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CRITERIA FOR MEASUREMENT OF MICROBIAL GROWTH AND ACTIVITY IN SOIL

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Summary—Changes in CO_2 evolution, phosphatase and urease activity and ATP contents were related to bacterial and fungal biomass determined microscopically during glucose mineralization at different concentrations of mineral nutrients. Similar results were obtained in a sandy loam and a clay soil except that in the clay the increase in microbial and enzyme activities were delayed. Higher initial rates of CO_2 evolution were noted after the addition of P to a glucose and N amended soil at C:P ratios greater than 30:1. Increases in phosphatase activity coincided with increases in bacterial and fungal populations only in treatments without inorganic P. Peak rates of CO_2 evolution preceded biomass production by 18–24 h, therefore, CO_2 evolution rates did not show a correlation on normal regression analysis with biomass. Soil ATP content was influenced by P concentrations and soil type. ATP was therefore not a specific indicator of biomass in the detailed studies where P concentrations and sequential growth of bacteria and fungi were major factors. Soil urease increased with bacterial and fungal populations. It did not respond to P other than through microbial biomass and was highly correlated with microbial biomass.

The results show that no one measurement of microbial biomass or activity is sufficient to interpret microbial growth in the soil system. Each of the criteria measured were sensitive to specific conditions affecting biomass and activity.

INTRODUCTION

Procedures to measure rates of nutrient transformations in complex systems such as soil require an understanding of microbial biomass, activity and enzyme production. Microbial biomass can be determined by microscopic observation of bacterial (Nikitin, 1973) and fungal populations (Parkinson, 1973). Respiration measurements and enzyme contents have been determined in many soils both in the field and the laboratory, but correlations between activity measurements are usually low (Ross, 1973). Recently, ATP which is a common molecule for energy packaging and transport has been suggested both as a direct index of biomass (Lee *et al.*, 1971; B. S. Ausmus, unpublished Ph.D. Thesis, University of Tennessee, 1973; Ausmus, 1973) and as a criterion for evaluation of microbial activity (Greaves *et al.*, 1973). The availability of microscopic counting techniques and methods for soil ATP content as well as urease and phosphatase activity made possible a detailed analyses of these biomass and growth activities in a laboratory soil system.

Laboratory investigations of microbial biomass and activity following the addition of easily degradable substrate have simplified conditions such that mathematical descriptions of the relationship between microbial growth and soil C and N turnover are possible (McGill *et al.*, 1973; Wagner, 1975). In this study experiments were conducted under laboratory conditions to relate CO_2 evolution, urease, phosphatase and ATP to microbial biomass.

MATERIALS AND METHODS

Soils

Two grassland Dark Brown Chernozemic soils with neutral pH and 2% organic C, but different textures, were used. Bradwell is a fine sandy loam, while Sutherland is a clay. Both soils contain 10 μg $NaHCO_3$-extractable inorganic P g^{-1} soil. Bradwell contained 15.6 μg and Sutherland 10.4 μg of $NaHCO_3$-extractable organic P g^{-1}.

Soil incubation experiments

Two series of experiments were run. In series I, Bradwell fine sandy loam was subjected to the following treatments (g^{-1} dry soil);

(1) control with 0.17 mg $NaNO_3$-N and 0.05 mg KH_2PO_4-P
(2) 1.5 mg glucose-C, 0.17 mg N and 0.05 mg P
(3) 3 mg glucose-C, 0.17 mg N and 0.05 mg P
(4) 3 mg glucose-C., 0.17 mg N

Duplicate treatments consisting of 500 g soil were sampled at intervals by the removal of 25 g moist soil. A portion was used for enzyme assay and ATP content. The remaining subsample was frozen until analysed for bacterial and fungal biomass.

