Pergamon

Soil Biol. Biochem. Vol. 28, No. 4/5, pp. 427–432, 1996

PII: S0038-0717(96)00004-1
0038-0717/96 $15.00 + 0.00

MEASURING BACTERIAL AND FUNGAL SUBSTRATE-INDUCED RESPIRATION IN DRY SOILS

CINTHIA K. JOHNSON,[1]* MERLE F. VIGIL,[1] KENNETH G. DOXTADER[2] and WILLIAM E. BEARD[1]

[1]Central Great Plains Research Station, USDA-ARS, Akron, Colorado 80720, U.S.A. and [2]Department of Soil and Crop Sciences, Colorado State University, Fort Collins, Colorado 80523, U.S.A.

(Accepted 19 January 1996)

Summary—The substrate-induced respiration inhibition (SIRIN) method of Anderson and Domsch for partitioning bacterial and fungal contributions to soil respiration was modified for application to dry soils. This new method also provided a comparative basis when measuring SIRIN in soils of different moisture contents. Soil was incubated under optimum moisture conditions (55% water-filled pore space) to maximize microbial activity and to ensure homogeneous incorporation of substrate and inhibitors into soil. Soil samples were packed to a uniform bulk density prior to measurement of CO_2 evolution by gas chromatography. Glucose (3 mg g^{-1}) was added together with streptomycin (0.5 or 1.0 mg g^{-1}) and/or cycloheximide (15 mg g^{-1}) for selective respiratory inhibition. The procedure included conditioning for 16 h at 4°C, followed by 1.5-h equilibration and 2-h incubation. The method yielded consistent and reproducible CO_2 respiration measurements for soils from a semi-arid region having gravimetric moisture contents ranging between 7.5 and 23.2%. Method sensitivity was not sufficient to detect variations in the fungal-to-bacterial ratio due to management practice for the soil under study. Measured fungal-to-bacterial ratios of 29:1 and 15:1, for conventionally and no-till managed soil, were not significantly different at a probability level of 5%. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Anderson and Domsch (1973, 1975) partitioned prokaryotic and eukaryotic respiration by applying selective inhibitors to the physiologically-based substrate-induced respiration (SIR) method. This method, substrate-induced respiration inhibition (SIRIN), has been used to assess bacterial and fungal contributions to glucose mineralization in the rhizosphere (Nakas and Klein, 1980), hydrocarbon mineralization (Song *et al.*, 1986) and soil respiration following herbicide application (Wardle and Parkinson, 1990b). Tate (1991) combined chloroform fumigation–incubation with SIRIN to evaluate bacterial and fungal response to fumigation.

However, SIRIN is not applicable to dry soils because most microbial cells in dry soil are dormant (Söderström, 1977; Ingham and Klein, 1984), and SIRIN measures only physiologically-active biomass. Van de Werf and Verstraete (1987a, 1987b) reported that microbial biomass, measured by SIR without water addition, was substantially smaller than estimated by fumigation–incubation using moistened samples. They concluded that the SIR method measures only metabolically-active, glucose–responsive biomass. Wardle and Parkinson (1990a) found that antibiotic inhibition of SIR was positively correlated with initial water content when it was less than 55% but independent of the original water content for samples adjusted to 55% prior to testing. Clearly, appropriate soil moisture helps to maximize microbial respiration, thereby increasing the proportion of microbial biomass measured and response to inhibitors in SIRIN studies. Adjustment of soil moisture content may be important not only for analyzing dry soil, but also for providing a comparative basis when measuring SIRIN in soils of different moisture contents.

Some investigators have moistened soil prior to SIRIN analysis, although none have developed a reliable procedure for calculating the relative proportion of fungal-to-bacterial respiration. Stamatiadis *et al.* (1990) compared direct counts of total and metabolically-active fungi and bacteria with corresponding CO_2 evolution rates using SIRIN; soils were amended with chemicals suspended in sufficient water to bring water-filled pore space (WFP) to 60%. Unusually long incubation times (1, 3 and 10 d) were used in their experiments, and no information for determining appropriate antibiotic concentrations or calculating fungal-to-bacterial ratios was given. West (1986) developed a method for SIRIN using water addition, although it yielded highly variable inhibition among replicates for arable soils. This may have occurred because: (1) the 2:1 water-to-soil ratio used (2 ml glucose and antibiotic solution plus 1 g dry-weight soil) was too high for maximum aerobic respiration, and (2) high biocide concentrations

*Author for correspondence.

caused non-target effects according to the criteria of Anderson and Domsch (1975).

There exists no adequate SIRIN procedure for use in dry soils. Additionally, present procedures do not involve the adjustment of soil moisture to maximize microbial respiration and so can not be used for comparing soils with differing amounts of moisture. Therefore, our main objective was to modify the SIRIN method of Anderson and Domsch (1975) to permit the determination of fungal-to-bacterial biomass ratios in soils of various moisture contents. A secondary objective was to examine the sensitivity of the method. Because soil management is believed to affect the relative proportion of fungi to bacteria, the method was applied to two samples of the same soil type, one under conventional and one under no-till management.

