Plant and Soil 101, 189–195 (1987)

PLSO 6909

Growth measurements of terrestrial microbial species by a continuous-flow technique

CHARLES W. HENDRICKS[1], ELDOR A. PAUL[2] and PAUL D. BROOKS
Department of Plant and Soil Biology, University of California, Berkeley, CA 94720, USA
Present addresses: [1]Environmental Research Laboratory, US Environmental Protection Agency, Corvallis, OR 97333, USA and [2]Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824, USA

Received 10 June 1986. Accepted October 1986

Key words: biomass, CO_2 measurement, continuous-flow, fungi, glucose utilization, microbial growth, respiration, soil bacteria

Abstract

A continuous nutrient flow system has been developed to measure microbial activity in soil with various concentrations of added substrate. The system consists of a thin soil layer through which substrate was added continuously over periods up to 4.5 days. Substrate utilization was determined by effluent analysis. Respiration was measured manually by injecting a sample into a gas chromatograph or automatically by coupling the growth chamber to a computer-controlled gas sampling valve. This permitted respiratory CO_2 to be measured by the gas chromatograph at intervals selected by the investigator. Software controlling the valve and gas chromatograph not only automated gas phase sampling, but also provided a scan of CO_2 evolution and a preliminary data summary. This included the date and time of sample, peak height, and percent CO_2 in the gas phase. Data for growth on glucose using a microbial population native to a California annual grassland soil demonstrated that the direct cell count and respiratory techniques for biomass estimation give comparable results. This procedure provides the potential for detailed analyses of substrate utilization in studies of the growth and maintenance of soil microorganisms.

Introduction

Traditional methods of estimating microbial growth in terrestrial systems have relied upon the batch or static culture of organisms in various media or in aliquots of soil (Alexander, 1986). These techniques have provided considerable information about the types and numbers of organisms, their ability to grow on various substrates, and the rates of physiological and biochemical transformations. However, static cultures develop growth conditions where the physiological state of the culture is constantly changing due to the depletion of substrate and the accumulation of metabolites.

Attempts have been made to control the physiological state of microorganisms in soil culture (Stotzky, 1958). Perhaps the best known and most widely used of the procedures is the "Perfusion Technique" (Lees and Quastel, 1946) developed to study soil nitrification. This technique relies on perfusing soil with a source of nitrifiable material, thereby overcoming some of the difficulties associated with batch cultures. Specifically, the water content of the soil column can be kept at levels above saturation throughout the course of the experiment. Macura (1961) modified the perfusion apparatus by replacing the circluation feature with the incremental addition of substrate. Using the modified apparatus, data collected in nitrification studies (Macura and Kunc, 1965) were similar to those reported earlier by Lees and Quastel (1946).

Both the perfusion and the incremental flow procedures operate at moisture levels greater than saturation and rely on air-saturated substrate to maintain aerobiosis within the column.

The development of methodology to measure rates of nutrient transformation in soil and to mathematically express microbial growth on easily degradable carbohydrates (Nannipieri *et al.*, 1978; Paul and Voroney, 1984) has led to a requirement for growth measurements at predetermined soil moisture stresses. The use of automated CO_2 measuring devices (Brooks and Paul, 1987) can be combined with the analysis of eluate from a soil culture to determine the fate of microbial metabolites in this complex growth matrix. We have developed a continuous-flow procedure that permits control of the physiological state of the culture and the moisture level while making it possible to measure unutilized substrate. In this study, laboratory experiments were conducted to relate evolution of CO_2 and utilization of substrate to the production of biomass using both static culture and our continuous-flow techniques.

The objectives of this study were to develop continuous-flow methodology for the culture of soil microorganisms, to compare those results with corresponding data from static cultures, and determine the extent to which the system could be automated.

Materials and methods

Soil

The soil used in this study was a Mollic haploxeralf of the Argonaut series from an oak-grass savannah in the lower Sierra foothills of California. These soils are formed from highly weathered basic igneous rocks in a Mediterranean type climate. This site typically receives 60 cm annual rainfall. The soil pH was 5.9; organic carbon, 2.6%; phosphate, 2.1%; and nitrogen, 0.22%. The carbon/nitrogen ratio was 12 (Smith and Paul, 1985).

