_2$ with respect to Time

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