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Utility of radiotracer activity measurements for subsurface microbiology studies

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Summary

Radiotracer activity measurements were conducted on subsurface sediments collected from the Savannah River Plant, Aiken, SC. Sediments were aseptically extruded from stainless steel core liners into a nitrogen flushed glove bag. Subsurface materials were immediately inoculated into aerobic and/or anaerobic tubes for time course experiments. Mineralization experiments utilized ^{14}C-2-acetate and ^{14}C-UL-glucose, while radiotracer uptake experiments included ^{14}C-1-acetate incorporation into lipids and ^{3}H-methyl-thymidine incorporation into microbial DNA. Microbiological activity of subsurface sediments varied 5 orders of magnitude between dense compacted clays and water-bearing sands. Aquifers tens of meters beneath the earth's surface exhibited activities greater than some of the near-surface soils. Radiotracer techniques proved to be sensitive, reproducible and applicable to field implementation. Agreement was observed between water abundance, sand content and microbial activities.

Key words: Activity measurement; Subsurface sediment; Mineralization; Aquifer

Introduction

Dogma has held that microbial activities in sediments were restricted to the surface centimeters [1]. Recent studies have suggested the presence of large and diverse microbial communities in groundwaters [2–5]. Microbiology of groundwaters and terrestrial subsurfaces is receiving increased interest in light of groundwater and vadose zone contamination and impending loss of substantial portions of our potable water resources [6, 7]. Reclamation of some contaminated subsurface waters may be relegated totally to microbial processes necessitating detailed assessment, and monitoring of microbial activities and potentials in subsurface environments.

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Examination of the ecology and physiological potentials of indigenous subsurface microorganisms is an integral aspect of biological remediation of contaminated groundwaters. Estimates of microbial biomass and diversity, carbon and electron flow, catabolism, growth and nutrient status are necessary, but missing pieces of data. Techniques must be applicable to on-site utilization during field sampling. Once the ecology of subsurface systems is examined, strategies for rectification can be developed. The monitoring of subsurface chemistry and microbial activities is imperative for controlling, reclamation and proper maintenance of subsurface environments.

This report focuses on the utility of radioisotope activity analyses as measures of catabolism and anabolism. The radioisotope experiments were sensitive in that they delineated microbial activity between lithologic formations within a given well, formations between wells, differences among wells, i.e., zones of severe contamination or zones of high nutrient loading. Techniques described were suited to field application and proved successful at evaluating microbial activities spanning 6 orders of magnitude.

Materials and Methods

Gases, chemicals and isotopes

Nitrogen and N_2:CO_2 (90:10%) were greater than 99.9% pure. In the laboratory all gases were passed through copper-filled Vycor furnaces (Sargent-Welch Scientific Co., Skokie, IL) to remove traces of oxygen. All chemicals used were of reagent grade and were obtained from Mallinckrodt (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Resi-analyzed glass-distilled solvents and reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). [^{14}C-1-]-Acetate (56 mCi/mmol), [^{14}C-1,2-]-trichloroethylene (10 mCi/mmol) and [methyl-^{3}H]-thymidine (79.9 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [^{14}C-2-]-Acetate (56 mCi/mmol) and [^{14}C-UL-]-glucose (2.8 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL).

Description of experimental site

The Savannah River Plant (SRP) is a 768 square kilometer area set aside by the U.S. government in the 1950s for the production of national defense material. In 1958 an unlined surface impoundment was constructed in the M-area for settling metal processing wastes. Waste waters entering the basin also contained substantial quantities of solvents common to the metal finishing industries. For over 2 decades liquids overflowed and leached through the basin contaminating the local subsurface with short-chained chlorinated hydrocarbons. Principal among the contaminants was trichloroethylene (TCE) which is presently greater than 1 mg/l in several subsurface formations including some water-bearing zones. Five core-holes were examined in this study. Well MSB-805 was approximately 10 m from the basin boundary, while other wells were 1–5 km from the basin (Fig. 1). Retrieval of subsurface core materials began during the fall of 1984.

