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A PHYSIOLOGICAL METHOD FOR THE QUANTITATIVE MEASUREMENT OF MICROBIAL BIOMASS IN SOILS

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Summary—A method is described for the rapid and objective estimation of the amount of carbon in the living, non-resting microbial biomass of soils. The method, which is based on the initial respiratory response of microbial populations to amendment with an excess of a carbon and energy source, was quantified using an expanded version of Jenkinson's technique.

The simultaneous application of the two methods to 50 soil samples showed a highly significant correlation ($r = 0.96$) between both. From this correlation it could be deduced that at 22°C, a substrate-induced maximal respiratory rate of 1 ml $CO_2 \cdot h^{-1}$ corresponds to *c.* 40 mg microbial biomass C. Evidence supporting these results was obtained from pure culture studies. The various soil types investigated were collected from agricultural as well as forest sites and they contained between 15 and 240 mg microbial C·100 g dry soil^{-1}. The respiratory method provides reproducible estimates of biomass size within 1–3 h after soil amendment. It can be combined without difficulty with a selective inhibition method for determination of bacterial and fungal contributions to soil metabolism.

INTRODUCTION

The increased interest in the role of soil microorganisms in nutrient and energy-flow relationships in natural as well as man-manipulated environments has emphasized the need for simple and objective methods for measuring the size of microbial populations in solids.

Among the methods currently available are those involving direct counting, in which microorganisms can be variously stained (Nicholas and Parkinson, 1967; Babiuk and Paul, 1970; Schmidt, 1973; Trolldenier, 1973; Söderström, 1977) and observed by different optical methods (Casida, 1968; 1971; 1976; Frankland, 1974) or those involving soil extractions, in which enzymes (Kuprevich and Shcherbakova, 1971; Skujins, 1967), cell components peculiar to cell walls of certain groups of microorganisms (Millar and Casida, 1970; Steubing, 1970; Swift, 1973), or components characteristic to living cells (Lee *et al.*, 1971; Ausmus, 1973) are extracted and quantitatively assayed. Other methods include those based on the disintegration of the soil, followed by separation and collection of the microbial cells (Balkwill *et al.*, 1975; Faegri *et al.*, 1977), or physiological methods, in which the respiratory activities of substrate-supplemented habitats are used to estimate relative proportions of actively metabolizing biomasses (Wright and Hobbie, 1966; Anderson and Domsch, 1973; 1975), and finally a new method, in which the carbon bound in the microbial biomass is released by microbial mineralization and thereby provides a means for calculating its weight (Jenkinson, 1966; Jenkinson and Powlson, 1976b).

Each of the biomass methods mentioned has its limitations. The microscopic methods require skilled personnel, and often present difficulties in distinguishing between living and dead cells; in addition, a number of assumptions have to be made before counts can be converted into weights. The extraction methods often fail to fully extract the desired chemical and the relative recovery often depend on substrate characteristics or the type of extraction procedure used. Also, the quantity of a particular cell component can vary considerably with growth conditions and within different members of a microbial population. The ratio of a specific component to the actual biomass is, therefore, not necessarily a constant. With respiratory methods thus far described, a factor which links respiration rates to biomass weights has not yet been derived.

Among these various methods for biomass measurement, Jenkinson's (1966) approach, which has recently been examined in detail (Shields *et al.*, 1973; Jenkinson, 1976; Jenkinson and Powlson, 1976a,b; Jenkinson *et al.*, 1976; Powlson and Jenkinson, 1976; Anderson and Domsch, 1978), appears to give the most direct estimation of actual weight. The approach of Anderson and Domsch (1973; 1975), in which the respiration of the substrate-stimulated biomass is measured, allows relative biomass measurements to be made, but does not give information regarding the weight of the biomass. In view of the speed and simplicity of the respiratory technique, an attempt was made to calibrate these measurements, using the method of Jenkinson and Powlson (1976b), to provide a simple, objective, and rapid method for measuring the quantity of biomass in soils.

