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A New Method for Monitoring Cellulose and Starch Degradation in Soils

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The guidelines (FEDERAL REGISTER 1978) proposed by the Environmental Protection Agency for registration of crop pesticides state that microbial function studies must include data on the effects of a pesticide on nitrogen fixation, nitrification, and degradation of cellulose, starch and protein. These function tests must be sensitive, rapid, easily executed, versatile and capable of monitoring metabolism of microorganisms in a soil sample.

Because a suitable method was not currently available for monitoring cellulose and starch degradation, we have developed a function test based on radiorespirometry, a methodology used extensively in our laboratory for extraterrestrial life detection (LEVIN 1962, LEVIN and STRAAT 1976a, LEVIN and STRAAT 1976b), clinical identification of microorganisms (SCHROT et al., manuscript in preparation) and rapid detection of bacteria in water (LEVIN et al. 1956) and in blood (SCHROT et al. 1973). This rapid and sensitive technique is readily applicable to monitoring starch and cellulose metabolism in soils. The method described herein quantatively measures microbial cellulose degradation by monitoring metabolically evolved $^{14}CO_2$ following addition of ^{14}C-labeled cellulose or starch to the soil sample.

MATERIALS AND METHODS

Reactions are conducted in a double vial, similar to that described by BUDDEMEYER (1974). The inner, reaction vessel is a small, cylindrical, sterilized glass vial to which the soil sample and the ^{14}C-labeled substrate are added. The outer container is a standard liquid scintillation glass vial into which is placed a 7.9 x 4.2 cm fluor strip cut from Whatman #1 filter paper. Prior to adding the strips to the outer vials they are immersed in concentrated dioxane counting cocktail (8 g PPO, 0.2 g POPOP, 100 g naphthalene and 200 ml dioxane) until saturated, and dried on a wire rack in a fume hood for 10 minutes. The strips are rolled and then added to the vials such that they line the inside wall; the potentially toxic dioxane fumes are removed by placing the vials under vacuum desiccation for forty-eight hours. Following evacuation, the scintillation vials containing the strips can be stored in the dark at room temperature. Under these conditions, they remain stable for approximately two weeks. During a reaction, these strips interact with beta

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particles of $^{14}CO_2$ metabolically evolved in the inner vial, thus, soil metabolism can be monitored by placing the entire reaction vial (i.e., inner and outer vials) in a liquid scintillation counter and continuously counting for one minute intervals as a function of time.

The counting efficiency of the technique was determined with a solution of $NaH^{14}CO_2$ (New England Nuclear) by placing 0.1 ml containing 0.1 or 0.2 µCi in the inner vial; 1.0 ml of H_2SO_4 was then added through the rubber stopper seal to cause quantitative release of $^{14}CO_2$. The observed cpms were compared to those observed when 0.1 or 0.2 uCi of $NaH^{14}CO_2$ was placed in a conventional scintillation cocktail (Oxyfluor, New England Nuclear). From this count ratio, the counting efficiency of the fluor strips in the double vial technique was determined to be 23-25%.

To conduct a test, 0.5 g of soil sample is weighed into each sterile inner vial which is then placed in the center of a fluor strip-containing scintillation vial and held in place by a small amount of silicone grease. The reaction is initiated by inoculation of each inner vial with 100 µl aliquots of ^{14}C-cellulose or ^{14}C-starch. (^{14}C-cellulose was obtained from New England Nuclear at a specific activity of 6.5 µCi/mg and solutions were prepared to contain 10 µCi/ml. ^{14}C-starch was obtained from New England Nuclear at a specific activity of 1.5 mCi/mg and solutions were prepared to contain 5 µCi/ml.) After the cellulose or starch addition, outer scintillation vials are immediately sealed with rubber stoppers and placed in a liquid scintillation counter where gas evolved from the inner vial diffuses to the outer vial, interacts with the fluor strips, and is monitored by continuous counting.

In using the test to determine the effects of a pesticide or other inhibitor on metabolism, both active and heat-sterilized soil samples are inoculated with the selected concentrations of inhibitor contained in 0.1 ml. Soil samples are then thoroughly mixed by shaking, aliquots are weighed into inner vials which are placed in the outer scintillation vials, and ^{14}C-substrate is added. In the case of long term studies, the remainder of the soil samples is incubated at room temperature for future sampling.

