

## **FIELD APPLICATION OF THE DETERMINATION OF BACTERIAL NUMBER**

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Abstract  
Statement of the Problem

### ABSTRACT

The six-component extractant developed in this laboratory [Webster *et al.* (1984)] has been divided into two mixes; this eliminated the precipitate formation that occurred in the complete mix when it cooled. It was the separation of phosphoric acid and EDTA that prevented this precipitation. Even though a precipitate formed in the completed extractant, it did not affect extraction of ATP from soil samples. The detergent polyoxyethylene 10 lauryl ether was demonstrated to function as well as Lubrol (which is no longer readily available) in the extractant. Extraction procedures using either a Brinkmann Polytron and wrist action shaker (in the laboratory procedure) or a Waring blender (for the field procedure) were equivalent. A detailed protocol is described that works well in the field. The procedure was made field-applicable through the use of a portable generator, desktop high-speed centrifuge, and a normal laboratory luminometer. Temperature variations influenced the activity of firefly luciferase and was the most important factor in achieving absolute equivalence in extraction of soil samples and the determination of ATP. The process could be greatly improved through the use of an air-conditioned laboratory trailer or van to provide the required temperature control.

### STATEMENT OF THE PROBLEM

Water, a compound essential for life and the major constituent of living matter, is a resource that must be protected from pollution. Much of our nation's fresh water reserve is ground water; for example, Bitton and Gerba (1984) estimate that 90-95% of the drinking water for rural America is ground water and that in all some 100 million Americans drink ground water. Since ground water is vulnerable to contamination and is difficult to restore once contaminated, means to protect this vital natural resource must be found quickly. Wastes and pollutants must be disposed of so that they do not become hazards to the environment, especially water. Water pollution is a state, regional, national, and international problem.

Three phenomena determine the transport and fate of pollutants in soil and in the subsurface environment (Dunlap and McNabb, 1973). These phenomena are sorption, abiotic chemical alteration, and biotic changes produced by microorganisms and/or their enzymes (a scheme for these interactions is presented in Fig. 1 with labeling of the insult, environment, and resource). Biological characterization of soil and subsurface environments will provide information on the processes influencing pollutant transport, transformation, and fate. An understanding of the ecological subtleties of these environments is essential for both the protection and restoration of surface and ground waters. Microbial activity, at least for the subsurface environment, is the most important of these three processes in eliminating pollutants (McNabb and Dunlap, 1975). Thus, microbial numbers and their metabolic activity in soil and subsurface materials will determine the persistence or disappearance of chemicals that might pollute.

## Statement of the Problem

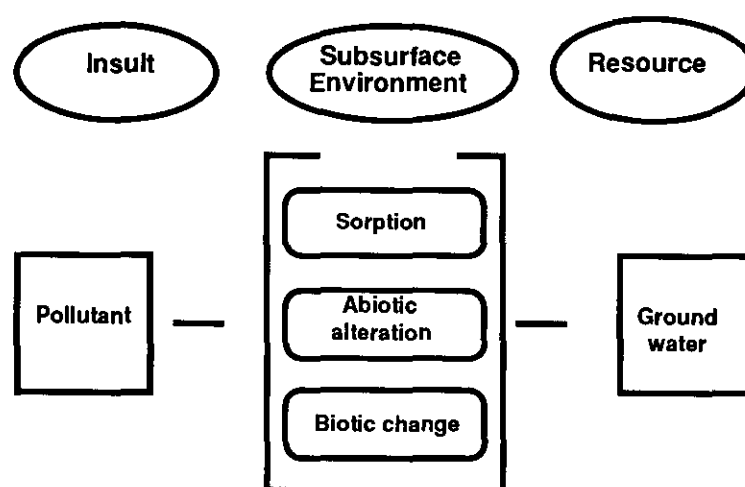


Figure 1. The Fate of Pollutants in Soil and The Subsurface Environment.

This laboratory (Webster *et al.*, 1984, Wilson *et al.*, 1986, Vaden *et al.*, 1987, and Balkwill *et al.*, 1988) has established the validity of ATP measurements for determining the number of bacteria in soil and subsurface samples. The comparison of the ATP method with other chemical indicators in the study done with others (Balkwill *et al.*, 1988) shows that several different chemical determinations will provide an estimation of the number of bacteria. The ease and sensitivity of the ATP determination make it a most promising test for routine field application. There is experimental evidence establishing a correlation between ATP content and pollutant degradation (Wilson *et al.*, 1986a and unpublished observations).

Enumeration of bacterial alone is not sufficient to elucidate the quantity of metabolically active biomass (see Jensen, 1989). We have shown (Webster *et al.*, 1992) that determination of the adenylate energy charge gives a rapid means of determining the metabolic activity of microorganisms such as those present in some contaminated soils at Traverse City, MI. This determination will reflect the current metabolic activity. ATP measurement predicts the potential for metabolism (Wilson *et al.*, 1986a).

Van de Werf and Verstraete (1987) have applied the mathematical analysis of a biokinetic modeling technique to estimation of the active soil microbial biomass via respiration determinations. This method depends on the utilization of specific substrates and therefore might be compromised by unknown substrate specificities. In addition a 2 week equilibration period in the dark was used before an incubation for 120 h, which makes this determination unsuitable for rapid field measurement of bioremediation potential.

Biorestitution of aquifers contaminated with organic compounds is a cost-effective means for restoration (Wilson *et al.*, 1986b and Lee *et al.*, 1988). A means to determine the number of bacteria and their metabolic status yields important information on the restoration process (Webster *et al.*, 1992).