Sampling techniques

Professional Service Industries, Inc. (Jackson, SC), provided the core drilling serv-

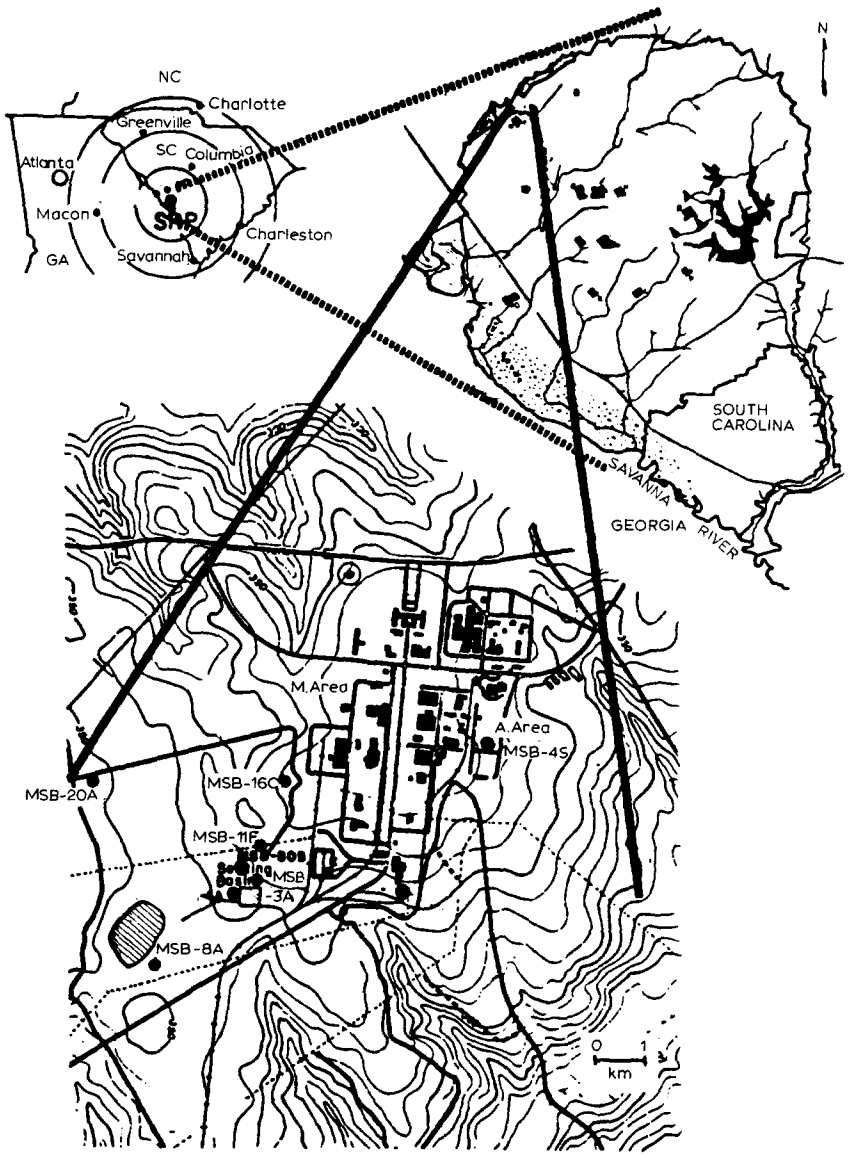

Fig. 1. Map of MSB well clusters.

ices. Monitoring wells were cored to a depth of approximately 100 m and screened in the major aquifer which provided potable water. Coring at site MSB-805 ceased in a dense clay formation approximately 25 m above the major aquifer. Quik-gel (NL Baroid/NL Industries, Inc., Houston, TX), a sodium bentonite viscosifer drilling fluid, was used as the drilling mud. Undisturbed sediment materials were obtained using a Pitcher Barrel (Pitcher Drilling Co., Palo Alto, CA), Shelby tubes or a Dennison

corer (Acker, Scranton, PA) depending on sediment type. All sediments were collected in core liner tubes constructed of either brass or stainless steel which were steam cleaned prior to use. The Dennison corer utilized a stainless steel basket to retain unconsolidated sands. Soft clays were obtained using a 76 cm stainless steel core tubes with the Shelby type sampler. The Pitcher corer was well-suited to obtain more consolidated materials or dense clays. Several samples required multiple attempts and a variety of samplers in order to recover consistently greater than 85% of the sediment material.

