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UAF radiorespirometric protocol for assessing hydrocarbon mineralization potential in environmental samples

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Abstract

Following the EXXON *Valdez* oil spill, a radiorespirometric protocol was developed at the University of Alaska Fairbanks (UAF) to assess the potential for microorganisms in coastal waters and sediments to degrade hydrocarbons. The use of bioremediation to assist in oil spill cleanup operations required microbial bioassays to establish that addition of nitrogen and phosphorus would enhance biodegradation. A technique assessing 1-^{14}C-n-hexadecane mineralization in seawater or nutrient rich sediment suspensions was used for both of these measurements. Hydrocarbon-degradation potentials were determined by measuring mineralization associated with sediment microorganisms in sediment suspended in sterilized seawater and/or marine Bushnell-Haas broth. Production of $^{14}CO_2$ and CO_2 was easily detectable during the first 48 hours with added hexadecane levels ranging from 10 to 500 mg/l of suspension and dependent on the biomass of hydrocarbon degraders, the hydrocarbon-oxidation potential of the biomass and nutrient availability. In addition to assessment of the hydrocarbon-degrading potential of environmental samples, the radiorespirometric procedure, and concomitant measurement of microbial biomass, has utility as an indicator of hydrocarbon contamination of soils, aqueous sediments and water, and can also be used to evaluate the effectiveness of bioremediation treatments.

Introduction

Following the EXXON *Valdez* oil spill on March 24, 1989, the University of Alaska Fairbanks (UAF) developed and implemented a procedure to measure numbers and mineralization activity of hydrocarbon degrading microorganisms in marine sediments of Prince William Sound and the Gulf of Alaska. In the summer of 1990, the protocol was slightly modified to assess the effectiveness of bioremediation on beaches contaminated by the EXXON *Valdez* spill (Lindstrom et al. 1991). The biomass measure developed for the protocol was a miniaturized most probable number (MPN) assay for surfactant producing microorganisms called Sheen Screen (Brown & Braddock 1990). We describe here the rationale, experimental justification and utility of the portion of the protocol which measures the potential for microorganisms in environmental samples to mineralize hydrocarbons.

Rationale

Biodegradation testing of organic compounds of limited solubility has been investigated for regu-

latory purposes (Boethling 1984; Fogel et al. 1985) for modelling (Chakravarty et al. 1972) and for determining physiological mechanisms of oil biodegradation (Aelion & Bradley 1991; Heitkamp & Cerniglia 1987; see Leahy & Colwell 1990). Typically, biodegradation tests measure CO_2 evolution, biochemical oxygen demand or loss of the parent compound after incubation of a known concentration of that compound under defined conditions (Fogel et al. 1985). The UAF radiorespirometric protocol is designed to measure the hydrocarbon mineralization activity of microorganisms in environmental samples. Ideally, the activity of the microorganisms would be measured under conditions completely independent of any of the complex factors which regulate their hydrocarbon mineralization activity in situ. Such measures are often called 'biodegradative potential' (Bartha & Atlas 1987; Aelion & Bradley 1991). This radiorespirometric assay is therefore designed to minimize all of the complex factors regulating microbial hydrocarbon metabolism (including hydrocarbon availability) except the in situ microbial biomass and the potential to degrade hydrocarbons in each sample.

The rate of $^{14}CO_2$ (r*; dpm/da) production from a specific radiolabelled hydrocarbon is a function of the overall rate of CO_2 production (R; μg/da), and the specific activity of the added radiotracer (A*/(Sn + A); dpm/μg), where A* is the total radioactivity added, Sn is the in situ hydrocarbon concentration (μg/g sediment or ppm in water) and A is the concentration of hydrocarbon added with the radiolabelled hydrocarbon. Therefore:

$$r^* = \frac{A^*}{(Sn + A)} \times R \qquad (1)$$

The actual rate of CO_2 production from all carbon sources in the sample (R) may, in turn, also be a function of hydrocarbon 'availability' represented by (Sn + A), initial biomass, and the respiratory activity of that biomass. Finally, the respiratory activity is controlled by incubation conditions which dictate the growth rate limiting factor.

