MICROBIAL SURVEY OF AN ACID SLUDGE PIT AND THE SURROUNDING AREA

By

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CHAPTER I

INTRODUCTION

Microorganisms are able to metabolize natural and xenobiotic compounds for energy and growth (24). Bioremediation makes use of microbial degradative activities for restoration of hydrocarbon-polluted environments (2). All bioremediation methods depend on having appropriate bacteria present that can degrade specific contaminants. Use of native microorganisms to metabolize contaminants is generally more effective than introducing microorganisms (seeding). This is because it is difficult for outside organisms to adapt effectively to a new environment. They must compete with indigenous microorganisms that are highly adapted to their particular soil environment and therefore the outside organisms are at a selective disadvantage (14).

One of the main goals of this research was to assay for bacteria living in acid sludge pit materials. This would indicate that bioremediation of the organic sludge might be carried out by the indigenous population. Because of the highly acidic environment present in the pit, seeding would probably be ineffective.

The study site was located at the Kerr-McGee facility in Cushing, Oklahoma. We conducted studies in two different areas at the facility. One area encompassed the acid sludge pit and its direct surroundings. The sludge pit was composed of sulfuric acid and other materials, organic and inorganic, that were generated in the oil refining process.

The other area of interest was a land farming site. The land farm was made up of material from the sludge pit that had been tilled into the surface of the soil in an effort to bioremediate the material. We were interested in this area because the vegetation growing on top of the land farm was distinctively different than the vegetation of the immediate surrounding area, i.e. no grass was present on the land farm but it was present in the surrounding area. A control area was chosen outside the land farm area.

A second goal was to estimate the microbial population around and in the sludge pit and in the land farm site, and compare it to the number of bacteria in the control area. The purpose of this comparison was to determine if the numbers of bacteria present in the land farm and in the areas surrounding the sludge pit were lower than in the control area, which might be an indication of soil toxicity.

We determined the pH of each soil sample and the extent of organic contamination. We also calculated the number of bacteria in the soil samples using Acridine Orange Direct Counts (AODC) and direct plating on various media aerobically and anaerobically. Using AODC we confirmed the

presence of bacteria living in the sludge pit. We were unsuccessful at culturing the bacteria from the sludge pit.

To research the metabolic activity of the bacteria in the sludge pit we performed several analyses. We determined that the bacteria in the pit were respiring using a fluorescent redox probe, 5-cyano-2,3-ditolyl tetrazoleum chloride (CTC). In addition we extracted ATP from the bacteria in the sludge pit. The presence of ATP provided further indication that microorganisms were living in the sludge pit. Oxygen uptake assays carried out on samples from the sludge pit were inconclusive. Finally, we attempted to measure the amount of CO_2 produced by the microbial population by adding ^{14}C glucose as a carbon source.

Our results showed that bacteria were present in the sludge pit and that they were metabolically active. We also determined that in the areas around the sludge pit and in the land farm, there was no significant difference in the number of bacteria present relative to our control area and there was no detectable organic contamination at these sites. Further studies need to be done to determine if the bacteria present in the sludge pit have the ability to degrade the contaminants present.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Microorganisms have the ability to metabolize natural and xenobiotic compounds for energy and growth (24). The process of bioremediation involves using microorganisms to degrade toxic organic compounds into non-toxic products. Bioremediation, which makes use of natural microbial degradative activities has become a major method employed in restoration of hydrocarbon-polluted environments that (2). A classic example of successful biormediation is the cleanup of crude oil from the *Exxon Valdez* in Prince William Sound, Alaska.

Successful attempts to bioremediate environments are based on (1) a detailed understanding of the geohydrology of the site, (2) knowledge of the characteristics of soil and establishing the nutrient status and redox conditions of the site, (3) the identity, concentrations, and locations of both organic and inorganic contaminants at the site, (4) the presence of indigenous microorganisms that are able to degrade the contaminant(s) of interest, and (5) the implementation of techniques to enhance microbial activity by modifying the chemical and/or physical environment at the site (8).

The above information is critical, because environmental rates of hydrocarbon degradation are limited by the enzymatic capability of the indigenous hydrocarbon-degrading microbial populations and by the various environmental factors listed above (2,8). The enzymatic capability of each microorganism determines what classes of hydrocarbons it can break down. Degradation of different classes of hydrocarbons may be carried out by different populations of microorganisms. Foght et al. reported that the microorganisms that degrade aromatic hydrocarbons may be distinct from those that attack aliphatic hydrocarbons (12).

The environmental factors that influence degradation in the soil include the concentrations of molecular oxygen and available nitrogen and phosphate that are present (23). Molecular oxygen is important because the initial steps in the biodegradation of most hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases which require molecular oxygen (2). In soils and groundwaters, oxygen is often the limiting factor. However, recently the microbial degradation of oxidized aromatic compounds such as benzoate (29) and chlorophenols (6) has been shown to occur under anaerobic conditions. Recent evidence also indicates that microorganisms are capable of metabolizing unsubstituted and alkyl substituted aromatics, including benzene, toluene, and xylene in the absence of

molecular oxygen (15). Knowing the concentrations of nitrogen and phosphorus is important because they are required for bacterial growth (10).

Other environmental factors that influence microbial degradation include water activity and pH. The availability of water is important because water is required for growth of bacteria and is required in the degradation of some hydrocarbons (15). Another important consideration in microbial degradation is pH. Most heterotrophic bacteria favor a near-neutral pH. Extremes in pH are therefore expected to decrease species diversity (23). Verstraete et al. reported a near doubling of the rate of biodegradation of gasoline in an acidic soil (pH 4.5) by adjusting the pH to 7.4 (30). Dibble and Bartha observed an optimal pH of 7.8 for the mineralization of oily sludge in soil (10). In summary, because of the importance of the type of bacteria present and the environment, petroleum hydrocarbons can persist indefinitely under one set of conditions, whereas under another set of conditions the same hydrocarbons can be completely biodegraded within a relatively short period of time.

Bioremediation technology takes these environmental factors into consideration. The two general approaches to bioremediation are environmental modification, such as fertilization and aeration, and addition of adapted hydrocarbon degraders by seeding (11). An example of environmental modification is land treatment (or farming).

In land treatment, the contaminated soil is fertilized, irrigated and tilled to increase the availability of nutrients, moisture, and oxygen to the soil microorganisms (27). The organisms used are most often the indigenous populations. This technology has been used successfully throughout the United States, especially at petroleum refinery sites treated under Resource Conservation and Recovery Act (RCRA) and also with creosite-contaminated sludges and soils (27). Wang and Bartha recently studied the effects of bioremediation by landfarming on residues of fuel spills in soil of 2-3 ml of fuel/mg soil (32). In 4-6 weeks the land farm remediated enough of the contaminated soil to support plant growth. Detoxification was complete in 20 weeks. In another study, Warith et al. used environmental modification to bioremediate soil that was contaminated with approximately 350 ppm polyaromatic hydrocarbons (PAH) (33). They maximized bacterial metabolism by fertilizing with nitrogen and phosphorus, and daily tilling of the soil. The pH was maintained at around 7. After 50 days the amount of PAH was reduced to 46 ppm (33).

Another environmental modification method is *in situ* treatment. This is commonly used for contamination in the subsurface and ground water. The process involves the addition of small amounts of ammonia and phosphate, and large quantities of an oxygen source like hydrogen peroxide (27). This is accomplished by injecting nutrient-enriched

solutions into the contaminated zone through a series of wells or trenches, and recovering groundwater down gradient.

All bioremediation methods depend on having appropriate bacteria present. Bossert and Bartha have compiled a list of 22 genera of bacteria that can degrade hydrocarbons (5). If appropriate organisms are not present then they may be introduced into the surface and subsurface environment by seeding. There are many problems with this methodology in soil. One important question is whether such specialized organisms can survive in the new environment (24, 2, 5, 14). These microorganisms must adapt to a different environment and compete with indigenous microorganisms. Indigenous microbial populations are highly adapted to their particular soil environment and therefore would be expected to have a competitive advantage over the seed organisms (14). Other potential problems include inadequate concentrations of the chemical of interest, the presence of inhibitory substances, predation, preferential metabolism of competing organic substrates and insufficient movement of the seed organism within the soil (14).

There are examples of some effective seeding experiments. *Arthrobacter* sp. capable of utilizing isopropyl-N-phenylcarbamate in culture were also able to degrade the herbicide in soil (7). Also a strain of *Pseudomonas cepacia* able to grow on 2,4,5-trichlorophenoxyacetic acid also degrades the pesticide in soil (21).

This research project involves examining an acid sludge pit that contains approximately 60% hydrocarbons and 25% sulfuric acid and determining if there are bacteria living in it. If there are bacteria present they may have the ability to degrade the waste under the correct environmental conditions. As explained above, using indigenous bacteria to degrade the compounds present is better than using seed organisms. This site is particularly challenging because of the high acid concentration. Therefore any bacteria that live in the sludge pit must be acidophilic.

Highly acidic environments are toxic to most bacteria but there are known acidophilic bacteria. *Thiobacillus acidohphilus* and *T. cuprinus* are mixotrophic acidophiles that can obtain energy from the oxidation of reduced sulfur compounds or from other organic substrates (16). *T. thioxidans* is an acidophile that oxidizes sulfur. *T. ferroxidans* and *T. prosperus* are iron oxidizing bacteria. Most of these bacteria have been cultured from environments contaminated by acid mine drainage. Some can grow at a pH less than 2, for example *T. ferrooxidans* and *Leptospirillum ferrooxidans* (16).

Site Background

The Kerr-McGee site is located approximately two miles north of Cushing, Oklahoma. It was operated as an oil refinery from 1917 to 1966. From 1917 to 1952 an acid process used to purify greases produced a waste sludge

composed of 20%-30% sulfuric acid and 50-70% hydrocarbons and inorganics. Kerr-McGee stored the waste in three sludge pits. One of the pits has an area of ten acres and is 9-20 ft deep. Two smaller pits together cover 4 acres and are 8-10 ft deep. The bottoms of the pits are composed of clay and diatomaceous earth. The pH of these pits was estimated to be below 2.

