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MICROBIAL BIOMASS MEASUREMENTS AT THE PAWNEE SITE: PRELIMINARY METHODOLOGY AND RESULTS

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INTRODUCTION

This report describes studies of microbial form and function at the Pawnee site of the Grassland Biome Program of the U. S. International Biological Program. The major objective of the investigations was to develop sampling and analytical procedures for the estimation of microbial biomass. A secondary area of interest was to measure microbial respiratory activity in soil, with the aim of relating this to microbial biomass values.

Research was conducted in two related areas:

1. Direct estimation of microbial biomass in soil.
2. Development of an ATP assay as an indirect measurement of biomass.

1. DIRECT ESTIMATION OF MICROBIAL BIOMASS

Introduction

Measurements of the biomass of soil microorganisms are necessary for an understanding of nutrient cycling and distribution in the grassland environment. Direct counting techniques are used to measure total biomass, since plate count procedures give results which generally cannot be related to biomass.

The objective of the present study was to determine the biomass of soil bacteria and fungi in selected soils of the Pawnee site. Variations in the biomasses of these two groups of microbes with soil depth and with season were investigated.

Methods

Soil cores (2.5 cm diameter) were collected with a hydraulic coring unit. Generally 2 to 4 cores were collected at each site. The soil

samples were brought to the laboratory within two hours, where they were analyzed immediately or stored in a refrigerator until the time of analysis.

Bacterial biomass values were derived from estimations of cell numbers in soil. Bacterial counts were made by means of two methods. Initial studies were made using a Petroff-Hausser counting chamber. The soil was diluted with water to a level such that each small square of the counting chamber contained on the average of four to 10 bacterial cells. Fifty randomly selected squares were counted, using an oil immersion objective and brightfield or phase contrast conditions. In later studies, a modification of the Witkamp smear technique was used (Witkamp, 1966). An aliquot of soil suspension was smeared over an area of 1 cm^2 on a microscope slide. The slide was examined directly under phase contrast or stained with rose bengal and viewed using brightfield conditions. Fifty randomly selected fields were counted.

With both techniques, 10 slides were prepared and counted for each soil suspension. The two procedures gave roughly comparable results, although the Petroff-Hausser counting chamber yielded slightly lower counts. Total numbers of bacteria per g of soil were computed.

With an eyepiece micrometer, it was determined that for 100 randomly selected bacterial cells in soil, the average radius and length were 0.40 $\pm$ 0.04μ and 1.0 $\pm$ 0.02μ, respectively. Bacterial volumes and dry weights were calculated as described in Table 1.

Fungal biomass values were derived from estimations of mycelium length by a modification of the method of Thomas, Nicholas, and Parkinson (1965). Soil samples were diluted with a dilute agar solution. An aliquot of the suspension was placed on a ruled slide so as to cover an area of 2.5 cm^2. The thin agar film was examined under 200 power magnification with or

without staining by phenolic aniline blue. The hyphae observed were traced on paper using a camera lucida attachment on the microscope. A planimeter was used to measure the total hyphal length per field. Total mycelial length per gram of soil then was calculated.

It was determined that the average radius of 40 hyphal filaments was 1.1 ± 0.2μ. Fungal hypal lengths, volumes, and weights were calculated as shown in Table 2.

Results

Soil samples were collected north of microwatershed Number 2 in an area which has had no grazing by cattle since 1939. The soil was of the Ascalon series, probably Ascalon sandy loam.

Table 3 indicates bacterial numbers and dry weights in soils collected at three dates during summer 1969. Fungal mycelial lengths and dry weights for these same soil samples are presented in Table 4. From data given in these two tables, microbial biomass values were calculated to depths of 15 and 30 cm (Table 5).

Discussion

For both bacteria and fungi there was a decrease in biomass values with soil depth. The decline was much more apparent with fungi than with bacteria. A significant seasonal trend also was observed. The biomass values for each group of microorganism decreased from July to September.