By adding a relatively large amount of hydrocarbon substrate to each sample to be assayed, A in equation (1) will be large enough to make r* primarily dependent on A, rather than Sn, for all but the most grossly contaminated samples (Seki 1976). By incubating the microorganisms from the sediments in nutrient-rich medium under identical conditions for relatively short periods of time, the effect of most other external factors, including microbial growth rate, is minimized. Therefore, r* is the 'potential' for the microbial community derived from a sediment sample to metabolize the particular hydrocarbon added in the assay (A). Those microbial communities with high r* imply exposure to and acclimation (higher biomass and/or activity) to the same petroleum components in nature as added in the assay (A) (Bauer & Capone 1988). The protocol can also be used to measure bioremediation treatments by incubating suspensions of microorganisms from sediments in nutrient unamended water or seawater, or microorganisms in water samples with no amendments. Such a modified protocol was used to measure the effectiveness of various bioremediation treatments following the EXXON *Valdez* oil spill. Measured either way, r* is not a measure of in situ hydrocarbon mineralization rates.

The mineralization potential of marine sediments for hexadecane, phenanthrene, naphthalene, benzene and benzo[a]pyrene has been assayed based on the above rationale. For this report we describe justification for a specific protocol to assess the mineralization potential of hexadecane which was mineralized to the greatest extent in Prince William Sound following the spill of crude oil and has been used as a paradigm for linear alkanes in a variety of environmental samples (Caparello & LaRock 1975; Seki 1976).

Materials and methods

Materials

[1-^{14}C]-hexadecane and n-hexadecane were obtained from Sigma Chemical Company (St. Louis, MO). The specific activity of the radiochemical varied with the particular experiment (see results). The source material had a specific activity of 5 mci/mmol.

Samples

Beach sediment samples were collected from various places in Prince William Sound, Alaska during 1989 and 1990. Some assays were done within a few hours of sample collection on board ship or at a remote laboratory. Other experiments (including respirometry) were done during the winter of 1990–91 at the University of Alaska with composite samples from various heavily oiled sites (> 5 grams of total petroleum hydrocarbons per kilogram of material).

Radiorespirometry

A fraction of each of the sediment samples was mixed 1 : 10 (w/w) in a sterile 500 ml bottle containing Bushnell-Haas broth (Difco, Detroit, MI) amended with 2–5% NaCl (BH) or sterile filtered seawater from Prince William Sound. After vigorous shaking by hand for one minute, 10 ml aliquots of the sediment suspension were pipetted into several sterile 40 ml precleaned glass incubation vials fitted with Teflon-lined septa (I-Chem Research, Hayward, CA). Triplicate vials were prepared for each condition and time of measurement. Each vial was injected with various amounts of radiolabelled hexadecane (in acetone). The resulting initial concentration of added hydrocarbon for the specific protocol is 100 μg/vial (100 μg/g wet sediment; 10 ppm of suspension).

After injecting several of the vials with radiolabelled hexadecane, one ml of 10N NaOH was injected into one vial of each series of vials at time zero to stop microbial activity and trap CO_2. The remaining vials in each series were incubated at room temperature without shaking for various time periods (48 hours according to protocol) before addition of NaOH.

To recover $^{14}CO_2$ in each killed sample, a procedure roughly analogous to that described by Marinucci & Bartha (1979) was followed. The sample was first acidified with 1.5 ml of 12N HCl and then purged for 15 minutes with N_2 gas (30 ml/min). An in-line Harvey trap (Harvey Biological Supplies, Hillsdale, NJ) containing 15 ml of acidified toluene effectively scavenged unoxidized or partially oxidized volatile hydrocarbons purged from the sample along with the CO_2. After passing through the Harvey trap, the gaseous stream was bubbled through a standard liquid scintillation vial containing 10 ml of CO_2-sorbing phenethylamine cocktail. The radioactivity in the vial was counted in a Beckman Model LSC 1800 liquid scintillation counter (Beckman Instruments, Irvine, CA) with automatic quench correction.