Soil samples were collected from a depth of 0–4 cm in the above study site, moistened and incubated for 5 days at room temperature (25–27 C) and then screened through a 2-mm sieve. Aliquots of the prepared soil were stored at 5 ± 2 C until used.

Continuous-flow culture apparatus

The continuous-flow culture apparatus was constructed from a Nucleopore (Pleasanton, CA)*. Twin-lok membrane holder (47-mm diameter) with a filter holder adapter attached to provide additional headspace. A Millipore (Bedford, MA) fiberglass prefilter was used to support a thin layer of soil. The 13.0 g (dry wt) of soil used in each growth experiment formed a 3 to 4-mm layer when the device was in operation.

After adding soil, the apparatus was placed into a wide-mouthed Mason jar (500 ml) and sealed with a new dome lid. The lids were fitted with Tygon tubing used for adding substrate, removing effluent, and sampling the gas phase (Figure 1). A Lachat (Mequon, WS) peristaltic pump regulated substrate addition and effluent removal. The gas phase was sampled by periodically pressurizing the vessel with air using an automated valve assembly (Brooks and Paul, 1987). For 12 h prior to the addition of substrate, a modified Johnson's solution (Johnson, 1957) lacking additional nitrogen was passed through the soil (9.6 ml h^{-1}) to pack it and to achieve the desired vacuum on the effluent side of the device to maintain the soil at a field capacity moisture level. The concentration of the modified Johnson's solution (μM) was: $MgSO_4 \cdot 7H_2O$, 250; KH_2PO_4, 500; Kcl, 13; H_3BO_4, 6.25; $MnSO_4 \cdot H_2O$, 0.50; $ZnSO_4 \cdot 7H_2O$, 0.50; $CuSO_4 \cdot 5H_2O$, 0.13; H_2MoO_4 (as 85% MoO_3), 0.13; Fe-EDTA, 5.00. This solution also provided essential micronutrients for cell growth where nutrients were being continuously flushed from the system.

Automatic sampling valve

The 36-port sampling valve used in this investigation was operated by an IBM computer (Boca Raton, FL) fitted with a Dascon I control board and SRA01 and STA01 modules (Metrabyte Corp., Stoughton, MA). This system operated the valve motor and switched solenoids that controlled air flow and gas chromatograph (GC) sampling phases of CO_2 analysis. The control board and BASIC software also provided preliminary calcula-

* Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

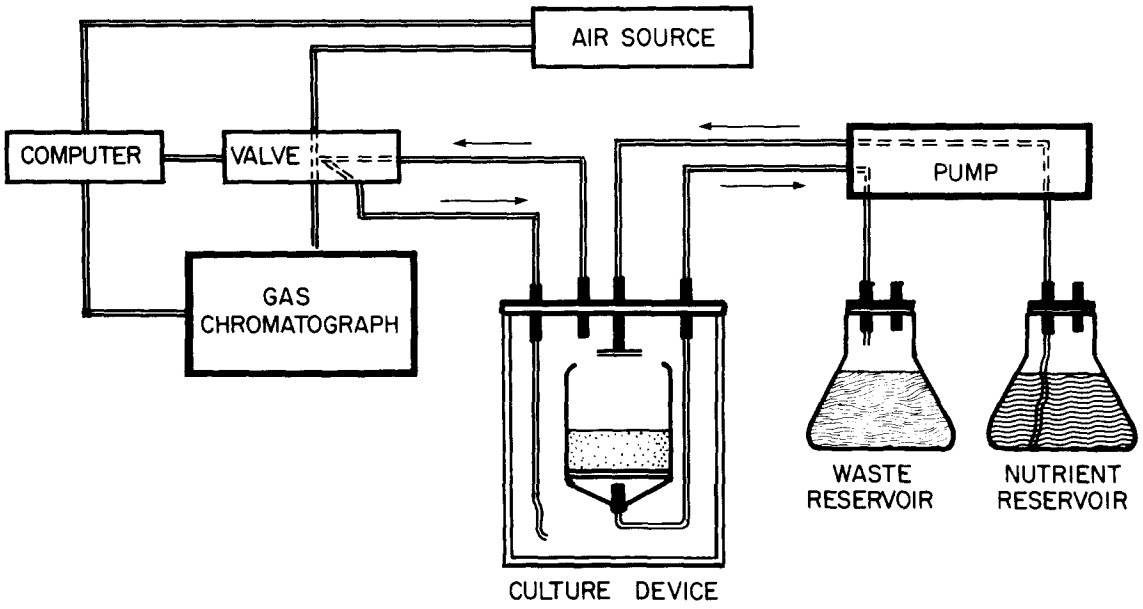

Fig. 1. Schematic diagram of the automated continuous-flow system.

tions of the CO_2 data and the storage and retrieval of this information by the computer (Brooks and Paul, 1987).