The direct count procedure does not differentiate between living and dead cells. Thus, it is not possible to comment on the physiological significance of the biomasses measured in this study. It is clear from the data, however, that a considerable quantity of organic matter is present in soil in the form of microbial tissue. It is generally held

that such tissue is about 50% C and 5% N (Alexander, 1961). On this basis it can be calculated that for the soil under investigation, 25 to 40 g of carbon and 2.5 to 4 g of nitrogen/m^2 to 30 cm depth is present in the form of microbial cells.

2. DEVELOPMENT OF AN ATP ASSAY AS A MEASURE OF BACTERIAL BIOMASS

Introduction

The determination of microbial numbers or biomass in soil usually has been carried out by one of two procedures: direct microscopic examination, or plate counts of viable cells. Both methods are laborious and time-consuming, and give results which often are difficult to interpret. With the direct count procedure, it is not usually possible to differentiate between living and dead cells; the method tends to overestimate numbers of viable cells. The plate count method, on the other hand, is subject to the criticism that not all viable organisms will produce colonies on a single medium or under a given set of environmental conditions; this may result in an underestimation of bacterial numbers. In addition, the importance of certain fungi and streptomycetes which produce large numbers of spores in soil may be overemphasized by the plate count procedure.

A new technique for determining bacterial biomass has been proposed and utilized by Levin et al. (1968), and by Hamilton and Holm-Hansen (1967). The method is based on the measurement of adenosine triphosphate (ATP). These workers have reported that, for the bacterial species they tested, the ratio of ATP to cell mass is fairly constant. This constancy has been observed throughout all phases of growth of the tested microorganisms. If the ATP content per cell were truly constant, it would be possible to estimate bacterial numbers on the basis of ATP measurements. Among the

advantages of the ATP technique would be its great simplicity, as well as its potential for providing a more accurate measure of the mass or numbers of viable microorganisms in soil.

The objective of the present study was to assess the suitability of ATP measurements as indices of bacterial biomass in liquid culture and in soil.

Methods

A firefly luciferin-luciferase enzyme system was used to assay for ATP. In the presence of this nucleotide triphosphate, the luciferase system emits light, the amount being proportional to the level of available ATP. Light production was quantitatively measured, using a Nuclear of Chicago Mark I liquid scintillation counter. The reaction mixture contained an aliquot of test solution, Tris buffer (pH 7.4, 0.05*M*), and 0.05 ml of firefly extract (Sigma Chemical Company, St. Louis, Mo.). Total volume of the mixture lacking the firefly extract was 1.0, or 3.0 ml. The reaction was carried out directly in a liquid scintillation vial. Counting was begun 30 sec. after the addition of the final component of the mixture (usually firefly extract). Counts were taken for 0.1- or 0.3-min. intervals.

Bacterial ATP was extracted by boiling cells collected on Millipore membrane filters for 5 min. in 0.05 *M* Tris (pH 7.4). For the analysis of liquid cultures of bacteria, between 1.0 and 5.0 ml of culture fluid were filtered. For studies of bacteria in soil, a sterile soil was inoculated with a pure culture of bacteria. The soil was diluted with sterile water, and aliquots of the suspensions were filtered. Viable counts of soil and liquid culture were made, using standard dilution plate techniques and Plate Count Agar (Difco).

Results

A typical standard curve for the analysis of ATP by the firefly enzyme method is presented in Fig. 1. As can be seen here, the method is extremely sensitive and is linear over a wide range of ATP concentrations.

A number of bacteria were isolated from soils of the Ascalon series at the Pawnee site. These isolates were grown in a glucose-mineral salts liquid medium. At 12-hr intervals, the cultures were analyzed for ATP and numbers of viable cells by the dilution plate technique. Table 6 summarizes data on the ATP content per viable cell in these cultures. For each bacterial isolate, the range in ATP levels during the course of growth over a 10-day period is indicated. It is clear from these results, and from additional data from the analysis of nearly a dozen other soil bacteria, that on a cellular basis, ATP concentrations are fairly constant among different strains throughout all phases of growth. The mean ATP content per cell for all bacterial strains tested was 1.6×10^{-9} µg.

