

## The use of fluorescein isothiocyanate in the determination of the bacterial biomass of grassland soil<sup>1,2</sup>

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Quantitative recoveries of bacteria added to a number of soils were obtained using fluorescein isothiocyanate (FITC) as a stain. Enzyme fluorescence, using fluorescein diacetate or dibutylate, could not be adapted for the routine counting of metabolizing organisms in soil. Staining with rose bengal gave recoveries of 60-80%.

Soil-extract dilution plates revealed  $4 \times 10^7$  bacteria/g in the 0-10 cm layer in April and  $27 \times 10^7$  in October. One-third of this number was observed in the 20-30 cm layer. Actinomycete colonies added another 20% to the counts at the 0-30 cm depth but constituted 70% of the total colonies from the 90-120 cm depth. Microscopic counts indicated  $2.2-4.6 \times 10^9$  organisms/g of surface soil. The number of bacteria decreased in a linear fashion with depth but no relation was found between the numbers obtained by direct microscopy and those by plate counting. However, each of the methods showed a high relationship between the size of the bacterial population present at each depth on the different sampling dates.

The observed bacteria, actinomycete spores, and hyphal segments averaged  $0.6 \times 1 \mu$ . On this basis, the biomass in the top 30 cm, as determined by direct microscopy, ranged from 30 to 76 g/m<sup>2</sup> (dry weight basis). This implies, considering the amount of available energy, that the individual cells have enough energy to divide only a few times each year. Direct microscopic counting yields a quantitative picture of the organisms present but cannot separate between inactive cells, spores, and metabolizing organisms. Data from plate counts probably give a truer indication of metabolically active cells.

### Introduction

Microorganisms play a predominant role in the geocycle of nutrients and energy and in the degradation of environmental pollutants. An understanding of carbon and nitrogen transformations in nature must be based on a knowledge of the bacterial, fungal, and actinomycete populations present. The study of the role of microorganisms in ecosystems and the forthcoming search for life on other planets (10) has been largely responsible for the increased interest in the development and reevaluation of techniques for studying the biomass, ecology, and activity of organisms in the soil (19).

Investigations of the numbers and types of bacteria in natural environments such as soil have been based primarily on plate and microscopic counts. A wide divergence in numbers usually is found between the two techniques (3). The determination of the total population of bacteria, so that the biomass can be calculated, can be accomplished only by a direct counting technique. An applicable method must be rapid, must be reasonably simple to use, and must accurately assess the number of bacteria present. Ideally, it should differentiate the live organisms from the

dead. Microscopic techniques ranging from classical staining techniques (15), fluorescent staining (13), immunofluorescence (5, 17), stereoscan electron microscopy (6, 7), and autoradiography (1) to infrared film photography (2) have been used to date.

The recent development of fluorescent dyes which can be attached either to enzyme substrates or specific protein conjugates, such as isothiocyanates, has made direct fluorescent staining of the biomass feasible. After staining, the fluorescent organisms can be readily differentiated from the non-fluorescent clay particles. The following study reports procedures for the direct observation of bacteria in soil by staining soil smears with fluorescein isothiocyanate (FITC). The effect of season and depth of sampling was determined by the use of both microscopic and dilution plate counts.

### Materials and Methods

#### 1. Methods of Sampling

Soil cores of 6.9-cm diameter were obtained from plots on the Sceptre clay soil at the Matador Field Station of the Canadian Committee of the International Biological Programme (CCIBP). Cores were sectioned into 10-cm lengths and transported in polyethylene bags to the field laboratory where they were immediately composited and subsampled so that five soil samples were used for further analysis.

<sup>1</sup>Saskatchewan Institute of Pedology Publication No. R38.

<sup>2</sup>Canadian IBP No. CCIBP-35.

## 2. Soil Bacterial Suspensions and Soil Smears

Five methods of dispersion were tested: trituration (22), sonification (18, 23), high-speed blender, mechanical shaking, and hand shaking. The method adopted for routine use consisted of placing 20-g soil samples in a Waring Blender containing 190 ml of sterile distilled water and dispersing them at maximum speed for 2 min. Subsamples were removed for either plate counting or direct microscopy.

For microscopic examination, 0.01 ml of the  $10^{-2}$  dilution were removed and spread over a 1-cm<sup>2</sup> area of a microscopic slide. The air-dried smears were then slightly heat fixed.

## 3. Preparation of Staining Solution

The staining solution consisted of 0.25 ml of 0.5 M sodium carbonate buffer (pH 9.6), 1.1 ml of 0.01 M potassium phosphate buffer (pH 7.2), 1.1 ml of 0.85% saline, and 1.0 mg of FITC (Mann Research Laboratories, New York). The solution was mixed at room temperature and used immediately or stored in the dark at 4 C for a maximum of 6 h.

