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Microscopic Counting and Adenosine 5'-Triphosphate Measurement in Determining Microbial Growth in Soils

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A microscopic technique utilizing dispersion of fungal hyphae in a Waring blender, filtration through membrane filters (Nucleopore Corp.), and counting on a fluorescence microscope was developed for counting fungal hyphal biomass. Nonfluorescent staining techniques of the soil-filter preparation did not give quantitative recoveries. Water-soluble aniline blue, which binds to the β-1,3-glucans of the fungal cell wall, made visualization of the hyphae by fluorescence possible. A range of fungi added to soil were quantitatively recovered. Adenosine 5'-triphosphate (ATP) was extracted from soil by lysis of the organisms with $CHCl_3$ in $NaHCO_3$, which prevented adsorption of the organic phosphorus to the soil colloids. Centrifugation and removal of $CHCl_3$ was followed by dilution with pH 7.8 tris(hydroxymethyl)aminomethane buffer. ATP concentrations were measured by using the luciferase-luciferin light reaction. Since $NaHCO_3$ interfered to some extent with this reaction, the standards were made up in equivalent mixtures of tris(hydroxymethyl)aminomethane buffer and $NaHCO_3$. Recovery of ATP was rapid and quantitative in a range of soils. Measurement of the ATP and bacterial and fungal numbers in an incubated soil showed that fungal and bacterial population increases were delayed by phosphorus deficiency. Microbial populations were not affected at a later date. The ATP content of the soil system was reduced by phosphorus deficiency throughout the incubation period. This indicated that ATP could be altered without major changes in the microbial populations.

In soil, microbial enzymes are the agents of nutrient transformations, and the living biomass is a repository of nutrient elements. Yet soil microbial activity has traditionally been measured separately from microbial biomass, and their interrelationship has not been accurately defined. However, the interdependence of enzyme activity and microbial growth means that procedures used to quantify rates of nutrient transformations must be used concurrently rather than individually. The most useful approach for studying microbial growth and nutrient turnover by soil microorganisms would appear to include simultaneous measurements of microbial biomass, adenosine 5'-triphosphate (ATP) content, respiration, and specific enzyme activity with time in soil after treatments, including both energy and mineral nutrient restrictions.

Available microscopic techniques used for biomass include the Jones and Mollison (15) agar slide technique with either phenol aniline blue (14) or phase-contrast microscopy (11, 25). Fluorescence which can separate the microorganisms from the soil clay-mineral and organic complexes has been used with acridine orange (27, 28), primulan (6), fluorescein isothiocyanate (FITC) (2, 10), and 1-anilino-8-naphthalene sulfonic acid (21).

Determination of biomass in soil or sediment by extraction of ATP has been suggested using H_2SO_4 (1, 17), butanol-octanol (26), and boiling $NaHCO_3$ (3).

In this study, methods for estimating fungal hyphae and soil ATP content were tested. For microscopic work, preference was given to fluorescence because of its ability to distinguish between organisms and the clay-soil matrix. This report shows the development of a fluorescent-filtration technique for fungal hyphae. It also includes methodology for an ATP assay based on the use of $CHCl_3$ to lyse cells and $NaHCO_3$ as an extracting agent.

MATERIALS AND METHODS

Soils. A number of soils having specific characteristics that could interfere with ATP analysis were tested. Growth studies were conducted with a Bradwell fine sandy loam, sampled from a fallow site and air dried before sieving (0.5 mm).

Microbial counts. The method of Babiuk and Paul (2) was used for bacterial smear preparation,

FITC staining, and counting on a fluorescence microscope, except that the soil smear was divided into an outside edge representing 5% of the area and an inside area representing 95%. Five fields per smear were counted in the outside area; 15 were counted from the inner area. Four smears were counted per treatment.

Fungal counts. A weighed sample of soil (5 g) was suspended in water (500 ml). This suspension was blended for 5 min at high speed in a Waring blender. Portions (0.2 ml) of the soil-water suspension were placed in test tubes containing 10 ml of prefiltered (0.4 μm) water. The tubes were shaken by hand, and the material was filtered through a 25-mm Nucleopore filter (Nuclepore Corp., Pleasanton, Calif.) with a pore size of 0.4 μm.