Two lines of evidence suggest that dead cells contain little or no ATP. In old bacterial cultures containing a high proportion of dead cells, ATP levels still were highly correlated with plate-count estimates of viable cells. Bacterial populations killed with heat or chemical agents, or as a result of dessication, contained no detectable ATP.

Bacterial growth in soil was studied, using the plate-count procedures and the ATP method. Table 2 presents data obtained from an experiment in which sterile samples of Haxton silt loam were inoculated with several bacterial strains. The soil was amended with a dilute solution of glucose and ammonium nitrate. After an incubation period of five days, the soil cultures were extracted with sterile water and the extracts analyzed for ATP and viable organisms. Measured ATP levels in the soil were converted

to cell numbers on the basis of the previously determined value for ATP content per cell. A close correlation between bacterial numbers estimated from dilution plates and from ATP determinations is apparent from these data. Uninoculated, sterilized soil contained no detectable ATP.

The kinetics of bacterial growth and death in soil were followed in an experiment with isolate B-4, a *Bacillus* sp. The results of this study are presented in Fig. 2. The bacterium was allowed to grow for four days in sterile soil amended with glucose and ammonium nitrate. On the fourth day, the cultures were treated with a small amount of chloroform to kill a portion of the cells.

The plate count procedure revealed an increase in the bacterial population followed by a decline as a result of the toxic action of the chemical. ATP concentrations in the soil closely paralleled the changes in cell numbers as determined by counts of viable cells. ATP levels here are expressed as μg/gm of soil.

Discussion and Research in Progress

It appears that the ATP procedure may be satisfactory for the estimation of biomass of soil bacteria. The method is simple and rapid; as many as a dozen soil samples may be extracted and analyzed in an hour. The major shortcoming of the technique in its present form lies in the uncertainty of its ability to measure accurately the fungal component of the soil microflora. This shortcoming might limit its usefulness in determining the biomass of mixed microbial populations found in natural soil samples. Efforts now are being directed toward correlating ATP levels with fungal biomass. In addition, procedures are being developed which should allow the fractionation of mixed populations of soil micro-

organisms so that separate biomass determinations can be made for bacteria and fungi.

LITERATURE CITED

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Table 1. Example of calculation of bacterial biomass.

A.	Average length of a bacterial cell*	1.0μ
B.	Average radius of a bacterial cell*	0.4μ
C.	Average volume of a bacterial cell (πxB^2xA)	$0.501\mu^3$
D.	Average density of a bacterial cell**	$1.1 g/cm^3$
E.	Average weight of a bacterial cell (D x C)	5.52×10^{-13} g/cell
F.	Number of bacteria/g of dry soil*	1×10^9 cells
G.	Total wet weight of bacteria/g of soil (E x F)	5.52×10^{-4} g/g of soil
H.	Average % of dry weight of a bacterial cell**	20%
I.	Dry weight of bacteria/g of soil (G x H)	1.10×10^{-4} g/g of soil
J.	Density of soil**	$1.1 g/cm^3$
K.	Dry weight of bacteria/cm^3 of soil (I/J)	$1.0 \times 10^{-4} g/cm^3$
L.	Dry weight of bacteria/cm^2 to depth of 10 cm (10xK)	$1.0 \times 10^{-3} g/cm^2$
M.	Dry weight of bacteria/m^2 to depth of 10 cm (10^4xM)	$10 g/cm^2$

* Measured value.

** Assumed value.

Table 2. Example of calculation of fungal biomass.