Retrieved sediments were removed from the drill line and immediately carried into the mobile microbial ecology laboratory (MMEL) where they were removed from the core liners using an extruding device (Model P-107, Soiltest, Evanston, IL). Sediments exiting the core liners automatically entered a N_2-gas flushed bag constructed from 3 layers of polyethylene slotted for gloves, core liner and a sample removal port. In latter experiments a portable anaerobic chamber equipped with airlock and gloves (Coy Laboratory Products, Ann Arbor, MI) was used. Sediments were placed into sterile whirlpak bags for lyophilization and aerobic experiments, into N_2-gas flushed tubes for anaerobic experiments, or into N_2-gas flushed canning jars for storage.

Time course experiments

Sediment aliquots were inoculated for aerobic and anaerobic activity experiments on-site within 30 min of core extrusion. All isotope solutions (1–50 μCi) were frozen prior to use, thawed and transferred with gas-tight syringes (Hamilton Co., Reno, NV). Time course experiments were performed in duplicate using sterile polypropylene centrifuge tubes for aerobic isotope incorporation experiments and anaerobic crimp top tubes (Bellco Glass Co., Vineland, NJ) for anaerobic experiments. All incubations were at ambient temperature which was similar to the in situ temperature of 20–25 °C.

Acetate incorporation experiments contained 2 g sediment, 5.0 μCi of ^{14}C-acetate and 1.0 ml sterile distilled water. At t_0 and appropriate time points duplicate incorporations were inhibited with 3.0 ml methanol and frozen. In subsequent studies biological inhibition was accomplished by the addition of a phosphate-buffered chloroform-methanol solution which enabled delineation of lipid type (data not shown).

Thymidine incorporation experiments contained 1.0 g sediment, 30 μCi of ^{3}H-methyl-thymidine plus 2 nmol of nonradioactive thymidine in 1.0 ml of sterile distilled water. At appropriate time points duplicate incorporation tubes were inhibited by the addition of 3.0 ml 80% ethanol and samples frozen at -20 °C. Preliminary time course experiments consisted of 10 incubation times from 30 min to 1 month. Time points of 0, 30 min, 2 h, 8 h, 1 d and 4 d, generally provided linear rates within 3 or more points.

Mineralization experiments contained 2 g sediment, 1.0 ml sterile water and 2.0 μCi of carrier-free ^{14}C-2-acetate or ^{14}C-UL-glucose in 25 ml crimp top tubes. Time course experiments were performed in duplicate with 10 time points ranging from t_0, 1 h and up to 1 month. At selected time points duplicate tubes were inhibited with 0.5 ml of 2.0 M sodium hydroxide.

Analytical procedures

In the laboratory acetate incorporation experiments were thawed and sediments ex-

tracted by a modification [8] of the single-phase chloroform-methanol method of Bligh and Dyer [9]. The lipid fraction was evaporated to dryness and portions were counted by scintillation counting to determine the amount of radioactivity incorporated into microbial lipids. The earliest time points yielding measurable results were used to calculate a linear rate which was extrapolated to dpm/d. Lipids analyzed from groundwater samples were concentrated onto 0.2 μm polycarbonate filters (Nucleopore, Pleasanton, CA) previously extracted with methanol. The quantity of lipids was subsequently determined by the hexane-isopropanol extraction procedure previously described [10]. Lipid biomass from subsurface sediments were extracted by a modification of the Bligh and Dyer procedure [8, 9] including fractionation of neutral lipids, glycolipids and phospholipids on silicic acid columns.

Thymidine incorporation experiments were subjected to microbial lysis in 2 ml 0.3 M sodium hydroxide containing 1% sodium dodecyl sulfate, 10 mM thymidine and 1% humic acid. After heating at 110 °C for 4 h the supernatants were collected and dialyzed as previously described [11, 12]. Radioactivity incorporated into macromolecules, i.e. DNA, was determined using scintillation counting. Controls were conducted to insure that dialysis was sufficient and to verify that radioactivity retained in the dialysis tubing (molecular weight cutoff of 3550 °A) was sensitive to DNAase and additional dialysis.

Radioactive ^{14}C-carbon dioxide and ^{14}C-methane from mineralization time course experiments were examined by the gas chromatography-gas proportional counting technique described by Nelson and Zeikus [13]. A Packard 417 gas chromatograph (GC) equipped with a thermal conductivity detector was connected to a Packard 894 gas proportional counter. The GC operated at 85 °C, helium carrier gas at a flow of 45 ml/min with a 0.32 cm $\times$ 1.83 m stainless steel column packed with carbosieve (80–100 mesh). One h before analysis tubes were acidified with 0.5 ml of 6 M hydrochloric acid.