MATERIAL AND METHODS

Soils

Soils A to I (Table 1), collected from the upper 10 cm of agricultural plots, and soil K from the upper 10 cm of a grassland, were passed through a 2 mm

Table 1. Soil characteristics

Soil	Type	C_t (%)	N_t (%)	C/N	pH (KCl)
A	Para brown earth	0.778	0.100	7.9	4.95
B	Sandy brown earth	0.953	0.110	8.7	5.15
C	Brown podzol	1.252	0.115	10.9	5.45
D	Basic brown earth	1.282	0.118	10.9	7.05
E	Pseudogley	2.388	0.230	10.4	4.80
F	Chernozem	2.583	0.202	12.8	6.90
G	Pseudogley	2.593	0.240	10.8	5.85
H	Sandy brown earth	2.793	0.330	8.5	5.25
I	Degraded chernozem	2.933	0.151	19.4	7.10
J	Clay-rich brown earth	3.125	0.259	12.1	4.05
K	Peat soil	26.374	2.200	12.0	5.65
L	Acid brown earth	39.200	1.400	28.0	3.80

sieve to remove larger roots and animals. Soil J and soil L were thoroughly mixed composite samples of the L, F and H layers of a mixed oak and a spruce forest, respectively. All soils were stored moist at 22 ± 0.5°C for at least 10 days before use.

Amendment experiments

In the course of the experiments, soil samples (100 g dry wt for soils A–I, 20 g dry wt for soils K and L) were amended with a carbon and energy substrate. The desired quantity (see below) was placed in a glass mortar with 0.5 g talcum, ground to a fine powder, poured onto and then blended into the moist soil using a hand-operated electric mixer (equipped with a single blade: Esge Co., Type M 301) operated at *c.* 1600 rev min^{-1} (medium speed) for 25 to 30 s. The soil was held in a 1 l plastic beaker during mixing. With respect to homogeneous distribution, this method proved to be superior to application of the substrates in aqueous solutions.

Measurement of soil respiration

The soil samples were poured into 25 × 4 cm plastic tubes, stoppered at both ends with polyurethane foam rubber plugs, and connected to a continuously operating "Ultragas 3" CO_2 analyzer (Wösthoff Company, Bochum, FRG) for incubation at 22 ± 0.5°C. Once each hour, CO_2-free air was drawn through the soils for 20 min and the rate of CO_2 production was measured for 10 min.

Measurement of microbial carbon

With slight modifications, the method of Jenkinson and Powlson (1976b) was used. In this method, the microbial cells in soil are killed by fumigation with alcohol-free $CHCl_3$, and then subjected to mineralization by reinoculating the soil with a small amount of the original soil and incubating for 10 days at a constant temperature. Controls consist of identically incubated but non-chloroformed soil samples. The amount of microbial C is calculated from the difference between the CO_2-C evolved from $CHCl_3$-fumigated and non-fumigated samples.

Since the C in the freshly killed cells will not be totally mineralized within 10 days, the amount released must be corrected to 100%. To do this, the C mineralized from the microflora is divided by a value (*k*-factor) which is equal to the portion of C released during mineralization of known quantities of $CHCl_3$-killed biomass during 10 days. Jenkinson (1976), working at 25°C with 12 species of microorganisms and one soil, determined the *k*-factor as *c.* 0.5 (= 50% mineralization); Anderson and Domsch (1978), who worked at 22°C and used 12 (radio-labelled) species of bacteria and 15 (radio-labelled) species of fungi added to 4 different soils suggested a *k*-factor of 0.411 (= 41.1% mineralization). This latter factor was used for the biomass calculations made in the present study.

In addition to use of a different *k*-factor, three other modifications of the Jenkinson and Powlson (1976b) method were made: (1) 100 g (dry wt) samples of soil were used rather than 250 g, (2) a continuously operating respirometer was used instead of air-tight jars containing alkali CO_2 traps, and (3) hourly rates of CO_2 evolution were determined instead of a single measurement at the end of the 10 days incubation. Experimental details of the soil fumigation are given in Anderson and Domsch (1978).

Measurement of the CO_2 to biomass-C relationship in pure culture experiments

Static cultures of 14 species of soil fungi were cultivated in 100 ml of medium which contained glucose ($10\,g \cdot l^{-1}$) and NH_4NO_3 ($1\,g \cdot l^{-1}$). At intervals selected by reference to growth curves of each organism, cultures were harvested by filtration and washed with copious amounts of K_2HPO_4 buffer (pH 7.0). Harvested cells were divided into 8 samples of approximately equal wet weights: 5 of these samples were resuspended in 100 ml portions of buffer for respirometry; and 3 samples were collected on preweighed filter paper for dry weight determinations. The samples (between 5–40 mg dry weight) used for respirometry were supplemented with 250, 500, 1000, 2000 or 4000 μg glucose · ml $buffer^{-1}$ and incubated in wash flasks at 22 ± 0.5°C on the above mentioned respirometer. Samples used for dry weight determinations were subsequently oxidized to determine C content (Leco Corporation Carbon Determinator, St. Joseph, Michigan, USA).