Fungal recovery. Cultures of fungi were blended at high speed for 3 min. The blended cultures (5 ml) were transferred to a container with 5 g of soil. Four replicates as well as fungal and soil controls were used (12). Twenty fields were observed on four filters per replicate. Water-soluble aniline blue (BDH Chemicals, Poole, England) (0.1% [wt/vol] in 0.2 M K_2HPO_4, pH 8.0) was filtered to remove undissolved dye crystals. Fungal filters were stained for 30 min on glass slides. Filters were air dried and dry mounted on glass slides under large cover slips, using fingernail polish for fastening. Counts were made within 1 day after staining, although adequate fluorescence could be observed for some time after staining.

Acridine orange and phenolic aniline blue stain preparation followed established procedures (23). Staining with 1-anilino-8-naphthalene sulfonic acid used the technique published by Mayfield (21).

Fluorescent material stained with water-soluble aniline blue was observed with a Carl Zeiss universal microscope equipped with an HBO 200 burner, BG12/4-mm exciter, and no. 50 barrier filter. Fungi stained with phenolic aniline blue were observed under bright-field illumination. Fungal hyphal lengths were measured by using an eyepiece grid.

ATP extraction. H_2SO_4 extraction (17) and boiling $NaHCO_3$ were tested. The technique finally adopted for the extraction of ATP from soils and microbial cultures is outlined in Fig. 1. $NaHCO_3$ was found to have an inhibiting effect on the light-emitting reaction. It was necessary to dilute the extract as much as possible and to assay both the standards and the soil extracts in mixtures containing the same amounts of tris(hydroxymethyl)aminomethane (Tris) and $NaHCO_3$. Final ATP concentrations were reported, taking into account a 150:1 dilution per g of wet soil and moisture content of the soil.

RESULTS

Statistical accuracy in microscopic counting depends on the number of units counted per field. The membrane filter technique used for direct counts of fungi hyphae (12) provided the needed concentration of soil and fungal hyphae. Phenolic aniline blue, Millipore filters (Millipore Corp., Bedford, Mass.), and bright-field illumination were first tested in estimations of the fungal hyphae of several soils. There was masking of hyphae by the soil particles. Dilution to lower the amount of occluding soil reduced the lengths of hyphae per field so that large numbers of replicates were necessary. Fluorescent stains specific to the organic com-

5 g of soil, 50 ml of 0.5 N $NaHCO_3$, pH 8.5, 15 ml of $CHCl_3$, blend in Waring blender for 1 min at high speed, add 75 ml of 0.5 N $NaHCO_3$, pH 8.5,, blend for 1 min at high speed.

Transfer 15 ml to centrifuge tube, centrifuge (3,500 $\times$ g for 4 min at 2°C).

Transfer 5 ml of aqueous solution (without disturbing $CHCl_3$ or soil) to Erlenmeyer flask (50 ml).

Remove all traces of $CHCl_3$; connect Erlenmeyer flasks to vacuum (water pump) and shake in water bath (60°C, 1 to 5 min) until large $CHCl_3$ bubbles are pulled off (aqueous $NaHCO_3$-ATP solution bubbles are smaller).

After evacuation, cover the flasks with parafilm and store in a freezer (−40°C).

For analysis, thaw and add 0.1 N Tris buffer (pH 7.8) until mixture equals 30 ml.

Add 250 ml of water to luciferase from firefly tails (50 mg/vial), shake, leave in dark for 24 h, centrifuge (15 min, 10,000 $\times$ g), keep supernatant cool (ice) until assay.

Dilute concentrated ATP standards (10 μg/ml) to at least 6 concentrations (range, 0.002 to 0.100 μg of ATP/ml) with $NaHCO_3$ (0.5 N, pH 8.5) and Tris (0.5 N, pH 7.8). Diluted standards should contain the same ratio of $NaHCO_3$-Tris as the extracting solution.