Soil smears were stained for 3 min with FITC solution and then washed in 0.5 M sodium carbonate buffer (pH 9.6) for 10 min and in 5% sodium pyrophosphate for 2 min. The smears were immediately mounted in glycerol (pH 9.6) and observed with a Zeiss microscope equipped with an Osram HBO 200 mercury lamp, exciter filter (BG 12/4 mm), and a barrier filter (No. 50 or Schott OG4).

Rose bengal staining involved placing soil smears in a staining rack containing phenolic rose bengal solution consisting of 1 g rose bengal and 0.03 g anhydrous CaCl<sub>2</sub> in 100 ml of a 5% aqueous solution of phenol. The staining rack and contents were then placed in a hot-water bath (80–90 C) for 1 h. After this they were examined under the microscope using phase contrast objectives.

## 4. Preparation of Soil Extract Agar (SEA) and Plating

One liter of water mixed with 1 kg Sceptre soil was autoclaved for 30 min at 121 C. The mixture was then filtered using glass fiber paper. The extract was diluted to 1 liter and used shortly after preparation. To prepare the solidified medium, 15 g of agar and 0.2 g of K<sub>2</sub>HPO<sub>4</sub> were added to a liter of extract. The pH was adjusted to 6.8. The SEA was dispensed in 30-ml quantities into 25 × 150 mm test tubes and autoclaved. One tube was used for pouring each plate.

The dispersed soil was diluted to the required dilution ( $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  g of soil/ml of water) and 0.1-ml portions spread on the solidified agar with the aid of a glass rod. Five plates were used for each dilution. The plates were incubated for 14 days at 22 C before counting of the bacterial and actinomycete colonies.

## Results

The soil under study was a well-aggregated Dark Brown Chernozemic clay soil. The addition of dispersing agents, such as sodium metaphosphate, has been reported useful in aiding dispersion, especially in well-aggregated soils

(22). Incomplete dispersion occurred if no dispersing agent was added to hand-shaken samples (Fig. 1). Consequently, the number of bacteria was underestimated by the plate count. A slight decrease in numbers at 1% sodium metaphosphate may indicate that this concentration was toxic (4).

Figure 1 indicates that the more drastic methods of dispersion, such as sonification and the blender treatment, were little affected by the addition of a dispersing agent. Considering the ease of operation and reproducibility, the blender (without a dispersing agent) was used for subsequent analyses.

The techniques tested for microscopic examination included a number of stains. The Jones and Mollison technique was not found applicable to this soil. The large amount of clay prevented accurate assessment of the numbers in the relatively deep agar suspension. Phenol aniline blue (8), acridine orange (20), and erythrosin (21) did not adequately differentiate the microorganisms from the soil particles and debris.

Esterified dyes, such as fluorescein diacetate or dibutylate, theoretically should be very useful for the direct microscopic examination of bacteria. The stain does not fluoresce unless an active esterase has removed the side groups, such as the acetate or butyrate. Using this stain it should be possible to differentiate between metabolizing and non-metabolizing organisms. The polar, fluorescein acetate is said to enter the cell. Within the cell the esterase removes the side chain resulting in fluorescence. Since the fluorescein is non-polar, concentration takes place with the result of increased fluorescence of the organisms (16).

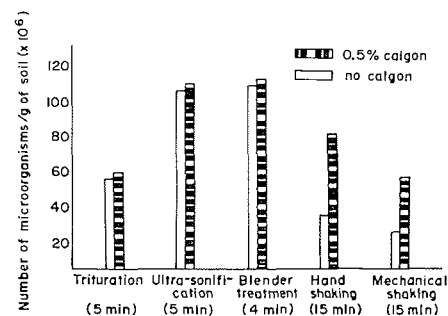


FIG. 1. Comparison of different methods of dispersion with and without a dispersing agent.

It was found that bacterial cells more than 4 h old were relatively impermeable to the fluorescent stain. Permeability increasing agents, such as trypsin and toluene, did not prove successful enough during this investigation to allow this technique to be used on a routine basis.

Staining of the soil smears with rose bengal (15) gave recovery of 61% of the added *Pseudomonas fluorescens* and 81% of the *Bacillus subtilis* (Table I). The low recovery of the small

organisms, such as *Pseudomonas fluorescens*, indicated that some of the organisms, even smaller than *Pseudomonas*, normally found in soil would probably be overlooked. The results obtained in the field by this technique during 1967 are shown in Table II.