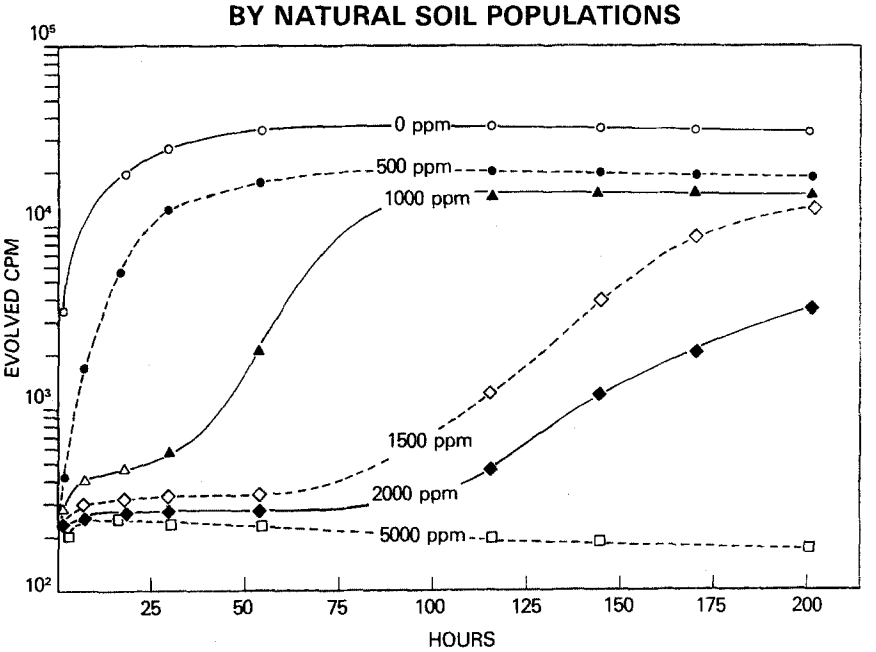
RESULTS AND DISCUSSION

The ability of the radiorespirometric method to detect microbial cellulose metabolism in soil samples is illustrated in Figure 1. As shown, upon addition of ^{14}C-labeled cellulose to the soil sample, $^{14}CO_2$ is immediately evolved. Although the reaction appears essentially complete by 20 hours, controls indicate that some gradual $^{14}CO_2$ evolution continues to occur. However, this is counterbalanced by absorption of $^{14}CO_2$ by the rubber stoppers such that a plateau is apparent. (Note that absorption is not a problem for incubation periods less than 24 hours. For longer times, an alternative to correcting for absorption is the use of rubber stoppers presoaked with $^{14}CO_2$

which minimizes the absorption phenomenon.) When corrected for counting efficiency and $^{14}CO_2$ absorption, the total $^{14}CO_2$ evolved in the reaction mixture containing only soil and ^{14}C-labeled cellulose corresponds to 0.4 μCi, or 40% of total ^{14}C-cellulose added.

Figure 1

PHENOL INHIBITION OF ^{14}C-CELLULOSE DEGRADATION BY NATURAL SOIL POPULATIONS



Each reaction mixture in a Buddemeyer double vial contained 0.5 g soil sample and 0 ppm (o——o), 500 ppm (●----●), 1000 ppm (▲——▲), 1500 ppm (◇----◇), 2000 ppm (◆——◆) or 5000 ppm phenol (□------□), as indicated. Each reaction was initiated by the addition of 100 μl ^{14}C-cellulose (UL) containing 1 μCi and 0.154 μg cellulose. Evolved radioactivity was monitored as a function of time. (Data are uncorrected for 30 cpm background.)

The effects of an inhibitor on the system have been demonstrated using phenol, a standard disinfectant. Upon addition of increasing amounts of phenol immediately prior to addition of ^{14}C-labeled cellulose (Figure 1), a reduction in the total $^{14}CO_2$ evolved is observed and the kinetics of $^{14}CO_2$ evolution are altered. Progressive inhibition is observed with increasing phenol concentration until microbial metabolism is totally inhibited at 5000 ppm phenol. At intermediate phenol concentrations (1000 ppm, 1500 ppm, 2000 ppm), it is of interest that the cellulose-degrading populations show metabolic recovery from

initial inhibition. Thus, after about 50-100 hours of inhibition, significant amounts of $^{14}CO_2$ begin to evolve. The resulting differential inhibition as a function of time is presented in Table 1.

