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ATP and Adenylate Energy Charge Determinations on Core Samples from an Av-Fuel Spill Site at the Traverse City, Michigan Airport

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In 1980, residents of East Bay Township in Grand Traverse county, Michigan complained of foam and odor in water drawn from domestic wells. The Michigan Department of Public Health determined that the wells were contaminated with potentially hazardous levels of organic compounds. The Michigan Department of Natural Resources determined that the chemicals probably came from the Cherry Capitol Airport in Traverse City, Michigan. The Cherry Capitol Airport (TVC) is a joint use civil airfield–U.S. Coast Guard air station; it is the home base of Coast Guard aircraft that perform search and rescue missions for eastern Lake Michigan. In 1982 the U.S. Geological Survey made a geologic and hydrologic study of this area and of its ground water contamination (Twenter *et al.*, 1985). The suspected source of ground water contamination was the discharge of aviation (av) fuel through a failed flange on a fuel line from a fueling bay equipped with self-service fuel pumps. The flange failure was discovered in December 1969 after an 8-h loss of 2500 gal of JP-4 jet fuel (JT Wilson, *personal communication*). This site, a demonstration site for the EPA, has been the subject of extensive study and remediation (Wilson *et al.*, 1986a, Wilson *et al.*, 1986b, Wilson *et al.*, 1987; Ostendorf *et al.*, 1989; Ostendorf, 1990; Ostendorf *et al.*, 1991, Wise *et al.*, 1991; and Hutchins *et al.*, 1991).

This report details a biochemical characterization of subsurface core samples from the pollution plume at the Traverse City site. Biomass measurements were made using adenosine triphosphate (ATP) quantitation and metabolic potential determinations using adenylate energy charge (AEC) measurements. The use of ATP as a measure of biomass in environmental samples was introduced by Holm-Hansen and Booth (1966). Wiebe and Bancroft (1975) suggested that AEC measurements could aid in characterization of growth by microbial communities in environmental samples. Karl (1980) has reviewed the use of nucleotide measurements to indicate biomass and metabolic activity.

MATERIALS AND METHODS

The subsurface material was obtained by the Robert S. Kerr Environmental Research Laboratory of the U.S. Environmental Protection Agency (RSKERL). The samples were stored at 15 °C until used. Lula V cells (10^7/100 g sample were used to determine recovery (Balkwill and Ghiorse, 1985). The top one inch of material was removed from the jars and discarded; then 10-g samples of the material

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were taken in duplicate. The samples (10 g) were homogenized in 100 ml of 0.1 % sodium pyrophosphate for one min. A volume of 0.1 ml and appropriate dilutions were plated on PTYG plates as described by Balkwill and Ghiorse (1985). The colonies were counted after 48 and 72 h incubation at room temperature under aerobic conditions.

The extraction procedure and extractant developed by Webster *et al.* (1984) and modified for use with subsurface material (100 g samples) by Webster *et al.* (1985) were used.

ATP was measured in a 500-µl mixture with 50 µl of sample; 100 µl of Analytical Luminescence Laboratory's firefly luciferase (Firelight); 50 µl of Tricine buffer, pH 7.8, containing 25 m*M* Tricine, 5 m*M* $MgSO_4$, 1 m*M* EDTA, and 1 m*M* dithiothreitol; and water. The bioluminescence was determined on a Lumac/3M Model 2010A Biocounter for 10 s. An internal standard of 0.1 ng of ATP was added to the assay mixtures containing the experimental samples to determine the extent of inhibition, if any, of the assay itself (there was about 40% inhibition; addition of an internal standard of Lula V cells to aliquots of the samples before extraction corrected for the inhibition and any losses during extraction). See Webster and Leach (1980) for optimization of the firefly luciferase ATP assay.

