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Quantification of Bacterial and Fungal Contributions to Soil Respiration

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Summary. 1. The theoretical principles and experimental procedures of a method for the direct measurement of the relative contributions of bacterial and fungal populations to soil respiration were described.

2. Differentiation was based on the selective inhibition of protein synthesis in procaryotic and eucaryotic cells by the antibiotics streptomycin and actidione.

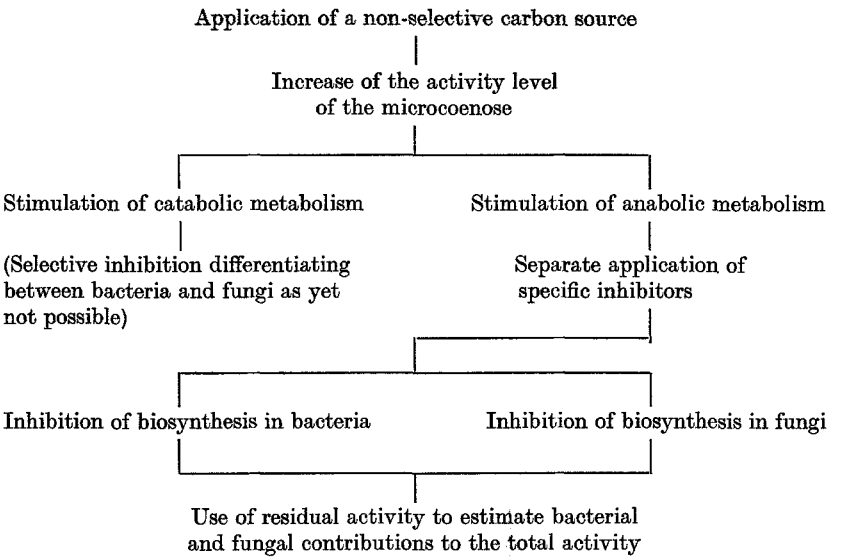
3. Using an agricultural soil as an example, it was shown that the bacterial and fungal contributions were ca. 22% and 78%, respectively.

4. The possible advantages and limitations of the method for soil ecological studies are discussed.

Microorganisms from quite different systematic categories degrade organic materials in soil. There are, however, very few substantiated data concerning the relative contributions of the various groups of organisms to the total transformations which occur in the microcoenose. Earlier studies concerning the composition of the soil microflora led mainly to floristically interesting groupings which only in exceptional cases allowed arrangement into metabolic groups. In contrast to this, measurements of overall metabolic processes of the soil have given information concerning the soil population in its entirety, but have not given information concerning individual populations. Calculation of microbial biomasses, to be sure, can give a basis for comparison of weights of different populations, but the difference between living-metabolically active, living-inactive, and dead biomass remains uncertain. In addition, because of the influence of size of organisms on their metabolic rates, no clear relationship exists between total biomass and intensity of metabolism.

In the method described below, an attempt was made to directly measure the bacterial and fungal contributions to the total metabolic activity of the soil. It was hypothesized that through the use of selective inhibitors, anabolic processes of the representative portions of the microbial population could be blocked, and that within a relatively short

period of time, especially before the development of successive populations, an estimation of the relationship of the active bacteria and fungi to one another would be possible. A preliminary description of this method has been presented elsewhere (Domsch, 1968; Anderson and Domsch, 1973). The principle of the method is shown in Scheme 1.



Scheme 1. Principle of the selective inhibition of metabolically active populations of bacteria and fungi in soil

Materials and Methods

1. Inhibitors

The bactericides used were streptomycin sulfate (Bayer) and terramycin (active ingredient, oxytetracycline: Pfizer). The fungicides were actidione (85—100% active ingredient, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide = cycloheximide: Upjohn Co.) and jadit (100% n-butyl-4-chlorsalicylamide: Farbwerke Hoechst). Inhibitors were diluted with a carrier material, talcum, and then mixed into soil. The talcum to soil ratio was 0.5 g to 100 g. Concentrations were based on dry weight of soil or wet weight of culture media.