Time-zero controls were processed the same way and all of the time zero data for all of the suspensions were averaged. This mean (in units of 'quench corrected dpm') was subtracted from all of the mineralization potential samples, independent of source, to yield a 'corrected dpm' value. Positive controls showed that the purging system would recover greater than 99% of $^{14}CO_2$ from radiolabelled bicarbonate processed as if it were a sediment sample. The potential for carryover between samples was monitored by running blank controls through the purging line periodically. Blank controls run in this manner always fell within the range for 'time-zero' control samples.

In addition to evaluating the parameters of the assay described, we purposely modified the above radiorespirometry protocol to test its efficacy.

Respirometry

A series of sterile 160 ml serum vials was supplied with various amounts of hexadecane dissolved in acetone. All vials were placed under a fume hood and the acetone allowed to fully evaporate. To these vials, 40 ml of 1 : 10 sediment suspensions in BH broth or sterile, filtered seawater was added.

Duplicate serum vials for each treatment were sealed with rubber stoppers and incubated at room temperature on a gyrotory shaker at 130 rpm. After 48 hours, all vials were killed with 3 ml of 12 M phosphoric acid. Control vials were killed at 0 hours.

After acidification, the vials were shaken for an additional 24 hours, and then 3 ml samples of headspace gas were withdrawn from each vial and analyzed for CO_2 on a Shimadzu gas chromatograph

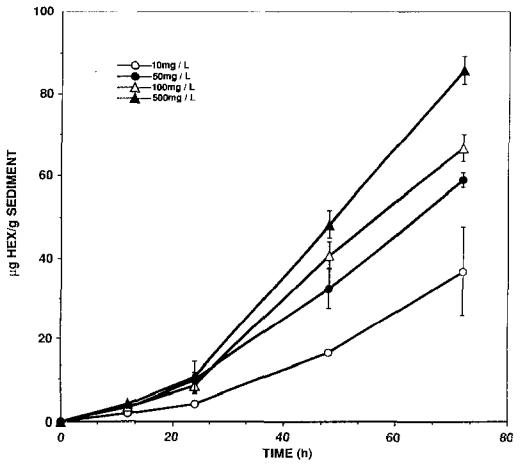

Fig. 1. Mineralization of various concentrations of added ^{14}C-hexadecane to 1 : 10 seawater suspensions of heavily oiled beach material from Prince William Sound, Alaska (composite sample). Each suspension contained 1.1×10^6 hydrocarbon degraders per gram of added sediment at time 0 (see text).

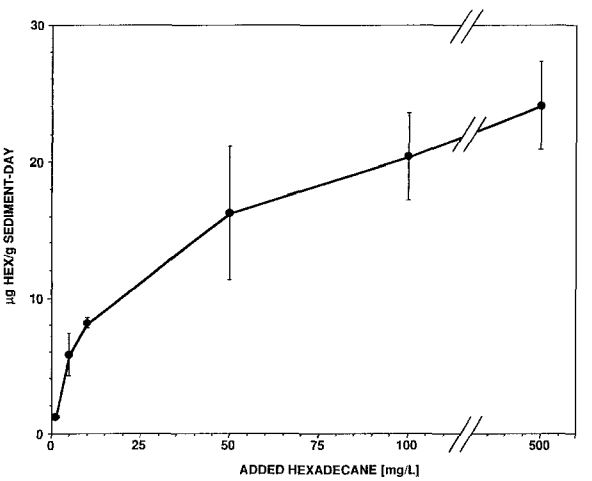

Fig. 2. Rate of ^{14}C-hexadecane mineralization as a function of added hexadecane concentration. Rates were calculated from data in Fig. 1 at 48 hours incubation and based on added hexadecane (A) rather than total linear alkanes in the suspension (Sn + A).