THEORETICAL CONCEPT

In preliminary experiments, it was found that constant-temperature soil samples could be caused to respire at maximal rates by amending these with an

excess of readily-mineralizable substrate. Since the response maxima so obtained were found to be different from soil to soil, but were the same for replicates of any single soil, it was hypothesized that under identical conditions of incubation, the initial, substrate-induced, maximal respiratory responses were correlated to the actual size of the living, non-resting microflora, and because of this, could be used to measure their sizes in different soils.

As a prerequisite to the use of initial response maxima for biomass measurements, two conditions had to be fulfilled: (1) a substrate had to be selected which would be available to the majority of the soil microorganisms, and (2) a constant had to be derived which would allow conversion of relative metabolic measurements into microbial biomass weights. The sequence of experiments conducted to attain these goals are shown in Fig. 1. The results of the experiments are given in the following sections.

RESULTS

Physiological measurement of biomass response

Selection of an appropriate carbon and energy source. Besides being compatible with the metabolic requirements of a high proportion of the soil microflora, a substrate suitable for the present purpose should be: (i) water-soluble, to allow its rapid dispersion to as many microhabitats as possible; (ii) relatively complex in structure, to prevent its mineralization by bound or free soil enzymes, and (iii) non-toxic, to avoid adverse side effects when added to soil in quantities in excess to those which can be immediately metabolized.

Four substrates were tested to determine which induced the highest rate of soil respiration. The substrates were added at concentrations of 250–4000 $\mu g \cdot g$ (dry wt) $soil^{-1}$ and the rates of CO_2 evolution per unit soil were determined 1 h after amendment. The results of these experiments (Fig. 2) show that at substrate saturation, both glucose and casamino acids caused response rate maxima of 1.51 ml $CO_2 \cdot 100\,g\ soil^{-1} \cdot h^{-1}$. It was assumed from this, that both of these carbon and energy sources were being mineralized by equally sized, and most probably similar microbial populations, and that either substrate would have been of value for further experiments. However, because of uncertainties in regard to the composition of different batches of casamino acid mixtures, glucose was chosen. Besides being available in pure form, the uptake mechanisms and the pathways by which this compound can be degraded are well defined, and one or more pathways for its mineralization have been found to exist in most known taxa of aerobic soil microorganisms.

Determination of substrate-induced maximal initial respiration rates. Soil samples were amended with a series of glucose concentrations and the initial rates of mineralization were determined. Results obtained with three selected soils, which exemplify the results obtained in all experiments, are shown in Fig. 3. A comparison of the three soils shows that maximal res-