A.	Radius of microscope field*	0.08 mm
B.	Area of field ($\pi \times A^2$)	$2.01 \times 10^{-2} mm^2$
C.	Area of agar film*	$4.5\ cm^2$
D.	Number of fields / agar area (100C/B)	2.24×10^4
E.	Average length of mycelium/per field*	0.0990 cm
F.	Total length of mycelium/agar film (DxE)	2.23×10^3 cm
G.	Volume of agar film*	0.5 ml
H.	Dilution factor*	10
I.	Total length of mycelium/g of soil (FxH/G)	4.46×10^4 cm
J.	Average diameter of mycelium*	2.2×10^{-4} cm
K.	Total volume of mycelium/g of soil ($IxJ^2x\pi$)	$1.69 \times 10^{-3}\ cm^3$
L.	Average density of mycelium**	$1.1 g/cm^3$
M.	Total wet weight of mycelium/g of soil (KxL)	1.86×10^{-3} g
N.	Average % dry weight of mycelium**	20%
O.	Total dry weight of mycelium/g of soil (MxN)	3.72×10^{-4} g
P.	Density of soil**	$1.1 g/cm^3$
Q.	Dry weight of mycelium/cm^3 of soil (O/P)	$3.38 \times 10^{-4} g/cm^3$
R.	Dry weight of mycelium/cm^2 to depth of 10cm (10xQ)	$3.38 \times 10^{-3} g/m^2$
S.	Dry weight of mycelium/m^2 to depth of 10 cm (10^4xR)	$33.8 g/m^2$

* Measured value.

** Assumed value.

Table 3. Bacterial numbers and biomass in Ascalon sandy loam at various sampling times.

Sampling Depth (cm)	Number of Cells/g of Soil ($\times 10^9$)	Bacterial Dry Weight (mg/g of Soil)
July 20		
0- 3	1.23 ± .21*	0.12
6-10	1.08 ± .15	0.11
20-25	1.05 ± .14	0.10
50-60	1.02 ± .16	0.10
70-90	0.990 ± .11	0.10
August 20		
0- 3	1.12 ± .11	0.11
6-10	0.921 ± .085	0.092
20-25	0.915 ± .10	0.092
50-60	0.857 ± .097	0.086
70-90	0.802 ± .091	0.080
September 15		
0- 3	0.91 ± .11	0.091
6-10	0.754 ± .097	0.075
20-25	0.662 ± .069	0.066
50-60	0.557 ± .065	0.056
70-90	0.446 ± .066	0.045

* Standard deviation.

Table 4. Fungal mycelium lengths and biomass in Ascalon sandy loam at various sampling times.

Sampling Depth (cm)	Length of Mycelium (m/g of soil)	Dry Weight of Mycelium (mg/g of soil)
July 20		
0- 3	423 ± 67	0.32
6-10	186 ± 29	0.14
20-25	83 ± 12	0.061
50-60	85 ± 14	0.066
70-90	58 ± 9	0.044
August 20		
0- 3	324 ± 47	0.25
6-10	119 ± 23	0.091
20-25	60 ± 9	0.046
50-60	61 ± 11	0.047
70-90	55 ± 9	0.042
September 15		
0- 3	282 ± 39	0.22
6-10	97 ± 14	0.074
20-25	55 ± 7	0.042
50-60	48 ± 8	0.037
70-90	39 ± 6	0.029

* Standard deviation.

Table 5. Bacterial and fungal biomass in Ascalon sandy loam at various sampling times.

Sampling Time	Soil Depth	Bacterial Dry Weight*	Fungal Dry Weight*
July 20			
	15 cm	17 ± 2**	34 ± 4
	30 cm	32 ± 3	50 ± 6
August 20			
	15 cm	13 ± 1	23 ± 3
	30 cm	26 ± 2	35 ± 5
September 15			
	15 cm	11 ± 1	19 ± 2
	30 cm	22 ± 2	29 ± 3

* g/m^2 to indicated depth.

** Standard deviation.

Table 6. ATP content of soil bacteria.

Isolate	µg ATP per cell ($\times 10^{9}$)	
	Range	Average
B-1-1	0.8 - 1.1	1.0
B-1-2	1.7 - 2.1	1.9
B-1-3	1.3 - 1.9	1.6
B-4-A	0.9 - 1.4	1.2
B-5-A	1.5 - 1.9	1.7

Table 7. Number of cells developing in inoculated samples of sterile soil.

Isolate	Number of Cells ($\times 10^{-8}$)	
	Plate Count	ATP Method
B-1-1	8.51	7.90
B-1-2	3.93	4.20
B-1-3	5.14	5.04
B-4-A	3.05	2.74
B-5-A	2.03	2.43

5-day incubation period.