Add ATP solution (0.2 ml) to 0.4 ml of enzyme supernatant in a glass scintillation vial. Place vial in a JRB model II photometer which integrates the light emitted over a 1-min time interval after a 15-s delay. Subtract controls (solution of $NaHCO_3$-Tris, without ATP) from each value to establish the standard curve.

FIG. 1. *ATP extraction and assay.*

ponent under investigation will delineate hyphae. Several fluorescent stains were therefore tested. Water-soluble aniline blue specific to the polymer β-1,3-glucan (7) found in fungal cell walls (4) has been used to differentiate callose tissue (9).

Millipore filters, made from cellulose, bound water-soluble aniline blue and showed autofluorescence. Nucleopore filters made from polycarbonate have a uniform surface and limited autofluorescence. These were subsequently used throughout the experiment. A comparison of water-soluble aniline blue fluorescence and phenolic aniline blue with bright-field illumination (Table 1) gave higher hyphal length measurements for the fluorescent technique on the three soils tested. The β-1,3-glucans to which the dye binds have been reported absent from cell walls of some phycomycetes. A qualitative study of 18 species of soil fungi included: *Fusarium, Trichoderma, Aspergillus, Phycomyces, Helminthosporium, Cladosporium, Epicoccum, Penicillium, Stachybotrys, Phoma, Paecilomyces, Absidia, Sordaria*, and sterile dark forms. These showed no obvious difference in staining of 2- and 60-day cultures grown in nutrient broth. Although many of the senescent cultures had formed heavily pigmented cell walls, aniline blue gave adequate fluorescence.

Under our conditions, acridine orange staining of older hyphal fragments resulted in poor fluorescence. The stain also bound to clay, creating background interference. The stain 1-anilino-8-napthalene sulfonic acid, said by Mayfield (21) to react with proteins but not soil particles, was adsorbed to the filter and clays. It was found to be a good bacterial stain in light-textured soils. FITC, the stain with which we have had a great deal of experience, also is adsorbed to soil particles and requires careful washing procedures. These were not tested on the sulfonic acid stain. FITC has in past work been shown to be satisfactory for bacterial smears. However, we found that this protein stain does not stain all fungal hyphae.

TABLE 1. *Measurement of hyphal lengths with phenolic aniline blue under normal light and water-soluble aniline blue under fluorescence*

Soil	Texture	Hyphal length (m/g)	
		Phenolic aniline blue	Water-soluble aniline blue
Bradwell	Sandy loam	238	348
Sutherland	Clay	291	430
Oxbow	Sandy loam	440	606

To quantify recovery efficiency, six fungal species were added to separate soils (Table 2). All additions including *Phycomyces delbrückii* were recoverable. The latter organism did not show as bright a fluorescence as the others. The heavily pigmented walls of *Helminthosporium sativum* and *Cladosporium sphaerospernum* did not affect their fluorescence. Testing the length of time after which added fungi could be isolated from the soil did not show any difference for 1.5 h, after which time there was an increase in total hyphal lengths attributed to growth (data not shown).

ATP extraction from soil. The literature indicates that present techniques for ATP extraction are best adapted to either acid soil (1), saline sediments (3, 18), or coarse-textured soil (26). A test designed for routine analyses also should not have unwieldy aspects. Exchange resins and volatile extractants requiring fume hoods or charcoal elution were considered to limit the usefulness of an extraction technique.

Boiling, 0.1 N $NaHCO_3$ (Table 3) resulted in recovery of 50 to 70% of the ATP added as microbial cells. Experimentation indicated that the variability in ATP extraction was due to incomplete lysis of the microfloral components of soils. Superheating the extract by adding ethylene glycol to the heating bath and heating the tubes under slight pressure did not improve recovery efficiency, nor did chelating agents or higher concentrations of $NaHCO_3$.