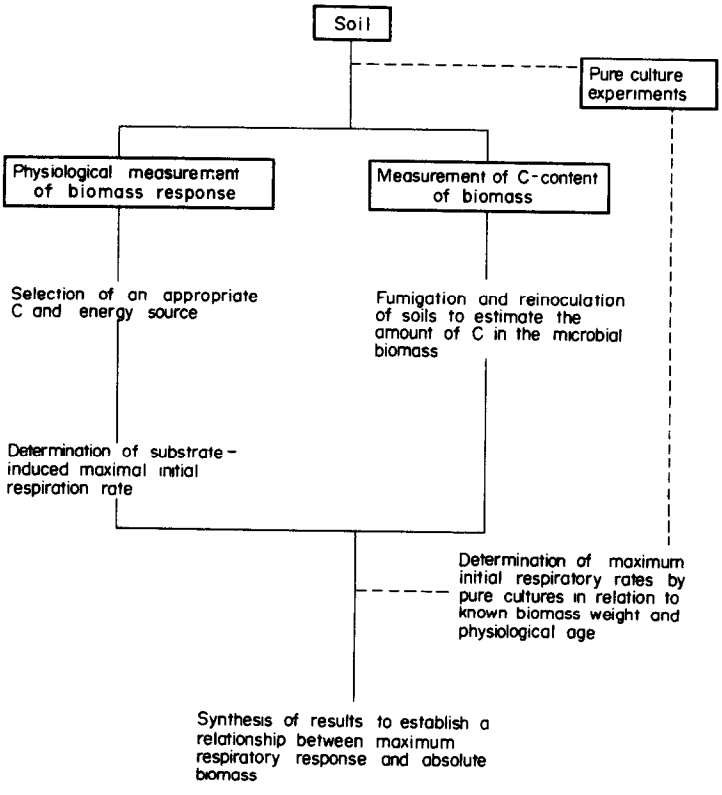

Fig. 1. Experiments conducted to derive a physiological method for measurement of microbial biomass size in soil.

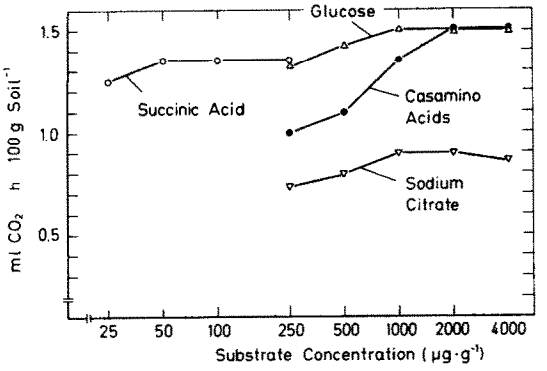

Fig. 2. Response of the microflora in soil C to various carbon-energy substrates. Measurements made after 1 h incubation at 22 ± 0.5°C.

piration rates are reached in soils L, F and B at 4.34, 2.01 and 0.67 ml $CO_2 \cdot 100\,g\ soil^{-1} \cdot h^{-1}$, respectively. In terms of relative biomass, soil L contained 6.4 times and soil F contained 3.0 times as much biomass as soil B. The absolute biomasses of these soils are compared in the following section.

Of particular importance in these experiments is selection of the mineralization rate which best represents the size of the original microbial population. Figure 4, which shows mineralization curves having initially negative, zero or positive slopes, demonstrates the selection of rate maxima from each curve type. A positive tendency in any of these curve types has been interpreted as indicating biomass synthesis (Anderson and Domsch, 1975); therefore, measurements are best made before this tendency occurs. For soils displaying curves of type II and III, 1 h after substrate addition has been found to be the most suitable time. Most of the soils tested (8 out of 12) had type II curves. Soils with large biomasses, high organic matter and relatively high moisture levels often showed type I curves (soils C, F and L). In these soils, readings were taken when the slope became zero. It is currently not known why initial negative slopes occur but it is thought that substrate-accelerated death of cells, a phenomenon discussed by Dawes (1976), might be related to this observation. For curve type III, which was observed with a single soil (soil C), readings after the first hour were accepted as the closest approximation.

Measurement of the C-content of the microbial biomass

In addition to measurement of the substrate-induced maximal initial respiration rate of each soil, the C incorporated in the microbial biomass of each was simultaneously determined. The results obtained with three of the soils, which exemplify those obtained in all experiments conducted, are shown in Fig. 5. Of these curves, the curve demonstrated by soil F is ideal for measurement of soil microbial biomass by the fumigation-reinoculation method. In this case, the CO_2-C evolved from the fumigated soil was greater than that from the non-fumigated sample almost immediately after reinoculation, and, shortly after 240 h of incubation, the respiration of both the fumigated and the non-fumigated control samples approached unity. For soil F, the CO_2-C from 3–240 h totalled 32.6 mg, and the biomass C was equal to $32.6/0.411 = 79.3\,mg \cdot 100\,g$ (dry wt) of $soil^{-1}$.

Examining the curves for soils L and B in Fig. 5, it can be seen that the strict use of cumulative CO_2 data, such as would be obtained through use of incubation as suggested by Jenkinson and Powlson (1976b), would lead to errors in biomass determinations due to the negative values at the beginning or at the end of the incubation. To avoid these errors in the present investigation, net respiration curves were prepared for each experiment, and only the values where the respiration of the fumigated sample was greater than the control were used. For soil B, therefore, the calculation period was between 4 and 170 h and the biomass C was equal to $10.5/0.411 = 25.5\,mg \cdot 100\,g\ soil^{-1}$. For soil L, the period was between 23 and 260 h and the biomass C was equal to $70.7/0.411 = 172.0\,mg \cdot 100\,g\ soil^{-1}$. Comparison of these values to one another, as was done with the rate measurements of the physiological method, shows that soil L has *c.* 6.7 and soil F has *c.* 3.1 times as much biomass C as soil B.