(GC-8A) fitted with a 0.5 ml injection coil and a thermal conductivity detector. A 2 m, 1/8" stainless steel column, packed with PoraPack-N, was used at 40° C. Both injection and detector temperatures were set at 90° C. The detector current was stabilized at 140 mA for two hours prior to injection of samples. Helium was used as a carrier gas with a flow rate of 20 ml/min. CO_2 concentrations were calculated based on a standard atmospheric CO_2 concentration of 340 mg/l.

Biomass

The number of hydrocarbon-oxidizing microorganisms in each sample was determined using the Sheen Screen most probable number technique (Brown & Braddock 1990). While no technique to enumerate specific metabolic types of microorganisms in natural systems is absolute, the Sheen Screen technique, which uses disruption of an oil sheen to indicate the presence of hydrocarbon-metabolizing microorganisms in various dilutions of sample gives, consistent results that are appropriate for relative comparisons among sources of material.

Results and discussion

The first consideration in the design of a mineralization radioassay is the need to detect significant mineralization in a reasonable time frame that is both appropriate to the kinetic rationale and sensible with respect to availability of isotopes and other materials for the number of samples to be processed. Ideally, the assay should provide a measure of the hydrocarbon-degrading activity of the biomass present in a given sample. Figure 1 shows the amount of hexadecane mineralized in seawater suspensions of oiled sediment as a function of added hexadecane and time of incubation. The lowest concentration of hexadecane that we were able to add with the isotope was 0.1 ppm due to the specific activity of the ^{14}C-hexadecane (5 mci/mmol).

The source of the oiled sediment used for the data in Fig. 1 was a composite from several oiled beaches in Prince William Sound. The number of hydrocarbon degraders in the composite was determined to be 1.1×10^6/ g by the Sheen Screen method (Brown & Braddock 1990). Figure 1 illustrates a slight lag (as contrasted to acclimation) followed by linear production of $^{14}CO_2$ from added hexade-

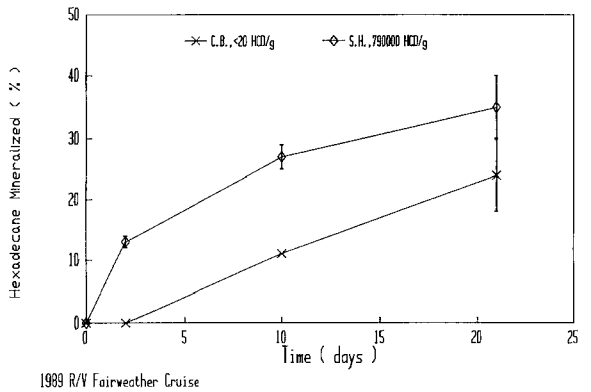

Fig. 3. Mineralization of 10 mg/l ^{14}C-hexadecane added to 1 : 10 BH suspensions of beach material from Columbia Bay (×---×) and Snug Harbor (◇---◇), Alaska. Columbia Bay material was not oiled and had fewer than 20 hydrocarbon degraders per gram of sediment in the suspension. Snug Harbor material was heavily oiled and contained 7.9×10^5 hydrocarbon degraders per gram of sediment in the suspension.

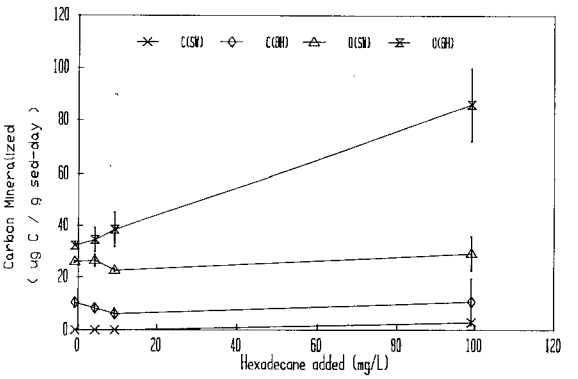

Fig. 4. Mineralization of oil and oil plus various concentrations of added hexadecane in 1 : 10 BH and seawater SW suspensions of beach material used in Figs 1 and 2 (oiled) and from Port Fidalgo, Alaska (not oiled; C). The beach material from Port Fidalgo had fewer than 20 hydrocarbon degraders per gram of sediment in the suspension.