Soil incubation experiments

Two types of experiments were run to test the effectiveness of the continuous-flow system. The first series was designed to test the nutrient flow and CO_2 collection portions of the automated system. Mason jars were modified to accept a small rubber septum so that the gas phase could be sampled daily and manually injected into a gas chromatograph (Varian, Model 90P) for CO_2 measurement a gas-tight syringe. This system used a Poropak Q column and a thermoconductivity detector. Known CO_2 concentrations were used to calibrate and system unit standards were run after every fifth sample.

β, D-glucose (Sigma Chemical Company, St. Louis, MO) was added to the static culture jars in concentrations ranging from 0.05 to 10.0 mg C g^{-1} dry soil, and ammonium sulfate was added to make an equivalent C:N ratio of 10:1. Both the glucose and ammonium sulfate were prepared in modified Johnson's solution. Carbon and nitrogen were aded to the continuous-flow cultures in the same concentration and proportion, but the pump speed and tubing size are adjustable to permit delivery of a particular concentration over 1 day. For the experiments reported here, the flow rate was 9.6 ml day^{-1} using 0.57 mm diameter tubing. A tension of 0.01 KPa vacuum was maintained on the effluent side of each continuous-flow culture to keep soil moisture near field capacity.

The second series of experiments used the automatic CO_2 sampling valve of Brooks and Paul (1987). Carbon (β, D-glucose) was added at the rate of 10 mg C of both static and flow-through cultures was sampled automatically every 4 hrs and analzyed for CO_2. In both series of experiments, reported CO_2 values were calculated as the mean of at least two cultures.

At the beginnng of each experiment and periodically throughout the course of each run, influent and effluent samples and soil from static cultures were collected from at least two cultures and measured for residual glucose using the Glucostat technique (Sigma Chemical Company, St. Louis, MO). Samples were analyzed in dulplicate within an hour after collection. Liquid samples were analyzed directly, but glucose was extracted from the soil of static cultures by the method of Brookes *et al.* (1985).

Biomass determinations

At specific intervals during an experiment, representative static and continuous-flow cultures were terminated and biomass determinations were made. Bacterial numbers in samples were estimated by the technique of Babiuk and Paul (1970). This procedure utilizes fluorescein isothiocyanate (FITC) stain and requires a fluorescence microscope to visualize and count the bacteria. Fungi were counted with the bright field microscope, and smears were prepared and stained with phenolic analine blue (Paul and Johnson, 1977). Bacterial numbers and fungal hyphal lengths were measured using an eyepiece grid in the microscope and, for both the fungal and bacterial determinations, 20 fields were counted from each of two smears per culture sampled.

Biomass estimates using the Respiratory Response Method (Anderson and Domsch, 1978; Smith *et al.*, 1985) were made concurrently with the bacteria and fungi counts. These analyses were conducted in 50-ml Erlenmeyer flasks using 5 g of soil (wet wt) from both the static and the continuous-flow cultures. β, D-glucose (500 ug/g soil) was added to each flask and incubated at 22 $\pm$ 1 C for 2 h, after which headspace CO_2 was sampled through a rubber septum and measured by GC analysis.

Since visual observations showed fungal mats developing on the surface of the continuous-flow cultures receiving 10 mg C gm^{-1} dry soil d^{-1}, samples were taken at the beginning and end of an expermient, dried for 5 d at room temperature (25 27 C) coated with platinum, and observed with an ISI, DS-130 (30 KV) scanning electron microscope.

Results

The continuous-flow culturing system consists of two parts: the nutrient cycling portion, and the CO_2 sampling and analytical components (Fig. 1). The pump speed regulated nutrient flow so further control by the computer was not necessary.