Relationship between maximum initial respiration rates and absolute biomasses

Soil studies. The relationship between CO_2 rates and the absolute biomasses of soils A through L is shown in Fig. 6. The significant correlation ($r = 0.96$) indicates that the microbial biomass C of soils can be readily estimated by determining the maximum initial respiration rate following glucose addition and substituting this value into the equation $x = 40.4\ y + 0.37$, where y = maximum initial rate of respir-

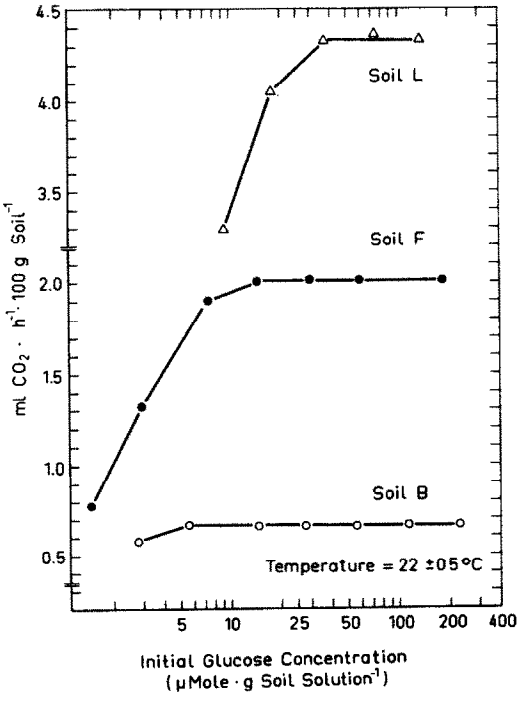

Fig. 3. Response of the microflora in three different soils to different concentrations of glucose. Incubation temperature 22 ± 0.5°C; rate measurements made for soil L after three, and soils F and B after 1 h incubation.

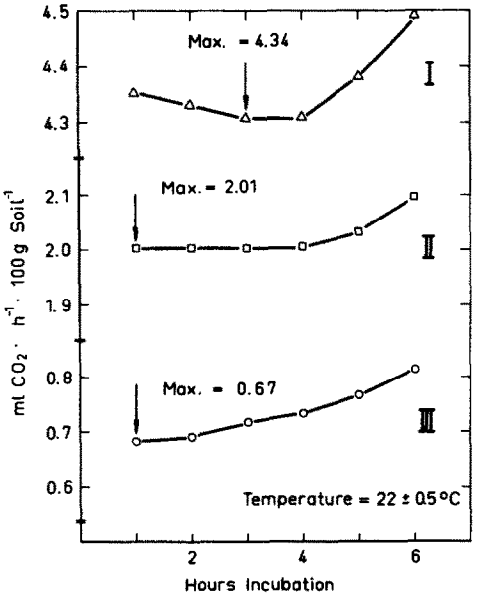

Fig. 4. Types of rate curves from glucose-amended soil samples. Arrows indicate the hour best representing the initial maximum response of the existent biomass.

ation, expressed in ml CO_2·unit soil^{-1}·h^{-1}, and x = mg microbial C·unit soil^{-1}. This equation is based on mineralization data between 0.35 and 6.50 ml CO_2·100 g soil^{-1}·h^{-1}. It is only valid in this particular form if the temperature of the soil during measurements is kept at 22°C. Substitution of y = 1 into the equation and solving for x indicates that 40 mg biomass C produces *c.* 1 ml CO_2·h^{-1}, or 1 mg biomass C releases 25 μl CO_2·h^{-1}.

Pure culture studies. To examine the overall feasibility of the above *in situ* correlation between stimulated respiration and biomass, experiments were conducted with pure cultures of fungi to determine the maximum initial rates at which known weights of cell material, harvested during various stages of cultural growth, would respond to amendment with glucose.