Chloroform has been used successfully as a lysing agent of the soil biomass (13) and to lyse

TABLE 2. *Recovery efficiency of added fungal mycelia with Nuclepore filters and water-soluble aniline blue fluorescence*

Fungus	Soil association	Hyphal length (m/g)			Recovery (%)
		Added	Soil	Total	
Helminthosporium sativum	Bradwell	271	274	521	91
Fusarium oxysporum	Bradwell	310	274	582	100
Aspergillus sydowi	Bradwell	919	303	1,286	104
Trichoderma viride	Sutherland	580	432	1,038	104
Phycomyces delbrücki	Sutherland	468	407	913	108
Cladosporium sphaerospernum	Sutherland	428	403	806	94

TABLE 3. *ATP extraction from soil with boiling $NaHCO_3$*

Soil	ATP[a] (μg/g of soil)			Recovery (%)
	Soil	Added	Recovered	
Oxbow	1.50	3.95	3.45	49
Sutherland ..	1.12	1.75	2.15	59
Bradwell	0.84	1.64	1.97	69

[a] ATP was added as whole cells of *Enterobacter cloacae*.

cultures for ATP assay (8). $NaHCO_3$ was therefore tested in conjunction with $CHCl_3$ as an ATP extractant. Figure 2 shows the effect of varying amounts of $CHCl_3$ on recovery of added ATP. Each extraction was done with 5 g of soil and 50 ml of cold 0.1 N $NaHCO_3$. There was an increase in recovery percentage until $CHCl_3$ represented one-third of the extracting solution.

Because $NaHCO_3$ has an inhibitory effect on the luciferase enzyme reaction, its replacement with a less inhibitory buffer would ensure higher assay sensitivity. However, Tris buffer in combination with $CHCl_3$ or Tris-bicarbonate produced by neutralizing Tris with CO_2 did not have the extracting efficiency of $NaHCO_3$.

The technique used for further study involved pretreatments of each gram of soil with 10 ml of $NaHCO_3$, lysis with 3 ml of $CHCl_3$, and then addition of a further $NaHCO_3$ solution equivalent to 15 ml/g. After centrifugation, the $NaHCO_3$-water and $CHCl_3$ showed clearly defined phase separation, with the ATP being present in the aqueous layer.

The soils in Table 4 represent a cross-section of soils known to have characteristics that could limit extraction efficiency. Texture had little effect on extraction. Thus, these differ from those for H_2SO_4 extraction, where clay contents had a strong influence (1). Organic matter, pH, and the free-aluminum content did not have a great effect at 0.5 N $NaHCO_3$. The luciferin-luciferase system used to measure ATP contents in soil has been found to yield a standard error of 5 to 10% if four replicates are used. Recovery values for the Weyburn and Haverhill soils were low when 0.1 N $NaHCO_3$ was used. The specific problem in these soils at this concentration is not known, but using 0.5 N $NaHCO_3$ overcame the problem.

Microbial biomass and ATP measurement in an incubated soil. The usefulness of the developed experimental techniques in measuring microbial growth in soil was determined in an incubation experiment. In soils amended with glucose, the ATP content increased rapidly after a lag time of 6 h (Fig. 3). Amendment with phosphorus plus glucose gave rise after 30 h to ATP levels 30% higher than with glucose alone. The phosphorus-amended soil also maintained its ATP level at a higher concentration for 12 days. The variation between soil replicates was greater than that found for the ATP assay alone and was attributed to variations in microbial growth as well as assay error.

Bacteria increased rapidly (Fig. 4) after a 6-h lag time, with similar patterns being observed in the presence of glucose with or without added phosphorus, except that added phosphorus resulted in earlier growth of the microorganisms. Ladd and Paul (16), using the same soil (Bradwell) with similar treatments, found that 96% of the added glucose had been utilized by 18 h. At this time in our experiments, bacterial populations were peaking. The changes in bacterial numbers, determined microscopically over 12 days, were approximately the same for both treatments if both primary and secondary populations were considered. Total numbers increased between three and four times the original population (from 1.5×10^9 to 5×10^9 bacteria/g of soil).

Behara and Wagner (5) found that the bacterial population of a glucose-amended soil increased more quickly than did the fungi. McGill et al. (20) found that after addition of acetate, the fungal population increased more rapidly, whereas a field study with glucose showed simultaneous growth of both populations (24, 25). In this study with added glucose and phosphorus, the fungi (Fig. 5) were delayed, peaking at approximately 48 h. This was halfway between the two peaks noted for the bacteria.