2. Soil

The soil used, which was taken from the A-horizon of a brown podzol on sand loess above glacial outwash sand (Südgelände, Forschungsanstalt für Landwirtschaft, Braunschweig), had the following characteristics: C_t = 1.256%; N_t = 0.115%; pH (KCl) = 5.40; maximum water holding capacity = 36.2 g H_2O/100 g dry soil. Particle size distribution: 2000—630 µm; 6.6%; 630—200 µm; 20.9%; 200—63 µm; 14.0%; 63—20 µm; 34.4%; 20—6.3 µm; 11.2%; 6.3—2 µm;

2.9%; <2 μm; 9.8%. The soil was collected, stored for 24 h at 22° C, and then passed through a 2 mm sieve. Water content of the sieved soil was 40—45% of the maximum water holding capacity.

3. Organisms

Fungi were isolated from washed soil particles as described by Gams and Domsch (1967).

4. Measurement of CO_2 Production from Soil

For measurement of CO_2 production from soil, 100 g samples were filled into plexiglass tubes (25 cm long, 4 cm i.d.), which were connected to an "Ultragas 3" CO_2 analyser (Wösthoff Co., Bochum). The soil samples were either unsupplemented (control), supplemented with glucose, or supplemented with glucose plus inhibitor(s). Glucose was mixed into soil after it had been homogeneously mixed in a mortar with talcum. Even distribution was obtained when the glucose-talcum mixture (0.125 g talcum/100 g soil) was shaken with the soil in an inflated plastic bag. Glucose supplemented soil was divided into aliquots which were treated with talcum only, or talcum + the desired quantity of inhibitor(s).

5. Measurement of CO_2 from Pure Cultures

Soil (500 g) was poured into a 1 l flask and autoclaved for 120 min at 1.4 atm on 3 consecutive days. After 5 to 7 days of storage, the sterile soil was brought to 40—45% of the water holding capacity and simultaneously inoculated with either a spore suspension or a homogenate of mycelium. To prepare mycelial homogenates, 14—21 day old liquid cultures were washed twice with sterile water and then homogenized for 60 sec in 50 ml of sterile water. Soil cultures, which were incubated for 14 to 21 days (22°C), were emptied into sterile inflated plastic bags, thoroughly mixed by shaking, treated with glucose (1000 ppm), and then divided into aliquots for mixture with inhibitors followed by measurement of CO_2 production as described above. The purity of the soil cultures was checked by spreading small samples onto nutrient agar in petri plates at the beginning and at the end of each experiment.

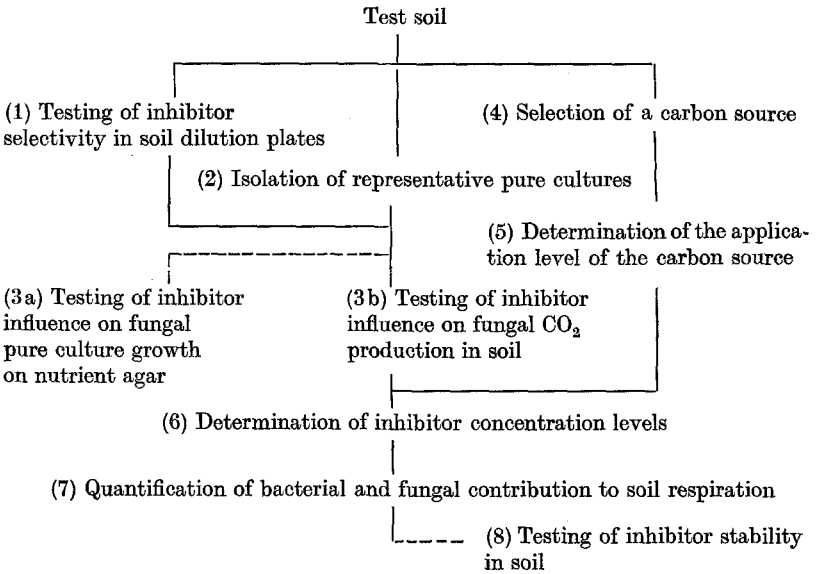
6. Growth of Colonies on Inhibitor Treated Agar

In preliminary experiments to test the effects of the inhibitors on bacterial and fungal populations, soil suspensions were mixed at 45 °C with malt agar which contained, in solution, suspension, or as an emulsion, the antifungal or antibacterial substances. Controls consisted of soil suspended in untreated agar. Five replicate plates were prepared for each variable. Incubation was at 22°C for 5 and 14 days after which fungal and bacterial (plus actinomycete) colonies, respectively, were counted. Fungal colonies were counted from $1:2\times10^5$ dilutions and bacterial colonies were counted from $1:2\times10^6$ dilutions.