The results of these experiments are shown in Table 2. It can be seen that glucose supplemented cells which were harvested in the earlier phase of growth release more CO_2 per mg C than those harvested in the later phases. In the early, middle and late linear and the stationary phases, the averages for the 12 fungi were 42, 23, 20 and 18 μl CO_2·mg C^{-1}·h^{-1}, respectively. The average for all phases taken together was 26 μl CO_2·mg C^{-1}·h^{-1}. This average, which is the same as the value obtained from soil studies (25 μl·mg C^{-1}·h^{-1}), supports the feasibility of the linear regression obtained in the *in situ* investigations.

DISCUSSION

The potential use of respiratory response rates to estimate the sizes of microbial biomasses in natural habitats was indirectly suggested by Drobnik (1960) and Novák (1973) for soils, and directly suggested by Wright and Hobbie (1966) for bacterial populations in aqueous environments. None of these studies, however, offered a direct means for converting respiratory values into actual biomass weights.

In the present investigation, a practical and direct means has been derived for this conversion. The major advantages of this method are its objectivity and its simplicity. After adding increasing amounts of glucose to soil, the maximum initial respiratory

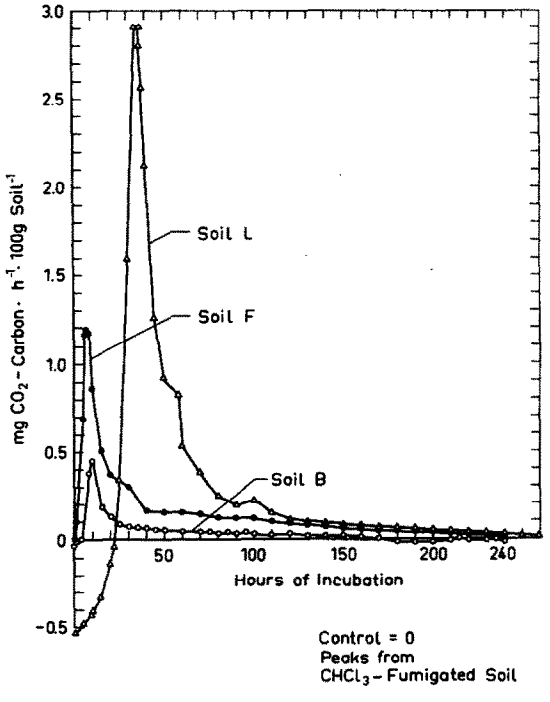

Fig. 5. Types of net respiration curves showing the release of CO_2-carbon from $CHCl_3$-fumigated, reinoculated soils.

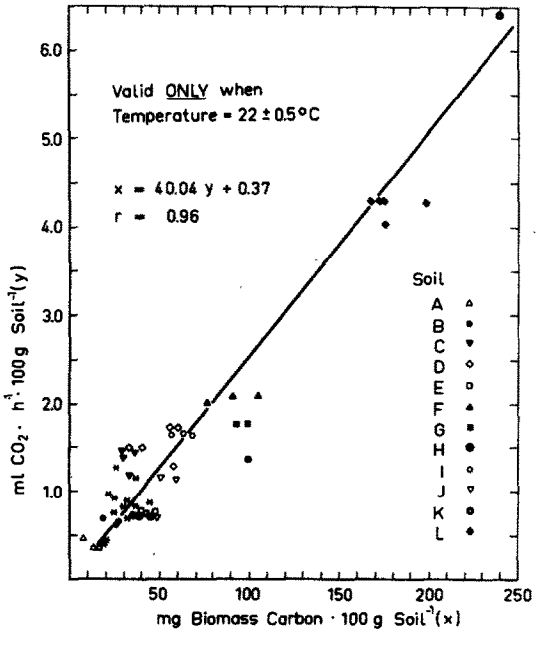

Fig. 6. Relationship between substrate-induced maximum initial respiration rates and absolute microbial biomasses of soils. Symbol * shows samples of soil C which had been pre-incubated to change the size of the microbial biomass.