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## Background

### ATP as a Biochemical Indicator

Specific chemical substances involved in microbial life processes reflect the quantity of living material present in a sample and the metabolic status of the organisms. These chemical substances are: 1) a source of reducing power, which is NADPH; 2) a source of energy, which is ATP; and 3) ten specific biosynthetic building blocks (Atkinson, 1977). ATP plays a central role in metabolism and, because of the close regulation of biochemical mechanisms, it is maintained at a fairly constant concentration. It is rapidly degraded upon an organism's death; ATP in a typical living microorganism has a half-life of 1 s. A human uses and synthesizes his/her body weight of ATP daily (McElroy, 1972) and one-sixth of all enzymes require ATP or a related adenine-containing cofactor. Ever since Holm-Hansen and Booth (1966) proposed ATP as a measure of biomass in sea water, much research has established that cellular nucleotides such as ATP are relevant indicators of biomass and metabolic activity (Karl, 1980). Figure 2 shows the linear relationship between ATP concentration and bacterial number.

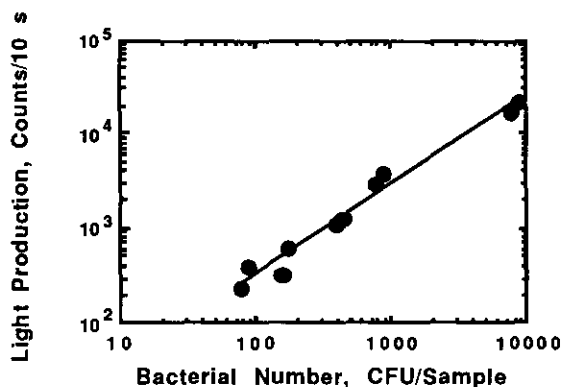


Figure 2. A Plot of ATP Content and Bacterial Colony-Forming Units.

The biochemical significance of ATP has led to the development of many methods for its determination (Leach, 1981). The most specific and sensitive determination of ATP is based on measurement of light production catalyzed by firefly luciferase (Leach, 1984). This laboratory has used firefly luciferase in environmental research for more than seventeen years.

### The Adenylate Energy Charge Concept

Observations that the ratio of two of the adenylates (ATP, ADP, and AMP) controlled enzymatic reactions led to formulation of the adenylate energy charge concept by Atkinson and Walton (1967). The adenylate energy charge is  $(AEC) = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$ . The AEC is a parameter measuring the energy status and metabolic potential of cells (Ball and Atkinson, 1975). Wiebe and Bancroft (1975) proposed that determination of the adenylate energy charge (at several times during an experiment) would measure the growth rate in natural microbial communities. Their research established the validity of using energy charge measurements for determination of a population's metabolic status. Cellular metabolic regulation maintains the concentrations of the adenylates within a narrow range; therefore, marked decreases in AEC reflect environmental stress. Actively growing cells have AECs in the range of 0.8-0.95; stationary cells maintain AECs of about 0.6; and

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senescent cells have AECs below 0.5. Holm-Hansen and Karl (1978) have described methods for the application of biomass and adenylate energy charge determinations to environmental samples. While AEC determinations are not reported in this paper, they could easily be done using the appropriate enzymes, substrates, and cofactors by using the soil extract.

## Application of ATP Determinations to Soil and Subsurface Samples

If ATP is to be used as an indicator of surface and subsurface biological activity, there must be: 1) procedures for extracting all ATP from the sample material so that it can be measured and 2) sensitive and specific analytical methods for the determination of ATP. The chemical structure of ATP influences its extraction and recovery from environmental samples. The aromatic rings are planar and tend to stack with other aromatic molecules. Hydrogen bonds can also occur. The base portion has basic properties while the phosphate portion is acidic -- meaning that the behavior of ATP will be influenced by pH. The polyphosphate portion can interact with metal ions and contains the "high energy" of anhydride linkages which is available for biochemical functions. It also reacts with phosphate-binding sites. Ribose, a 5-carbon sugar, supplies reactive hydroxyl groups for polymerization when ATP and dATP serve as precursors of the polynucleotides. It is very difficult to extract ATP from soil or subsurface material quantitatively. Most extractants applied to soil were originally developed for extraction of ATP from microbial cultures *in vitro* or from water samples and therefore do not address the difficulties inherent in soil or subsurface samples (Jenkinson and Ladd, 1981).

This laboratory (Webster *et al.*, 1984 and Vaden *et al.*, 1987) has developed an extractant mixture to block the action of the interfering substances and reactions in soil and subsurface material. Some of the difficulties that an analyst faces in measuring microbial ATP from soil are illustrated in Figure 3. The difficulties that must be overcome include: obtaining quantitative release of ATP and preventing losses of the released ATP through base stacking and hydrogen bonding, phosphate binding, interaction with protein or enzymes that either bind or degrade ATP, and nonspecific interactions. Each 10 ml of the extractant developed in this laboratory contains 1.2 g urea, 2 g DMSO, 1.8 mg adenosine, 0.5 g Lubrol or 0.2 g polyoxyethylene 10 lauryl ether, and is 2 N in H<sub>3</sub>PO<sub>4</sub> and 0.02 M in EDTA. The phosphoric acid provides acid for extraction of ATP from cells and inactivation of proteins, and phosphate to saturate the phosphate-binding sites and to complex and/or precipitate metal ions in the sample. EDTA chelates metal ions and thus prevents inhibition of luciferase, and aids in lysis of the bacterial cells. Adenosine serves to saturate sites that would bind ATP by hydrogen bonding or by base-stacking interactions. Urea denatures enzymes that might degrade ATP and prevents undesirable hydrogen bonding. Lubrol or polyoxyethylene 10 lauryl ether and DMSO remove bacterial cells from surfaces and aid in lysis. Polytron treatment (a combination of homogenization and ultrasonic treatment) is used to remove bacteria from surfaces, ensure adequate mixing, and disrupt cells. The reagents and method were tested on six different Oklahoma soils and yielded a recovery of 99 % of the ATP from added *Escherichia coli* cells (Webster *et al.*, 1984). When compared with the 12 best methods suggested by previous studies, this extractant and procedure yielded the greatest amount of ATP and the highest energy charge from soil (Webster *et al.*, 1984 and Vaden *et al.*, 1987).