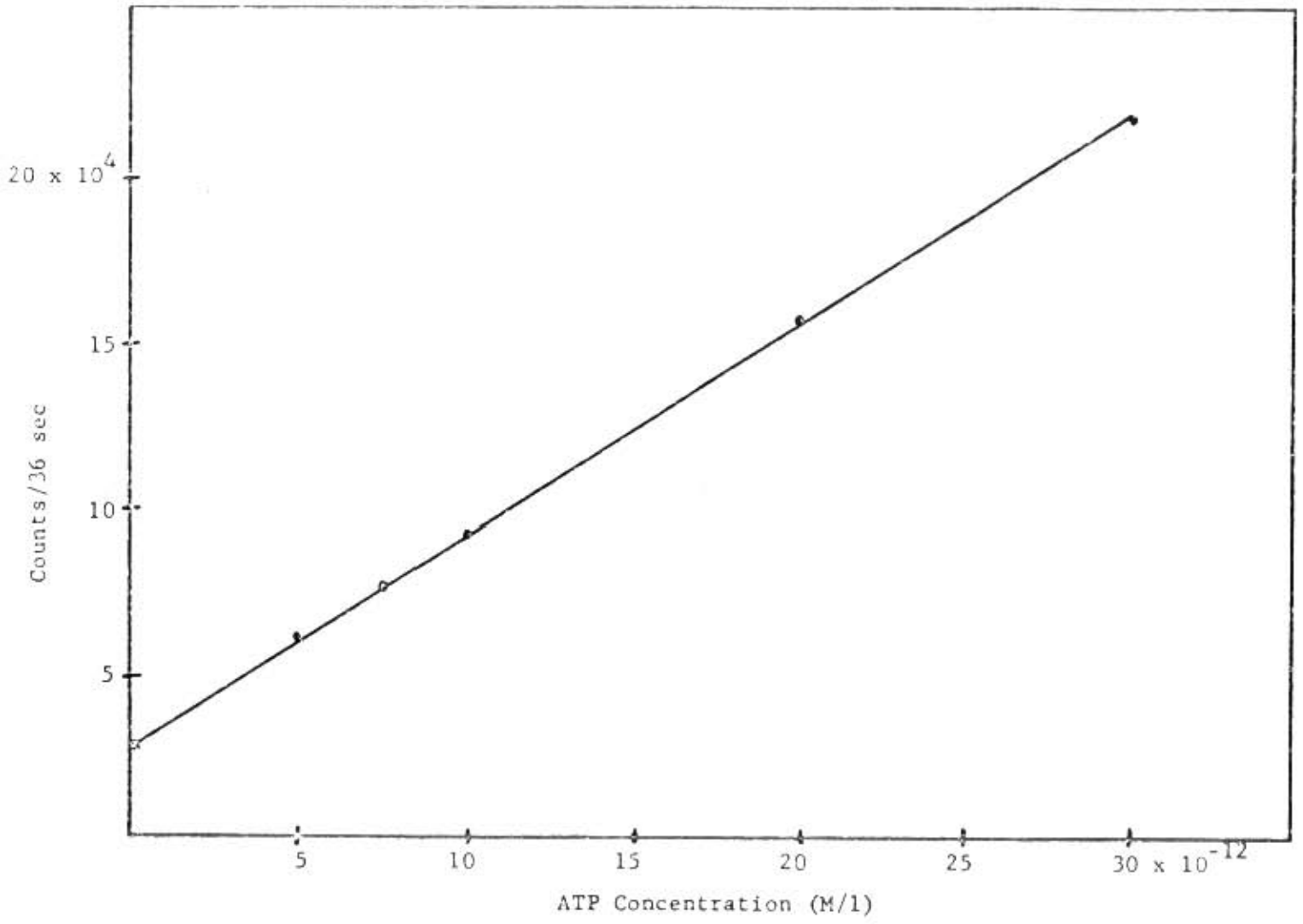

Figure 1. Standard ATP calibration curve relating light production, in counts per 36 sec, to ATP concentration.