Inhibitor influence on growth of pure cultures of fungi was investigated by inoculation of mycelial discs into petri plates. Six replicate plates were prepared per variable. Radial growth was measured after control cultures had grown to within 0.5 cm of the plate edges.

Experimental Results

The series of experimental steps conducted in this study are outlined in Scheme 2. The details of each experiment are presented in this section.



Scheme 2. Flow diagram of the experiments used in the selective inhibition method

1. Testing of Inhibitor Selectivity in Soil Dilution Plates

The results of the experiments (Table 1) indicated that actidione was highly selective for fungi; the first significant effects of the compound on the numbers of bacteria appearing on the plates were noted at concentrations of 2000 ppm. In contrast to this, jadit had strong inhibitory effects on both populations at concentrations as low as 250 ppm. Neither streptomycin nor terramycin decreased the numbers of fungi. They did, however, decrease bacterial (plus actinomycete) numbers almost completely at all concentrations tested.

2. Isolation of Representative Pure Cultures

The following fungi, which together represent 46.3% of the total population, were isolated from the test soil: *Phoma eupyrena* (9.1%); *Mortierella* sp. II (6.9%); *Coniothyrium fuckelii* var. *sporulosum* (4.8%); *Mortierella elongata* (4.2%); *Cladorrhinum foecundissimum* (3.9%), *Verticillium nigrescens* (3.9%); sterile mycelium DS VII (3.0%); *Mucor hiemalis* (2.7%); *Trichosporiella cerebriformis* (2.7%); *Rhinocladiella mansonii* (2.7%); *Fusarium sambucinum* (2.4%).

Fungi were chosen for pure culture experiments since definition of their populations is less complicated than in the case of bacteria.

Table 1. Test for the selectivity of inhibitors in dilution plates. Results give the average number of colonies ± the standard deviation for 5 replicate plates

	Inhibitor concentration (ppm)					
	0	250	500	1000	1500	2000
Actidione						
Bacteria	101.6 ± 10.7	—	100.8 ± 7.8	101.2 ± 2.0	92.8 ± 7.2	83.6 ± 6.0
Actinomycetes	14.2 ± 2.9	—	12.2 ± 2.3	16.2 ± 1.6	12.4 ± 2.1	9.8 ± 2.9
Fungi	15.8 ± 3.1	—	0	0	0	0
Jadit						
Bacteria	111.6 ± 6.7	8.8 ± 3.7	5.2 ± 3.7	—	—	—
Actinomycetes	10.8 ± 2.4	0	0	—	—	—
Fungi	14.5 ± 2.3	0	0	—	—	—
Streptomycin[a]						
Bacteria	82.4 ± 7.5	1.6 ± 1.5	0.4	1.6	1.4	—
Actinomycetes	8.2 ± 0.8	0	0	0	0	—
Fungi	13.8 ± 1.0	13.6 ± 1.5	16.4 ± 2.9	14.2 ± 1.7	15.8 ± 1.3	—
Terramycin[a]						
Bacteria	82.4 ± 7.5	0	0	0	0	—
Actinomycetes	8.2 ± 0.8	0	0	0	0	—
Fungi	13.8 ± 1.0	14.0 ± 1.1	18.4 ± 1.9	19.2 ± 2.9	15.6 ± 1.1	—

[a] Streptomycin and terramycin experiments were conducted simultaneously.