Nannipieri *et al.* (1990) reviewed the methods for measuring biological activity in soil. These methods include measurement of: respiration, dehydrogenase activity, ATP, adenylate energy charge, rates of RNA and DNA synthesis, and heat production. Ciardi and Nannipieri (1990) compared methods for measuring ATP in soil and concluded that the Webster *et al.* (1984) method was the best. As with any complicated environmental

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system, the determination of a single parameter will give the condition only at the time of the measurement and will not cover all circumstances.

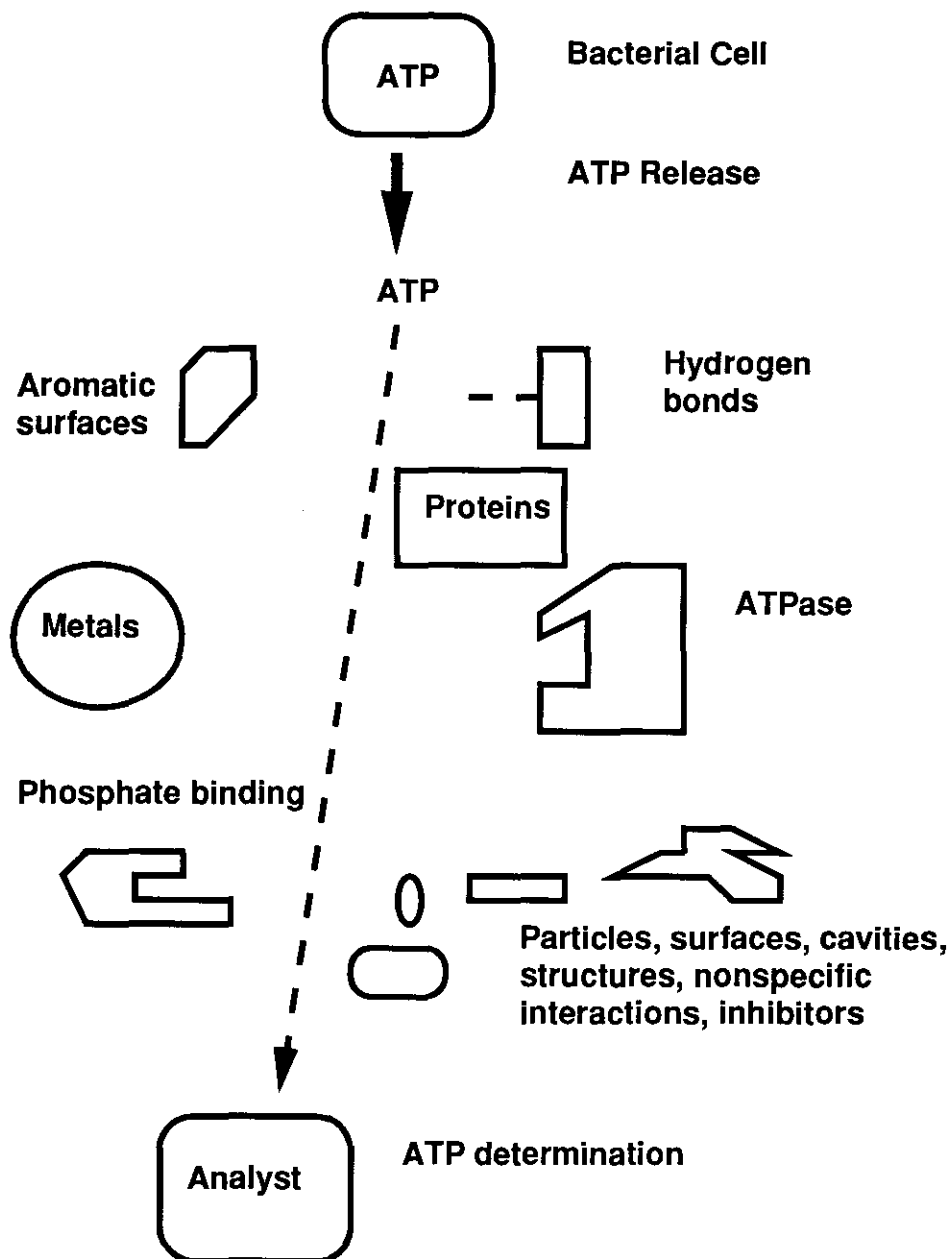


Figure 3. Potential Difficulties in Measuring Microbial ATP from Soil Samples

Webster *et al.*, (1985) have applied this extraction procedure and extractant (see Webster *et al.*, 1984) to samples of subsurface material. Also the number of bacteria in the subsurface samples was determined by microscopic counting after Acridine Orange staining; the proportion of cells capable of respiration was determined by 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction. This measure of metabolic activity was correlated with the ATP found in the subsurface samples.



## Objectives of the Project

Subsurface samples from Oklahoma and Texas contain  $10^6$ - $10^7$  cells per g of subsurface material (depths of 2-9 m). Both methods showed that usually between 1 and 10 % of the cells were metabolically active. The ATP levels in subsurface material were indicative of the ability of the material to biotransform toluene (Wilson *et al.*, 1986). When ATP contents were  $<0.05$  ng/g, biotransformation of toluene was not detectable. At intermediate ATP concentrations (0.16-0.37 ng/g), the biotransformation of toluene averaged 21% of the original concentration per week. At ATP concentrations  $>0.5$  ng/g, the degradation of toluene exceeded 90% of the original concentration per week. Thus ATP measurement can determine when significant numbers of metabolically active bacteria exist in soil and subsurface material with the potential to modify pollutants.

## OBJECTIVES OF THE PROJECT

Completion of the development and demonstration of the field applicability of a bioluminescence procedure for determination of the ATP extracted from soil and subsurface materials will provide a rapid means of determining microbial numbers in soil and subsurface samples. The ATP content reflects the biological potential of subsurface material to degrade pollutants. The application of methods for determining the adenine energy charge of soil and subsurface microbes would allow measurement of the general metabolic status of the microorganisms. This assessment of metabolic potential of soil and subsurface microorganisms will help identify environments where natural bioremediation is occurring or could occur, where pollutants have overtaxed or could overtax the biodegradative potential, and a range of conditions in between. The methods developed and technical information obtained will provide a basis for less costly disposal procedures and ensure a better quality environment by protecting a key natural resource, water.