Several support bases for the soil culture layer were evaluated, including various pore sizes of membrane filters, glass wool, filter paper, sand, and glass fiber prefilters. Of these supports, the inert glass fiber prefilters provided the required strength and unobstructed flow. Glass Mason jars of several sizes were also evaluated; the 500-ml jars were found most effective for our use. However, the size of the jar used will depend on the number of samples taken per hour, the amount of CO_2 evolution, and the sample size accommodated by the valve and gas chromatograph.

Glucose concentrations of 10, 1.0, and 0.1 mg C g^{-1} dry soil d^{-1} were used in the evaluation of the continuous-flow procedure; initial glucose concentrations for the static cultures were 10, 1.0, and 0.1 mg C g^{-1} dry soil (Fig. 2). The constant addition of substrate to the continuous-flow cultures resulted in an increase in respiration throughout the experiment, as expected. CO_2 evolution from the static cultures increased and then declined as substrate was depleted. Initially, however, all cultures were respiring at the same rate.

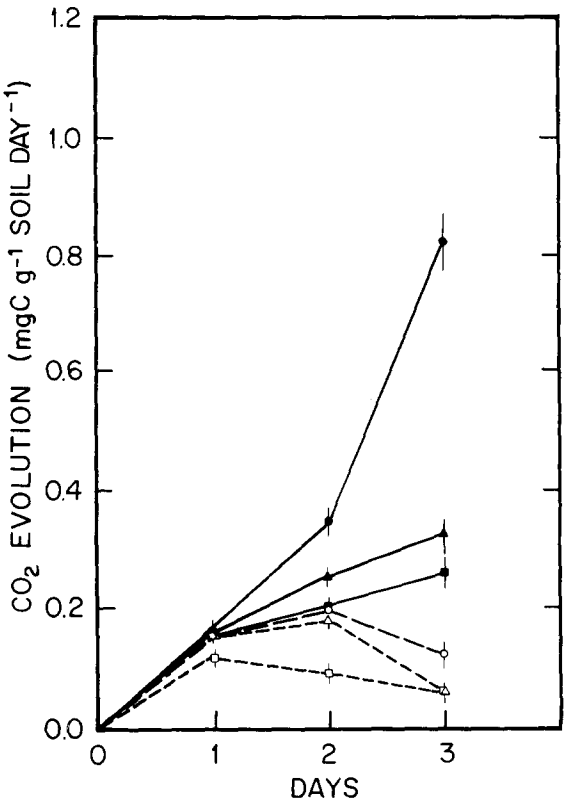

Fig. 2. Daily CO_2 evolution values for continuous-flow and static growth in manual CO_2 injection system. *Solid* and *open points* are shown for continuous-flow and static data, respectively, for growth on glucose at 10 mg C (●), 1.0 mg C (▲), and 0.1 mg C (■) g^{-1} dry soil. (Data points are the mean of at least 2 replications $\pm$ SEM.)

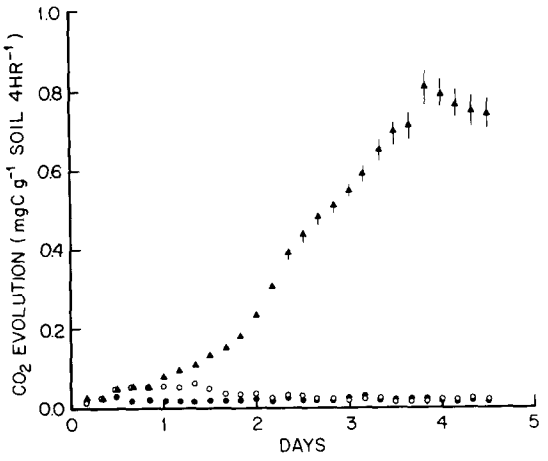

Fig. 3. CO_2 evolution values for continuous-flow (▲) static (○) and control (●) cultures for growth on glucose (10 mg C g^{-1} dry soil d^{-1}) using the automated system. (Data points are the mean of at least two replications ± SEM.)