In series II, the Bradwell and Sutherland soils (250 g) were mixed with 3 mg g^{-1} of glucose-C, 0.17 mg g^{-1} N and 5 P concentrations: KH_2PO_4-P at 0, 0.05, 0.10, 0.15 and 0.3 mg g^{-1} dry weight of soil. The control soil received water only. Soil and solutions were mixed, brought to field capacity and equilibrated 12 h at 4°C before incubation at room temperature. At intervals, 10 g moist soil were removed and analysed. In both series, 50 g of each of the above treatments were added to 500 ml Erlenmeyer flasks, connected to an aeration train supplied with CO_2-free air. Incubation was at room temperature and evolved CO_2 was trapped in towers containing glass beads and 20 ml of 1.0 M NaOH.

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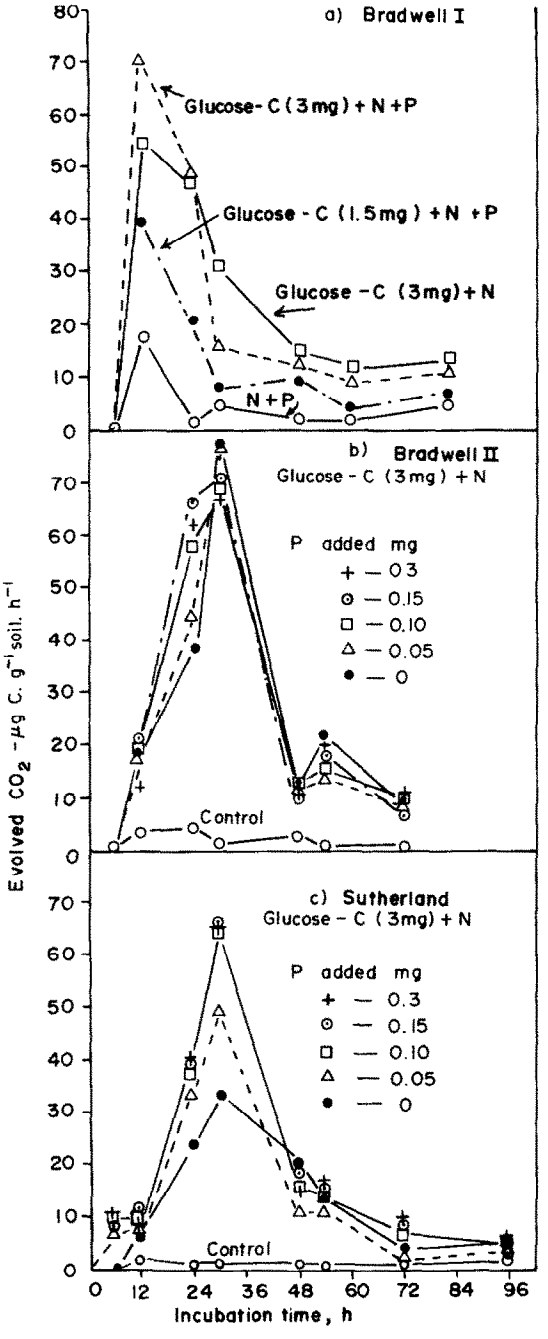

Fig. 1. CO_2 evolution rates in amended soils. (a) Bradwell I: Effect of glucose-C and P addition on CO_2 evolution from a sandy soil. (b) Bradwell II: Effect of varying concentrations of P. (c) Sutherland: Effect of varying concentrations of P in a clay soil.

Enzyme assays

Phosphatase. Three ml 0.1 M, pH 6.5, maleate buffer, and 1 ml 0.03 M *p*-nitrophenyl phosphate were added to 1 g moist soil. After incubation for 20 min at 37°C, the tubes were placed on ice and 1 ml 5 mM $CaCl_2$ and 4 ml 0.5 M NaOH were added. Absorbance was determined at 400 nm (Tabatabai and Bremner, 1969).

Urease. One ml of 3% urea and 2 ml 0.1 M pH 7.1, phosphate buffer were added to 1 g moist soil. After incubation for 30 min at 37°C in a water bath shaker, the tubes were placed on ice. Ammonia was extracted with 10 ml 2 M KCl. Two ml of 2.5% sodium phenate, 3 ml 0.01% sodium nitroprusside and 3 ml 0.02 M sodium hypochlorite were added to 4 ml of the filtrate. The mixture was shaken, incubated for 30 min in the dark, and absorbance measured at 630 nm (Fawcett and Scott, 1960). Activity was expressed as μmoles NH_4^+ produced by 1 g dry soil h^{-1}. Activities of phosphatase and urease were expressed after correction for two controls, i.e. without soil and without substrate.