The larger pit was in close proximity to Skull Creak which flows through the city of Cushing. It was a common occurrence for rainwater runoff to overflow the pit boundaries and contaminate the creek. To solve this problem Kerr-McGee made four runoff modifications. They created a 4 acre neutralization pond where the runoff from the pit could be neutralized before being discharged into the creek. They installed a French drain on the side of the pit closest to the creek. This allowed runoff to enter the drain and flow into the neutralization pond. On the other side of the pit a ditch was installed to collect the runoff which would then flow into the neutralization pond. The fourth thing Kerr-McGee did was divert Skull creek so it would not pass right next to the pit.

In the late 1980's Kerr-McGee, in an effort to biodegrade some of the acidic sludge, moved sludge from the acid sludge pits and tilled it into the soil to create a land farm site. Today, the vegetation growing on the land farm consists of fire weed and tumble weeds, not the grass that surrounds the land farm area. This change in

vegetation on the land farm is a possible indicator of soil toxicity in the land farm.

Research Goals

One of the goals of this research project was to determine if bacteria lived in Kerr-McGee's acid sludge pit. If bacteria were present, they might be able to degrade the contaminants present in the sludge under the correct environmental conditions. Another goal of this research project was to estimate the microbial population around the sludge pit and the land farm site and to compare it to the number of bacteria in a control area. This was done to determine if the number of bacteria present in the land farm is lower than the surrounding area which might be an indication of soil toxicity.

CHAPTER III

MATERIALS AND METHODS

Sample Collection

Soil samples were received from the Kerr-McGee Facility in Cushing, Oklahoma in June 1992. The soil samples came from the top twelve inches of soil from the sludge pit, areas northwest, northeast and southeast of the sludge pit, the land farm, and a control area. Approximately 750 gm of soil at each site was collected. The samples were stored at 4°C.

Gas Chromatography

Gas Chromatograph Operation

A Hewlett Packard HP5890A Gas Chromatograph with a flame-ionization detector was used to determine the amount of organic material at each site. Nitrogen was the carrier gas and hydrogen provided the flame. The nitrogen and air were delivered at 50 psi and the hydrogen was delivered at 34 psi. Operating conditions were: injector temperature was 200°C; oven temperature ranged from 35°C for an initial time of two minutes to a final temperature of 90°C for a final

time of two minutes. The temperature increased at a rate of 20°C per minute post injection. The graph parameters were as follows: chart attenuation was one; the chart speed was 1 cm/min; the area of rejection was 10,000 and the threshold was four.

Sample Preparation

All six soil samples collected were analyzed by gas chromatography. To prepare the soil samples for analyses, 0.1 gm of each soil sample was extracted with 350 µl of ethyl acetate in microcentrifuge tubes. The extracted samples were vortexed for 20 seconds and centrifuged for thirty minutes at room temperature. Using a 10 µl syringe, 1 µl of each soil extract was injected into the gas chromatograph. Each sample was analyzed three times. As a negative control, ethyl acetate alone was injected into the gas chromatograph. To determine the effectiveness of ethyl acetate as an extractant, 100 ppm toluene was added to the land farm sample and 100 ppm toluene was added to 350 µl of ethyl acetate. Both samples were mixed for 30 minutes. The land- farm/toluene sample was extracted with 350 µl of ethyl acetate. Both samples were then run through the gas chromatograph to determine the amount of toluene that could be extracted from the soil.

pH Determination

To determine the pH of each soil sample, 10 gm of soil was mixed with 10 ml of deionized water. The pH was taken using an Orion Research Digital Ion Analyzer.

Direct Count Acridine Orange Stain (AODC)

To perform direct counts using acridine orange, bacteria were collected on a 0.4 μm membrane filter by vacuum filtration and stained with acridine orange. The bacteria were then directly counted using an epifluorescence microscope. Under epifluorescence the bacteria generally fluoresce bright green while organic debris appears orange (13).

Sample Preparation

To determine the number of bacteria in each soil sample, 10 gm of soil was mixed with 90 ml of 0.1% sodium pyrophosphate for one hour. Each sample was diluted to 10^{-3} using 0.1% sodium pyrophosphate buffer. The samples from the sludge pit were not diluted. Three separate suspensions were made per soil sample.

Filter Apparatus Preparation

The filter apparatus was wrapped in aluminum foil and autoclaved for 20 minutes at 20 psi. Using sterile forceps, a 5.0 μm cellulose membrane filter was placed on top of the support screen of the filter apparatus. A 0.4 μm black

polycarbonate membrane filter was placed on top of the cellulose filter using sterile forceps. The cellulose membrane filter served as an under drain support and ensured equal distribution of particles across the black polycarbonate membrane. A schematic representation of the apparatus is depicted in Figure 1.

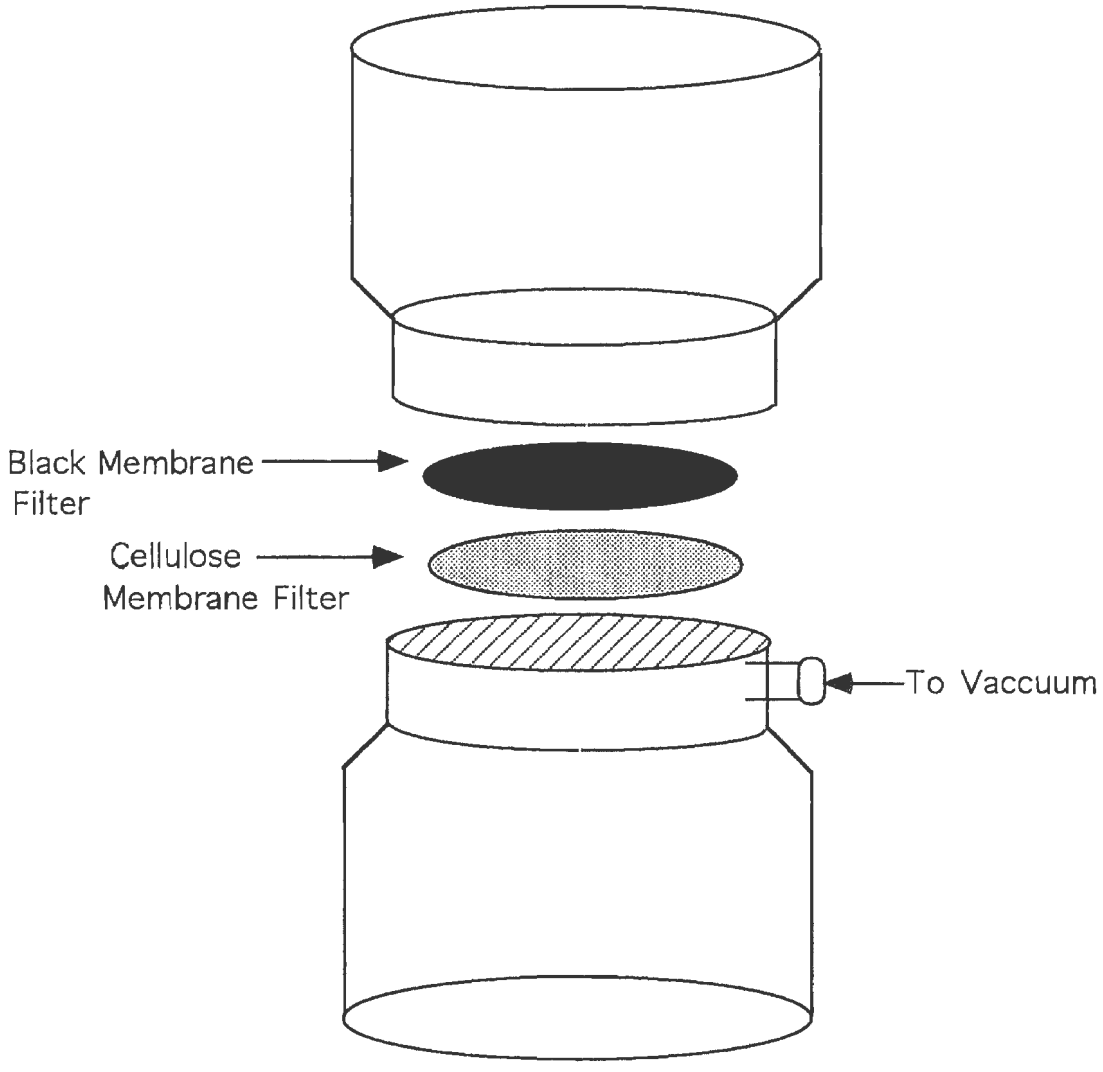
Staining Procedure

Each sample was filtered in order to trap the bacteria on the polycarbonate membrane. A volume of 0.01% Acridine orange solution sufficient to cover the filter was added and allowed to incubate for two minutes. The vacuum was then turned on and the sample was flushed with 5 ml of sterile deionized water. The black polycarbonate membrane filter was put on a glass microscope slide. One drop of nonfluorescing immersion oil was added to the membrane and a cover slip was overlaid. Cells were then observed using epifluorescence microscopy.

Controls

As a positive control to demonstrate the ability of acridine orange to stain bacteria and test the accuracy of this method for determining the concentration of bacteria, 1.0 ml of an *E. coli* culture containing 6.0×10^7 cfu/ml was added to the land farm soil suspension. The suspension was then diluted to 10^{-4} and stained. To ensure the 0.1% sodium pyrophosphate and the deionized water were sterile, the

Figure 1. Filter Appararus used for Acridine Orange Direct Counting and CTC Redox Probe


Black Membrane Filter
Cellulose Membrane Filter
To Vaccuum

solutions were stained with acridine orange and examined under the microscope.

Fluorescence Microscopy Procedure

Within fifteen minutes of staining, the samples were viewed on an Olympus Microscope BH2-RFC equipped with an epifluorescence illuminator using a mercury light source and BP-490 exciter filter and 0530 barrier filters. Ten fields per sample were counted.