A rapid, real-time, and field-applicable method for estimation of microbial metabolic potential would cut cost and time required for exploration and renovation of sites that are either polluted or might be used for disposal. The microbial biomass (bacterial number) can be determined by measurement of the ATP content (Karl, 1980, Webster *et al.*, 1985, and Jensen, 1989). ATP is the currency of energy exchange in living organisms; as a result of biochemical regulation there is a fairly constant amount of ATP per unit of cell mass. The bioluminescence assay for ATP using firefly luciferase is linear over 4-5 orders of magnitude of ATP concentration with a sensitivity of 50 femtograms and that assay has been optimized in this laboratory (Webster and Leach, 1980). The amount of ATP in a sample is proportional to the number of organisms (Webster *et al.*, 1985 and 1988, and see Fig. 2).

The research objectives are:

- A) to simplify the methods, reagents, and protocols used to extract nucleotides from microorganisms in soil and subsurface samples,
- and
- B) to demonstrate the applicability of these simplified and improved methods for determination of the extracted nucleotides from environmental samples in the field.

These two objectives are divided into the following specific aims:

1. To develop a procedure that can be applied easily under field conditions and constraints for the extraction of ATP from soil and/or subsurface material.

## Methodology

2. To optimize the bioluminescence assay of ATP for field application to environmental samples.
3. To develop conditions, forms of reagents, and a mixing protocol that can be applied easily in the field to yield an effective extractant.
4. To select and to demonstrate the applicability of equipment required for the extraction procedure and assay that can be used readily in the field.
5. To apply these determinations to environmental samples.

## METHODOLOGY

## Bioluminescence Measurement of ATP

ATP is measured in a 500- $\mu$ l reaction mixture with 50  $\mu$ l of sample; 100  $\mu$ l of Analytical Luminescence Laboratory's firefly luciferase/luciferin (Firelight<sup>®</sup>); 50  $\mu$ l of "complete" Tricine buffer, pH 7.8 containing 25 mM Tricine, 5 mM MgSO<sub>4</sub>, 1 mM EDTA, and 1 mM dithiothreitol; and water (Webster and Leach, 1980). The light production is measured on a Lumac/3M Model 2010A Biocounter for 10 s after initiation of the reaction by injection of Firelight<sup>®</sup>.

## Extraction Procedure

The extraction method is modified from that of Webster *et al.* (1984) and Vaden *et al.* (1987). The surface of the area to be sampled is scraped (about 1 inch deep) with a shovel to remove vegetation and debris. The soil is dug using a shovel with the material being taken within 12 inches of the surface. The soil samples are freed from visible roots, rocks, sticks, and any plant or animal material (such as worms). The soil is pulverized using a 4-pronged garden tool in a large tin can and then sifted through a flour sifter. Samples (100 g) of soil are weighed on a triple beam balance into a paper cup and immediately covered with aluminum foil. A portion of the freshly prepared extractant mixture (45 ml), 5 ml of M-9 medium (Anderson, 1940), 100 g sample, and 75  $\mu$ l of Antifoam A are added to the stainless steel semimicro jar of a Waring blender and the samples are blended for one min in 20-s intervals. After each 20-s treatment a sterile spatula is used to mix the contents of the jar. The contents of the blender are placed into a centrifuge tube and centrifuged for 20 min at 30,000  $\times$  g ( $15 \times 10^3$  rpm) in a Servall SS-1 centrifuge operated at ambient temperature (for the laboratory procedure a Sorvall RC-5B centrifuge was used at 4 °C). The supernatant solution is removed and diluted 1:10 with 0.1 M Tricine buffer pH 11.2 (use of this buffer bypasses the titration procedure previously used, but the samples must be checked with Phenol Red indicator to determine if the final pH is between 7.0 and 8.0). To check the pH, 50  $\mu$ l of Phenol Red solution, 1.8 ml of 0.1 M Tricine buffer pH 11, and 200  $\mu$ l of sample are mixed in a tube and the color observed. The pH is adjusted with ethanolamine to 7.6 (orange color) if needed. Since Phenol Red inhibits firefly luciferase, the samples used for ATP determination are neutralized by adding the experimentally determined amount of ethanolamine without the indicator. The neutralized samples are stored on ice until analyzed.

## Findings

## FINDINGS

## Precipitate Formation in the Extractant.

Depending on the temperature and length of time from mixing the components of the extraction mixture, a precipitate can form. Mixtures were made omitting one of the six components of the extractant. The temperature of the solution was measured and the mixtures were observed to determine if and when precipitation occurred. Table 1 shows that precipitation in the complete mix of six components occurred within 2 h when the mixture had cooled to 40 °C. In the five-component mixes only the one that contained both EDTA and H<sub>3</sub>PO<sub>4</sub> showed precipitation. This occurred within 6.5 h and the solution had cooled to 24 °C. Therefore, it is the interaction of EDTA and H<sub>3</sub>PO<sub>4</sub> that results in precipitation.

Table 1. Solution Stability of Various Combinations of the Components of the Extractant

Mix contents	Temperature, °C		Time, h	Result
	Starting	Ending		
Adenosine, urea, DMSO, H <sub>3</sub> PO <sub>4</sub> , EDTA	42	24	6.5	Precipitation
Adenosine, urea, DMSO, H <sub>3</sub> PO <sub>4</sub>	42	24	24	Clear
Lubrol	52	25	72	Clear
Adenosine, urea, DMSO, H <sub>3</sub> PO <sub>4</sub> , EDTA, Lubrol	59	40	2	Precipitation
Adenosine, urea, DMSO, H <sub>3</sub> PO <sub>4</sub> , Lubrol	59	25	72	Clear
Adenosine, urea, DMSO, Lubrol, EDTA	59	25	72	Clear

The extractant was prepared as described in the detailed protocol (see Appendix 1) with several combinations of components. Three of these mixes omitted one component, one mix omitted two components, and the other solution contained only the detergent. The temperature of the solution was determined after the components were combined. On the day of preparation the solutions were observed at half-hour intervals and thereafter daily. At the last observation the temperature was again measured.