ATP determinations

ATP was extracted with 0.5 M $NaHCO_3$, pH 8.5, after lysis with $CHCl_3$ (Paul and Johnson, 1977). Extracts were stored at −40°C until analysed by the luciferin-luciferase method. CO_2, enzyme activities and ATP determinations were carried out in duplicate.

Direct counts

Bacterial numbers were determined by fluorescence microscopy after staining the bacteria with fluorescein isothiocyanate (Babiuk and Paul, 1970) and the fungi with water-soluble aniline blue (Paul and Johnson, 1970) and the fungi with water-soluble aniline blue (Paul and Johnson, 1978).

Regression analysis included each independent variable separately starting with the one that resulted in the greatest reduction in the residual sum of squares. Dependent variables were: CO_2, ATP, urease and phosphatase activities. Independent variables were: fungi, bacteria and fungi + bacteria. Biomass was determined by weighing microscopically determined volumes of representative soil fungi and bacteria (T. L. Degenhardt, University of Saskatchewan, personal communication).

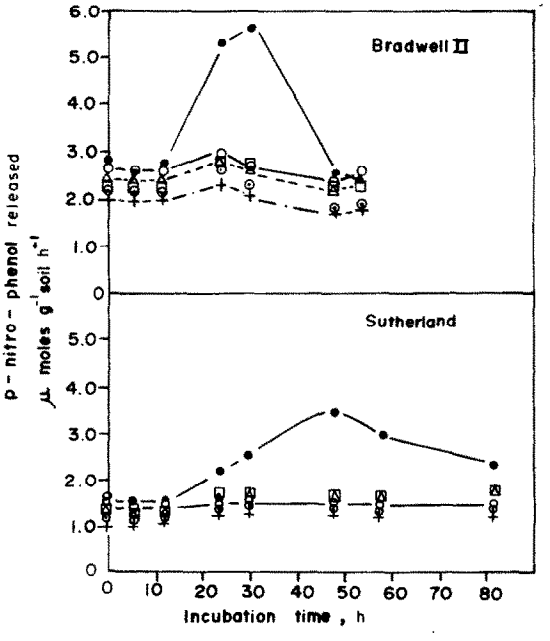

Fig. 2. Phosphatase activity in a sandy soil (Bradwell) and clay soil (Sutherland) after amendment with 3 mg glucose-C and P at various concentrations: +—0.3 mg, ○—0.15 mg, □—0.10 mg, △—0.05 mg, ●—0 mg P.