cane. Such results have been typical for hundreds of oiled sediment and water samples from both marine and freshwater environments assayed in this manner. When the daily rate of $^{14}CO_2$ production is calculated after two days of incubation from the data in Fig. 1, the relationship between mineralization rate and added hexadecane concentration is observed (Fig. 2). The selection of 48 hours for rate calculations is made for illustrative purposes only and is not based on kinetic principles. However, the data in Figs 1 and 2 show that for environmental samples of mixed microbial populations of hydrocarbon degraders, 48 hour incubation with ^{14}C-hexadecane over a range of concentrations will result in significant levels of $^{14}CO_2$ production. Shorter incubation increases the likelihood of lag phase interference, and longer incubation will increase the likelihood of increased $^{14}CO_2$ production through 'acclimation' of the natural population to hexadecane.

Figure 3 illustrates that environmental samples with undetectable numbers of hydrocarbon degraders (< 20/g) will acclimate and mineralize hexadecane upon prolonged incubation (enrichment), while environmental samples with high numbers of hydrocarbon degraders (7.9×10^5/g) will produce significant $^{14}CO_2$ from hexadecane within 48 hours.

Figure 3 shows the utility of the protocol for assessing which environmental samples have been exposed to linear alkanes. Those 'acclimated' for hexadecane mineralization show high potentials after only 48 hours of incubation in nutrient-rich suspensions. Figure 3 also illustrates that through the addition of 10 ppm hexadecane, an experimentally significant percentage of the added hydrocarbon is mineralized. The rate of $^{14}CO_2$ production (r*) in Fig. 3 is clearly a function of biomass *and* its hexadecane biodegradation potential and not any other resource or physical constraint since all samples were incubated under the same conditions in nutrient-rich (BH broth) suspensions.

Figure 4 illustrates that ^{14}C-hexadecane mineralization reflects carbon mineralization as measured by total CO_2 production within 48 hours from seawater and nutrient amended suspensions. The source of the heavily oiled sediment used for the data obtained in Fig. 4 was the same as that used for the data reported in Figs 1 and 2. The clean sediment was not oiled and had fewer than 20 hydrocarbon degraders/g. Carbon mineralization observed in seawater suspensions of oiled sediment appears to be nutrient-limited with C mineralization rates unaffected at increased hexadecane concentrations (Fig. 4). These results do not indicate that added hexadecane is not mineralized in the seawater suspensions (see Fig. 1), only that increases in total C