To evaluate the continuous-flow culture apparatus coupled with the automated valve and GC, evolved CO_2 was automatically measured every 4 h from cultures receiving 10 mg C g^{-1} dry soil day^{-1}. Respiratory rates increased logarithmically for 2.5 days, and reached a maximum of 0.82 mg C $4\,h^{-1}\,g^{-1}$ dry soil at day 4. The maximum respiration rate observed for the static cultures was 0.05 mg C $4\,h^{-1}\,g^{-1}$ dry soil (Fig. 3).

Both continuous-flow and static culture devices were sacrificed daily for glucose and microbial biomass analysis (Tables 1 and 2). Respiration data (Table 1) are expressed as 24-h values obtained from the integration of the 4-h data (Fig. 3). In the upper log phase of growth (3-d), 57% of the input glucose concentration remained in the effluent of the continuous-flow culture apparatus. Of the 43% utilized by the microorganisms, 62% evolved as CO_2 and approximately 38% of the carbon converted to cell mass. Glucose was virtually depleted from the static cultures by the third day.

The general trend of microbial growth from the direct cell count and respiratory response biomass techniques is similar (Table 2). We feel that the more favorable growth conditions occurring in the continuous-flow apparatus resulted in a dramatic increase in the fungal population. This observation

Table 1. Glucose utilization by microbial cells in the continuous-flow (CF) and static (S) culture apparatus

Time (day)	Growth condition	Respiratory rate (mg C g^{-1} dry soil d^{-1})	Glucose remaining (mg C g^{-1} dry soil)
0	Control	0.00 ± 0.002[b]	10.0 ± 0.00
1	CF	0.34 ± 0.09	8.0 ± 1.07
	S	0.20 ± 0.06	5.7 ± 0.82
2	CF	0.89 ± 0.13	5.4 ± 0.80
	S	0.26 ± 0.07	4.1 ± 0.59
3	CF	2.66 ± 0.42	5.7 ± 0.79
	S	0.13 ± 0.04	0.3 ± 0.04
4	CF	4.26 ± 0.63	5.5 ± 0.82
	S	0.10 ± 0.02	ND[a]

[a] ND = not detected.
[b] ± SEM.

Table 2. Biomass estimates based on direct cell count and respiratory response methods for glucose (10 mg C g^{-1} dry soil) added to soil samples under both continuous-flow (CF) and static (S) conditions

Time (day)	Growth condition	Direct cell count method					Respiratory method
		No. cells (g^{-1} dry soil × 10^9)	Hyphal length (m g^{-1} dry soil)	Biomass (mg C g^{-1} dry soil)			Total biomass (mg C g^{-1}) dry soil)
		Bacteria	Fungi	Bacteria	Fungi	Total	
0	Control	1.83 ± 0.93	107 ± 23	0.12 ± 0.06	0.10 ± 0.04	0.22 ± 0.10	0.62 ± 0.25
1	CF	3.33 ± 1.53	299 ± 59	0.23 ± 0.11	0.28 ± 0.10	0.51 ± 0.21	1.17 ± 0.42
	S	2.19 ± 0.79	299 ± 59	0.15 ± 0.09	0.28 ± 0.11	0.43 ± 0.20	0.97 ± 0.38
2	CF	3.89 ± 1.98	567 ± 126	0.27 ± 0.11	0.53 ± 0.18	0.80 ± 0.29	1.89 ± 0.77
	S	3.03 ± 1.45	288 ± 65	0.21 ± 0.12	0.27 ± 0.09	0.48 ± 0.21	1.10 ± 0.42
3	CF	5.46 ± 2.07	3,770 ± 829	0.38 ± 0.20	3.52 ± 0.64	3.91 ± 0.84	4.17 ± 1.04
	S	4.78 ± 1.86	567 ± 113	0.33 ± 0.18	0.53 ± 0.21	0.86 ± 0.39	0.70 ± 0.26
4	CF	3.51 ± 1.40	5,620 ± 1,193	0.24 ± 0.12	5.25 ± 1.09	5.49 ± 1.21	7.24 ± 1.66
	S	3.29 ± 1.74	288 ± 56	0.22 ± 0.11	0.27 ± 0.11	0.49 ± 0.22	0.64 ± 0.21

[a] ± SEM.