TABLE 1

INHIBITION OF CELLULOSE DEGRADATION BY PHENOL

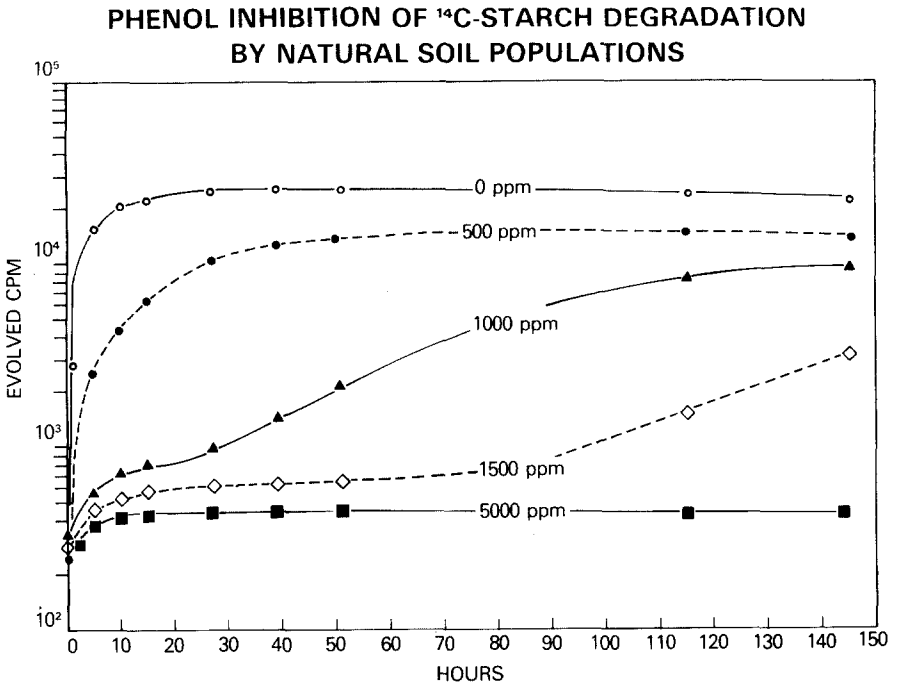
ppm phenol	% inhibition of cellulose degradation at 17 hours	% inhibition of cellulose degradation at 200 hours
500	72.5	44.0
1000	97.6	56.5
1500	98.4	60.3
2000	98.6	89.1
5000	98.7	99.5

Percent inhibition of cellulose degradation by phenol was calculated by comparison of detected cpm from untreated soil and detected cpm from phenol treated soils.

Figure 2 demonstrates the usefulness of the radiorespirometric method for monitoring microbial degradation of ^{14}C-labeled starch in soil samples. As shown, upon addition of ^{14}C-labeled starch to the soil sample in the absence of phenol, $^{14}CO_2$ is immediately evolved until the amount of $^{14}CO_2$ present in the reaction vial reaches a plateau equivalent to approximately 45% of the substrate added. Addition of varying amounts of phenol to the system similarly results in altered kinetics of $^{14}CO_2$ evolution. As observed for cellulose degradation, recovery of starch-degrading populations is observed over time at phenol concentrations of 1000 ppm and 1500 ppm whereas complete toxicity is apparent at the highest level of the inhibitor (Figure 2).

These data illustrate the potential of the radiorespirometric method as a functional test for monitoring microbial cellulose and starch degradation in soil samples. In addition to measuring percent inhibition as a function of inhibitor concentration at a specified point in time course, the technique can also be used to examine altered kinetics of $^{14}CO_2$ evolution. Further advantages of this double vial system are its sensitivity, rapidity of response, ease of execution, the capability for continuous monitoring of metabolic activity with no further sample manipulation, and the ability to test metabolic activity directly in the soil rather than in a simulated culture system.

Figure 2



Each reaction mixture in a Buddemeyer double vial contained 0.5 g soil sample and 0 ppm (o——o), 500 ppm (●----●), 1000 ppm (▲——▲), 1500 ppm (◇-----◇), or 5000 ppm phenol (■——■), as indicated. Each reaction was initiated by the addition of 100 µl ^{14}C-starch (UL) containing 0.5 µCi and 0.335 µg starch. Evolved radioactivity was monitored as a function of time. (Data are uncorrected for 30 cpm background.)

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