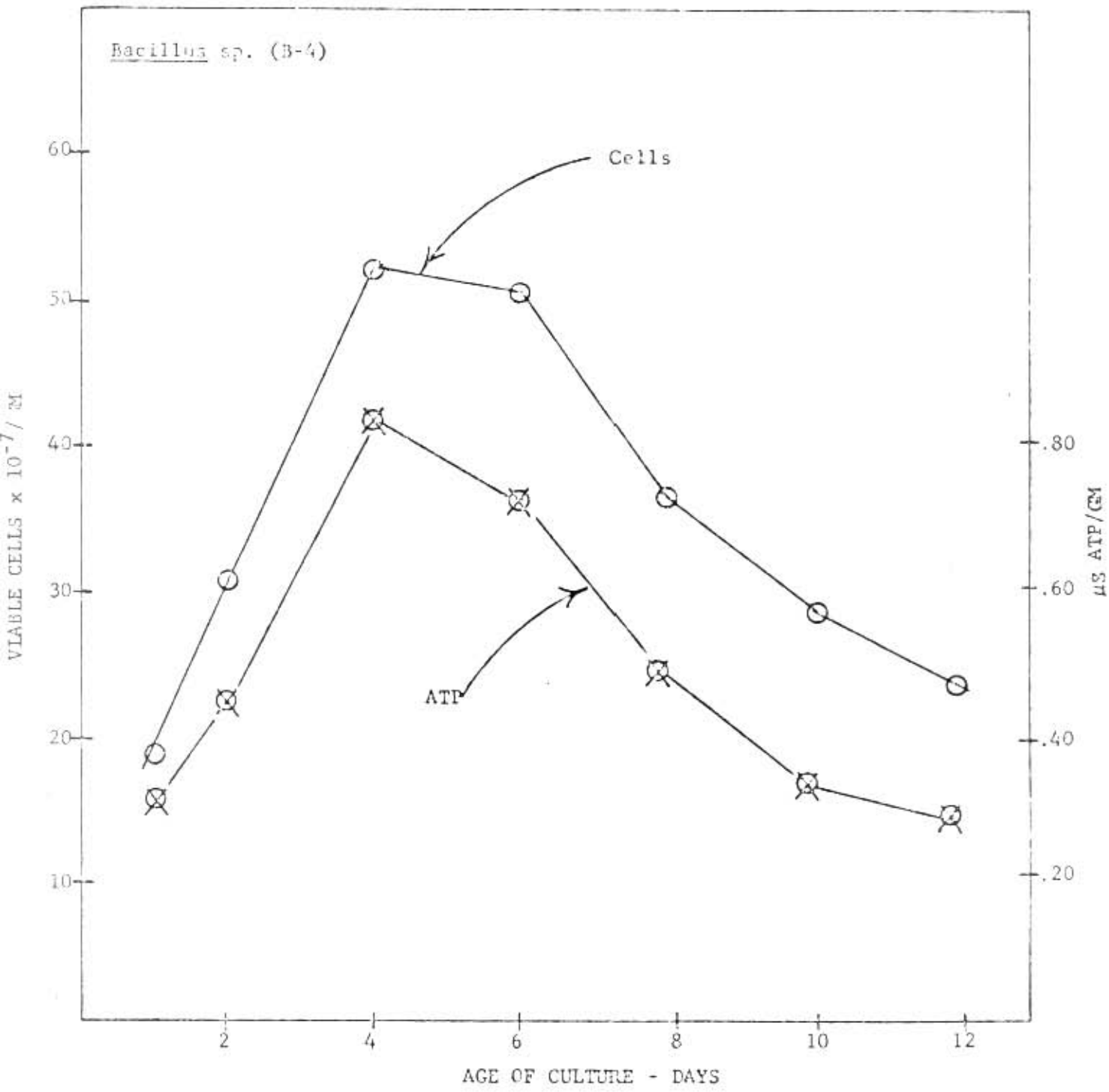

Figure 2. ATP levels and numbers of viable cells in autoclaved soil inoculated with bacterial isolate B-4. The culture was treated with chloroform at 4 days.