Fluorescein isothiocyanate (FITC) conjugates with the proteins present on the exterior surface of microbial cells. The excess stain can be washed off and extraneous fluorescence quenched with

TABLE I

The recovery of microorganisms, added to a soil suspension (Sceptre), using rose bengal as a stain

Type of organism	Number of bacteria per gram of soil			Recovery, %
	Original 10 <sup>9</sup>	Added 10 <sup>9</sup>	Observed 10 <sup>9</sup>	
<i>B. subtilis</i>	1.4	1.9	2.6	81
<i>P. fluorescens</i>	1.9	1.7	2.2	61

TABLE II

Direct count of bacteria in Sceptre clay soil, 1967

Ecosystem	Date	Number of organisms per gram of soil			
		0-15 cm		75-90 cm	
		Mean 10 <sup>9</sup>	S.D. 10 <sup>9</sup>	Mean 10 <sup>9</sup>	S.D. 10 <sup>9</sup>
Virgin	August 11	2.1	0.2	—	—
	September 27	2.0	0.2	1.2	0.2
Cultivated	August 11	2.1	0.3	—	—
	September 27	2.0	0.3	1.2	0.2

TABLE III

The recovery of bacteria, added to a soil suspension, using fluorescein isothiocyanate as a stain

Soil type and texture	Organism added	Number of bacteria per gram of soil			Recovered, %
		Original 10 <sup>9</sup>	Added 10 <sup>9</sup>	Observed 10 <sup>9</sup>	
Sceptre HvC	<i>B. subtilis</i>	3.83	2.13	5.78	96.9
Sceptre HvC	<i>A. aerogenes</i>	3.83	2.22	5.98	98.8
Sceptre HvC	<i>P. fluorescens</i>	3.83	2.57	6.38	99.7
Sutherland C	<i>B. subtilis</i>	3.44	1.97	5.35	98.8
Sutherland C	<i>A. aerogenes</i>	3.44	2.05	5.56	101.3
Sutherland C	<i>P. fluorescens</i>	3.44	2.37	5.77	99.3
Bradwell L	<i>B. subtilis</i>	2.51	1.90	4.36	98.8
Bradwell L	<i>A. aerogenes</i>	2.51	1.97	4.46	99.5
Bradwell L	<i>P. fluorescens</i>	2.51	2.28	4.59	95.8
Asquith LS	<i>B. subtilis</i>	1.96	2.10	4.00	98.3
Asquith LS	<i>A. aerogenes</i>	1.96	1.97	3.71	94.5
Asquith LS	<i>P. fluorescens</i>	1.96	2.35	4.24	98.4

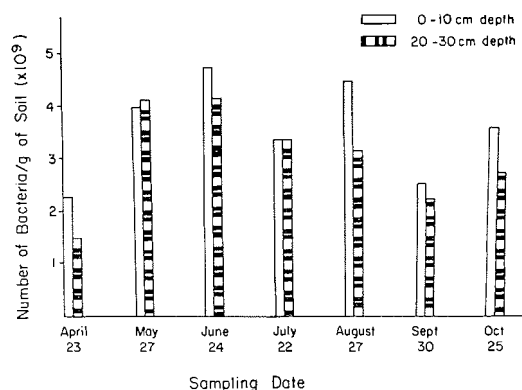


FIG. 2. The change of bacterial numbers, as determined by direct microscopic counting using fluorescein isothiocyanate as a stain, in a virgin Sceptre soil at various sampling dates in 1968.

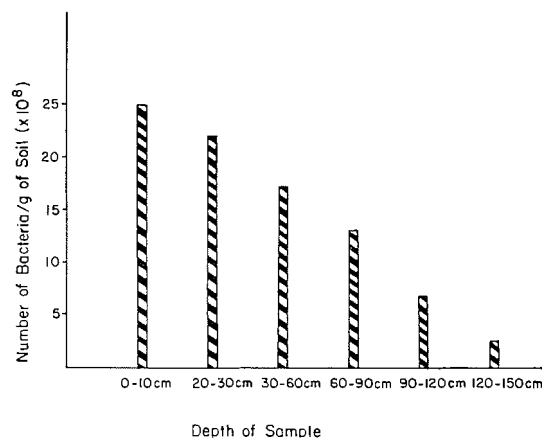


FIG. 3. Bacterial numbers at various depths as determined by direct microscopic counting.