Additional support

Geological, hydrological, drilling and safety consultations were provided by E. I. duPont de Nemours and Co., and Professional Services Industries. Chlorinated hydrocarbon analyses of sediments obtained during the coring of wells were performed by Environmental Testing and Certification Inc. (Edison, NJ).

Results

In previous studies our laboratory has observed microbial activities and signature lipids in extreme environments including subsurface sediments [2, 11]. In this study we examined the usefulness of radioisotopic analyses as measures of microbial activities in subsurface sediments. Microbial activities between 5 wells and formations within wells were examined at the Savannah River Plant (SRP) in Aiken, SC.

Initial experiments examined colony forming units (CFU) of microorganisms cultured from wells screened at the various groundwater depths (Table 1). Well MSB-805 was located 10 m from the settling basin in the vicinity of highest TCE contamination. Well MSB-4A was 10 m from an adjacent side of the settling basin. Other wells were located 1–5 km from the basin. As shown in Table 1, well MSB-805 exhibited the fewest

TABLE 1

MICROBIAL COLONY FORMING UNITS, DIVERSITY AND BIOMASS ESTIMATES FROM M-AREA GROUNDWATERS

Sample MSB #	Colony forming units/ml (CFU/ml)	Diversity (observed morphology)	PLFA (pmols/20 l)	Biomass estimate from PLFA (CFU/ml)
805	7.0×10^{2}	4	<10	<10
4A	2.3×10^{3}	11	8900	4.0×10^{3}
11A	N.D.	N.D.	70	3.0×10^{1}
11F	3.3×10^{3}	>20	N.D.	N.D.
16A	N.D.	N.D.	240	1.0×10^{2}
16C	1.5×10^{3}	>20	1100	5.0×10^{2}
20A	3.6×10^{4}	>20	N.D.	N.D.

N.D. = Not determined; PLFA = phospholipid fatty acids.
All 'A' samples are from the shallow aquifer screen zone, approximately 30 m beneath the surface. Site 4 borders the trichloroethylene contaminated area, other sites are 1 – 5 km distant.

CFU and the lowest microbial diversity. The colony morphologies were all fungal and were not morphologically similar to types observed in other SRP groundwaters. Phospholipid data concurred that biomass was sparse in well MSB-805, while well MSB-4A exhibited more CFU, increased diversity including bacteria, and measurable phospholipid fatty acids (PLFA). There was general agreement between CFU, diversity and PLFA but these analyses did not directly relate to microbial activities nor was information gained about the abundance of specific populations within the microbial community.

Phospholipids represent a sizable fraction of cell wall lipids and are indicative of viable biomass and community structure in many environments [2, 8, 10, 11]. From this study phospholipids in subsurface sediments accounted for less than 1.0% of the total organics recovered from the described lipid extraction procedures (Table 2). Phospholipid concentrations ranged from greater than 10000 pmol/g in surface soils to less than 0.3 pmol/g in subsurface clays. Phospholipid concentrations of 2–100 pmol/g were typical, and corresponded to approximately 10^{4}–10^{6} microorganisms/g sediment which agreed with enumeration studies (Phelps et al., submitted). Although the large pool of hydrocarbons and sedimentary remnants of lipids masked the phospholipid fractions, these results suggest that phospholipids from deceased biomass were hydrolyzed to neutral lipids or degraded.

Previous studies in freshwater and marine sediments [12, 14–16] demonstrated the utility of using radiotracers to measure microbial activities of microorganisms. Prior to the implementation of radioisotope experiments in subsurface sediments we determined that microbial components could be recovered from sediment materials (Table 3). Cells were grown in the presence of ^{14}C-1-acetate, lyophilized and determined to contain 250000 dpm of lipid materials per 10 mg cell mass. Cell biomass totaling 1.5×10^{6} dpm was mixed into sediments for 1 h and then lipids were extracted from the sediments and radioactivity determined by liquid scintillation counting. Greater than 90% of the radioactive lipid was recovered from controls containing no sediment.