Table 2. Relationship between respiratory response and physiological age of mycelia incubated at 22 C in glucose-buffer solution

	Phases of cultural growth							
	Early linear		Middle linear		Late linear		Stationary	
	Days*	μl CO_2**	Days	μl CO_2	Days	μl CO_2	Days	μl CO_2
Fusarium sambucinum	2	22	6.5	44	7.5	45	9	43
	2	16	6.5	40	7.5	44	9	38
Microdochium bolleyi	3	14	8	10	11	10	14	7
Mortierella elongata	2	15	7	3	10	3	14	4
M. hyalina	2	43	4	26	7.5	16	11	12
	3	34	5	21	7.5	15	9	13
M. zonata	3	73	6	10	9	5	13	6
Paecilomyces carneus	3	78	5	28	10	31	14	21
Penicillium herquei	3	19	4	10	8	8	23	8
P. janthinellum	1.5	26	7	25	9.5	25	11	27
	2	22	6	30	9.5	15	14	14
Phoma eupyrena	3	75	6	18	9	14	22	7
Trichoderma hamatum	1.5	51	2.5	42	4	28	4.5	25
T. koningii	2	63	7	26	10	25	15	28
	3	46	7	21	9.5	20	11	21
Verticillium nigrescens	4	62	8	15	14	22	20	5
	4	59	11	19	15.5	20	16.5	19

* Days of incubation.

** μl $CO_2 \cdot$mg $C^{-1} \cdot h^{-1}$.

response is determined. To convert this response rate into a biomass unit, the value obtained is entered into a regression equation, where y is equal to the respiratory response in ml $CO_2 \cdot$unit soil$^{-1} \cdot h^{-1}$ and x is the biomass C in mg·unit soil^{-1}.

The major technical errors which can be made when using this method are incubation of soil samples at temperatures other than 22°C, which would modify the above equation, or measurement of respiration rates after changes in the size of the biomass have occurred.

By combining this method with the selective inhibition technique described by Anderson and Domsch (1973; 1975), it is possible to estimate the total living biomass of soils, and then *roughly* partition this into the sizes of the bacterial and fungal populations. The first step in the selective inhibition technique is identical to the glucose concentration experiments described in the present study: therefore, it can be immediately used to estimate the size of the total microbial biomass. For soils F, H and J, which have previously been investigated by the selective inhibition method, it has been determined that the total biomasses were c. 30, 89 and 93 mg biomass C·100 g soil^{-1}, respectively. Using the bacterial to fungal ratios for these soils (Anderson and Domsch, 1975), which were 1:4 for soil H, 1:9 for soil F and 1:4 for soil J, it can be further determined that the ratios of prokaryotic (bacterial) to eukaryotic (fungal) biomass in these soils were c. 6:24, 9:80 and 19:74 mg biomass C·100 g soil^{-1}, respectively.

Shields *et al.* (1973), who used microscopic methods to compare soil microbial biomasses in a brown chernozem soil for a period of 104 days, found that the average bacterial to fungal C was *c.* 6.8:43.4 mg·100 g soil^{-1}. Although these values are not directly comparable to those obtained with the chernozem used in this study (soil F), they are similar in range and proportion and indicate that results obtained by careful microscopic counting and by the current method are, within limits, comparable.

The correlation coefficient of $r = 0.96$ indicates that the use of Jenkinson's method for calibration of the respiratory technique is an acceptable means for converting *in situ* respiration values to microbial biomass weights. Nevertheless, a number of soils did quite substantially deviate from the ideal regression line. One possible reason for deviation is that microbial populations in different soils might consist predominantly of either young cells, which would give higher than "average" CO_2 production per unit biomass C, or aged cells, which would give lower values. With most soils, however, it can be assumed that the course of normal population dynamics will create "average" situations in which living cells of all physiological ages, including resting stages, are present. A second possible source of deviation is that microfaunal elements (protozoa, nematodes) contribute to the respiratory response. This is unlikely, however, and will probably be of little consequence to the overall immediate response to glucose amendment. A further source of deviation is the yet unexplored response of rootlets: for this reason they must be carefully removed from the soil. In regard to deviations resulting from Jenkinson's method, mineralization of resting microbial cells as well as $CHCl_3$-killed micro-

fauna, seeds or rootlets may add to the CO_2 evolved during mineralization of the fumigated microbial biomass.

The soils investigated in the present study ranged in C content from 0.78 to 39.2%, in pH values from 3.8 to 7.1, and in biomass from 15 to 240 mg·100 g soil^{-1}. Some of the methods cited in the introduction would not be applicable under these conditions, and it seems to be an argument in favour of the above proposed physiological method that it can be readily used to measure biomass in a great variety of soils.

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