Table 2. Influence of inhibitors on fungal CO_2 production and growth

	Inhibition in % of the untreated control in the presence of							
	Actidione 2000 ppm		Jadit 250 ppm		Streptomycin 1000 ppm		Terramycin 1000 ppm	
	CO_2 [a]	growth [b]	CO_2	growth	CO_2	growth	CO_2	growth
Aureobasidium bolleyi	100	100	88	100	15	11	46	14
Phoma eupyrena	100	100	28	100	6	5	8	35
Mortierella sp. II	82	100	61	100	68	34	82	61
Coniothyrium fuck. var. *spor.*	100	32	74	100	0	7	0	32
Mortierella elongata	100	100	95	100	13	14	14	45
Cladorrhinum foecundissimum	100	100	100	100	81	5	63	54
Verticillium nigrescens	100	100	97	100	3	16	0	58
Mucor hiemalis	100	100	78	100	4	8	0	14
Trichosporiella cerebriformis	100	96	64	100	17	11	67	44
Rhinocladiella mansonii	41	16	31	100	11	3	16	1
sterile mycelium DS VII	100	—	63	—	8	—	20	—
Fusarium sambucinum	100	100	55	100	10	7	31	92

[a] Calculations based on total CO_2 produced up to time of peak maximum in control cultures.
[b] Calculations based on radial growth of colonies on nutrient agar.

3. Testing of Inhibitor Influence on Fungal Pure Culture Growth and CO_2 Production

In preliminary experiments, 64 experimental compounds, as well as 18 commercially available fungicide formulations, were tested for their selectivity at geometrically increasing concentrations between 10 and 200 ppm, against selected soil fungi and soil bacteria. In the majority of experiments, the highly fungicidal compounds were also found to be strongly bactericidal. The list of potentially suitable fungicides was thereby narrowed to actidione and jadit.

The results of the experiments testing the effects of actidione, jadit, streptomycin, and terramycin on the growth and CO_2 production of the fungi isolated from the test soil are shown in Table 2. The percent inhibition of CO_2 production from pure soil cultures of fungi was calculated from curves such as those shown for *Mucor hiemalis* in Fig. 1. For this purpose, the net CO_2 produced from the beginning of the experiment to the peak maximum for the glucose supplemented soil was determined. The results indicated that both jadit and actidione were highly effective inhibitors of fungal growth. The greatest inhibition of fungal CO_2 production, however, at the concentrations tested, was by actidione. Both of the antibacterial antibiotics had slight effects on growth and CO_2 production of some of the fungi. Since the streptomycin had least effect on CO_2 production, it was chosen for use in further experimentation.

4. Selection of the Carbon Source

In preliminary experiments it was shown that reduction of CO_2 evolution by streptomycin or actidione was most accurately measured when the soil was simultaneously supplemented with a carbon source. Since both antibiotics act by inhibition of protein synthesis, enough of a substrate was added to induce, at least for a short period of time, a steady and increasing rate of biosynthesis. Secondly, enough was added to allow distribution in as many microhabitats as possible. Glucose, which is the most common monosaccharide occuring in the soil, was chosen as a supplement since the majority of soil fungi and bacteria described in the literature can use it as a source for energy and growth.

5. Determination of the Application Level of the Carbon Source

The reaction of the test soil to various quantities of glucose is shown in Fig. 2. In all supplemented samples, an immediate increase in net CO_2 production was evident. A steady and increasing CO_2 production curve, which lasted for 11 h, was obtained by addition of 1000 ppm glucose. Microbiological analysis of the 1000 ppm supplemented soil showed