## Effectiveness of the Extractant Containing the Precipitate.

While the occurrence of precipitation is aesthetically unpleasant, the question remained whether that precipitation interferes with the ability of the extractant to function. A sample of the complete extractant mixture was prepared and its extracting ability was determined for a week. The effectiveness of the aging extractant was compared with that of freshly made extractant (see Table 2). There was an insignificant difference (paired t statistic  $F(t) = 0.9$ ) between the two reagents over the 5-day work-week experiment.

## Findings

Table 2. Aging of Extractant

Day	Cells		Soil		Sample assayed Soil + ATP Extractant		Soil + Cells		ATP
	Fresh	Aged	Fresh	Aged	Fresh	Aged	Fresh	Aged	
	1000 counts per 10 s								
1	9.0	7.3	12.4	10.4	17.8	15.4	18.5	16.9	11.7
3	9.5	8.3	10.5	9.8	15.5	14.7	17.5	17.5	10.5
4	5.6	5.5	8.6	8.9	13.7	13.6	13.9	16.5	10.9
5	7.3	5.9	9.6	10.5	14.7	16.0	18.2	18.1	9.4
Average	7.9	6.8	10.3	9.9	15.4	14.9	17.0	17.3	10.6

For a work-week period (5 d) the extraction effectiveness of freshly prepared extractant or extractant that had been prepared on day 1 (aged) were compared (a precipitate was present in the aged extractant after day one). The aged extractant was left at room temperature for the experimental period.

#### Replacement of Lubrol.

Sigma Chemical no longer supplies Lubrol PX; therefore, we performed optimizing experiments to select a non-ionic detergent that would be functionally equivalent to Lubrol for the extraction soil ATP. Several detergents were tested at different concentrations. An extractant containing polyoxyethylene 10 lauryl ether at 0.2 % in place of Lubrol extracted  $12,354 \pm 207$  counts (average of two experiments each with duplicate samples and quadruple assays) from soil while the extractant containing 0.5 % Lubrol extracted  $10,974 \pm 509$  counts. The t statistic that the two means are different was significant at  $F(t) = 0.995$ .

#### Preparation of Stable Stock Solutions of Extractant.

Even though precipitation did not appreciably alter the effectiveness of the extraction, for field application of the extraction procedure it would be useful to have the extractant already prepared in separate solutions of the components that could be mixed at the site. Two different mixtures were prepared in which the interacting components (EDTA and  $H_3PO_4$ ) were kept separate and mixed with a mixture of all the other components at the time of the extraction. Table 3 shows results obtained with these two mixes over a work-month. Both mixes were equally effective in extracting ATP from sample of a soil and there was no decrease in effectiveness during the 28-day period of the experiment showing that the extractant materials were stable.

## Findings

Table 3. Extraction Using Divided Mixes Kept for a Month

Day	Counts per 10 s				
	Mix 1 - Soil	Mix 2 - Soil	Mix 1 Soil + ATP	Mix 2 Soil + ATP	ATP
0	8,740±076	8,848±256	13,803±089	13,630±167	8,558
3	9,073±344	10,408±235	14,148±255	16,198±386	9,894
7	11,505±477	11,371±555	18,233±552	17,355±450	11,779
14	9,904±504	9,787±364	15,015±287	15,011±449	9,239
21	14,512±213	13,688±444	21,736±375	20,183±340	14,831
28	13,276±279	12,731±547	19,830±319	19,446±667	12,204

Two separate mixes of the extractant were prepared; each had one component (EDTA or H<sub>3</sub>PO<sub>4</sub>) separate. The mixes were stored at room temperature and completed on the indicated days. A standard soil sample (Eskridge soil) was extracted and the amount of ATP measured. A standard amount of ATP was added to the extracted sample to determine if there were any production of assay inhibitors during the storage. The response of the Firelight® to an ATP sample is also shown. This allows for determination of any differences in the enzyme response.

Mix 1 contained: Part A: Adenosine, urea, DMSO, H<sub>3</sub>PO<sub>4</sub>, Lubrol; Part B: EDTA.

Mix 2 contained: Part A: Adenosine, urea, DMSO, Lubrol, EDTA; Part B: H<sub>3</sub>PO<sub>4</sub>.

#### Equivalence of Laboratory and Field Extraction Procedures in the Laboratory.

The extraction procedure as originally developed in the laboratory (Webster *et al.*, 1984) used a Brinkmann Polytron combination homogenizer and sonic oscillator and a wrist action shaker. While both could be used in the field, we had previously used a Waring blender for field extraction (Webster *et al.*, 1985). To establish that the two different mixers were equivalent we compared the extraction of ATP from a soil. With the Polytron and wrist action shaker we extracted  $11,631 \pm 154$  counts per 10 s count period from a soil sample and using the Waring blender we extracted  $12,052 \pm 279$  counts per 10 s count period from that same soil. The two procedures yield equivalent results, but the use of the Waring blender simplifies field operation.

#### Field Operation of the Photometer.

The key instrument in the ATP-measuring procedure is the bioluminescence photometer. Battery-powered photometers are available, but we did not have one. Since we needed electrical power for the operation of the centrifuge and Waring blender, we tested the Lumac/3M Biocounter Model 2010A with a portable generator. Figure 4 compares the standard dose-response curves obtained with Firelight® and varying concentrations of ATP in the field by using the portable generator as the source of electrical power and in the laboratory by using standard electrical power.