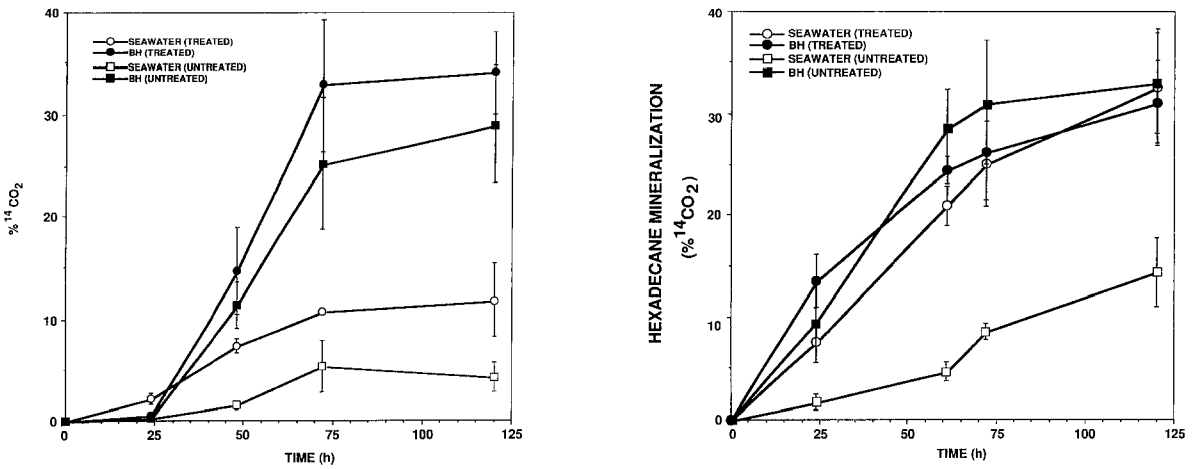

Fig. 5. Mineralization of 10 mg/l ^{14}C-hexadecane in 1 : 10 (5a) or 1 : 100 (5b) seawater (open symbols) and BH suspensions (closed symbols) of untreated oiled beach material from Disk Island in Prince William Sound, Alaska before (□, ■) and four days after (○, ●) fertilizer application to the beach.

mineralization rates are not detected. In addition to substantially more CO_2 being produced from oiled sediment without added nutrients than clean sediment without added nutrients, Fig. 4 shows that when high levels of nutrients are present in the sediment suspensions, only the microorganisms associated with the oiled sediment show increased CO_2 production at elevated concentrations of added hexadecane. This confirms that the conditions used for the radiorespirometry protocol are useful for sensitive measurements of mineralization activity as a function of nutrient availability as well as of biomass (Fig. 3).

Figure 5 shows how the protocol was used to interpret the effectiveness of fertilizer treatments of beaches in Prince William Sound oiled after the EXXON *Valdez* spill. Significantly more of the 10 mg/l hexadecane added to nutrient enriched (BH) suspensions of untreated oiled beach sediment was mineralized than those sediments suspended in seawater (Fig. 5a). Samples taken from the fertilized portion of this beach (four days after fertilizer application) showed no difference between seawater and nutrient-rich suspensions (Fig. 5a). The number of hydrocarbon degraders did not increase significantly four days after fertilization of this beach, thus the increase in r* could not be attributed directly to biomass (within the limits of resolution of the Sheen Screen '5 tube' MPN method).

Figure 5b shows results from incubations with more dilute suspensions of Disk Island beach sediment with 10 mg/l of added ^{14}C-hexadecane. The results indicate that, for this particular sediment suspension, available nutrients rather than biomass are probably the major contributing factor to increased hexadecane mineralization potential after fertilization.

Finally, experiments have shown that large additions of Prudhoe Bay weathered crude oil (2.5 g/l) to suspensions of oiled sediment and the acetone carrier have little or no effect on hexadecane mineralization (Data not shown). Other factors such as alternate carbon sources, sediment or water type, incubation conditions, etc., may also affect data interpretation. Thus, as with any protocol, once established, the specific procedures must be rigorously followed. While those procedures will vary depending on the process and question being investigated (i.e. fuel oil vs. crude oil mineralization potential, terrestrial or aqueous, fertilized vs. unfertilized, etc.), each sample must be handled identically for each protocol.

Conclusions

The UAF radiorespirometric protocol was developed for assessing hydrocarbon mineralization potential of microorganisms in sediments and water of Prince William Sound, Alaska. The protocol, when using nutrient-amended suspensions, has proven ideal for detecting 'acclimated' biomass of hydrocarbon degraders (Braddock et al. 1990) and when using seawater suspensions, for assessing the effectiveness of bioremediation of oil fouled beaches (Prince et al. 1990). While distinguishing between increased potential as a function of increased 'active' biomass and increased potential as a function of availability of a limiting resource can be problematical for individual samples incubated without added nutrients, the need to know why potentials vary is often not as important as the ability to detect differences in mineralization potentials.

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