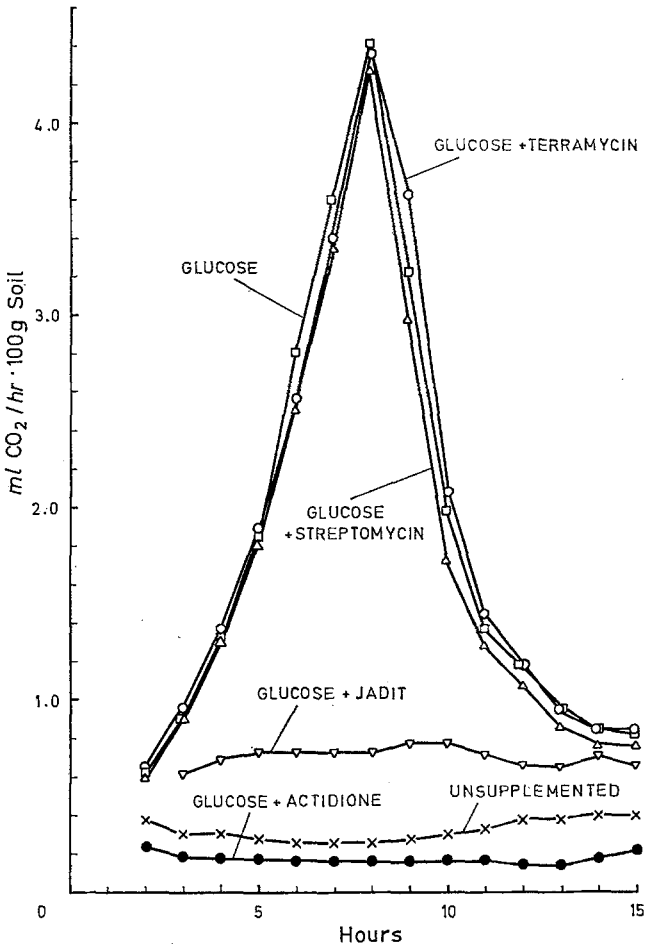


Fig. 1. Influence of streptomycin (1000 ppm), terramycin (1000 ppm), jadit (250 ppm) and actidione (2000 ppm) on CO_2 production from glucose (1000 ppm) supplemented cultures of *Mucor hiemalis*

that the increase in CO_2 production was directly related to increase in bacterial and fungal biomass.

6. Determination of Inhibitor Concentration Levels

Inhibitor concentration levels were selected as follows: first, an experiment was conducted in which a mixture containing 1 part antibacterial to 2 parts antifungal antibiotic was added in increasing quantities to glucose supplemented soil aliquots. The results (Fig. 3) indicated that at the combined application rate of 1000 ppm streptomycin plus

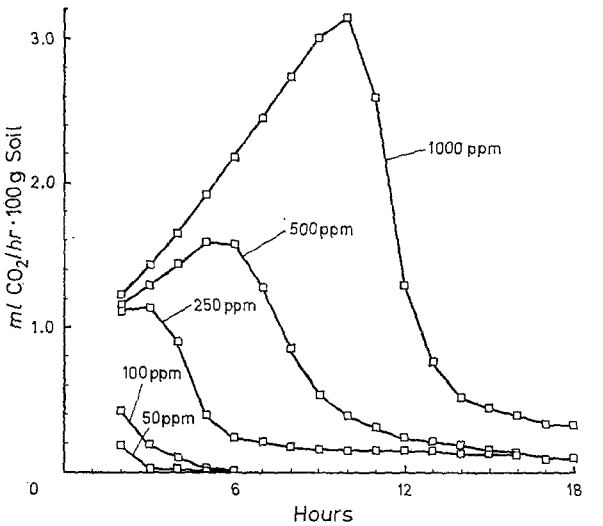


Fig. 2. CO_2 production from soil samples to which increasing quantities of glucose (50 to 1000 ppm) were added

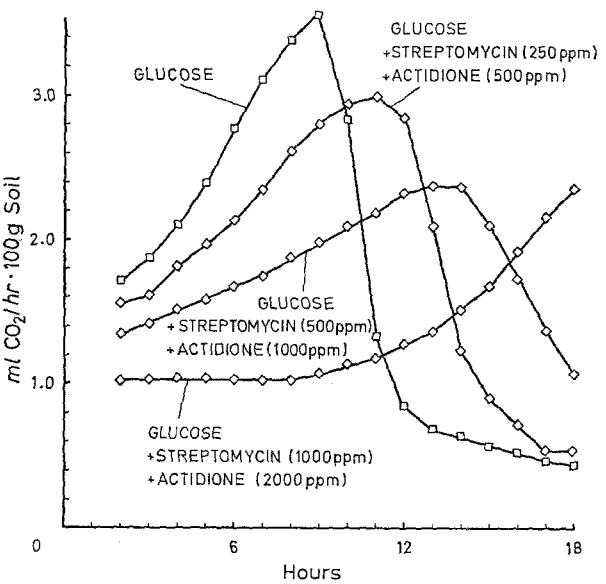


Fig. 3. Preliminary selection of inhibitor concentrations by application of increasing quantities of a streptomycin/actidione (1:2) mixture to glucose (1000 ppm) supplemented soil