AEC was determined by modifications of the procedures described by Ball and Atkinson (1975) and Lundin and Thore (1975). The reaction mixtures were set up as described by Holm-Hansen and Karl (1978) with the substitution of Tricine buffer for phosphate buffer and the inclusion of KCl (required for pyruvate kinase). There is less inhibition of light production from ATP with Tricine buffer than with phosphate buffer. Two-hundred-µl aliquots of the samples to be analyzed were incubated separately with 50 µl of four different buffered enzyme and substrate solutions. Reaction mix A, for the determination of ATP, contained 75 m*M* Tricine, pH 7.5; 5 m*M* $MgCl_2$; and 0.0125 m*M* KCl. Reaction mix B, for determining ATP + ADP, contained in addition to the contents of tube A, 0.5 m*M* phosphoenolpyruvate (Sigma P 7002) and 20 µg of pyruvate kinase (Sigma P-1506, the ammonium sulfate suspension was centrifuged in a Beckman Microfuge, the $(NH_4)_2SO_4$-containing solution removed, and replaced with an equal volume of 20 m*M* Tricine buffer, 2 m*M* dithiothreitol, and 0.1% bovine serum albumin). Tubes A and B were incubated for 30 min at 30 °C. Reaction mix C, for determining ATP + ADP + AMP, contained in addition to the contents of tube B, 25 µg of adenylate (myo)kinase (Sigma M 3003, an ammonium sulfate suspension of myokinase, was centrifuged 10 min in a Beckman Microfuge and the pellet suspended in 20 m*M* Tricine, 2 m*M* dithiothreitol, and 0.1 % bovine serum albumin). The C tubes were incubated for 90 min at 30 °C. All tubes were placed in a boiling water bath for 3 min to stop the reactions. The tubes were chilled in ice, then brought to room temperature just before being assayed for ATP. The AEC was calculated as described by Ball and Atkinson (1975). Mixtures of the adenylates yielding energy charges from 0.25 to 0.75 were included as controls. Table 1 shows results for these control determinations which establish the suitability of the modified procedure and document the standard deviations for AEC measurements.

Table 1. Adenylate energy charge determinations

Mixture of nucleotides	Adenylate Energy Charge	
ATP — ADP — AMP	Known	Found
1 — 1 — 0	0.75	0.75 ± 0.01
1 — 0 — 1	0.50	0.49 ± 0.00
0 — 1 — 1	0.25	0.26 ± 0.01
1 — 1 — 1	0.50	0.48 ± 0.02

RESULTS AND DISCUSSION

In June 1986 RSKERL personnel obtained core material under sterile conditions from a site (designated as Site Q) that is 750 ft from the source of the pollution at TVC and directly in the path of the plume. Some of this material was shipped in an ice-chest to Stillwater and arrived with some of the Blue Ice® coolant still frozen. There were six samples taken at depths of 17, 20, 24, 30, 35, and 45 ft. Bacterial numbers were determined via plate counts as described in the Materials and Methods section. Aliquots (100 g) of the core material were extracted and both ATP and AEC were determined as described in the Materials and Methods section.

Table 2 contains the results from the above described experiments with selected chemical analyses for the alkylbenzenes of the samples taken at this location at two different times (1985 and 1986). These other chemical analyses are used in correlation and discussion of the data. The chemical analysis data on the alkylbenzenes are from an unpublished manuscript by D Kampbell, J Wilson, B Bledsoe, J Armstrong, and J Sammons of EPA, Ada.

Table 2. ATP and adenylate energy charge measurements on Traverse City samples

Sample	Depth ft	Total alkylbenzenes mg/liter			ATP ng/g	AEC	Cells		
		'85	'86	Metabolism % decomposed			CFU	ATP	% Active
44Q5	17	11.82	0.273	97.7	1.28	0.71	2.3×10^5	2.1×10^5	91
44Q4	20				0.64	0.71	3.8×10^5	1.1×10^5	29
44Q3	24	3.15	0.149	95.3	0.24	low	9.9×10^5	4.0×10^4	4
44Q2	30	0.043	0.083	None	0.19	0.45	2.0×10^5	3.2×10^4	16
44Q1	35		0.0053		0.14	low	1.9×10^5	2.3×10^4	12
44Q0	45	0.032	0.0028	91.2	<0.02	0.34	2.4×10^5	$<3 \times 10^3$	4

Although the spill occurred in 1969, it was not discovered until 1979. JT Wilson (*personal communication*) believes that the microorganisms present in the soil were successfully metabolizing the hydrocarbons from the av fuel as they were

solubilized in the ground water and flowed from the source until about 1980. A significant fraction of the hydrocarbons were still bound to sand and gravel of the aquifer material when the samples described in this paper were taken in 1986. From 1978 to 1980 the city was installing a large sanitary sewer on the shore line of the lake. Removal of water from the construction site drew the water table down 2 ft below the seasonal low. The hydrocarbons of the av fuel were distributed over a much greater volume (3-D space) as they rode the water table. When the sewer work was completed, the water table rose again, which caused contamination of a much greater sectional area of the subsurface environment with the av fuel through which the ground water flowed to transport the pollutants. This larger plume contained more av fuel than the microorganisms could metabolize under the environmental constraints of oxygen tension, etc. and the pollutants were thus transported until they appeared in the water from the domestic wells.