TABLE 2

QUANTIFICATION OF LIPID FATTY ACIDS FROM CORE SAMPLES

Sample		Neutral (N), Glyco-(G) and Phospho-(P) lipids (pmol/g dry weight)			Fraction of P-lipid (%)	Comments
well	depth (m)	N	G	P		
MSB-36	13	1960	1060	26	0.85	moist clayey sand
	50	1610	1320	21	0.71	sandy clay
	69	1420	4370	8.8	0.15	dry sandy clay
	90	1950	670	0.5	0.02	dry clay
	93	3060	330	88	2.5	saturated clayey sand
	96	3840	1080	125	2.5	saturated sands
MSB-805	3	1950	936	14	0.48	sand
	6	364	270	25	3.7	moist sand
	29	1960	2070	3.6	0.08	dry sand
	43	1300	369	9.2	0.54	dry sandy clay
	69	525	257	22	2.7	moist sands
	72	2390	322	27	0.98	moist sands

Ester linked, lipid fatty acids were analyzed from 40 g of lyophilized core samples by capillary chromatography after a Bligh and Dyer extraction and silicic acid column fractionation. Results are expressed in pmol/g dry weight.

Clays retained approximately one-half of the radioactive lipids, while sands retained 30%. Sandy and silty clays were intermediate in their binding. These results indicate that sediments bind lipid materials making them unavailable to chloroform-methanol extraction. However, binding would account for few differences noted between environments or sediment type in this study.

Experiments were performed to compare the level of acetate incorporation into microbial lipids and thymidine incorporation into DNA. Values were expressed as dpm/d based upon rates of the earliest duplicate time points yielding discernible

TABLE 3

RECOVERY OF ^{14}C-LIPIDS FROM SEDIMENTS

	Percent of total ^{14}C-lipids bound to sediments	
	Experiment 1	Experiment 2
Sand	30	25
Sandy clay	36	44
Silty clay	41	50
Clay	51	53
Control	0	0

Cells were grown in the presence of ^{14}C-1-acetate. Approximately 1.5×10^6 dpm of lyophilized cells (2.5×10^5 dpm of which were lipid) were mixed into sediments for 1 h and extracted by the Bligh and Dyer method. Controls contained no soil.

results. In most instances 8 and 24 h time points were used. Results from well MSB-42 are shown in Table 4. In other experiments (data not shown) surface soils exhibited more than 10^6 dpm for both acetate and thymidine incorporations. At depths of 6 and 15 m activity measures decreased more than 2 orders of magnitude. Water-bearing tan sands between 27 and 43 m incorporated considerable acetate into lipids but formed little DNA from labeled thymidine. The water-bearing sands near 67 m depth exhibited high metabolism and growth. Moist sandy clays below the water-bearing zone were low in activity. The zone of lowest activity was the dense variegated clay which lies above the drinking water aquifer. In most variegated clays neither acetate nor thymidine activities were detected after 24-h incubations. The drinking water aquifer (100 m) exhibited considerable catabolism as shown by acetate incorporation but little growth as demonstrated by low levels of thymidine incorporation.

Acetate incorporation experiments were reproducible and duplicate tubes typically agreed within 15%. Sixty subsurface sediments analyzed at t_0, averaged 90 ± 18 dpm, including the background of the scintillation counter. Zero time points for the thymidine assay averaged 440 ± 180 dpm. Larger variability of thymidine incorporation experiments was in part related to dialyzing unused radioactivity from the samples. At longer time points the variability in the thymidine assay was considerable. Data from 10 subsurface well samples agreed in that activity measurements using radiotracers varied more than 5 orders of magnitude between formations. However, samples cor-

TABLE 4

INCORPORATION OF ^{14}C-1-ACETATE INTO MICROBIAL LIPIDS AND ^{3}H-METHYL-THYMIDINE INTO MICROBIAL DNA

MSB-42 (description)	Depth (m)	^{14}C-lipids (dpm)	^{3}H-DNA (dpm)
Surface clayey sands	7	310	6240
	13	552	33
	21	583	1470
Water-bearing tan sands	28	59400	3940
	38	19100	1070
	44	165000	5450
	50	700	1790
	57	502	16800
Water-bearing sands with clay lenses	64	165000	10200
	70	781000	24300
Moist sandy clays	76	1450	572
	83	N.D.	8890
	92	2810	8230
Variegated boundary clay	101	95	0
Drinking water aquifer	105	13000	451
	108	41600	0
	115	18500	341
	117	4150	N.D.

N.D. = not determined.
Data recorded as dpm/d calculated from linear rates of isotope accumulation during time course experiments.

responding to similar lithologies in other wells often exhibited activities in the same order of magnitude.