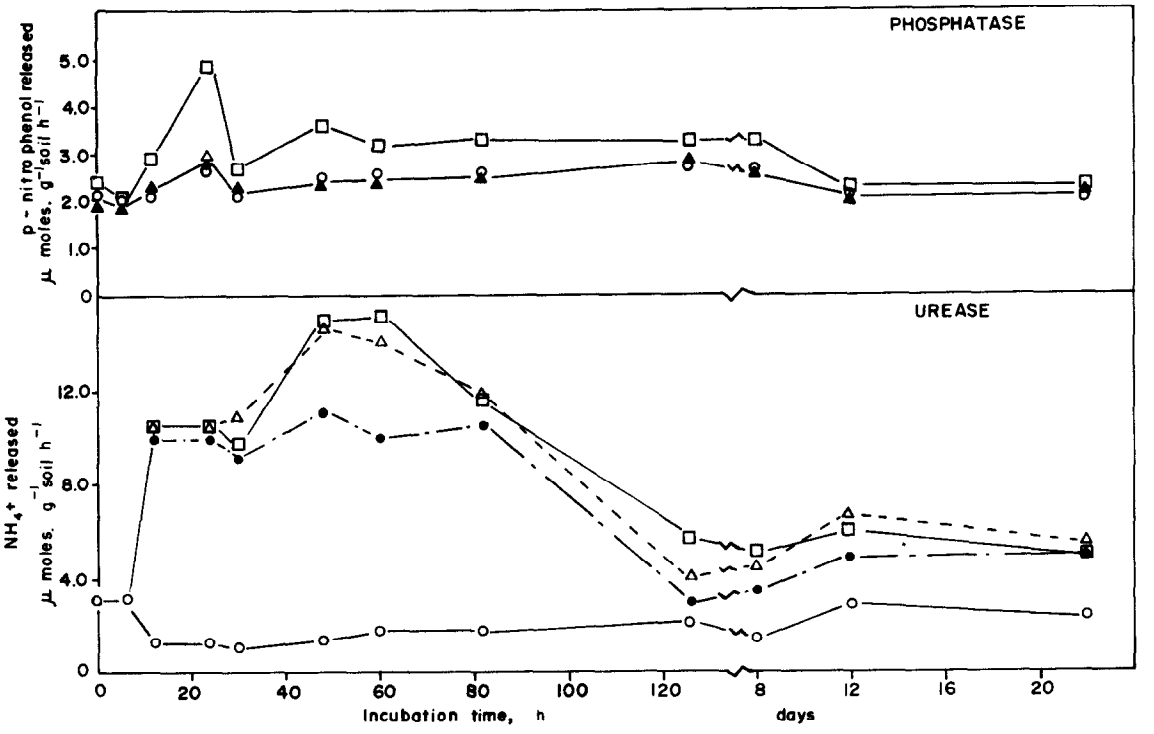

Fig. 3. Phosphatase and urease in Bradwell I soil at varying concentrations of C with and without P: ○—N + P, ●—1.5 mg C + N + P, △—3 mg C + N + P, □—3 mg C + N.

RESULTS

CO_2 evolution

The most widely used measure of soil activity is soil respiration either as O_2 uptake or CO_2 evolution. Soils amended with 3 mg glucose plus N and P had higher CO_2 evolution rates in the first 24 h than soils without P (Fig. 1a). The Bradwell soil without P, however, sustained a high CO_2 evolution rate for a longer period. After 82 h, both treatments showed equal CO_2 evolution rates. The total amount of CO_2 evolved from the addition of 1.5 mg glucose was 50% of the 3 mg g^{-1} treatment.

The differences in the time required for maximum CO_2 evolution in two identical treatments, Bradwell I (3 mg C + N + P) and Bradwell II (+0.05 mg P) in Fig. 1 is probably due to the influence of soil storage. Soil was air dried after sampling, and stored at room temperature in a closed plastic bag. Bradwell I was carried out after 1 month and Bradwell II after 2 months. Increased P enhanced initial CO_2-C evolution but did not affect total CO_2 (Fig. 1b). In the Sutherland clay soil, total CO_2 evolved at 0 and 0.05 mg P was lower than for other P treatments (Fig. 1c).

Enzyme activities

The addition of inorganic P repressed phosphatase activity in all concentrations in both soils (Figs. 2 and 3). Only the treatments with glucose at 0 mg P showed an increase in phosphatase activity. The newly synthesized phosphatase was not very stable in the Bradwell sandy soil. The enzyme activity in this P treatment equaled the control activity after 12 days (Fig. 3).

The increased urease activity after 12 h was sustained at a level of 10 μmoles NH_4^+ g^{-1} h^{-1} for 80 h in the 1.5 mg C treatment (Fig. 3). The addition of 3 mg C, either with or without added P, increased the urease activity to 14 μmoles g^{-1} h^{-1}. The first increase in urease activity corresponds to the rapid rise in bacterial numbers (Fig. 6), while the second increase, occurring at 48 h and at the higher glucose concentrations was coincident with a sharp increase in the fungal hyphal lengths. Urease activity after 8 days was stabilized at a value approximately double that of the control.