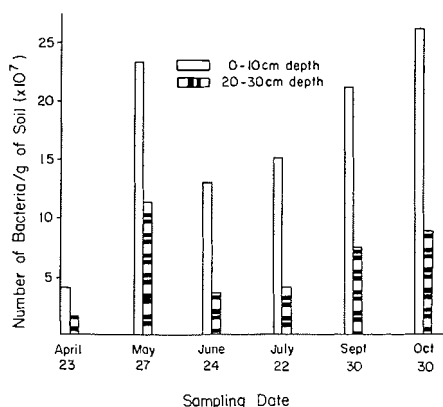


FIG. 4. The change of bacterial numbers, as determined by dilution plate method (SEA), in a virgin Sceptre soil at various sampling dates during 1968.

sodium pyrophosphate. Table III indicates that the FITC method accurately measured the number of bacterial cells in soils having clay contents ranging from 20 to 80%. With this stain, quantitative recovery of both large organisms (*Bacillus subtilis*) and small organisms (*Pseudomonas fluorescens*) was achieved. The FITC technique also was applied to buried slides. Bacterial cells, fungal mycelia, and spores, as well as filamentous actinomycetes, were readily visible.

The range in microbial numbers obtained upon monthly samplings and direct microscopic counts of the soil samples is shown in Fig. 2. The counts rose between April and May 1968 and then stayed relatively constant. The linear relationship between the number of organisms measured by microscopic counting and depth is shown in Fig. 3.

The number of organisms as estimated by the FITC direct method count in 1968 (Fig. 2) was considerably higher than the 1967 figures obtained using the rose bengal stain (Table II). However, the rose bengal method (Table I) counts only 60-80% of the bacteria in the soil, indicating that the actual number of bacteria present was similar in the 2 years.

Plate counts, on soil extract agar (Fig. 4), of the organisms present in the field fluctuated more throughout the year than did the direct count. The major differences between the two methods are a much higher number of organisms obtained by the direct method and the fact that the microscopic technique did not indicate as large a change in numbers of organisms with depth. Analysis of the data relative to depth and date of sampling indicated that there was no correlation between the two methods.

Using the direct count data only, a correlation coefficient of 0.83 was obtained between the 0- to 10- and the 20- to 30-cm depth. A correlation of 0.77 was obtained for microbial numbers in the two depths when the numbers throughout the summer were measured by the dilution plate. These highly significant correlations indicate that although the values obtained by the two methods are not related, the population changes measured by each method occur simultaneously at the different depths.

The relative number of actinomycetes developing on the plates increased from 20% of the total bacterial counts at the 0- to 30-cm level to

TABLE IV  
Biomass of bacteria and actinomycetes in a Sceptre virgin soil, dry wt, g/m<sup>2</sup>

Sampling depth, cm	Sampling time (1968)						
	April	May	June	July	Aug.	Sept.	Oct.
0-10	12.3	23.4	27.2	19.4	25.9	14.7	20.6
0-30	31.9	70.9	76.2	38.2	67.0	42.3	54.7

70% at the 90-120 cm depth. Since the streptomycete spores could not be differentiated from the normal bacterial cells by the microscopic method, a large proportion of the material counted at depth could be resting spores of actinomycetes. However, Kuster (9) has stated that the actinomycetes occurring at depth are present primarily as sterile mycelia. The use of the Waring Blendor to disperse the soil may break up the mycelia into fragments, which would then be counted as bacteria.

#### Discussion

The bacteria, the actinomycete hyphal bits, and spores counted by the FITC technique averaged  $0.6 \times 1 \mu$  in size. Using published figures (12) for the moisture content and density, it is possible to calculate the biomass (on a dry weight basis) of the total bacterial population (Table IV).

Soil may be looked upon as a continuous culture wherein substrate availability is the controlling factor during the summer. Microorganisms can live and reproduce only if available substrates are present. About 500 g of substrate per square meter per year was available to the microorganisms, considering aboveground parts, roots, and exudates at the Matador during 1968.\*

Microorganisms require a specific maintenance energy to replace unstable cell constituents (11). This energy can be described by the following formula:

$$dx/dt + ax = y(ds/dt)$$

where  $a$  = specific maintenance  $h^{-1}$ ,

$x$  = concentration of cells in grams,

$y$  = yield coefficient, grams of bacteria/gram of substrate,

$s$  = substrate for maintenance in grams.

Extrapolating from growth and nutrient concentration curves (14), an  $a$  value of  $0.001 h^{-1}$

seems a reasonable choice for our studies. Using this figure with the above formula and direct-count estimates for the number of bacteria, it was found that the microorganisms could reproduce only a few times a year since over half of the available energy is required for cell maintenance.

The large weight of bacterial material indicates that most of the bacteria in the soil at any one time must be in an inactive state. This is especially true if the biomass of bacteria present in the deeper depths and the other soil organisms are taken into account (3).