Direct Plate Counts

To determine the number of culturable bacteria, 10 gm of soil from the land farm, the control area, and areas northwest, northeast and southeast of the sludge pit were separately mixed with 90 ml of phosphate buffer in a 250 ml sterile flask and were shaken for one hour at room temperature. Dilutions of 10^{-4}, 10^{-5} and 10^{-6} were made in phosphate buffer. These dilutions were plated in triplicate on Peptone Trypticase Yeast Glucose Agar (PTYG), and Total Nutrient Agar (TNA) (Table 1) and incubated aerobically at 30°C for two days.

Liquid Enrichments and Direct Plate Counts from the Acid Sludge Pit

From the acid sludge pit suspension, 1.0 ml was removed and added to 9.0 ml of Ferrous Sulfate broth (19) or Soil Extract broth (3) (Table 2) in triplicate. Also, 1.0 ml of

the suspension was plated in triplicate on Ferrous Sulfate agar and Soil Extract agar and incubated at 30°C both aerobically and anaerobically for two weeks. In addition the acid sludge pit suspension was inoculated into Soil Extract Agar and Broth containing 0.05% yeast extract and incubated as described previously.

TABLE 1

MEDIA USED FOR DIRECT PLATE COUNTS

PEPTONE TRYPTICASE YEAST GLUCOSE AGAR		TOTAL NUTRIENT AGAR	
Glucose	10 gm	Tryptone	5 gm
Yeast Extract	10 gm	Yeast Extract	2.50 gm
Peptone	5 gm	Dextrose	1 gm
Trypticase	5 gm	NaCl	8.48 gm
$MgSO_4 \cdot 7H_2O$	0.6 gm	$CaCl_2$(1%)	20 ml
$CaCl_2 \cdot 2H_2O$	0.07 gm	Agar	20 gm
Agar	15 gm		

Distilled Water added to a final volume of 1 L

TABLE 2

MEDIA USED TO CULTURE BACTERIA FROM ACID SLUDGE PIT

FERROUS SULFATE BROTH (c)		SOIL EXTRACT BROTH (d)	
Ferrous Sulfate	50 ml	Soil Extract (b)	100 ml
Basal Salt/TSB(a)	700 ml	Distilled Water	900 ml
Distilled Water	250 ml		

a) Basal Salt/Tryptone Soy Broth (TSB) is composed of 0.9 gm of $(NH_4)_2SO_4$, 0.35 gm of $MgSO_4 \cdot 7H_2O$, 0.175 gm of TSB, and 500 ml of Distilled Water. Three separate broths were made having a pH of 2, 2.5 and 3 respectively and were autoclaved.

b) The soil extract was made by autoclaving a 1:2 suspension of soil from the sludge pit in distilled water for 2 hours, the extract was then centrifuged and the supernatant was collected. The final pH was then adjusted to match the pH of the soil.

c) To make Ferrous sulfate agar, ferrous sulfate, distilled water and 15 gm of Agar were autoclaved together and cooled to 55^{o} C. The basal salt/TSB was autoclaved separately and added to the sterile ferrous sulfate, distilled water and agar once it had cooled to 55^{o} C.

d) To make Soil extract agar, the distilled water and 15 gm of agar were autoclaved together and cooled to 55^{o} C. The soil extract was autoclaved separately and added to the sterile distilled water and agar once it had cooled to 55^{o}C.

Fluorescent Redox Probe

Sample Preparation

To determine whether the bacteria in the sludge pit were respiring, 1 gm of soil was incubated with 5 mM of 5-cyano-2,3-ditolyl tetrazoleum chloride (CTC) for 4 hours at 28°C with agitation. As in the AODC procedure, the samples were passed through a filter apparatus containing a 0.4 μM black membrane filter (Figure 1). The filters were then placed on a microscope slide; a drop of low fluorescing immersion oil was added to the filter; and a cover slip was overlaid.

As positive controls, *E. coli* cells were mixed with material from the acid sludge pit and *E. coli* cells alone were incubated with CTC. These controls demonstrated that CTC was an effective indicator of respiration.

Fluorescent Microscopy Procedure

The samples were viewed with a 100X oil immersion objective on an Olympus Microscope equipped with a mercury source. The filter combination used consisted of a blue (420 nm) excitation filter (Olympus model BP490) used in combination with a 590 nm barrier (cutoff) filter (Olympus model 0590). Actively respiring bacteria fluoresce bright red under epifluorescence (26).

ATP Extraction Procedure

An extraction was performed to measure the amount of ATP in the microorganisms in the acid sludge pit. To measure the amount of ATP extracted, a firefly luciferase enzyme assay was used. In this assay, the enzyme consumes ATP as a substrate and produces an easily measured quantity of light (22). The light produced was measured on a photometer.

The extraction buffer (Table 3) used in this study to recover ATP from the soil was developed by Webster et al. to facilitate ATP extraction (35). The phosphoric acid in the extractant served to extract ATP from the cells, inactivate proteins, saturate phosphate-binding sites, and to precipitate metal ions. The EDTA chelated metal ions and aided in bacterial cell lysis. The adenosine saturated ATP binding sites. The urea denatured enzymes that might degrade ATP. DMSO assisted in removing bacteria from surfaces and aided in lysis.

Extraction of ATP

To extract the ATP from the acid sludge pit, 25 ml of the extraction buffer (Table 3) was blended with 100 mg of soil from the pit for 60 seconds in a sterile Waring blender. The contents of the blender were centrifuged for 20 minutes at 50,000 X *g*. The supernatant was collected in sterile test tubes and put on ice. This procedure was

repeated with soil from the land farm area. The ATP levels in both soil types was measured three times.

TABLE 3

EXTRACTION BUFFER RECIPE

10N Phosphoric Acid	40 ml
10M Urea	40 ml
5 mg/mL DMSO	40 ml
5 mg/ml Adenosine	8 ml
1M EDTA	4 ml
1 gm Lubrol dissolved in 68 ml of water	

Preparation of Cells Used as Positive Controls

As a positive control to test for the percent recovery of ATP a known number of *E. coli* cells were extracted and the amount of ATP measured. *E. coli* cells were grown in 125 ml liquid cultures using LB medium (Table 4) overnight. The cell suspension was subcultured by adding 25 ml of culture into 25 ml of fresh LB and incubated for 90 min. on a shaker at 37° C. The cells were pelleted by centrifugation for five minutes at 50,000 X *g*. The supernatant was poured off and the cells were resuspended in 15 ml of M-9 buffer (Table 4). The suspension was then centrifuged for five minutes at

50,000 X *g*. The cells were then pelleted by centrifugation and the supernatant was poured off. The pellet was then resuspended in 18 ml of M-9. The optical density of the cells was then measured at 620 nm on a spectrophotometer to determine the number of cells, and the cells were put on ice.

In a Waring blender, 22.5 ml of extraction mixture was blended with 2.5 ml of cells for 30 seconds and centrifuged for 20 minutes at 50,000 X *g*. Also in a Waring blender, 22.5 ml of extraction mixture was mixed with 2.5 ml of cells and 100 mg of soil from the acid sludge pit and centrifuged for 20 minutes at 50,000 X *g*. The supernatants of both controls were collected in sterile test tubes and put on ice. As a negative control to ensure the M-9 was not contaminated, 22.5 ml of M-9 was added to 2.5 ml of extraction mixture in a sterile test tube. The tube was vortexed and put on ice. The amount of ATP extracted per cell was determined in the controls.

Neutralization of Samples

Because the optimum pH for luciferase activity was 7.8 (36) all samples were adjusted to that pH. To do this, 50 µl of Phenol Red was put into a small test tube with 200 µl of each extracted sample. To each sample, 1800 µl of 0.1M Tricine buffer (pH 8.5) was added. Phenol red at a pH of 7.8 was used as a color standard. If the solution turned pink, the pH was too high and a lower pH Tricine buffer was

tried. If the solution turned yellow, the pH was too low and 5N ethanolamine was added in drops. Once the appropriate pH of the buffer was determined, 200 µl of the extracted sample was combined with 1800 µl of the appropriate buffer. The adjusted samples were then stored on ice.

TABLE 4

MEDIA USED FOR ATP ASSAYS

LIQUID M-9 Recipe		LIQUID LB	
NH_4Cl	1 gm	Tryptone	10 gm
Na_2HPO_4	6 gm	Yeast Extract	5 gm
KH_2PO_4	3 gm	NaCl	20 gm
NaCl	5 gm		

Distilled Water added to a final volume of 1 L

Assay Preparation

The assay mixture contained 50 µl of each sample, 300 µl of sterile deionized water and 50 µl of tricine assay buffer (Table 5). As positive controls 50 µl of each soil sample extract was mixed with 200 µl of water, 50 µl of

tricine assay buffer, and 100 µl of 10 ng/ml ATP. To determine the activity of the enzyme, another positive control consisted of 100 µl of 10 ng/ml ATP, 250 µl of water and 50 µl of tricine assay buffer. As a negative control 50 µl of tricine assay buffer was added to 350 µl of water to ensure that no bacteria had contaminated the buffer or the water. All samples were stored on ice.

TABLE 5

TRICINE ASSAY BUFFER

1M Tricine	25 ml
1M $MgSO_4$	5 ml
0.1M DTT	5 ml
0.1M EDTA	5 ml
Distilled Water	92 ml

pH was adjusted to 7.75

Assay

In a dark room, 100 µl of reconstituted Luciferase enzyme was added to each assay mixture and the amount of light produced was determined on a Lumac photometer. The

amount of light was then compared to a standard curve relating ATP and light units.

To create the standard curve, dilutions of 1/200, 1/500, 1/1,000, 1/2,000, 1/5,000, 1/10,000, 1/20,000, 1/50,000, 1/100,000, 1/200,000, and 1/500,000 of 1 μg/500 μl ATP were made and read on a photometer (Appendix D).