Comparison of radiotracer incorporation experiments between formations in MSB-36A and MSB-42A are diagramed in Table 5. Four samples of the tan clays exhibited acetate incorporation into lipids averaging 365 dpm, while thymidine averaged 1130 dpm. The only measurable activity observed in 5 samples of clayey sands was 1 acetate value of 36 dpm. Variegated clays were similar in that most values were below background with the exception of 1 value of 1960 dpm for a thymidine series. From 14 clay samples, 8 acetate analyses exhibited background levels and the highest value was 700 dpm. Corresponding thymidine assays exhibited a high of 2000 dpm with 9 samples below background. Water-bearing sands exhibited activities which were several orders of magnitude greater. Wet tan sands had acetate incorporations of 24000–606000 dpm with an average of 229000 dpm. Thymidine activities ranged from 5400–11300 dpm with an average of 5530 dpm. The wet yellow sand consistently revealed the highest levels of subsurface activities with acetate and thymidine averaging 404000 and 136000 dpm, respectively. As evidenced by thymidine incorporation into DNA and subsequent microbiological culturing, this subsurface formation consistently showed the greatest amount of growth. The drinking water aquifer exhibited activi-

TABLE 5

COMPARISON OF ACTIVITY MEASUREMENTS BETWEEN SUBSURFACE SANDS AND CLAYS

Sediment type and depth	Well MSB-36A		MSB-42A	
	Acetate*	Thymidine*	Acetate*	Thymidine*
Clays				
Upper tan				
17 – 33 m	698	0	583	1470
Clayey sand				
33 – 47 m	N.D.	N.D.	0	0
			36	0
Dry variegated				
80 – 100 m	2830	0	95	0
Sands				
Wet tan sands				
27 – 43 m	27900	5820	19100	1070
			165000	5450
Wet yellow sands				
53 – 70 m	280000	6160	165000	10200
	522000	63300	781000	24300
Coarse sandy aquifer				
> 93 m	2680	2780	41600	0
	61100	3470	18500	341

N.D. = Not determined.

* Data recroded as dpm/d calculated from linear rates of isotope accumulation during time course experiments.

TABLE 6

MINERALIZATION OF ^{14}C-2-ACETATE AND ^{14}-UL-GLUCOSE TO $^{14}CO_2$ BY SUBSURFACE SEDIMENTS UNDER AEROBIC AND ANAEROBIC CONDITIONS

Sample	Aerobic		Anaerobic	
	Acetate*	Glucose*	Acetate*	Glucose*
Clays				
Clayey sand	9.2	17	11	38
Variegated	18	92	24	58
Water-bearing sands				
Yellow sands	678	291	N.D.	N.D.
Coarse aquifer	436	720	N.D.	N.D.
	1008	600	N.D.	N.D.
	984	558	12	38

N.D. = Not determined.
* dpm/d of $^{14}CO_2 \times 10^3$.
Experimental. Time course experiments were conducted in duplicate consisting of 7 time points from 1 h to 4 weeks. Tubes contained 1.0 ml sterile water, 2 g sediment, and 2.2×10^6 dpm carrier-free tracer. Tubes were incubated at ambient temperature under an air or nitrogen gas headspace. Results were analyzed using gas chromatography-gas proportional counting.

ties averaging 65000 and 17000 dpm acetate and thymidine, respectively. Water-bearing sands were generally 1000-fold more active with respect to acetate uptake than clays. Surface soils (data not shown) were $1.1–4.7 \times 10^6$ dpm for both acetate and thymidine, or 5 orders of magnitude greater than the clay subsurface materials.

Radioisotope mineralization experiments were performed to measure radiolabeled carbon dioxide or methane production from radiolabeled acetate or glucose. Time course experiments were incubated aerobically or anaerobically under N_2-gas. Time points were 0 h, 1 h, 8 h, 24 h, 7 d and 30 d. Data shown in Table 6 are reported as dpm of $^{14}CO_2$ accumulated per d. Accumulations of methane or radioactive methane were not detected. Clays exhibited nearly 2 orders of magnitude less activity than water-bearing sands. Values for the sands were calculated from 1 to 8 h time points during which no activity was observed from the clays. Values for clay samples were calculated from 7-d points and likely reflected enrichment of microorganisms. In other studies using 1-d time points negligible activity was noted in clays and differences between formations approached 6 orders of magnitude (data not shown). In no instance was the anaerobic activity significantly greater than the aerobic values.