Our data were obtained under one particular set of conditions; therefore, it is appropriate to define the limitations. The numbers of CFUs (colony-forming units) found by the plating procedure represent only the aerobic and facultative organisms because of the incubation conditions used during the plating. The number of cells based on the amount of ATP was calibrated by using the *Pseudomonas* sp. named Lula V that was isolated from a pristine subsurface site at Lula, OK (Balkwill and Ghiorse, 1985). The per cent of metabolically active cells was calculated by dividing the cell number found by the ATP determination by the total number of cells found from the plate count. This calculation overestimates the percentage of metabolically active organisms since the samples are anaerobic and anaerobic cells were not capable of forming colonies. Two of the samples yielded precipitates upon neutralization, which compromised the assays necessary for the AEC measurements. These samples are indicated by the term "low" in Table 2; no experiments were done to find a means of circumventing this difficulty. The total number of organisms determined by plating remained constant with increasing sample depth. The number of metabolically active cells as determined by the ATP measurements decreased with increasing sample depth. Also the AEC decreased with increasing depth indicating that cells at greater depths are less metabolically active. A representation of the metabolism of the alkylbenzenes is given by the percent of the alkylbenzenes that disappeared between November 1985 and July 1986. In the 17-, 24-, and 45-ft samples more than 90% of the alkylbenzenes were metabolized. In the 30-ft sample there was a doubling of the amount of alkylbenzenes, but this amount is low. The amount of alkylbenzenes decreased with increasing depth.

The use of subsurface material from a particular site to determine whether the natural population of organisms is capable of degrading the organic pollutants in question is a direct and quantifiable approach to the question of metabolic potential. Microcosm studies can be done with either anaerobic or aerobic conditions. Even the naturally occurring polluted ground water sample can be used to provide the substrates. Wilson *et al.* (1987) have shown that material from site C (located at the margin of the plume in a renovated zone) metabolized various alkylbenzenes, 99% of the original concentration disappearing within 2 w (the shortest time used) under aerobic conditions. The subsurface material and ground water at site C was aerobic, did not contain alkylbenzenes (indicating the absence of current pollution, and did not contain methane (evidence for the absence of past pollution and anaerobic restoration). Sterilized samples proved that this disappearance was biotic. Samples from site B also rapidly metabolized the alkylbenzenes aerobically. At this site the ground water contains alkylbenzenes, oxygen, and traces of methane. Site B is at the margin of the contaminant plume where aerobic

biorestoration is occurring. Samples from the heart of the pollutant plume (site A) are anaerobic, contain alkylbenzenes, and methane. Microcosms of this material can anaerobically metabolize the alkylbenzenes at a rate of 90% of the original concentration in 4 wk. There is difficulty with microcosm studies when the material from the site is saturated with pollutants. Samples from the heart of the pollutant plume at TVC contained an oily droplet phase that was demonstrable by microscopy (JT Wilson, *personal communication*). It took 7 bed volumes of water passing through the oil-saturated material from TVC to produce a decrease in toluene concentration was observed. Given the usual way of performing microcosm experiments, metabolism could be occurring but not be detectable. The large reservoir of hydrocarbons would mask the biorestoration. A direct measure of microbial biomass and metabolic activity is needed. ATP and AEC measurement can give that measure and establish if metabolically active organisms are present and are either metabolizing the compound or are resistant to any detrimental effect of the pollutants.

Prior studies have shown that the number of AODC (Acridine orange direct count) organisms is fairly constant with depth, but that the biorestorative potential varies. AODC measures total number of organisms and not their metabolic state. The plate counts show that there are large number of aerobic bacteria in the Q samples from TVC that do not vary significantly with depth. Previous studies have shown that biorestorative potential is reflected in the amount of ATP found (Wilson *et al.* 1986c). ATP and AEC determinations offer a rapid means of assessing the presence of microbes and their metabolic status in environmental samples.

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