MATERIALS AND METHODS

Soil

Between May and September 1992 surface soil samples (0–5 cm) were collected at the USDA-ARS Central Great Plains Research Station, located in Akron, Colorado. The soil collected, mapped as a Weld silt loam (fine, montmorillonitic, mesic, aridic Paleustoll), was obtained from a site receiving an average annual precipitation of 420 mm.

Two sets of soil samples were collected. Soil samples used for development of the SIRIN method came from a wheat–fallow rotation managed under conventional tillage and fertilized with 35 kg N ha^{-1}. The modified SIRIN method was then applied to a second set of samples, taken in early August, from long-term plots under conventional tillage (CT) and no-till (NT) management (fertilized with 44 kg N ha^{-1}). These plots were established in 1967 on land that had been under continuous cultivation since 1907 (Table 1); a minimum of 40 random samples was taken from each of three replicate CT and NT plots, composited, and mixed.

Soil samples were sieved (<2 mm), retaining the entire sample. Occasional large pieces of organic litter (>1 cm) were removed prior to analysis. Gravimetric moisture measurements, made on pre- and post-sieve samples, ranged from 7.5 to 23.2%. Soil samples were stored in heavy plastic bags at 4°C and all analyses were conducted within 2 weeks of soil collection.

Sample evaluation procedure

Soils were treated with streptomycin sulfate and cycloheximide (Sigma Chemical Company, Cleveland, Ohio), which served as prokaryotic and eukaryotic inhibitors, respectively. Solutions of the antibiotics were prepared in distilled H_2O and added dropwise with a 10-ml syringe to 60-g (dry wt.) soil samples spread on a plastic sheet. This application increased soil moisture to 53% WFP (Linn and Doran, 1984). The samples were mixed by gripping the edges of the plastic sheeting, cut through with a metal spatula, then mixed again. After transferral to 100-ml beakers, soil samples were covered with Parafilm and held at 4°C overnight (16-h conditioning).

Then, glucose was incorporated into the soil in solution, in 0.5 ml of distilled water, by mixing and packing the samples in thirds to a bulk density of 1.26 g cm^{-3}. The final soil water content was equivalent to 55% WFP. The Parafilm-covered samples were then allowed to equilibrate (0.5, 1.5 and 2.5 h times were evaluated) after which each 100-ml beaker was placed in a sealed 0.47-l (1-pt) Mason jar that served as a CO_2 collection chamber.

Following incubation at 22°C, CO_2 evolved was measured by assaying the gases in the jar headspace. A septum in each jar lid facilitated removal of a 0.5-ml sample with a 1-ml plastic syringe. CO_2 concentrations were measured using a Beckman GC-2 g.c. with an ultrasonic detection system.

Experimental approach

Our SIRIN procedure, designed to facilitate soil moisture amendment, was developed from the SIRIN method of Anderson and Domsch (1975), as modified by Beare *et al.* (1990) for application to moistened plant residue. This procedure consisted of a 16-h conditioning of soil samples with antibiotic solutions at 4°C, a period of equilibration following glucose amendment and a shortened incubation time. Equilibration times of 0.5, 1.5 and 2.5 h and incubation times of 1, 2 and 3 h were examined. The effects of conditioning along with varying equilibration and incubation times were evaluated for: (1) consistent and reproducible CO_2 evolution rates following the addition of glucose, and (2) maximum inhibition of respiratory activity in the presence of the inhibitors.

Determination of optimal glucose and inhibitor concentrations was made for each strategy according

Table 1. Selected properties of experimental soil

				NH_4HCO_3 — DTPA extractable					
		OM	TKN	P	K	Zn	Fe	Mn	Cu
Tillage[a]	pH	%		$\mu g\ g^{-1}$ soil					
CT	6.2	2.0	0.09	6.4	666	2.6	8.9	36.7	1.5
NT	5.8	2.6	0.11	10.3	680	1.3	9.3	50.2	1.5

[a]CT = conventional tillage and NT = no-tillage.

to criteria set forth by Anderson and Domsch (1975). Concentrations of glucose (0, 1, 2, 3, 4, 6 and 8 mg g^{-1} soil), streptomycin sulfate (0, 0.5, 1.0, 1.5, 2, 3,...8, 10 and 12 mg g^{-1}) and cycloheximide (0, 5, 8, 10, 12, 15 and 20 mg g^{-1}) were evaluated. The additivity ratios determined for each pair of inhibitor concentrations were ranked to identify the most suitable combinations.

Additivity ratios were calculated after Beare *et al.* (1990) as:

$$[(G - GC) + (G - GS)]/(G - GCS) = 1 \pm 0.05$$

Where:

G = mg CO_2 h^{-1} respired with glucose amendment only
GC = mg CO_2 h^{-1} respired with glucose + cycloheximide
GS = mg CO_2 h^{-1} respired with glucose + streptomycin
GCS = mg CO_2 h^{-1} respired with glucose + cycloheximide + streptomycin

RESULTS

Glucose concentration determination

The increase in respired CO_2 for glucose-treated soil was typically 1.5 to 4 times that of basal rates (no glucose amendment) for all equilibration times (Fig. 1). Substrate-induced respiration was greatest for soil samples amended with 3.0 mg glucose g^{-1}, plateauing at higher concentrations for all incubation and equilibration times; therefore, this concentration was used in all subsequent experiments.