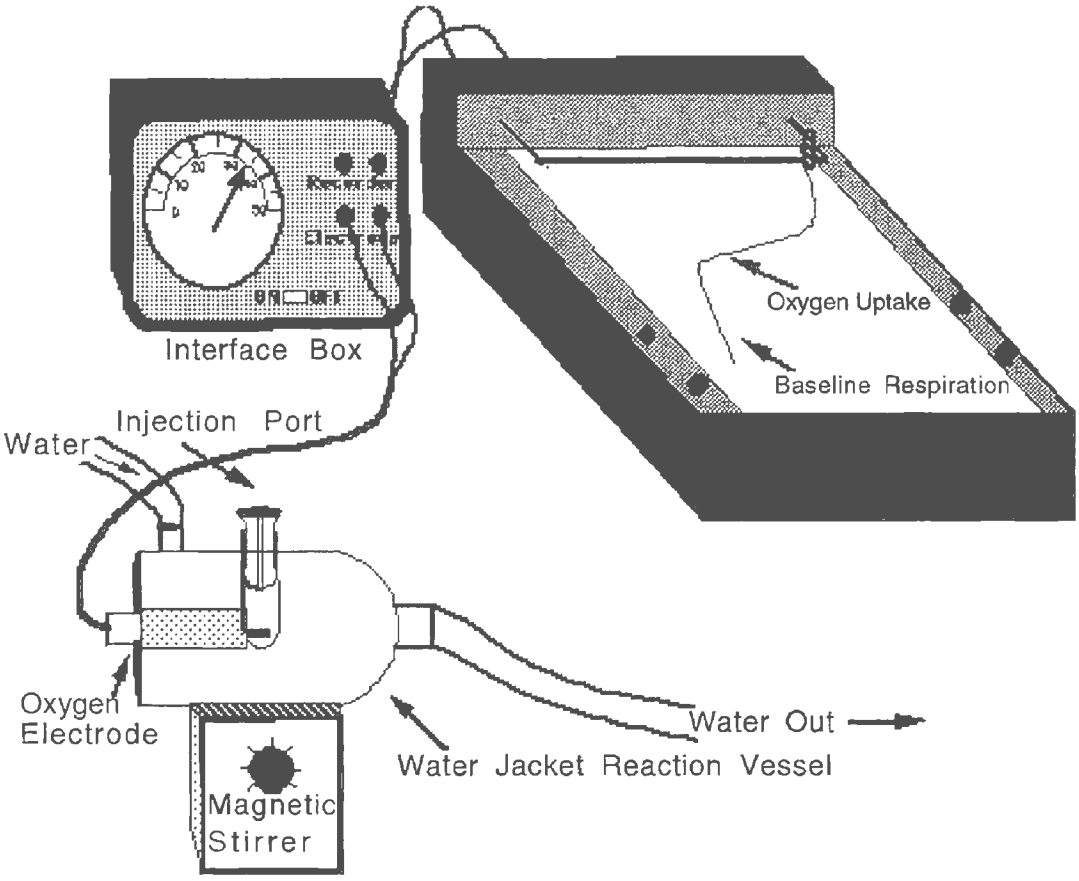
Oxygen Uptake

This assay was used to determine if oxygen was being used by bacteria in the sludge pit.

Apparatus

The equipment used in this assay is shown in Figure 2. A Clark-type oxygen electrode (Yellow Springs Instrument) was placed in the side of a water-jacket reaction vessel (Gilson Medical Electronics) which contained a chamber used to hold 2.0 ml of sample. The reaction chamber was fitted with a glass stopper to prevent the exchange of oxygen between the atmosphere and the sample during the assay. The oxygen electrode was connected to an interface box which allows the system to be calibrated using air-saturated water. Water has a saturation of 8.0 ppm oxygen at 22° C; this value was used to calibrate the electrode. As an additional check to ensure the electrode was functioning properly, a small amount of sodium hyposulfate was added to water and the oxygen concentration was measured. A reading of 0.1 ppm or less indicated that the electrode was

Figure 2. Oxygen Uptake Apparatus


Interface Box
Oxygen Uptake
Baseline Respiration
Injection Port
Water
Oxygen
Electrode
Water Out
Water Jacket Reaction Vessel
Magnetic
Stirrer

functioning properly. The oxygen permeable membrane covering the electrode was replaced when necessary.

Chart Parameters. The chart was set to move one inch every 30 minutes.

Sample Preparation

Two preparations per soil sample were made. One was prepared using 1 gm of soil and 9 ml of phosphate buffer. The other was prepared using of 1 gm of soil, 9 ml of phosphate buffer and 0.1 gm of sodium azide. The sodium azide was used to kill any bacteria present. All preparations were shaken at room temperature for one hour to form a slurry.

Assay

In the sample chamber, 200 µl of the slurry was mixed with 1800 µl of phosphate buffer. After 2 hours, 100 µl of 0.05% Casamino Acids was added and the reaction continued for 1 hour. As a negative control phosphate buffer was run alone.

^{14}C Uptake Assay

To determine if the bacteria present in the acid sludge pit were producing CO_2, ^{14}C labeled glucose was added to the soil and the amount of CO_2 produced was measured.

^{14}C Activity Determination

The total amount of ^{14}C glucose purchased was 50 μCi, having a specific activity of 2.3 mCi/mmole. The concentration of the stock solution was calculated to be 0.392 mg/mL (2.174×10^{-3} mM) (17).

A standard curve was generated to determine the counts per minute per ml of concentration of stock solution. Dilutions of 1/10, 1/25, 1/50, 1/100, 1/250, 1/1000, 1/5000, and 1/10,000 of the stock solution were made in triplicate. A 100 μl portion of the dilution sample was added to 400 μl of Ready Safe Cocktail (Beckman). The samples were then analyzed on the scintillation counter (Appendix C).

Assay

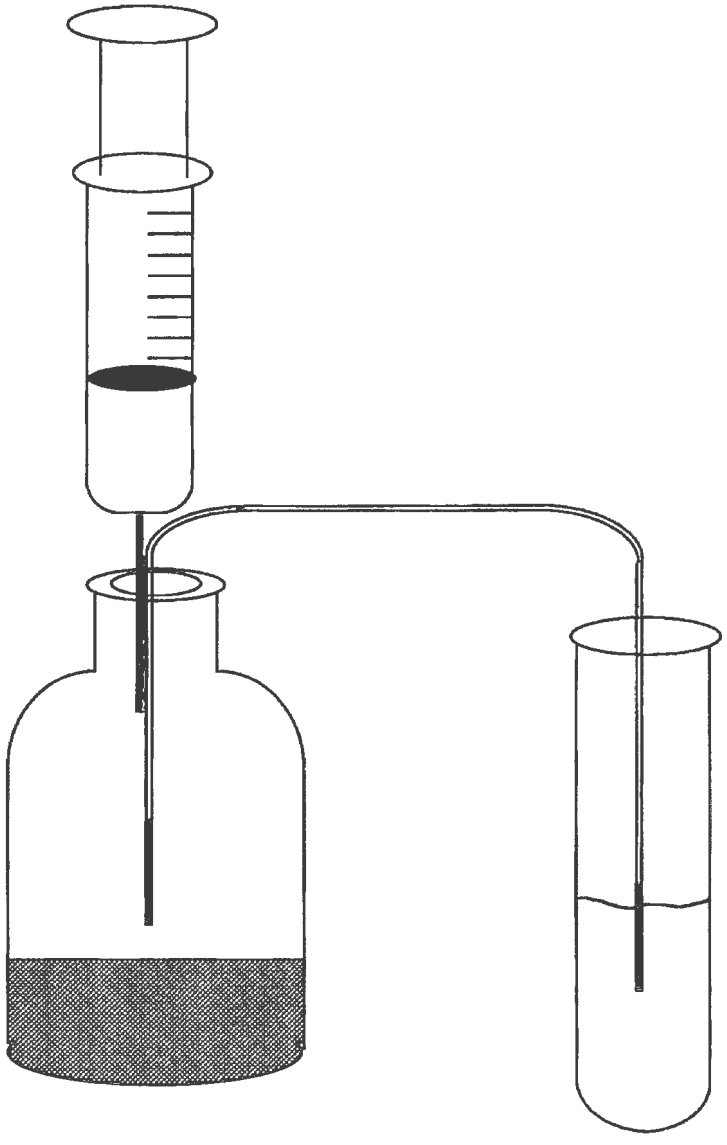
Two sterile serum bottles were each filled with 10 gm of soil from the sludge pit. One of the serum bottles had 1.0 gm of sodium azide added to it. Both samples received 100 μl of the stock solution. The serum bottles were then sealed and stored at room temperature for two weeks.

The ^{14}C labeled CO_2 that was produced was collected and measured. To collect the CO_2, 1 ml of solvable (New England Nuclear) was put in a test tube. A thin tube with one needle at each end was assembled. One end of the tube was placed in the serum bottle, and the other end was placed in the test tube. Using a syringe the serum bottle was then injected with air to displace the CO_2. The CO_2 was then captured in the solvable in the test tube (Figure 3). A 100

μl portion of the solvable containing CO_2 was placed into 400 μl of the Ready Safe cocktail. The amount of CO_2 was then measured on a liquid scintillation counter.

Approximately 2.5 ml of water was then added to each serum bottle, and7 the bottles were incubated another two weeks. The amount of CO_2 produced was then measured.

Figure 3. Carbon Dioxide Collection Apparatus used in ^{14}C Uptake Assay



CHAPTER IV

RESULTS AND DISCUSSION

The goals of this research project were to estimate the microbial populations around the sludge pit and the land farm area and compare them to a control site, and to determine if bacteria were living in the acid sludge pit. We also wanted to determine what accounted for the significant change in vegetation on top of the land farm.