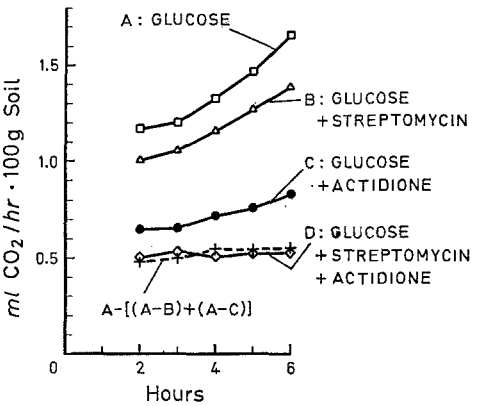


Fig. 4. Estimation of the quantitative relationship between bacterial and fungal respiration in glucose (1000 ppm) supplemented soil using streptomycin (1000 ppm) and actidione (2000 ppm). Percent bacterial respiration $= A-B/A-D$, percent fungal respiration $= A-C/A-D$

Table 3. Percent deviation of summation curves (Σ) from curves of soil samples supplemented with glucose + streptomycin + actidione (curve D). Σ = A-[(A-B) + (A-C)], where A = CO_2 production from soil + glucose, B = soil + glucose + streptomycin, C = soil + glucose + actidione. Inhibitor concentrations in D were varied in agreement with the concentrations in B and C

Hour	Actidione = 2000 ppm Streptomycin:			Streptomycin = 1000 ppm Actidione:		
	500 ppm	1000 ppm	2000 ppm	1000 ppm	2000 ppm	3000 ppm
2	−2	0	−16	+24	0	−20
3	−2	−3	−27	+18	+5	−41
4	−9	0	−40	+18	+5	−54
5	−9	0	−53	+21	0	−67
6	0	+9	−60	+21	+9	−70

2000 ppm actidione, the slope of the CO_2 production curve was zero for a period of at least an 8 h. This indicated that synthesis of new biomass, shown in the inhibitor free soil as a steadily rising CO_2 production curve, was strongly depressed. Next, starting with the application rates from the preceding experiment, a series of tests was conducted in which the concentration of one inhibitor was held constant while the concentration of the second inhibitor was varied. The basis of this series of experiments rested on the consideration that the sum of the effects of the inhibitors, when applied individually, should not exceed (or be less than) the sum of effects when the inhibitors were applied as a mixture. Large deviations

would indicate either insufficient quantities or loss of selectivity due to overdosages. The basic design for these experiments is shown in Fig. 4. In this example, the CO_2 loss caused by application of streptomycin (curve A minus curve B) plus that caused by application of actidione (curve A minus curve C) was equal to the CO_2 loss caused by addition of the inhibitors as a mixture (curve A minus curve D). The results of the experiments used to select inhibitor concentrations are given in Table 3. From this table, it is evident that either 500 or 1000 ppm of streptomycin, and 2000 ppm of actidione, were suitable for use with the test soil.

7. Quantification of Bacterial and Fungal Contributions to Soil Respiration

The data presented in Fig. 4, which were taken from 6 replicate experiments conducted over a 12-month period, were used to make a quantitative estimation of the relationship between the bacterial and fungal populations, which were capable of immediately responding to the introduction of glucose. The critical point in estimation of this ratio was that during the time chosen for calculation, major changes in the populations should not occur. For the intervals (hours 2 to 6) shown in Fig. 4, the bacterial (A—B/A—D) to fungal (A—C/A—D) ratios, in %, were: 24/76; 19/81; 22/78; 21/79; and 25/75. From these values, the average bacterial to fungal ratio could be calculated as 22/78%.

8. Testing of Inhibitor Stability in Soil

Since streptomycin (Pramer and Starkey, 1972) and actidione (Howe and Moore, 1968) can be degraded by microorganisms, tests were conducted to determine if appreciable CO_2 was formed from these compounds within the time limits of our experiments. For this purpose 100 g samples of otherwise unsupplemented soil were treated with 1000 ppm streptomycin or 2000 ppm actidione. The samples were incubated for 14 h after which the total CO_2 formed was found to be: unsupplemented soil, 7.2 ml; soil plus streptomycin, 6.7 ml; soil plus actidione, 7.7 ml. The deviations of the treated samples from the control were within the range of experimental error. Corrections for degradations of the inhibitors to CO_2 during the experiments were therefore not necessary.