The direct count is the only way of determining total bacterial biomass. Most of the organisms must, however, be in a resting state at any one time. Calculations of active biomass on the basis of the plate count, which was only  $\frac{1}{15}$  of that of the direct, results in an estimated division rate of bacteria much closer to that observed under laboratory conditions. Although direct microscopy must be used to estimate the total biomass, the plate count is probably a better estimate of metabolizing cells in the soil.

#### Acknowledgment

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1. BROCK, T. D., and L. M. BROCK. 1966. Autoradiography as a tool in microbial ecology. *Nature*, **209**: 734.
2. CASIDA, L. E. 1968. Infrared color photography: selective demonstration of bacteria. *Science*, **159**: 199.
3. CLARK, F. E. 1967. Bacteria in soil. In *Soil biology*. Edited by A. Burges and F. Raw. Academic Press, New York, pp. 15-49.
4. GOSSLING, B. S. 1958. The loss of viability of bacteria in suspension due to changing the ionic environment. *J. Appl. Bacteriol.* **21**: 220-226.
5. EREN, J., and D. PRAMER. 1966. Application of immunofluorescent staining to studies of the ecology of soil microorganisms. *Soil Sci.* **101**: 39-45.

\*Second Annual Matador Report, Saskatoon.

6. GRAY, T. R. G. 1967. Stereoscan electron microscopy of soil microorganisms. *Science*, **155**: 1668-1670.
7. HAGEN, C. A., E. J. HAWRYLEWICZ, B. T. ANDERSON, V. K. TOLKACZ, and M. L. CEPHUS. 1968. Use of the scanning electron microscope for viewing bacteria in soil. *Appl. Microbiol.* **16**: 932-934.
8. JONES, P. C. T., and J. E. MOLLISON. 1948. A technique for the quantitative estimation of soil microorganisms. *J. Gen. Microbiol.* **2**: 54-69.
9. KUSTER, E. 1967. The actinomycetes. In *Soil biology*. Edited by A. Burges and F. Raw. Academic Press, New York. pp. 111-127.
10. LEVIN, G. V. 1968. Experiments and instrumentation for extraterrestrial life detection. In *Advances in applied microbiology*. Edited by W. W. Umbreit and D. Perlman. Academic Press, New York. pp. 55-71.
11. MARR, A. G., E. H. NILSON, and D. J. CLARK. 1963. Maintenance requirements of *Escherichia coli*. *Ann. N.Y. Acad. Sci.* **102**: 536-548.
12. OGINSKY, E. L., and W. W. UMBREIT. 1959. Introduction to bacterial physiology. W. E. Freeman Co., San Francisco.
13. PITAL, A., S. L. JANOWITZ, C. E. HUDAK, and E. E. LEWIS. 1966. Direct fluorescent labeling of microorganisms as a possible life-detecting technique. *Appl. Microbiol.* **14**: 119-123.
14. PIRT, S. J. 1963. The maintenance energy of bacteria growing in cultures. *Proc. Royal Soc. London, Series B*, **163**: 224-231.
15. PRAMER, D., and E. L. SCHMIDT. 1967. Experimental soil microbiology. Burgess Publishing Co., Minneapolis.
16. ROTMAN, B., and B. W. PAPERMASTER. 1966. Membrane properties of living mammalian cells as studied by enzyme hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. U.S.* **55**: 134-141.
17. SCHMIDT, E. L., R. O. BANKOLE, and B. B. BOHLOOL. 1968. Fluorescent antibody approach to study of rhizobia in soil. *J. Bacteriol.* **95**: 1987-1992.
18. STEVENSON, I. L. 1959. The effect of sonic vibrations on the bacterial plate count of soil. *Plant and Soil*, **10**: 1-8.
19. STOTZKY, G. 1968. Relevance of soil microbiology to search for life on other planets. In *Advances in applied microbiology*. Edited by W. W. Umbreit and D. Perlman. Academic Press, New York. pp. 17-54.
20. STRUGGER, S. 1948. Fluorescent microscope examination of bacteria in soil. *Can. J. Res.* **26**: 188-193.
21. TCHAN, Y. T., and J. S. BUNT. 1954. Direct microscopy for study and counting of soil protozoa. *Nature*, **174**: 656.
22. ZVYAGINTSEV, D. G. 1966. Influence of soil dispersion and desorption of microorganisms on their quantitative estimate by the plate culture method. *Soviet Soil Sci.* 811-818.
23. ZVYAGINTSEV, D. G., and G. M. GALKINA. 1967. Ultrasonic treatment as a method of preparation of soil for microbiological analysis. *Microbiologia*, **36**: 910-916.