Gas Chromatography

This project began by determining the amount of organic contamination in each of the soils. Ethyl acetate was used to extract the organic compounds from the soil. We hypothesized that one of the reasons the vegetation was different on top of the land farm might be the presence of excessive amounts of organic contamination. The amount of organic contamination was determined by using a gas chromatograph with a flame ionization detector. The flame ionization detector responds to compounds which produce ions when burned in a hydrogen-air flame. This includes most organic compounds. The results were summarized in Table 6. As the table indicates, in general except for the acid sludge pit (Pit 5), no contamination was detected in any of

TABLE 6

REPRESENTATION OF THE ORGANIC CONTAMINATION IN EACH SOIL SAMPLE

	RETENTION TIME																
	1.09	1.32	1.34	**1.50**	**1.58**	1.65	1.85	**2.67**	3.05	3.12	**3.22**	3.60	6.19	7.39	7.58	8.09	8.25	10.58
Ethyl Acetate				X	X			XXXX			XX							
Southeast					X			XXXX	X		XX							
Northwest								XXXX			XX							
Northeast					X			XXXX			XX							
Pit 5	X	XXXX	XXXX				XX	XXXX				XXXX		XXX				XXXX
Control				X	X	XX		XXXX		X	XX							
Landfarm				X	X			XXXX	X		XX							
Toluene													XXXX		X	XX	XX	
EthylAc/Tolu					X			XXXX			XX		XXXX			X	X	
LF/EthAc/Tol		X			X			XXXX			XX		XXXX			X		

X= 10,000-100,000 (*)
XX= 100,000-1,000,000
XXX= 1,000,000-5,000,000
XXXX= >5,000,000

* Numbers represent area under the curve

Source of Contaminant is Ethyl Acetate

the soils tested. However, in the control area and the soil sample from the southeast side of the sludge pit, there were some peaks not attributed to ethyl acetate. The peaks were considered to be insignificant because there was no consistent pattern.

The lack of detectable organic contamination may be the result of the heavy rains Oklahoma received in June 1992 when these samples were collected. The rain may have caused contaminants to percolate deeper into the soil or to be washed away with surface water runoff.

pH Results

We determined the pH of all the soil samples. We wanted to determine if the acid sludge had migrated outside the confines of the pit. Such migration would be detected by a drop in pH. We also thought low pH might be responsible for the change in vegetation on the land farm area. As shown in Table 7 all the soils except for the sludge pit (Pit 5) had a pH of approximately 6.5. The pH of Pit 5 was extremely acidic with a pH of 1.2.

Soils with a pH of 6.5 are considered to be slightly acidic. Acid sensitive crops, like alfalfa, will not grow in soils with a pH of 6.5. Because the soil from the land farm had a similar pH when compared to the control area, we know that the pH of the land farm soil is not the cause of the different vegetation found on the land farm.

TABLE 7

pH OF SOIL SAMPLES

Soil Sample	pH
Northwest	6.49
Northeast	6.44
Southeast	6.51
Pit 5	1.2
Land Farm	6.42
Control	6.56

Bacterial Population

After determining the extent of contamination and pH of each soil sample, we determined the approximate number of bacteria at each site. The purpose of this was to determine whether there was a difference in the number of bacteria in the area surrounding the sludge pit and in the land farm when compared with the control area. We used both plate counts and Acridine Orange Direct Counts (AODC) to determine the bacterial population at each site. The media used in the plate counts were Total Nutrient Agar (TNA) and Peptone Trypticase Yeast Extract Glucose Agar (PTYG). These media were used by Balkwill and Ghiorse to characterize subsurface Bacteria in Oklahoma (3).

Plate counts generally detect only a small percentage of the actual number of bacteria (38, 28, 20, 3). Alexander in 1977 reported that, at best, artificial media are capable of detecting only 1-10% of the total number of soil microorganisms (1). This lack of detection is due to the fact that artificial media are inevitably highly selective and therefore underestimate microbial populations. Starved cells and oligotrophic microbes which are common in soils are notoriously difficult to culture because of their unique growth requirements (20). Acridine orange staining overcomes the problem of finding appropriate growth media by directly enumerating all bacteria, viable and non-viable, in the soil.

As discussed previously, the basic acridine orange staining procedure involved passing a known quantity of sample through a membrane filter. The filter was then stained with acridine orange and the microorganisms on the filter were observed using epifluorescence light microscopy. Acridine orange interacts with the nucleic acids of the bacteria. In general, when excited by blue light, the acridine orange dye fluoresces bright green if it is associated with an organism and fluoresces dim orange when it is associated with abiotic material (38).

Several limitations are associated with AODC. AODC staining will not differentiate between viable and non viable organisms (4). This is due to the process by which acridine orange interacts with nucleic acids. Inactive bacteria and bacteria with very low metabolic activity have mostly deoxyribonucleic acid (DNA) present and fluoresce green. This is because the rigid structure of the double helix allows fewer acridine orange molecules to attach; therefore the molecules do not interact with each other and the acridine orange fluoresces as a monomer (18). In contrast, if a large amount of ribonucleic acid (RNA) is present in the bacteria (i.e. if the bacteria are growing rapidly) it will fluoresce orange. This occurs because the random coiling of RNA allows the acridine orange molecules to interact with each other allowing the acridine orange to fluoresce as a dimer (18). Bacteria will also fluoresce orange if the DNA is broken down, (i.e. the cell dies). In

summary bacteria can fluoresce orange either because of a high concentration of RNA or because the cell is dead. Another limitation of acridine orange staining is that organisms must be removed from the surfaces of the soil to be stained (3).

The numerical results of the bacterial population survey are shown in Appendix A and B. A graphical representation of the results is shown in Figure 4. As expected, Figure 4 demonstrates that more bacteria were detected when acridine orange was used vs. the plate count method.

The results also indicate that there were no significant differences in the numbers of bacteria in the land farm (LF) and areas surrounding the acid sludge pit (Pit 5) when compared to the control area. This indicates that the land farm soil does not inhibit bacterial growth.

Figure 4 also shows that bacteria from the acid sludge pit could not be cultured on the media used but bacteria were detected using AODC. The morphology of bacteria found in the sludge pit was short rods (Figure 5). As shown in Figure 5, there were green and orange bacteria enumerated from the sludge pit. All bacteria were counted regardless of color, even though in this case the orange bacteria were probably dead. We make this assumption because these bacteria were living under very toxic conditions, therefore it is doubtful that they were growing rapidly. Also the photograph was taken eight months after the original samples

Figure 4. Number of Bacteria /ml of each Soil Sample. Calculated by Standard Plate Counts and Acridine Orange Direct Counts

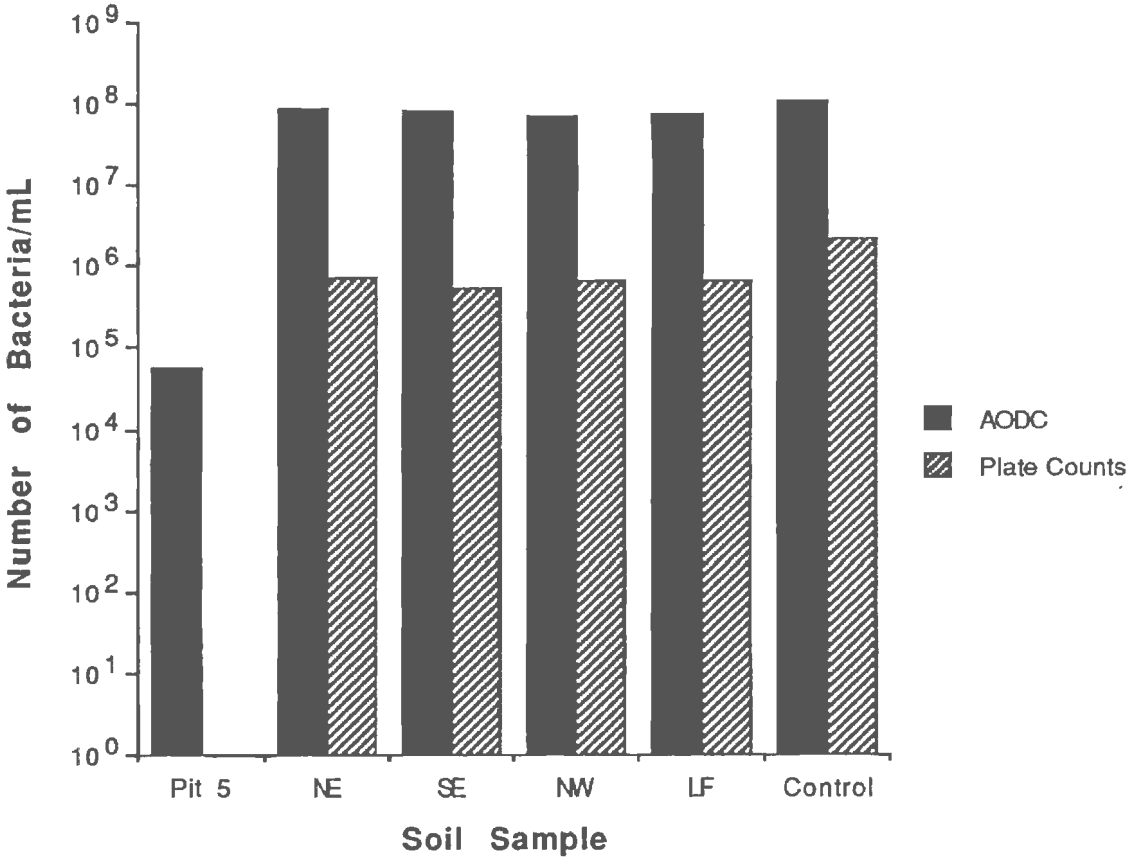

Number of Bacteria/mL
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Pit 5
NE
SE
NW
LF
Control
Soil Sample
AODC
Plate Counts

Figure 5. Photograph of Bacteria from Acid Sludge Pit. Bacteria are Stained with Acridine Orange and Magnified 100X.

were collected. The prolonged storage might have been detrimental to the bacteria.

These bacteria were probably very specialized in their growth requirements making it difficult to culture them on selective media. Another explanation of the failure to culture these bacteria was they may have been strict anaerobes. If that was the case, they would not have been able to grow because they were not cultured out under strict anaerobic conditions.

Because of the high amount of sulfuric acid present in the sludge pit, it is possible that the sludge pit could be home to sulfate-reducing bacteria. From looking at the morphology of the bacteria in the sludge pit under the microscope we know they have similar morphology (short rods) to the sulfidogen genus *Desulfomonile* (9). The *Desulfomonile* genus includes the bacterium *D. tiedjei*. *D. tiedjei* is unique in that it is the only known obligately anaerobic organism able to dechlorinate organic compounds (9). There are many other bacteria able to grow under sulfate reducing conditions, including members of *Desulfobacter, Desulfobacterium, Desulfococcus, and Desulfomonas* genera (9). None of these organisms, however, have been found in areas with an extremely low pH.