Amendment of soil with glucose, nitrogen, and phosphorus doubled the fungal population. Soil receiving all three amendments showed a faster increase in population and also a faster decline during the latter incubation period.

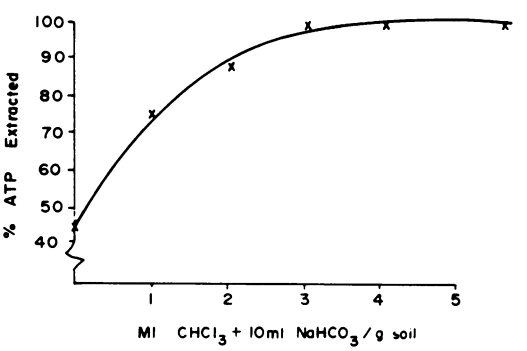

FIG. 2. *Effect of $CHCl_3$ concentration on ATP extraction in a Sutherland soil.*

TABLE 4. *Recovery of ATP[a] using two concentrations of $NaHCO_3$ from soils varying in texture, $CaCO_3$, Al, and organic matter*

Soil association	Characteristics	$NaHCO_3$ concn (N)	ATP content (μg/g of wet soil)			Recovery (%)
			Soil	Added	Total	
Sutherland	Clay	0.5	1.74	4.41	5.63	88
		0.1	1.89	3.40	5.15	96
Haverhill	Loam	0.5	1.80	1.23	3.13	100
		0.1	1.50	1.40	2.00	36
Oxbow	Loam	0.5	4.73	4.07	8.38	90
		0.1	4.55	3.95	8.30	95
Bradwell	Sandy loam	0.5	1.61	2.12	3.71	100
		0.1	1.60	1.80	3.55	105
Weyburn	Loam, 12% $CaCO_3$	0.5	0.77	0.68	1.42	104
		0.1	0.65	0.65	0.80	23
Blanket bog	20% organic matter, pH 6.7	0.5	1.16	1.62	2.38	75
		0.1	1.24	1.40	2.35	79
Sphagnum peat	Organic soil, pH 3.8	0.5	1.13	1.76	2.97	105
		0.1	1.22	1.45	2.75	105
Greensboro	pH 4.6, 2.6 μg/g, Al	0.5	0.11	0.38	0.54	113
		0.1	0.09	0.35	0.47	108
Saint Andre	pH 4.1, 7.0 μg/g, Al	0.5	0.31	0.43	0.96	151
		0.1	0.38	0.36	0.82	122

[a] ATP was added as whole cells of *Arthrobacter globiformis*, *Enterobacter cloacae*, or the hyphae of *Fusarium oxysporium*.

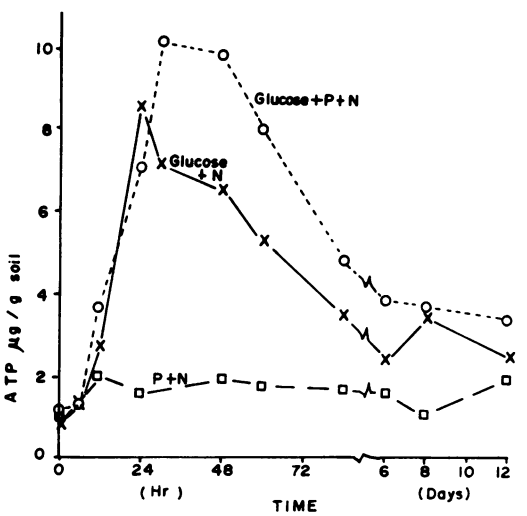

FIG. 3. *Changes in ATP levels of amended soil over a 12-day incubation period. Standard error of replicate means = 12% of mean.*