ATP

The soil receiving only N and P maintained ATP concentrations at approximately 2 μg ATP g^{-1} soil (Fig. 4). Soils amended with glucose showed a rapid rise in ATP after a 6 h lag, peaking after 24 h in the series I experiment shown in Fig. 4. The Bradwell series II experiment with varying concentrations of P and the Sutherland clay had shown slower increases in CO_2. These soils showed maximum ATP contents after 48 and 54 h, 24 h after the maximum CO_2 production rate (Fig. 5). ATP contents also lagged behind phosphatase (where it was not repressed) but coincided with urease values. Varying P concentrations at the same glucose concentration resulted in dramatically different amounts of ATP throughout the experiment in both soils. Numerous measurements of the ATP contents of soil indicate a standard error of 5–10% for conditions such as those reported above indicating that treatment effects were of significance.

The relationship between inorganic P and ATP was not transient. ATP pools in the amended soils were maintained at a higher level for at least 12 days (Fig.

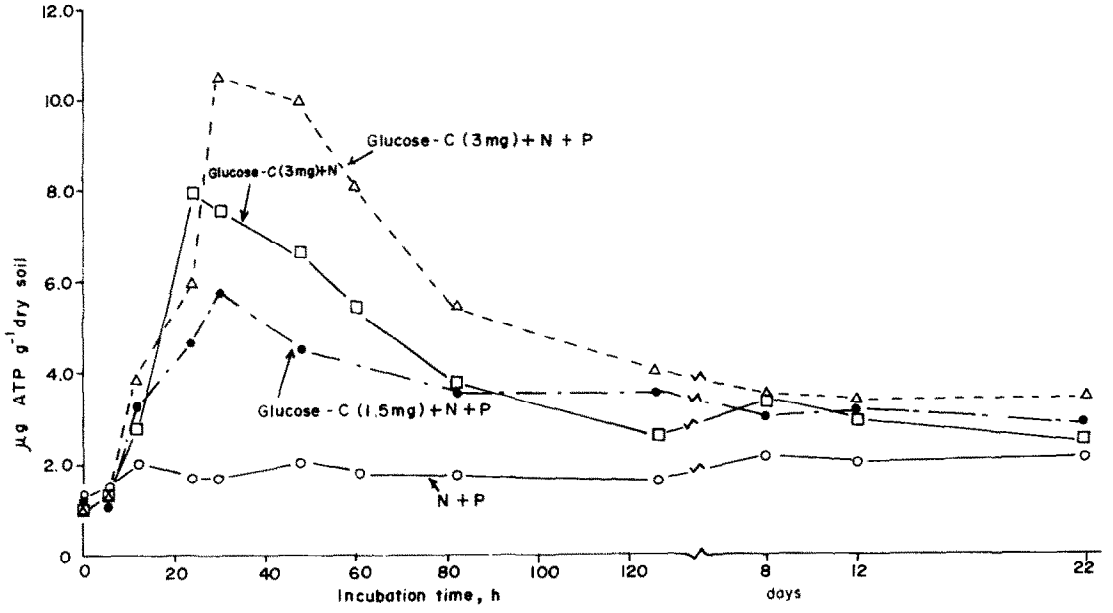

Fig. 4. ATP levels in Bradwell I during incubation after amendment with glucose-C, N and P.

4). In the Bradwell soil, doubling the P concentration from 0.05 to 0.1 mg g^{-1} resulted in an increase of 3 μg ATP g^{-1} soil (Fig. 5). P concentrations greater than 0.1 mg g^{-1} did not result in increased ATP levels indicating that the 30:1 C:P ratio supplied adequate P for ATP production. The Sutherland clay showed similar results but the magnitude of ATP increase was lower for all treatments.

Biomass

Bacteria increased four-fold from 0.12 mg to 0.47 mg at the higher glucose treatment with added N and P. The treatment with glucose + N but no P showed a two- to three-fold increase during the first 24–36 h followed by a secondary increase to 0.4 mg g^{-1} after 60 h. This second peak was attributed to microbial recycling of P and growth on secondary metabolites produced during the initial flush in the high energy but no P treatment. After this, the population decreased to a level lower than at the start. The addition of 1.5 mg C plus N and P resulted in an intermediate bacterial population.