Fluorescent Redox Probe

Because we were unsuccessful at culturing the bacteria from the sludge pit, we performed metabolic assays to find

out more about the bacteria living in the sludge pit. We first assayed for respiration using the redox probe 5-cyano-2,3-ditolyl tetrazoleum chloride (CTC). CTC when incorporated into bacterial cells allowed for direct epifluorescent microscopic enumeration of respiring bacteria (26). This procedure worked on the premise that in the oxidized state CTC was nearly colorless and nonfluorescent. CTC was converted by electron-transport activity into the fluorescent compound CTC-formazan. Bacteria containing CTC-formazan can be visualized by epifluorescent microscopy because they fluoresce bright red. We found that the bacteria in the sludge pit were respiring. This can be seen in Figure 6.

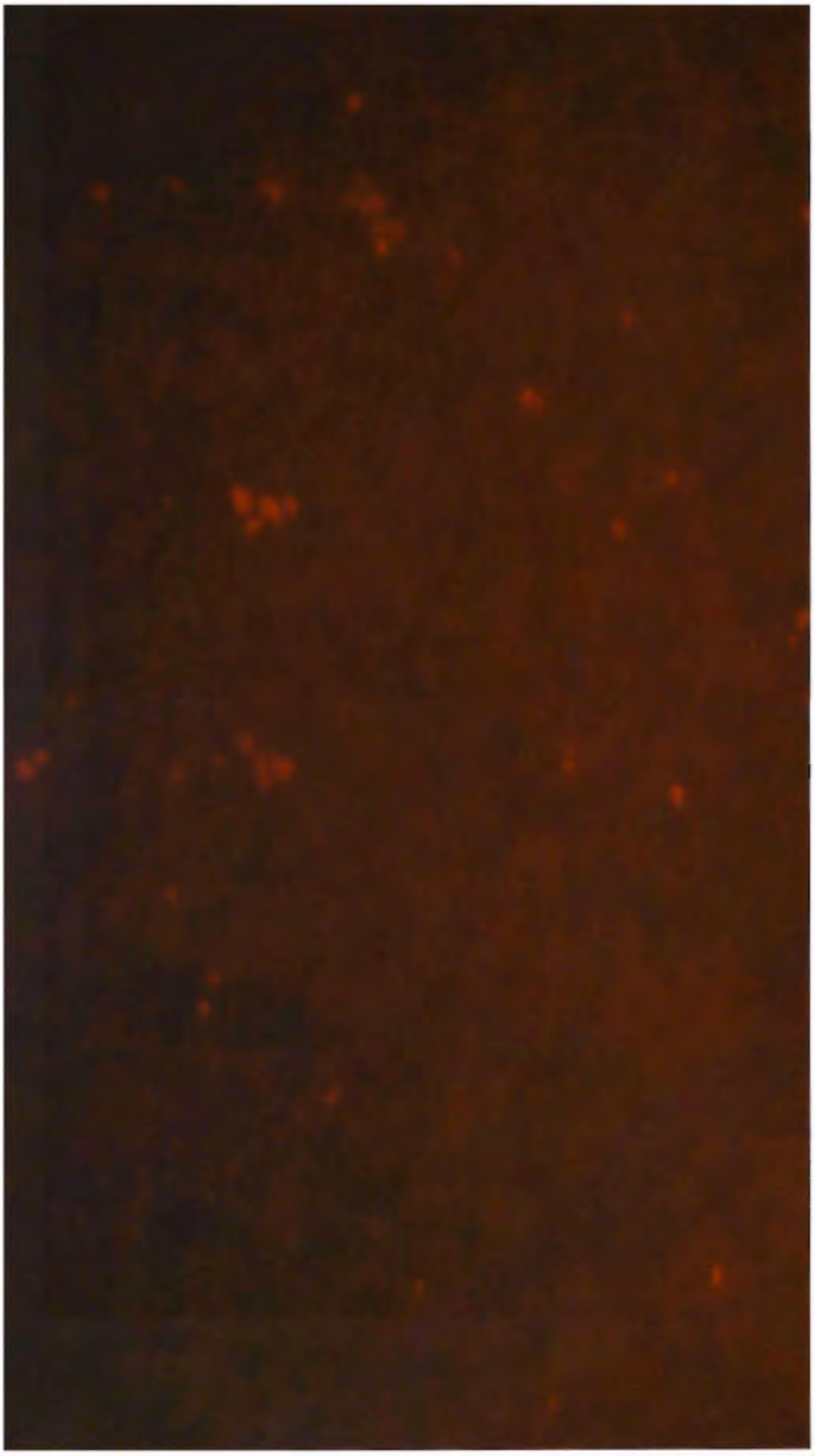
ATP Assay

Because CTC can be broken down under reducing conditions, to further support our claim that there were bacteria living in the acid sludge pit we performed ATP assays. ATP assays were performed using the enzyme luciferase which was isolated from fire-flies. In this assay luciferase acted upon luciferin (LH_2), oxygen and ATP to produce light. The amount of light produced was then measured on a photometer. The divalent metal ion Mg^{+2} functions as a cofactor. The principle reactions were:

1) ATP + Luciferin (LH_2)------> LH_2·AMP + PPi

2) LH_2·AMP + O_2-->AMP + CO_2+ oxyluciferin + Light (560 nm) (36).

Figure 6. Photograph of Respiring Bacteria from Acid Sludge Pit. Bacteria are Stained with CTC and Magnified 100X.



In general, the extraction of ATP from soil samples is more difficult than from liquid samples. The reasons for this are there may be less ATP present, there may be substances present that interfere with the extraction, and/or there may be substances present that interfere with the luciferase enzyme (36). As a control a standard bacterial suspension was mixed with the tested soil which allowed estimation of both extraction inhibition and assay inhibition (36).

Calculations (see Table 8) revealed approximately a 50% recovery of the ATP from the bacterial suspension. This is probably due to the fact that the material from the acid sludge pit was very difficult to break up making extractions difficult; also the extremely low pH of the acid sludge soil may have broken down some of the ATP that was extracted. Because of the low recovery we were unable to establish a correlation with the AODC counts, but since ATP was present we can positively state that there were bacteria living in the acid sludge pit. From the ATP Standard Curve (Appendix D) we recovered approximately 2.6 ng ATP/gm of soil from the acid sludge pit. As a comparison, in one study the amount of ATP present in uncontaminated garden soil that has a bacterial population of 7.0×10^6 CFU/mL has been calculated to be 480 ng/gm (36).

TABLE 8

ATP IN SOIL SAMPLES

	Background	ATP	Cells	Soil	Soil and Cells	Soil and ATP
	23	5600	6046	1065	3546	5898
	13	5903	6135	1123	3518	6235
	16	6417	5958	1082	3674	6586
Average:	**17**	**5973**	**6046**	**1090**	**3579**	**6240**

Percent Recovery of Cells: 3579/(1090+6046)= 50%
Percent Recovery of ATP: 6240/(1090+5973)= 88%

	Background	ATP	Cells	Soil	Soil and Cells	Soil and ATP
	12	5307	6154	1153	3465	6218
	14	5719	6064	1074	3754	6463
	13	5685	6052	1029	3653	6368
Average:	**13**	**5570**	**6090**	**1085**	**3624**	**6350**

Percent Recovery of Cells: 3624/(1085+6090)= 51%
Percent Recovery of ATP: 6350/(1085+5570)= 95%

	Background	ATP	Cells	Soil	Soil and Cells	Soil and ATP
	15	5408	6016	1031	3321	6156
	20	6002	6187	986	3754	6178
	13	5819	6253	995	3578	6054
Average:	**16**	**5743**	**6152**	**1004**	**3551**	**6129**

Percent Recovery of Cells: 3551/(1004+6152)= 50%
Percent Recovery of ATP: 6129/(1004+5743)= 91%

Oxygen Uptake Assay

Oxygen uptake assays determined if the bacteria in the sludge pit used oxygen. The sample chamber had a very small stir bar. Because soil particles present in the sample interfered with the stir bar we had to dilute the original sample 1/100.

Perhaps due to the large dilution, results were inconclusive. A slight depletion of oxygen was observed in the soil samples and in the soil samples containing sodium azide. The bacteria in the sludge pit consumed 0.1 ppm of oxygen/30 min, and the soil sample with sodium azide consumed 0.2 ppm of oxygen/30 min. This shows that there was some oxygen consumed but it might be due to chemical oxidation rather than bacterial oxidation. In conclusion these results might result from the large dilution of sample or the bacteria in the sample may be anaerobic.

^{14}C Uptake

The results from this assay show no detectable CO_2 production (see Table 9). These results could be explained in two ways. The bacteria might have been unable to metabolize the glucose or there might have been too few bacteria to produce detectable amounts of CO_2. Another possible explanation is that after nine months storage any bacteria that were present might have become non-viable.

TABLE 9

C-14 UPTAKE RESULTS

	Counts per Minute
Soil	32
Soil and Sodium Azide	29
Background	31
Soil and Water	64
Soil, Water, and Sodium Azide	42
Background	67

Conclusion

From the results of these experiments, we know that there were bacteria living in the sludge pit. This was demonstrated using AODC, which gave us a total bacterial count of 5.59E+04 CFU/ml. This result was confirmed using a CTC redox probe which demonstrated that the bacteria were respiring (Figure 6). Calculations of 2.6 ng ATP/gm of soil further confirms the presence of bacteria.

Additional confirmations of our results were sought by performing oxygen uptake assays and ^{14}C uptake assays. The oxygen uptake assays were inconclusive and the ^{14}C assays yielded negative results. This may be due to the fact that the bacteria were inactive due to the long storage time. Other potential problems with these assays were that glucose might have been an inappropriate substrate for the ^{14}C assay and that the bacteria might have been anaerobic which would make the oxygen uptake assay negative. We were unable to successfully culture the bacteria from the pit which might also indicate they were anaerobic.