Discussion

The method presented here is intended as an aid to the differentiation, quantification, and correlation of soil microbiological processes. It makes possible a reevaluation of the current evidence dealing with the ratios and relationships of bacterial and fungal metabolism in soils.

The current methods available for calculating bacterial and fungal biomasses or metabolic relationships include direct estimations using

counts of the numbers of organisms (Witkamp, 1963), or indirect calculations by extrapolation from metabolic indexes derived from pure culture experiments (Satchell, 1971). Statistical approaches by comparison of correlation coefficients have also been tried (Witkamp, 1966, 1973). These methods, however, have gained general acceptance only in special cases (Gray and Williams, 1971).

The present method was built on the premise that the metabolic activities of the microflora in the complex milieu of the soil could be entirely different than relationships observed when populations are isolated or otherwise separated from their natural habitat. Attempts were therefore made to develop a simple technique for measurement of the metabolism of either bacterial or fungal populations without their removal from the soil. The selective inhibition of respiration of either or both groups seemed a readily measurable and precise approach to this problem. Other examples of the use of inhibition of specific groups in microbiological studies are the blockage of NH_4^+-oxidation by 2-chloro-6-(trichloromethyl)pyridine (N-serve), the causal analysis of soil borne plant diseases (Haglund *et al.*, 1970), the selective isolation of microorganisms (Domsch and Schwinn, 1965; Tsao, 1970), and finally, the use of antibiotics in medicine.

The extensive overlap between the fundamental metabolic processes of bacteria and fungi (Ainsworth and Sussman, 1965; Anderson and Wood, 1969; Calvo and Fink, 1971) excluded the possibility of selectively inhibiting the total respiration of either group. Structural differences in the two cell types, however, allowed selective interference with essential anabolic processes, such as the inhibition of protein synthesis on procaryotic (bacterial) 70 S-type ribosomes by streptomycin (Jacoby and Gorini, 1967) or eucaryotic (fungal) 80 S-type ribosomes by actidione (Obrig *et al.*, 1971). In rapidly metabolizing and growing populations in soils, inhibition of protein synthesis is readily measured as a slow-down of increase in total respiration. These effects are thought to be due to disruption of synthesis of respiratory structures (Mahler *et al.*, 1968) or other as yet undefined secondary processes (Dubin *et al.*, 1963). Actidione, which in the present method is used to depress respiration of newly forming fungal biomass, has been also reported inhibitory to algae, protozoa, and other organisms having eucaryotic cells (Sisler and Siegel, 1967). Although these organisms are undoubtedly present in the soil, their contribution to the overall short term CO_2 production in response to glucose supplement is judged to be of little importance and has not corrected for in the model experiments.

Due to the limiting quantities of nutrients available in soils during most periods of the year, the general metabolism of active bacteria and fungi

is thought to be in a depressed state. To intensify both the catabolism and anabolism of these populations, and thus amplify the measurable effects of the selective inhibitors, an easily incorporated and readily degradable carbon source had to be added to the soil. Glucose was chosen for this purpose because of its general distribution (Gupta, 1967) and its apparent lack of activity as a depressor of enzyme synthesis (Macura and Kubátová, 1973) in soils. As discussed by Drobnik (1960), the primary oxidation of this substrate is attributable to both preexisting biomass (oxidative phase) and biomass formed in response to its addition (assimilative phase). After the assimilative phase is reached, the ratio between growth and maintenance metabolism appears to be independent of the glucose concentration applied (Stotzky and Norman, 1961; Freytag, 1964). Enough glucose was therefore added to induce a progressive and measurable increase in biomass production.

The duration of experiments in these studies was kept as short as possible to avoid confusing initial population ratios with those derived from the successive populations which would eventually develop in inhibitor treated soils (Ivarson and Sowden, 1959).

In application of the method, a number of considerations deserve attention. Since microbial populations in various soils differ in their reactivity to inhibitors (Harris and Woodbine, 1967; Williams and Davies, 1965) the possibility of resistance to streptomycin or to actidione should always be investigated. Similarly, the concentrations of the inhibitors, and their susceptibility to degradation to CO_2 should be retested for each soil. The time and methods of soil sampling should also be considered because of the distribution gradients of populations found at different times and in different soil horizons during the course of the year.

The use of this method for analysis of a variety of soil types will be reported elsewhere.

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