The fluorescence count of fungal hyphae stained in water-soluble aniline blue showed initial counts of 200 m g^{-1}, equivalent to 0.65 mg g^{-1} dry weight fungal mycelium g^{-1} soil. In the 3 mg C + N + P treatments, fungal weight increased to 0.95 mg dry weight by 24 h and 1.12 mg by 48 h. The addition of 1.5 mg C g^{-1} + N + P resulted in fungal hyphae biomass one-half that of the treatment with twice the added C.

Summation of the bacterial and fungal biomass indicated an initial peak at 12 h and another at 48 h for the high glucose + N + P treatment. The 3 mg glucose-C + N (0–P) showed slower growth rates with one peak at 24 h and another at 60 h. At this time, total biomass for both treatments was similar at 1.3 mg dry weight g^{-1} of soil. Initially the ratio of fungal to bacterial biomass was greater than 5:1. Due to the higher growth rate for bacteria, the fungi outweighed the bacteria by a factor of about 4:1 at 60 h. The lower glucose addition showed a similar two peak response attributable to a single peak in bacterial production and two peaks in fungi at 24 and 48 h. Continued incubation saw total biomass dropping below the initial level even though ATP and urease levels remained higher in the treated soil than in the control.

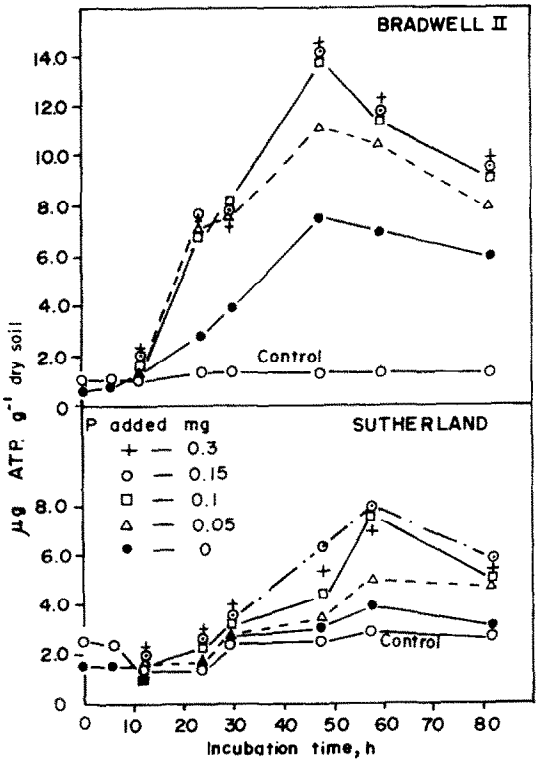

Fig. 5. Influence of varying concentrations of P on ATP contents of glucose amended Bradwell II and Sutherland soils.