The microbial population survey revealed that the number of bacteria in the land farm and around the sludge pit was approximately the same as the control area. We know that the pH and the presence of organic contaminants were not factors in causing a change in vegetation on the land farm. It is hypothesized that the change in vegetation might be the result of a lack of irrigation and fertilization of the land farm soil.

The presence of bacteria in the sludge pit is important because it indicates that bioremediation might be a feasible option to clean up the sludge pit. More studies need to be done determine the extent of the bacteria's ability to degrade the organic contents of the pit under different conditions.

Neutralization of the pit might make bioremediation more efficient. Neutralization would allow other bacteria that can not tolerate acidic environments to degrade the sludge. This was seen in a study done by Verstraete et al. (30). These researchers reported a near doubling rate of biodegradation of gasoline in an acidic soil (pH 4.5) by adjusting the pH to 7.4 (30). In our case, neutralizing the pit would be very expensive.

Fertilization of the sludge is also an option. The bacteria present might become more active if a plentiful supply of nutrients were available. However, even if all the organic waste was removed, the pit would still have to be neutralized. It is also important to remember that bioremediation will not remove inorganic material. Such material would have to be removed by another method.

LITERATURE CITED

1. Alexander, M. 1977. Introduction to Soil Microbiology. 2nd ed. John Wiley & Sons, New York.

2. Atlas, R.M. 1991. Microbial Hydrocarbon Degradation-Bioremediation of Oil Spills. J. Chem. Tech. Biotechnol. **52**:149-156.

3. Balkwill, D.L. and W.C. Ghiorse. 1985. Characterization of Subsurface Bacteria Associated with Two Shallow Aquifers in Oklahoma. Appl. Environ. Microbiol. **50**:580-588.

4. Balkwill, D.L., F.R Leach, J.T. Wilson, J.F. McNabb, and D.C. White. 1988. Equivalence of Microbial Biomass Measures Based on Membrane Lipid amd Cell Wall Components, Adenosine Triphosphate, and Direct Counts in Subsurface Aquifer Sediments. Microb. Ecol. **16**:73-84.

5. Bossert, I and R. Bartha. 1984. The Fate of Petroleum in Soil Ecosystems. p. 434-476. In: R.M. Atlas (ed.) *Petroleum Microbiology*. Macmillan Publishing Co., New York.

6. Boyd, S.A. and D.R. Shelton. 1984. Anaerobic Degradation of Chlorophenols in Fresh and Acclimated Sludge. Appl. Environ. Microbiol. **47**:272-277.

7. Clark, C.G., and S.J.L. Wright. 1970. Detoxication of isopropyl N-phenylcarbamate (IPC) and isopropyl N-3-chlorophenylcarbamate (CIPC) in soil and isolation of IPC-metabolizing bacteria. Soil Biol. Biochem. **2**:19-27.

8. Colberg, P.J.S. 1990. Role of Sulfate in Microbial Transformations of Environmental Contaminants: Chlorinated Aromatic Compounds. Geomicrobiology Journal. **8**:147-165.

9. DeWeed, K.A., L. Mandelco, R.S. Tanner, C.R. Woese, J.M. Suflita. 1990. *Desulfomonile tiedjei* Gen. Nov. and Sp. Nov. A Novel Anerobic Dehalogenating, Sulfate Reducing Bacterium. Arch. Microbiol. **154**:23-30.

10. Dibble, J.T. and R. Bartha. 1979. Effect of Environmental Parameters on the Biodegradation of Oil Sludge. Appl. Environ. Microbiol. 37:729-739.

11. Fiorenza, S., Duston, C.H. Ward. 1991. Decision Making-Is Bioremediation a Viable Option? J. of Haz. Mat. 28:171-183.

12. Foght, J.M., P.M. Fedorac, and D.W. Westlake. 1990. Mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene in crude oil; specificity among bacterial isolates. Can. J. Microbiol. 36: 169-175.

13. Ghiorse, W.C. and D.L. Balkwill. 1983. Enumeration and Morphological Charecterization of Bacteria Indigenous to Subsurface Environments. Dev. Ind. Microbiology 24:213-224

14. Goldstein, R.M., L.M. Mallory, and M. Alexander. 1985. Reasons for Possible Failure of Inoculation to Enhance Biodegradation. Appl. Environ. Microb. 50:977-983.

15. Grbic-Gallic, D. and T.M. Vogel. 1987. Transformation of Toluene and Benzene by Mixed Methanogenic-cultures. Appl. Environ. Microbiol. 53:254-260.

16. Harrison, A.P. 1984. The Acidophilic Thiobacilli and other Acidophilic Bacteria that Share their Habitat. Ann. Rev. of Microb. 38:265-292.

17. Hertzler, S.K. 1991. Rapid, Small-Volume Respirometry as a Substitute for the Five Day BOD Assay. Masters Thesis. Oklahoma State University.

18. Hobbie J.E., R.J. Daley and S. Jasper. 1977. Use of Nucleopore Filters for Counting Bacteria by Fluorescent Microscopy. Appl. Environ. Microb. 33:1225-1228.

19. Johnson, D.B. and S. McGinness. 1991. A Highly Efficient and Universal Solid Medium for Growing Mesophilic and Moderatrely Thermophilic Iron-Oxidizing, Acidophilic Bacteria. J. of Microb. Meth. 13:113-122.

20. Kieft, T.L. and L.L. Rosacker. 1991. Application of Respiration and Adenylate Based Soil Microbiological Assays to Deep Subsurface Terrestrial Sediments. Soil Biol. Biochem. 23:563-568.

21. Kilbane, J.J., D.K. Chatterjee, and A.M. Chakrabarty. 1983. Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia*. Appl. Environ. Microbiol. 45:126-129.

22. Leach, F.R., J.C. Chang, J.L. Howard, J.J. Webster, A.B. Arquitt, R. Merz, E.R. Doyel, P.T. Norton, G.J. Hampton, and J.Z. Jackson. 1985. Biochemical Methods for Detection of Subsurface Contaminants/Biomass. p. 241-269 In: C.H. Ward, W. Giger, and P.L. McCarty (ed.), *Ground Water Quality*. John Wiley & Sons, New York.

23. Leahy, J.G. and R.R. Colwell. 1990. Microbial Degradation of Hydrocarbons in the Environment. Microbiol. Rev. **54**:305-15.

24. McCarty P.L. 1991. Engineering Concepts for *In Situ* Bioremediation. J. of Haz. Mat. **28**:1-11.

25. Phelps, T.J., D. Ringlelberg, D. Hedrick, J. Davis, C.B. Fliermans, and D.C White. 1988. Microbial Biomass and Activities Associated with Subsurface Environments Contaminated with Chlorinated Hydrocarbons. Geomicrobiology Journal. **6**:157-170.

26. Rodriguez, G.G., D. Phipps, K. Ishiguro, and H.F. Ridgway. 1992. Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. Appl. Environ. Microb. **58**:1801-1808.

27. Ryan J.R., R.C. Loehr, and E. Rucker. 1991. Bioremediation of Organic Contaminated Soil. J. of Haz. Mat. **28**:159-169.

28. Stamatiadis S., J.W. Doran, and E.R. Ingham. 1990. Use of Staining and Inhibitors to Separate Fungal and Bacterial Activity in Soil. Soil Biol. Biochem. **22**:81-88.

29. Tarvin, D. and A.M. Buswell. 1934. The Methane Fermentation of Organic Acids and Carbohydrates. J. Am. Chem. Soc. **56**:1751-1755.

30. Verstraete, W., R. Vanloocke, R. DeBorger, and A. Verlinde. 1976. Modeling of the Breakdown and the Mobilization of Hydrocarbons in Unsaturated Soil Layers. p. 99-112. In: J.M. Sharpley and A.M. Kaplan (ed.) *Proceedings of the 3rd International Biodegradation Symposium*. Applоed Science Publishers Ltd. London.

31. Walton, K.C. and D.B. Johnson. 1992. Mucrobiological and Chemical Charecteristics of an Acidic Stream Draining a Dusysed Copper Mine. Environ. Pollu. **76**:169-175.

32. Wang, X., and R. Bartha. 1990. Effect of Bioremediation on Residues; Activity and Toxicity in Soil Contaminated by Fuel Spills. Soil Biol. Biochem. 22:501-506.

33. Warith. M.A., R. Ferehner, and L. Fernandes. 1992. Bioremediation of Organic Contaminated Soil. Haz. Waste & Haz. Mat. 9:137-147.

34. Webster, J.J., G.J. Hampton, and F.R. Leach. 1984. ATP in Soil: A New Extractant and Extraction Procedure. Soil Biol. Biochem. 16:335-342.

35. Webster, J.J., G.J. Hampton, J.T. Wilson, W.C. Ghiorse, and F.R. Leach. 1985. Determination of Microbial Cell Numbers in Subsurface Samples. Ground Water. 23:17-25.

36. Webster, J.J. and F.L. Leach. 1980. Optimization of the Firefly Luciferase Assay for ATP. J. Appl. Biochem. 2:469-479.

37. Webster, J.J., B.G. Walker, and F.R. Leach. 1988. ATP Content and Adenylate Energy Charge of *Bacillus stearothermophilus* During Growth. Current Microbiology. 16:271-275.

38. Wilson, J.T., J.F. McNabb, D.L. Balkwill, and W.C. Ghiorse. 1984. Enumeration and Characterization of Bacteria Indigenous to a Shallow Water-Table Aquifer. Ground Water. 21:134-142.