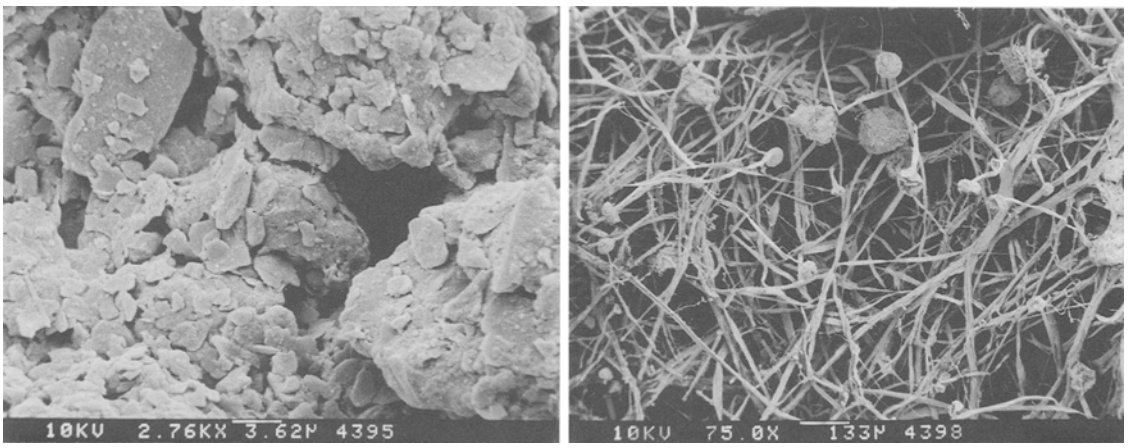

Fig. 4. Scanning electron microphotographs of soil from static and continuous-flow growth studies. Glucose concentratoins were 10 mg C g^{-1} dry soil and 10 mg C g^{-1} dry soil d^{-1}, respectively.

was confirmed by microphotographs that show the development of a fungal mat in the continuous-flow culture but not in the static culture (Fig. 4).

Discussion

Various types of soil column apparatus have been developed to study degradation of organic chemicals and process transformations by specific organisms. These include soil columns which could be periodically infused with water (or nutrient solutions) (Chahal and Wagner, 1965), the soil perfusion apparatus (Lees and Quastel, 1946) and the continuous cultivation apparatus developed by Macura (1961). Because of basic design limitations of these microcosms, soil moisture levels cannot be controlled, and the lengthy column produces a soil profile effect that results in differential sorption of ions such as NH_4^+ (Paul and McLaren, 1975). Studies of microbial growth are best performed under controlled moisture levels not greater than field capacity (Orchard and Cook, 1983). The use of thin soil layers and the effluent vacuum in this study demonstrated that soil moisture could be controlled and kept below 31 percent.

The results of this investigation have shown that a continuous-flow soil culture device can be constructed. Glucose utilization was not rate limiting since only 45% of the available glucose per day was utilized at the point of maximum CO_2 evolution. Nitrogen was probably not limiting either, since ammonium sulfate was applied at the ratio of 10 C:1 N. One can speculate that the rate-limiting factor was space, some unidentified micronutrient, or death and/or predation of the biomass (Pert, 1975; Jenkinson *et al.*, 1976; Nannipieri *et al.*, 1978; Jenkinson and Ladd, 1981). It is interesting to note that the bacterial biomass remained constant and little difference was observed between continuous-flow and static cultures. However, a substantial increase in the fungal component was observed, which suggests that these organisms have the ability to out-compete the other organisms when there is a continued excess supply of substrate. This phenomena also has been observed by others (Nannipieri *et al.*, 1978).

The error associated with the CO_2 and glucose analyses was approximately 15%. We suspect this error is associated with differences in the developing microbial flora from culture. This assumption is consistent with the observation that CO_2 data obtained from the automated valve and that obtained manually are not significantly different ($R^2 = 0.98$) (Brooks and Paul, 1986). The errors observed for the biomass estimates are higher than those associated with the chemical analyses. However, they are similar to those observed by others (Babiuk and Paul, 1970; Paul and Johnson, 1977). It may be possible to significantly reduce the error by homogenizing the sample prior to taking aliquots for bacterial and fungal estimates.