## Findings

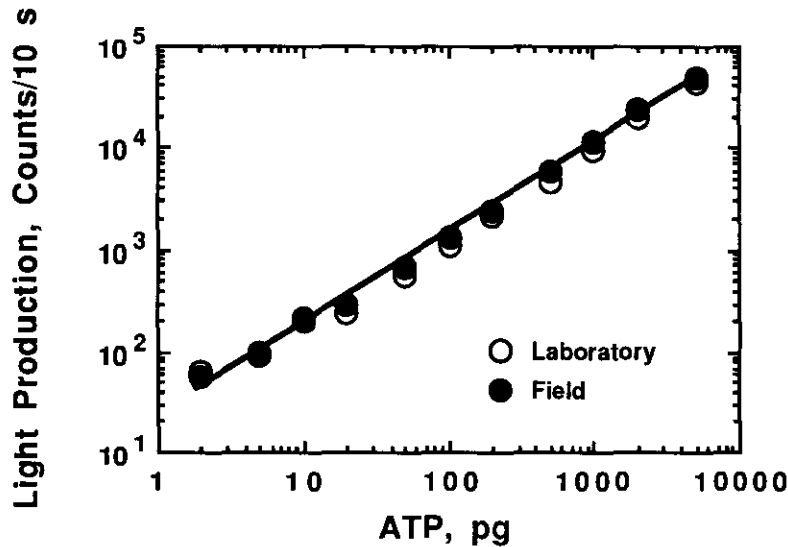


Figure 4. Equivalence of ATP Determination in the Laboratory and Field Using a Lumac/3M Luminometer. The field power source is a portable generator.

#### Comparison of Laboratory and Field Experimental Results.

Field experiments were done at two locations: Leach's backyard (located in the town of Stillwater on Eskridge Place (a street), hence the designation of Eskridge soil) and the Durham Ranch (located about 12 miles south and west of Stillwater, OK, on the west side of SE/4 Section 31 T 19N R1E, Payne County, Oklahoma). All electrical power was supplied from the portable generator at both sites and the experiments were done at the ambient temperature. Duplicate soil samples were analyzed in the field and in the laboratory. Table 4 presents the results. The quantities of ATP measured in the field are lower by 20-30 % than those measured in the laboratory. Firefly luciferase has a temperature optimum of 25 °C (Webster and Leach, 1980) and is unstable at higher temperatures (unpublished). The uncontrolled environmental conditions reduced the ATP response of firefly luciferase in the determination. The temperature during the October 20, 1992 test at Eskridge was 25 °C. For the Durham Ranch experiment the temperature was 35 °C in June 24, 1993.

## Discussion

Table 4. Comparison of Laboratory and Field Extraction

Experiment		Counts in 10 s			
		Cells	Soil	Soil + ATP	Soil + Cells
<b>A. Eskridge<sup>1</sup></b>					
Field					
	Run 1	2040±118	5203±363	14514±194	7369±439
	Run 2	2230±131	8775±251	9789±174	9454±513
	Average	2135	6989	12152	8412
Laboratory					
	Run 1	3695±187	8522±227	23502±712	9662±219
	Run 2	2329±121	8891±442	10327±156	10276±343
	Average	3012	8707	16915	9969
<b>B. Durham Ranch<sup>2</sup></b>					
Field					
	Run 1	3030±144	10565±0516	17417±0438	20195±609
	Run 2	2926±105	11582±1544	13180±1110	NE
	Average	2978	11074	15299	20195
Laboratory					
	Run 1	5321±241	16016±328	18276±121	19274±394
	Run 2	5336±312	15694±398	18986±587	17229±787
	Average	5329	15855	18631	18252

<sup>1</sup> The soil was obtained from a wooded area across Eskridge Place from Leach's backyard (the soil is designated Eskridge soil). Results from two experiments are shown. Replicate samples were taken to the laboratory and analyzed. The reported experiments were done in October 1992.

<sup>2</sup> Samples were taken from a cleared area in a pasture. The reported experiments were done in June 1993. NE = ran out of enzyme.

## DISCUSSION

When the extractant as originally formulated (Webster *et al.*, 1984) was stored a precipitate appeared. The appearance of the precipitate depended on the temperature of the solution. Table 1 shows that EDTA and H<sub>3</sub>PO<sub>4</sub> form the precipitate. Even when the precipitate has formed, the extractant is as effective as freshly prepared extractant at the extraction of ATP from soil samples (see Table 2). By adding either the EDTA or the H<sub>3</sub>PO<sub>4</sub> immediately before use of the extractant, the formation of the precipitation can be eliminated (Table 1). The five-component mixtures (omitting either EDTA or H<sub>3</sub>PO<sub>4</sub>) were stable and could be used for at least a month when completed with the missing component just before use (Table 3).

Potential problems in extracting ATP from soil samples include: 1) lack of quantitative release of ATP from the microorganisms, 2) failure to obtain complete and immediate inactivation of any ATP-destroying enzymes (a rapid quenching of all biological activity is required) 3) precipitation or trapping of the ATP that has been solubilized (base stacking, hydrogen bonding, binding via phosphate groups, binding to metals, or via ionic bonds and other nonspecific interactions), and 4) extraction or production of inhibitors that act in

## Discussion

the firefly luciferase assay. As described in the introduction section this extractant was designed and then optimized to eliminate or correct for any of those potential complications. The procedure and extractant was tested with a variety of soils and found effective; depending on the dilution that can be made before ATP assay, there may be an inhibition of the assay by the high ionic strength. Ciardi and Nannipieri (1990) compared several methods for extracting ATP from soil and conclude that the six-component mixture of Webster *et al.* (1984) is the best.

The field application of the extractant and the extraction procedure requires a source of electrical power for the operation of the luminometer, centrifuge, and Waring blender. A portable generator when equipped with an electrical noise filter provided suitable power for the operation of a Lumac/3M Model 2010A Biocounter. Figure 4 shows the equivalence of ATP standard curves obtained using the portable generator-produced power in the field and the commercially supplied power in the laboratory. The modifications in the procedure and extractant as described in the Findings section were accomplished without any decrease in the ATP extracted as compared to the original extractant and method.