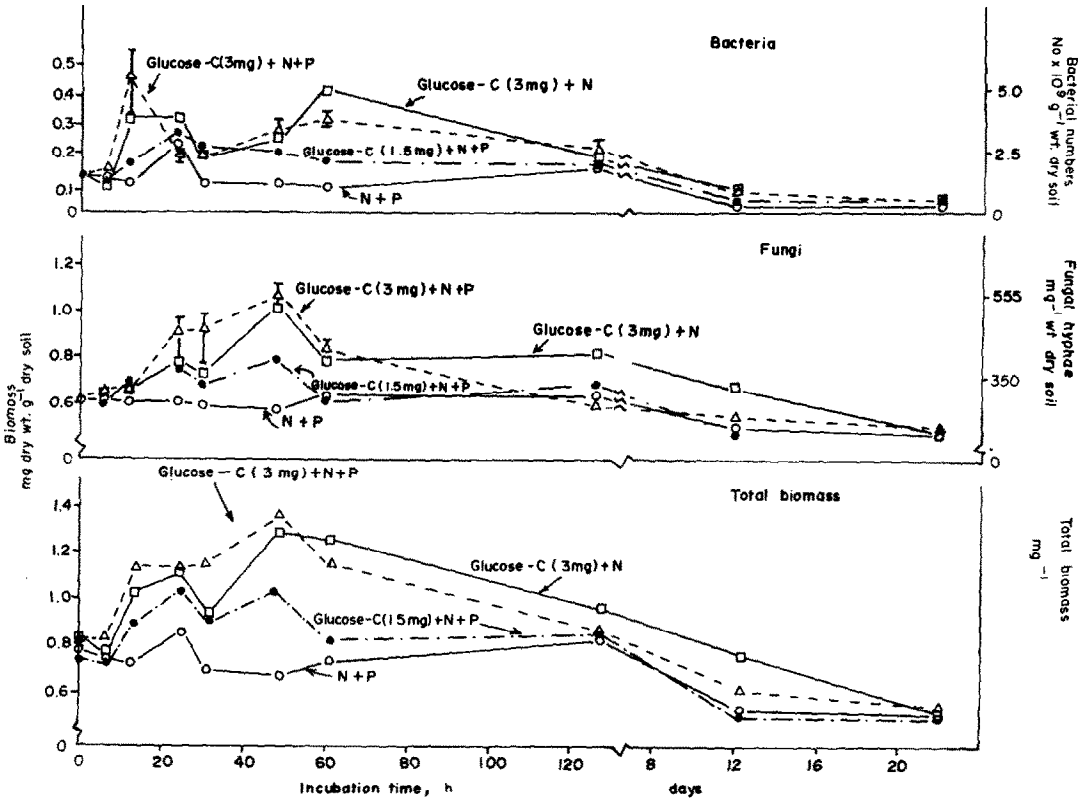

Fig. 6. Biomass of bacteria and fungi at varying concentrations of glucose with and without P in Bradwell I soil. I = standard deviation.

The concentration of ATP averaged 2.6 μmoles ATP g^{-1} biomass (Table 1) at the beginning of the experiment. The treatment receiving N + P but no energy supply varied with time and reached 8.56 μmoles ATP g^{-1} biomass after 22 days. The addition of glucose and 30 h incubation resulted in 19.2 μmoles ATP g^{-1} biomass expressed on a dry weight basis. Glucose + N resulted in less ATP (16.5 μmoles g^{-1}). Expression of ATP on a carbon basis using 48% C as representative of the biomass indicated a C:ATP ratio of 313 at zero time after prolonged storage of the soil. The C:ATP ratio dropped to 49 in the C + N + P treatment at 30 h and stabilized at 82:1 during the 12–22 day period.

DISCUSSION

Microbiologists studying the growth and activity of organisms in nature would very much like to be able to use one or two easily determined indicators of biomass or activity. Experimental conditions such as sand culture where there is little residual substrate for growth and the initial population is low show high correlations between the various microbial growth criteria (Witkamp, 1973). Most field studies with enzymes and respiration, however, show only moderate correlations (Kiss *et al.*, 1975).

Our results involving extensive sampling of two soils under laboratory conditions and detailed micro-

Table 1. ATP pool in Bradwell I soil expressed as μmole ATP g^{-1} of dry biomass.

Hours	N + P	Glucose-C (1.5 mg g^{-1}) +N + P	Glucose-C (3.0 mg g^{-1}) +N + P	Glucose-C (3.0 mg g^{-1}) soil) + N
0	3.38	2.40	2.31	2.30
6	3.97	3.70	3.27	3.82
12	6.02	8.57	7.65	5.58
24	3.96	9.75	13.15	16.02
30	4.78	13.82	19.18	16.51
48	6.10	9.75	14.83	10.67
60	5.08	16.37	14.26	9.36
126	4.15	8.20	10.14	5.48
Days				
12	7.46	14.54	10.59	7.96
22	8.56	11.97	14.73	10.89

scopic and biochemical analyses, indicate that as expected there are relationships between the various microbial activity and biomass indices.