Discussion

These results demonstrated the utility of radioisotope studies to measure the activities of microorganisms in terrestrial subsurface environments. Although CFU and lipid analyses served as indicators of biomass, they did not provide insight into the activities of the microorganisms in the environment and lacked specificity and sensitivity for the low-activity subsurface sediments. The described techniques were suitable for

water-saturated sediments containing diverse microflora [11, 12, 15, 16] or dense clays from which microorganisms were not cultured.

Growth, as evidenced by tritiated thymidine uptake into DNA, was examined. In parallel experiments, anabolism was noted by acetate uptake into lipids and catabolism was measured by the mineralization of acetate or glucose. Importantly, all techniques were amendable to field initiation enabling minimal manipulation prior to analysis. Remarkably, with single experimental designs it was possible to distinguish up to 6 orders of magnitude differences between formations within a core hole and to quantify differences between formations at different core holes. The radioisotope techniques have been used in separate studies to delineate zones of extreme contamination and toxicity (Phelps et al., submitted) and to show differences of microbial activities between wells. Activities were lowest when CFU and PLFA were negligible and appeared highest when CFU, diversity and PLFA were the greatest.

Activities were comparable between wells yet varied orders of magnitude between formations. Dense impermeable clay formations consistently exhibited negligible activities, while water-bearing sands appeared abundant in activities. Fifty meters distant from 1 well was an overhead discharge of treated waste water and microbial activities for the first 30 m depth were 2 orders of magnitude greater than shallow sediments of other wells in the area. All saturated zones exhibited activities at least an order of magnitude greater than unsaturated zones. Water availability and clay content appeared to be major controlling factors of microbial activities.

Although water samples are easier to obtain than aseptic sediments, evidence suggested that analyses of water also reflect the water delivery system. CFU and morphological diversity obtained from water may be indicative of the subsurface environment but to date analyses based on well water samples lack the specificity or sensitivity of sediment analyses. Examination of water samples does not facilitate comparisons between formations or lithology. Most noticeably well water systems drastically alter the subsurface environment by the presence of the pipe, increased flow, nutrient inputs from the drilling operation and enrichment of microbial growth throughout the delivery system.

When performing radioisotope activity measurements it is important to conduct time course experiments based upon shortest possible time frames to insure linearity of results. Additionally, the radiotracers should be carrier-free as well as free of competing substrates. For example, acetate solutions need to be free of ethanol to insure that isotope additions do not provide millimolar concentrations of an electron donor. Additions of large pool sizes of organics, water, or long incubation periods all increase the probability of enriching the microbial community. The addition of water is a no-win situation, since mixing of the isotope in clays is facilitated by additions of sterile water but incubations longer than 1 d often causes enrichment effects in many samples. This problem is addressed by using a variety of dilutions then back-calculating to the absence of any moisture as has been done to calculate the pH values for very acid soils [17].

Measurements of thymidine incorporation into DNA as described by Pollard and Moriarty [18] utilize isotope dilution experiments at each site. To perform isotope dilution studies at each subsurface site is not practical and repeat sampling is impossible. Several dilutions were conducted in preliminary experiments and the addition of

2 nmol per sample appeared best-suited to the wide range of habitats examined. Because the pool sizes were not determined we report the actual methods and dpm observed. Should pool sizes be determined then rates could be calculated as well as the rates of utilization or product formation.

The techniques described in this paper allowed measurement of microbial activities in subsurface environments spanning more than 5 orders of magnitude. Surprisingly, subsurface sediments hundreds of meters beneath the surface exhibited counts approaching 10% of those in surface soils. Contamination of subsurface sediments during sampling is of considerable concern but in this and other studies contamination by drilling fluids or manipulation appears slight ([19] and Fliermans et al., submitted). The ability to find samples of clays, which required the most manipulation, devoid of microbial activity or culturable microorganisms attested to reasonable aseptic sampling abilities. The development of more stringent aseptic sampling protocols, sensitive contamination markers and unobtrusive methods of sampling in-situ activities is under investigation.

The use of radioisotopes for assessing microbial growth, carbon flow, and metabolism have been successfully demonstrated in a variety of terrestrial subsurface habitats. The radioisotope techniques described herein were field-compatible, were initiated within minutes of sediment recovery, were amendable to aerobic or anaerobic conditions, were performed in time course experiments, and related well to geophysical or microbiological analyses. Radioisotope analyses for deep subsurface environments appear appropriate for assessing in-situ activities, evaluating the ecological processes occurring, and amendable to monitoring the rectification of contaminated subsurface environments.

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