The environmental conditions in the field have a potentially significant effect on results obtained in the field. In the field experiments reported herein there were no wind screens, shades, or other controlling of the field environment. The temperature optimum for firefly luciferase is 25 °C; the enzyme's activity is reduced by 20% when assayed at 30 °C. When firefly luciferase is incubated at 30 °C and then assayed at 25 °C it loses activity with a half life of ~1 h. Thus the decreased activity and increased denaturation of firefly luciferase limits its application on hot days (at 37 °C the half life is 20 min and this temperature is often reached during hot Oklahoma summer afternoons). A standard of ATP can be utilized to measure the activity of luciferase under the particular experimental conditions. In the laboratory it is possible to keep the extractant soil samples cold while centrifuging in a refrigerated centrifuge. This was not possible under our field conditions. If a laboratory-equipped van were used, the control over environmental conditions would be better. The wind and dust influence the comfort of the experimenter and provide an opportunity for contamination.

The results shown in Table 4 establish that this extractant and extraction procedure are applicable in the field. We observed good reproducibility of results within an experiment. However, the differences between the results obtained in the field and the laboratory vary more. We believe that this is due to the different environmental conditions between field and laboratory, especially the temperature. Much better agreement was obtained in the fall when the field ambient temperature was close to the laboratory's temperature.

Stanley (1986) reviewed the techniques for the extraction of ATP from microbial and somatic cells. He listed 8 criteria of an ideal extractant: 1) the extractant should penetrate the cell wall and membrane rapidly, 2) the extractant should extract the ATP rapidly, 3) the extractant should extract the target intracellular ATP pool completely, 4) the extractant should immediately and irreversibly inactivate all enzymes that either utilize or produce ATP, 5) the extractant should not break down ATP either in the short or the long term, 6) the extractant should not inhibit firefly luciferase, 7) the extractant should not change the kinetics of the enzyme (particularly if peak height is measured at a fixed time), and 8) the extractant should not extract interfering agents that influence either the enzyme or the measurement of light production. He classifies extractants as the follows: 1) boiling buffers, 2) dilute acids, 3) organic solvents, 4) surfactants, and 5) mixtures of the above. The particular circumstances of the experimental situation and sample nature determine which extractant is best. There is little consideration of soil or subsurface material. Van de Werf and Verstraete (1987) used a mixture comprised of Tris, EDTA, azide, and NRB (a



Conclusions and Recommendation  
Acknowledgment

detergent-containing solution supplied by Lumac for releasing bacterial ATP) for extracting ATP from soil. No comparisons were made with other extractant. However, they found that with a sandy clay loam soil only 84 % of the ATP was extracted from added *E. coli*. NRB activates firefly luciferase during limited exposures (<50-60 s) and then inactivates the enzyme during longer exposures (Jago et al., 1990). Jenkinson and Oades (1979) used a combination of trichloroacetic acid, sodium phosphate, and paraquat to extract microbial adenine nucleotides from soil. Paraquat competes for the ATP-binding sites and phosphate for the phosphate-binding sites. The nonselective herbicide paraquat is listed by the Merck Index (1989) as a poison.

### CONCLUSIONS AND RECOMMENDATIONS

The reagents and protocol for extraction of microbial ATP from soil samples have been simplified and the precipitation that was previously observed has been eliminated by dividing the stock solutions of the reagents. The extractant and procedure were applied in the field with good results. The protocol is described in detail and a checklist of materials needed for analysis is provided. Because of the temperature sensitivity of firefly luciferase, it is recommended that an air conditioned van or laboratory trailer be used for routine application of the method in the field.

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Appendix 1  
Detailed Protocol**Appendix 1: Detailed Protocol**

**Extractant Preparation** (Modified from Webster *et al.* (1984); Webster *et al.* (1985); and Vaden *et al.* (1987).

A Fisher Thermix stirring hot plate was used to warm and stir the solutions during solution preparation. When the laboratory is cool or after a period of time that allows for cooling of the solution, we have noted precipitation of material from the complete extractant. Therefore, the extractant is freshly prepared from two mixes.

**Water** -- The water used in all reagent preparation is treated via reverse osmosis (the building system), passed through two Barnstead ultrapure mixed-bed ion exchange columns (D8902), distilled in a Bellco glass still (a modified 5004 still with a 5004-30004 heater), collected and stored in sterile containers, filtered through a 0.22  $\mu$ m membrane filter into sterile bottles, and then autoclaved.

**Glassware** -- All glassware was washed in phosphate-free detergent, soaked in Pierce brand RBS,-pf, rinsed in RO water, and sterilized.

**Reagent Preparation****Mix A**

Detergent – Lubrol PX, Sigma (# L-3753). 0.5 g of Lubrol PX is dissolved in 34 ml of sterile water by warming to about 45 °C; the dissolved solution is kept on the edge of the hot plate so that the temperature remains about 35 °C. Or 0.2 g polyoxyethylene 10 lauryl ether, Sigma (# P 9769) is dissolved in 34 ml of sterile water by warming to about 45° C; the solution is kept on the edge of the hot plate so that the temperature remains about 35 °C.

Urea, EM Science (UX0065-5). 600 g (10 M) is dissolved in 500 ml of water (heat and stir), and the solution is diluted to 1000 ml and stored at room temperature.

Dimethyl sulfoxide, EM Science reagent grade (MX1458-5). Used at room temperature.

Adenosine, Sigma (A-9251). 2.5 g is dissolved in 350 ml of water, with stirring and heating until dissolved, 150 ml of water is added. The solution is dispensed into 100 ml prescription bottle and autoclaved. Store at room temperature.

Ethylenediaminetetraacetic acid, Sigma (ED-2SS). 37.22 g is dissolved in 100 ml of water with heat (pH adjustment if needed) and stirring; stored at room temperature.

**Mix B**

Phosphoric acid, J. T. Baker (0262-5). 228 ml of 85% (reagent grade) acid is mixed with 772 ml of water; store at room temperature.

To prepare the extractant, mix in the following order:

Mix A – 80 ml that contains

34 ml of warm Lubrol or polyoxyethylene 10 lauryl ether,  
20 ml of 10 M urea,  
20 ml of DMSO,  
4 ml of adenosine solution, and  
2 ml of 1 M EDTA.