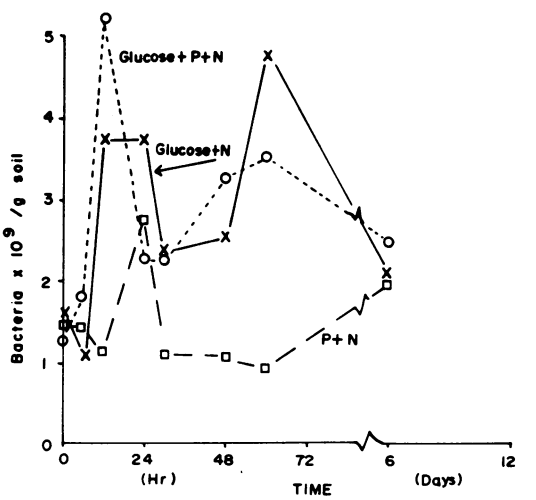

FIG. 4. *Changes in bacterial numbers of an amended Bradwell soil over a 12-day incubation period. Standard error for replicate means averages 9.5% of mean.*

DISCUSSION

The progress of microbial ecology is often hindered more by a lack of usable techniques than by concepts. This is now the case in soil microbial growth investigations. The Jones and Mollison (15) technique as modified by Nicholas and Parkinson (22) has proven a valuable if

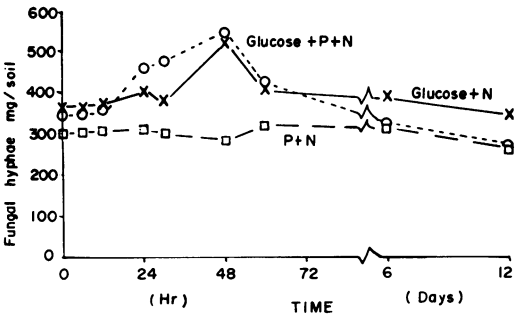

FIG. 5. *Changes in fungal hyphal lengths of an amended Bradwell soil over a 12-day incubation period.*

time-consuming tool in studies in soil biomass in the laboratory (14) and under field conditions (25). The major reason for preferring the water-soluble aniline blue technique outlined in this paper is that the use of low-fluorescence Nuclepore filters to concentrate fungi in conjunction with fluorescence has made it possible to count fungal hyphae at a speed that should enable its use in more microbiological studies.

The $NaHCO_3$ extraction technique depends on $CHCl_3$ to lyse microbial cells. In pure-culture studies, perchloric acid has been found to be slightly more efficient than chloroform (19). However, in a complex system such as soil, acidic extracting agents are susceptible to a large number of side reactions. They are neutralized by carbonates, react to some extent with organic matter, and coextract large concentrations of interfering sesquioxides. The removal of sesquioxides is laborious and results in lower recoveries. The recent use of charcoal to separate ATP from the acid and interfering agents and then elution of the ATP from charcoal (R. E. Hodson et al., personal communication) appears promising. It was not tested in our laboratory.

Probably no one technique will prove superior for all sediment-soil systems under investigation. The use of $CHCl_3$ to lyse the cells and $NaHCO_3$ to flood the soil colloidal system with an ion that interferes with the adsorption of ATP has been shown to be a fast and accurate method applicable to measurements of microbial growth in the soil system.

The addition of glucose, phosphorus, and nitrogen, followed by incubation, stimulated the bacterial population by a factor of 4 to 5 and stimulated the fungal hyphae twofold. Since there are more fungi than bacteria in the soil, overall biomass increase for the two organisms would be similar. The presence of phosphorus increased the growth rates of the populations during the initial stages after the lag phase, but did not greatly affect the microbial counts. Regression analyses of ATP versus microbial populations indicated no significant interactions in the N + P treatment alone. The addition of 1.5 mg of C and N + P yield a correlation coefficient of 0.67 (0.05% level of significance) for bacteria and fungi combined. Treatment with 3 mg of glucose-carbon resulted in a correlation coefficient of 0.81 (0.05% level of significance) for bacteria plus fungi in the presence of P + N. Without P there were no significant interactions. The presence of added P resulted in a 30% higher ATP content, which persisted throughout the experiment, indicating that the ATP level of the soil population was very sensitive to phosphorus levels.

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