The peak of CO_2 evolution preceded those of other measurements such as ATP and fungal biomass by 12–24 h. It, therefore, did not show significant correlations with the other criteria. CO_2 evolution rates were sensitive to P deficiencies during the 6–12 h active growth period but accumulated CO_2 evolution rates were similar in the Bradwell soil. Stotzky and Norman (1961) suggested that the initial concentration of essential inorganic nutrients determined the rate of utilization of added glucose, but the degree of utilization was controlled by the turnover of the nutrient elements. The CO_2 evolved during these experiments accounted for only 47% of the added C, 53% was stabilized as biomass or metabolites.

The increased phosphates activity coincided primarily with increased bacterial populations ($r = 0.73$) in the presence of glucose, but under conditions of P deficiency. In pure cultures, phosphatase is a repressible enzyme decreasing in content when microorganisms are transferred from deficient to normal P medium (Ihlenfeldt and Gibson, 1975).

Extraction of the incubated soil with 0.1 M $Na_4P_2O_7$ at pH 7 (Nannipieri *et al.*, 1974) resulted in 50–60% of the phosphatase activity occurring in the extracts. Two pH optima for phosphatase were obtained with the soils studied, one at 6.5 and another at 7.8–8.3. In the Bradwell soil, neutral phosphatase activity dominated, whereas the Sutherland soil had a high, broad pH optimum indicating the presence of alkaline phosphatase much of which was probably stabilized outside the cells. The phosphatase system is resistant to proteolytic enzymes while subunits are not (Cheng *et al.*, 1972; Schlesinger, 1965). Since the soil maintained a pH of 7 during the experiments, enzyme dissociation which occurs at the pH's below 6.0 would not occur. Some stabilization of extracellular enzymes is suggested since phosphatase activity in the soils did not drop as rapidly as biomass during the latter part of the incubation.

There was a strong positive correlation between biomass and ATP in the 1.5 mg and 3 mg glucose treatment amended with P ($r = 0.84^{++}$ and 0.81^{++}, respectively), but no correlation when P was omitted from the treatment, although the biomass was similar (Table 1). Forrest and Walker (1971) and Senez and Belack (1965) suggested that a lack of P uncoupled catabolism and anabolism lowering ATP pools. Our data show low ATP pools under P deficient conditions but equal CO_2 evolution and biomass.

Regression analyses showed the highest correlation with biomass occurred with urease which responded to both the early bacterial peak and the later final peak occurring at 60 h ($r = 0.94^{++}$ for total biomass). This enzyme system, present in a variety of organisms, was the only one not shown to be dependent on P concentrations other than through the action of P on microbial production of biomass.

The soil at zero time had on average of 2.6 μmoles ATP g^{-1} of microscopically determined biomass. This is equivalent to a C:ATP ratio of 313:1. The ATP pool g^{-1} dry weight of identified organisms rose by a factor of 2 in the N + P treatment but increased by a factor of 6–7 in the 3 mg – C + N + P treatment (Table 1). Great variations in the C:ATP ratio depending on P concentration have recently been noted for lake algae (Cavari, 1976) and it was suggested that this ratio would constitute a sensitive test for P deficiency. Our work also shows a sensitivity to available P.

In this study we varied soil type, substrate and P concentrations. N was maintained in excess. The early enhancement of CO_2 evolution rates by added P did not affect total CO_2 production or biomass. This together with the repression of phosphatase and the increased ATP to biomass ratios in the presence of added P indicate that sensitive techniques are available to measure microbial growth and activity in soil. However, any one of these indices must be used in conjunction with other tests to lead to a better understanding of the soil system. We conclude from our data that it is too simplistic to attempt to use only one or two indices as a general means of estimating biomass or activity in the soil system (Ausmus, 1973). The ratio of ATP to biomass measured microscopically should, however, be a reasonable indicator of activity at any one time. The biomass reported in this paper constitutes the bacteria and fungi only. Fungal spores, yeasts and microfauna are known to be present but were not included.

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