APPENDIXES

APPENDIX A

DIRECT PLATE COUNT RESULTS

TABLE 10

DIRECT PLATE COUNT RESULTS IN CFU/mL

	PTYG	TNA	
Landfarm	4.70E+05	5.70E+05	
	6.30E+05	5.30E+05	
	5.70E+05	1.20E+06	
	5.57E+05	7.67E+05	Average
		6.62E+05	Combined Average
Northeast	2.30E+05	8.80E+05	
	3.00E+05	1.00E+06	
	9.90E+05	7.50E+05	
	5.07E+05	8.77E+05	Average
		6.92E+05	Combined Average
Southeast	2.40E+05	4.10E+05	
	3.20E+05	1.08E+06	
	2.20E+05	8.50E+05	
	2.60E+05	7.80E+05	Average
		5.20E+05	Combined Average
Northwest	9.00E+05	9.60E+05	
	1.10E+05	8.90E+05	
	1.20E+05	8.30E+05	
	3.77E+05	8.93E+05	Average
		6.35E+05	Combined Average
Control	1.00E+06	2.00E+06	
	1.00E+06	3.00E+06	
	3.00E+06	3.00E+06	
	1.67E+06	2.67E+06	Average
		2.17E+06	Combined Average

APPENDIX B

ACRIDINE ORANGE DIRECT COUNT RESULTS

TABLE 11

ACRIDINE ORANGE DIRECT COUNT RESULTS

	Landfarm	Northeast	Southeast	Northwest	Control	Pit 5
Count	1.30E+04	1.80E+04	1.20E+04	1.70E+04	2.40E+04	1.00E+01
	1.00E+04	2.00E+04	1.40E+04	1.30E+04	2.20E+04	2.00E+01
	1.60E+04	1.00E+04	2.10E+04	1.50E+04	1.90E+04	0.00E+00
	1.50E+04	1.60E+04	1.80E+04	1.40E+04	2.20E+04	0.00E+00
	1.50E+04	1.60E+04	1.90E+04	1.20E+04	1.90E+04	0.00E+00
	8.00E+03	1.70E+04	1.60E+04	1.10E+04	1.60E+04	1.00E+01
	1.40E+04	1.20E+04	1.10E+04	1.20E+04	2.40E+04	1.00E+01
	1.30E+04	1.60E+04	1.40E+04	1.10E+04	2.00E+04	1.00E+01
	9.00E+03	9.00E+03	1.30E+04	1.30E+04	1.70E+04	1.00E+01
	1.90E+04	2.00E+04	9.00E+03	1.00E+04	1.80E+04	3.00E+01
Average	**1.32E+04**	**1.54E+04**	**1.47E+04**	**1.28E+04**	**2.01E+04**	**1.00E+01**
Number of Bacteria/mL	7.38E+07	8.61E+07	8.22E+07	7.16E+07	1.12E+08	5.59E+04

To calculate the number of bacteria/ mL:
N= a x n/ m x V

N= number of bacteria/mL
a= effective wet filtration area in sq. mm
m= area enclosed by the eyepiece reticule in sq. mm at magnification — a=1734 sq mm
n= mean count of bacteria present — m=.031 sq mm
V= total sample volume in mL — V=10 ml

APPENDIX C

C^{14} GLUCOSE EXPERIMENT

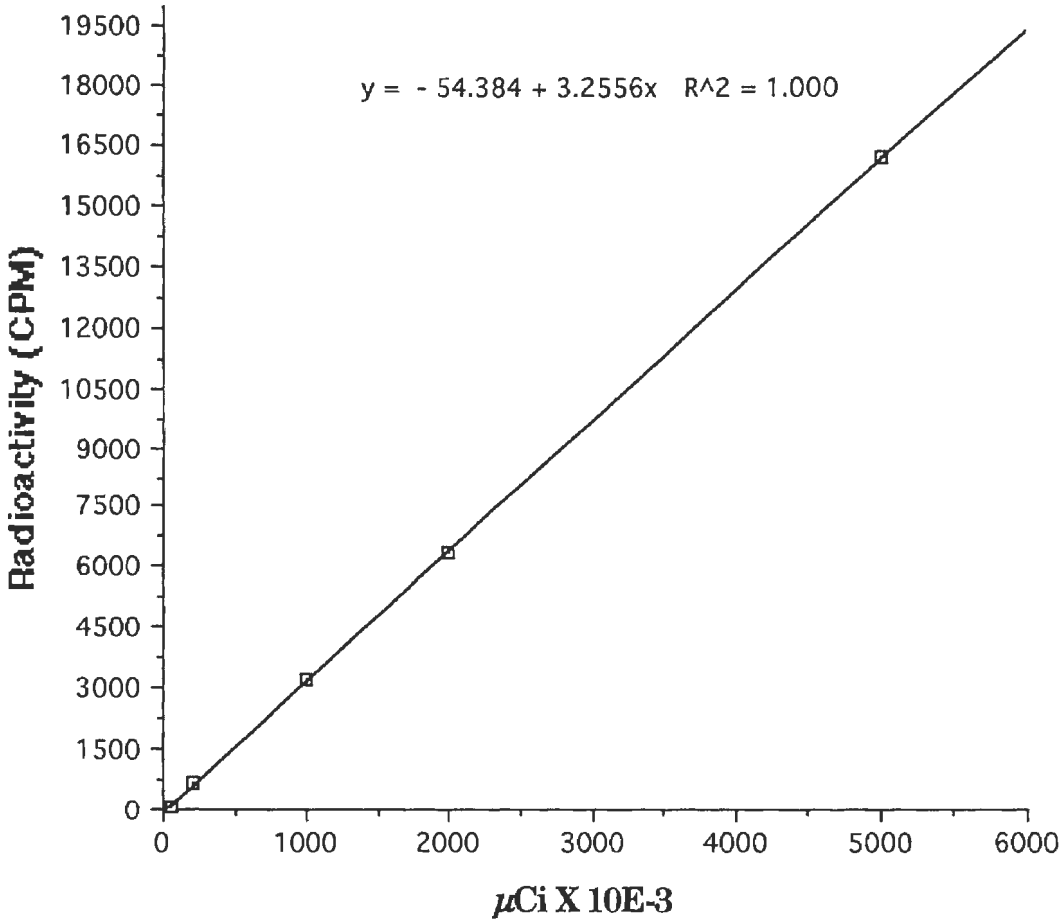
TABLE 12

DATA FOR C-14 GLUCOSE

STANDARD CURVE

Dilution of Stock	Concentration (uCi x .0001)	Concentration (umole x .0001)	Average CPM
10	5000	2174	16265
25	2000	870	6345
50	1000	435	3202
250	200	87	685
1000	50	21.74	90

Figure 7: C-14 Glucose Standard Curve



APPENDIX D

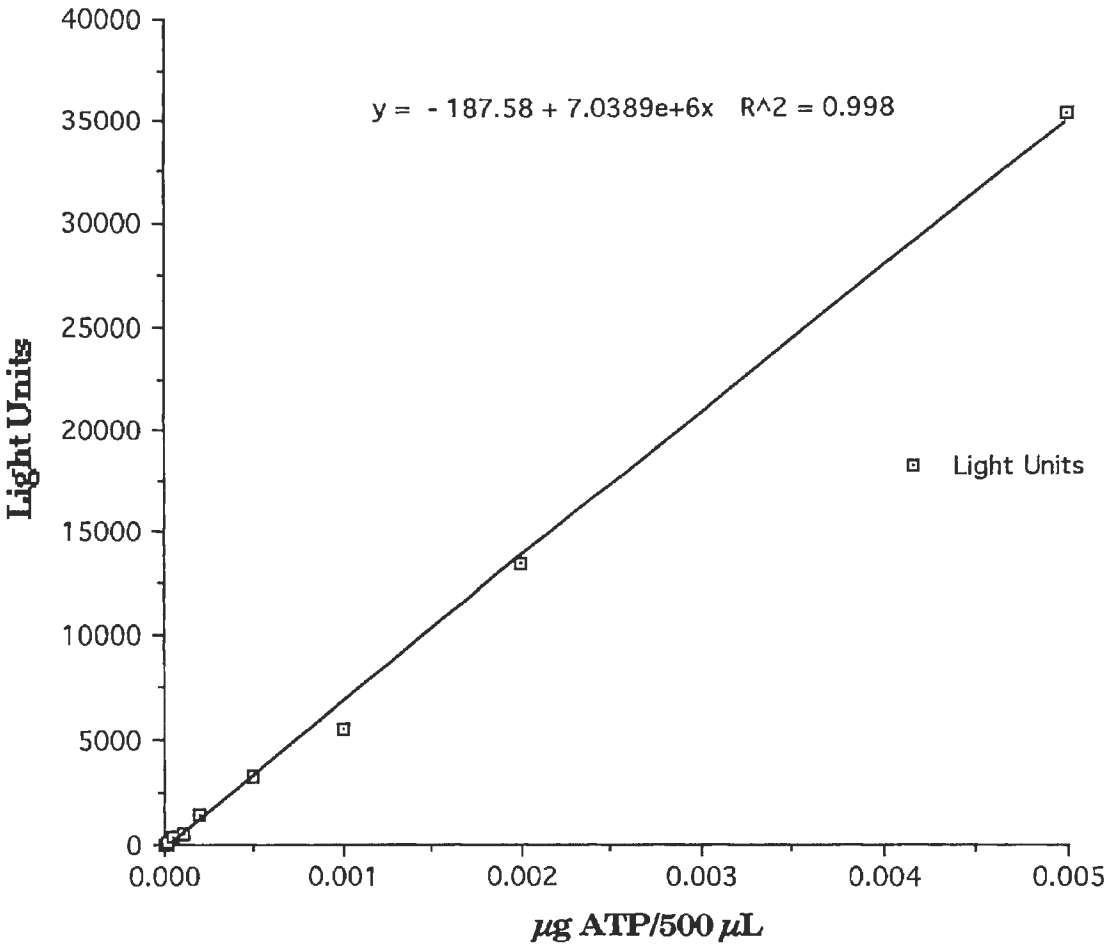
ATP EXPERIMENT

TABLE 13

DATA FOR ATP STANDARD CURVE

Dilution of Stock (-1)	ug ATP/500 uL	Average Light Units
500000	2.00E-06	40
200000	5.00E-06	48
100000	1.00E-05	64
50000	2.00E-05	167
20000	5.00E-05	377
10000	1.00E-04	594
5000	2.00E-04	1402
2000	5.00E-04	3352
1000	1.00E-03	5564
500	2.00E-03	13462
200	5.00E-03	35421

Figure 8: ATP Standard Curve



VITA 2

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