The development of the automatic valve by Brooks and Paul (1987) and the availability of a pump with good variable flow characteristics were a prerequisite to the construction of this con-

tinuous-flow culture system. We have successfully sampled at 24, 6, and 4 h intervals, but the maximum number of samples is governed by the time needed for analysis per sample and the total number of sample ports on the valve. The automatic valve system can perform CO_2 analysis as precise as present manual GC techniques thereby substantially increasing the number of analyses that can be performed. This system also has the unique advantage of side-by-side comparisons of cultures and offers better and more frequent sampling than can be achieved otherwise.

Acknowledgements

This work was supported by grant No. BSR-8306181 from the National Science Foundation and an IPA from the Environmental Protection Agency. We thank Jim Bartlett, UC-Berkeley, for processing and taking electron microphotographs of the soil layers.

References

Alexander M 1967 Introduction to Soil Microbiology. John Wiley and Sons, Inc., New York.

Anderson J P E and Domsch K H 1978 A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem. 10, 215–221.

Babiuk L A and Paul E A 1970 The use of florescein isothiocyanate in the determination of the bacterial biomass of grassland soil. Can. J. Microbiol. 16, 57–62.

Brookes P C, Landman A, Pruden G and Jenkinson D S 1985 Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method for measuring microbial biomass nitrogen in soil. Soil Biol. Biochem. 17, 837–842.

Brooks P D and Paul E A 1987 A new automated technique for measuring respiration in soil samples. Plant and Soil. 101, 183–187.

Chahal K S and Wagner G H 1965 Decomposition of organic matter in Sanborn field soils amended with ^{14}C glucose. Soil Sci. 100, 96–103.

Jenkinson D S and Ladd J N 1981 Microbial biomass in soil: measurement and turnover. *In* Soil Biochemistry. Eds. E A Paul and J N Ladd, pp 415–471. Marcel Dekker, Inc., NY.

Jenkinson D S, Powlson D S and Wedlerburn W M 1976 The effect of biocidal treatments on metabolism in soil. III. The relationship between soil and biovolume, measured by optical microscope, and the flush of decomposition caused by fumigation. Soil Biol. Biochem. 8, 189–202.

Johnson C M, Stout P R, Boyer T C and Carlton A B 1957 Comparative chlorine requirements of different plant species. Plant and Soil 8, 337–353.

Lees H and Quastel J H 1946 Biochemistry of nitrification in soil. I. Kinetics of, and the effects of poisons on, soil nitrification, as studied by a soil perfusion technique. Biochem. J. 40, 803–814.

Macura J 1961 Continuous flow method in soil microbiology. I. Apparatus. Folia Microbiol. 6, 328–334.

Macura J and Kunc F 1965 Continuous flow method in soil microbiology. V. Nitrification. Folia Microbiol. 10, 125–135.

Nannipieri N, Johnson R L and Paul E A 1978 Criteria for measurement of microbial growth and activity in soil. Soil Biol. Biochem. 10, 223–229.

Orchard V A and Cook F J 1983 Relationship between soil respiration and soil moisture. Soil Biol. Biochem. 15, 447–453.

Paul E A and Johnson R L 1977 Microscopic counting and adenosine 5-triphosphate measurement in determining microbial growth in soils. Appl. Environ. Microbiol. 34, 263–269.

Paul E A and McLaren A D 1975 Biochemistry of the soil subsystem. *In* Soil Biochemistry. Eds. E A Paul and A D McLaren. pp 1–36. Marcel Dekker, Inc., NY.

Paul E A and Voroney R P 1984 Field interpretation of microbioal biomass activity measurements. *In* Current Perspectives in Microbial Ecology. Eds. M J Klug and C A Ready, pp 509–519. Am. Soc. Microbiology, Washington, D.C.

Pert S J 1975 Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications, Oxford, England.

Smith J L, McNeal B L and Cheng H H 1985 Estimation of soil microbial biomass: an analysis of the respiratory response of soils. Soil Biol. Biochem. 17, 11–16.

Smith J L and Paul E A 1985 Process Controls and Nitrogen Transformations in Terrestrial Ecoystems, Progress Report 1984–1985, NSF Grant No. BSR-83-06181, University of California, Berkeley, CA.

Stotzky G, Ryan T M and Mortensen J L 1958 Apparatus for studying biochemical transformations in incubated soils. Soil Sci. Soc. Am. Proc. 22, 270–271.