Mix B – 20 ml of 10 N phosphoric acid

## Appendix 1 Detailed Protocol

### Other Reagents

Ethanolamine, Eastman, 5 and 10 N. Dilute 1 and 10 ml with 2.3 ml and 6.67 ml of water, respectively

#### Phenol Red

Dissolve 0.1 g of Phenol Red (Fisher Scientific, phenolsulfonphthalein) in 20 % ethanol  
Useful range pH 6.8 (yellow) –8.2 (red); desired pH 7.6 (orange)

Antiform A, Sigma (A-8267).

Firelight® brand of firefly luciferase/luciferin from Analytical Luminescence Laboratory

### Field Extraction Procedure

The soil samples dug from the surface with a shovel are freed of roots, rocks, sticks, plant materials, and animals (worms). The soil is pulverized with a 4-pronged garden tool and sifted through a flour sifter. Samples of soil (100 g) are weighed out on a triple-beam balance into paper cups then covered with aluminum foil. The extractant mixture (20 ml) and soil sample are added to the jar of a Waring blender and then are blended for a total of one minute in 20-s intervals. After each 20-s burst a sterile spatula is used to mix the contents of jar. The contents of the blender are placed into a centrifuge tube and centrifuged for 20 min at 30000 X g in a Sorvall SS-1 centrifuge operated at ambient temperature. The supernatant solution is removed and diluted 1:10 with 0.1 M Tricine pH 11.2 (this bypasses the titration procedure previously used, but the samples must be tested to determine if the final pH is between 7.0 and 8.0). The neutralized samples are stored on ice until analyzed.

### ATP Determination (Webster and Leach, 1980)

200 µl total volume  
100 µl Firelight® luciferase-luciferin  
50 µl buffer: 25 mM Tricine, 5 mM MgSO<sub>4</sub>,  
1 mM EDTA, & 1 mM DTT, pH 7.8  
50 µl sample

Measure light production in a 3M/Lumac Model 2010A Biocounter for 10 s.

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Appendix 1  
Detailed Protocol**Appendix 2: Equipment and Supply List**

## Centrifugation

- Servall SS-1 Superspeed centrifuge
- Superior Electric Powerstat 3PN116 Variac
- Servall 0-10 amp Ammeter
- 8 50-ml Nalgene polypropylene centrifuge tubes
- Ohaus Harvard Trip double-pan balance equipped with balanced cans for holding the tubes

## Electrical

- McCulloh MacPower Pro 2300E Generator
- Gasoline
- Oil
- 10 ft 3-wire extension cord
- Multiple outlet

## Accessories

- Stop watch
- Thermometer
- Notebook, pencil
- Procedure check list
- Kimwipes No 34155 by Kimberly-Clark
- Parafilm M from American National Can
- Aluminum foil, TV Brand, Fleming Foods
- Plastic trash bag
- Lawn chairs
- Sigma Antifoam A reagent (A 8267), 75  $\mu$ l per sample to prevent excessive frothing
- Ice chest and ice

## Glass/plastic/paper ware

- 18 X 150 mm test tube, Bellco Glass "Thro-away" Cat. No. 1711
- 13 X 100 mm test tubes, CMS, Cat. No. 339-283
- 6 oz Sweetheart cold drink cups, Image 00030

## Gilson Pipetman pipettors from Rannin

- One each
- P-20, P-200, P-1000, & P-5000
- An appropriate selection of sterilized tips

## Work surfaces

- 3 card tables
- Wooden box
- Plywood sheet

## Photometer

- Lumac/3M Model 2010A
- Cornell Dubilier "quietone" filter voltage stabilizer, Cat. No. 1F18
- Lumicuvettes, Lumac, Cat. No. 9200
- Cuvette rack, Lumac, Cat. No. 9205
- Cardboard box to cover and cut out direct sunlight to photometer
- Bactowash, Lumac Cat. No. 9206

Appendix 1  
Detailed Protocol

## Bacteria

*Escherichia coli* (Crookes strain, ATCC 8739) stock cells were maintained on Difco nutrient agar plates at room temperature for a week after overnight growth at 37 °C from a streak. Two generous loopfulls of bacteria were inoculated into 50 ml of Difco nutrient broth in a 125-ml Erlenmeyer flask. The flask was incubated overnight (16-18 h) at 37 °C with shaking (100 oscillations/min) in a New Brunswick G25 Controlled Environment Incubator Shaker. Another 125-ml Erlenmeyer flask containing 50 ml of nutrient broth was inoculated with 10 ml of the overnight growth. The flask was incubated for 1.5 h as before. The cells were harvested in sterile 50-ml polypropylene centrifuges tubes by centrifuging at 15,000 rpm for 30 min in a Sorvall RC-5B centrifuge. The cells were washed twice by suspension in M-9 medium [Anderson, E.H. (1940) *Proc. Nat. Acad. Sci. USA*, **32**, 120-128] and sedimented by centrifugation as before. The pelleted cells were suspended in 10 ml of M-9. The absorbance was determined at 630 nm and the concentration of bacteria determined using a standard curve. The cells were then diluted to yield a concentration of  $1 \times 10^8$  per ml with M-9 medium. These cells were used as the internal standard and were prepared fresh daily.

## Distribution of Functions to Tables

## First table – Sample analysis

- Lumac/3M Model 2010A Biocounter
- Voltage stabilizer
- Cardboard box to cover and shield luminometer
- Wooden box to level and provide firm base

## Second table – Sample processing

- Vortex mixer, Scientific Industries Vortex Genie
- Ohaus double-pan balance and cans for balancing centrifuge tubes
- Board for a firm base
- Kimwipes
- Phenol Red indicator
- Pasteur pipettes, VWR Scientific Cat. No. 14672-200
- Sterile spatulas and rubber policemen

## Third table – Sample and reagent preparation

- Waring blender Model FO 114, two semimicro stainless steel containers
- 2 50-ml graduate cylinders
- Ohaus Triple beam balance to weigh samples
- Aluminum foil to cover samples
- Paper cups for samples

## Soil sample preparation equipment

- Shovel
- Large can in which to store the soil
- Hand garden fork
- Large spoon
- Porcelain bowl
- Flour sifter, Bromwell 3 cup two wire agitator No. RCS-00039