Inhibitor concentration determination

Cycloheximide inhibited microbial respiration with all concentrations tested (5.0 to 30.0 mg g^{-1} soil) and inhibition tended to increase with incubation time (Fig. 2). Increasing equilibration time from 0.5 to 1.5 or 2.5 h reduced the variability in inhibition with respect to cycloheximide concentration and incubation time. Percentage inhibition was highest at cycloheximide concentrations greater than 10 mg g^{-1} soil.

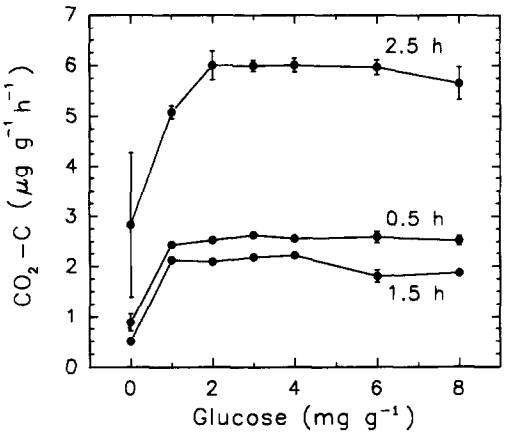

Fig. 1. Microbial respiration response to varied glucose concentrations, for three equilibration times, following 2-h incubation, for soil managed under conventional tillage. The error bars represent the standard error of the mean of replicate samples.

Streptomycin inhibition of SIR showed a higher degree of variability than cycloheximide (Fig. 2). Equilibration and incubation times of 0.5 and 1 h, respectively, resulted in increased respiration (negative inhibition) for streptomycin concentrations above 5 mg g^{-1} soil. Inhibition percentages for soil samples incubated for 2 h showed little change, while inhibitions following 3-h incubation were higher, although still inconsistent. Percentage inhibition values were more constant for soil samples incubated with streptomycin following 1.5-h equilibration, producing an inverse-sigmoidal inhibition response curve. With 1.5-h equilibration, the highest percentage inhibition was obtained after 2 h. Maximum inhibition (approximately 21%) occurred after 2 h for the samples receiving 10.0 mg streptomycin g^{-1} soil. Attempts to increase the percentage inhibition due to streptomycin by increasing equilibration time to 2.5 h proved ineffective.

Initial experiments with high concentrations of streptomycin (7.0 to 12.0 mg g^{-1} soil) coupled with 12 or 15 mg cycloheximide g^{-1} soil resulted in poor additivity, ranging from 1.17 to 1.33 (data not shown). Additivity was not improved when streptomycin concentration was maintained at 6.0 mg g^{-1} soil while adjusting cycloheximide concentration. However, by maintaining cycloheximide concentration at 15.0 mg g^{-1} soil and varying streptomycin concentration (3.0 to 6.0 mg g^{-1} soil), additivity exhibited marked improvement.

Method application experiment

Soil samples from conventionally-tilled (CT) and no-till (NT) treatments were amended with 15.0 mg cycloheximide g^{-1} soil, coupled with varying concentrations of streptomycin (0.5 to 4.0 mg g^{-1} soil) (Table 2). The procedure from method development experiments was followed, i.e. 16-h conditioning at 4°C, 1.5-h equilibration and 2-h incubation. Concentration pairs of 0.5:15.0 and 1.0:15.0 mg g^{-1} for streptomycin and cycloheximide, respectively, produced additivity ratios closest to 1.0 for both CT and NT soils. Due to the small proportion of bacteria found in this soil, some samples amended with small amounts of streptomycin (0.5 and 1.0 mg g^{-1} soil) demonstrated slightly negative inhibition. For additivity ratio calculations, negative inhibition was treated as having zero inhibition.

To calculate the fungal-to-bacterial biomass ratios for CT and NT soils, the percentage inhibitions for 8 replicate samples were averaged (4 each at 0.5:15.0 and 1.0:15.0). Replicate samples showed substantial

variability in inhibition resulting from soil amendment with streptomycin (1.7 ± 2.7% for CT and 3.0 ± 1.9% for NT), cycloheximide (48.7 ± 2.8% for CT and 42.9 ± 4.3% for NT) and streptomycin plus cycloheximide (50.8 ± 2.3% for CT and 46.0 ± 0.5% for NT). The fungal-to-bacterial biomass ratios, calculated from the observed respiratory inhibitions, were 29:1 for CT (standard deviation of 2.2) and 15:1 for NT soil (standard deviation of 5.4). An ANOVA indicated that the fungal-to-bacterial ratios were not significantly different at a probability level of 5%.

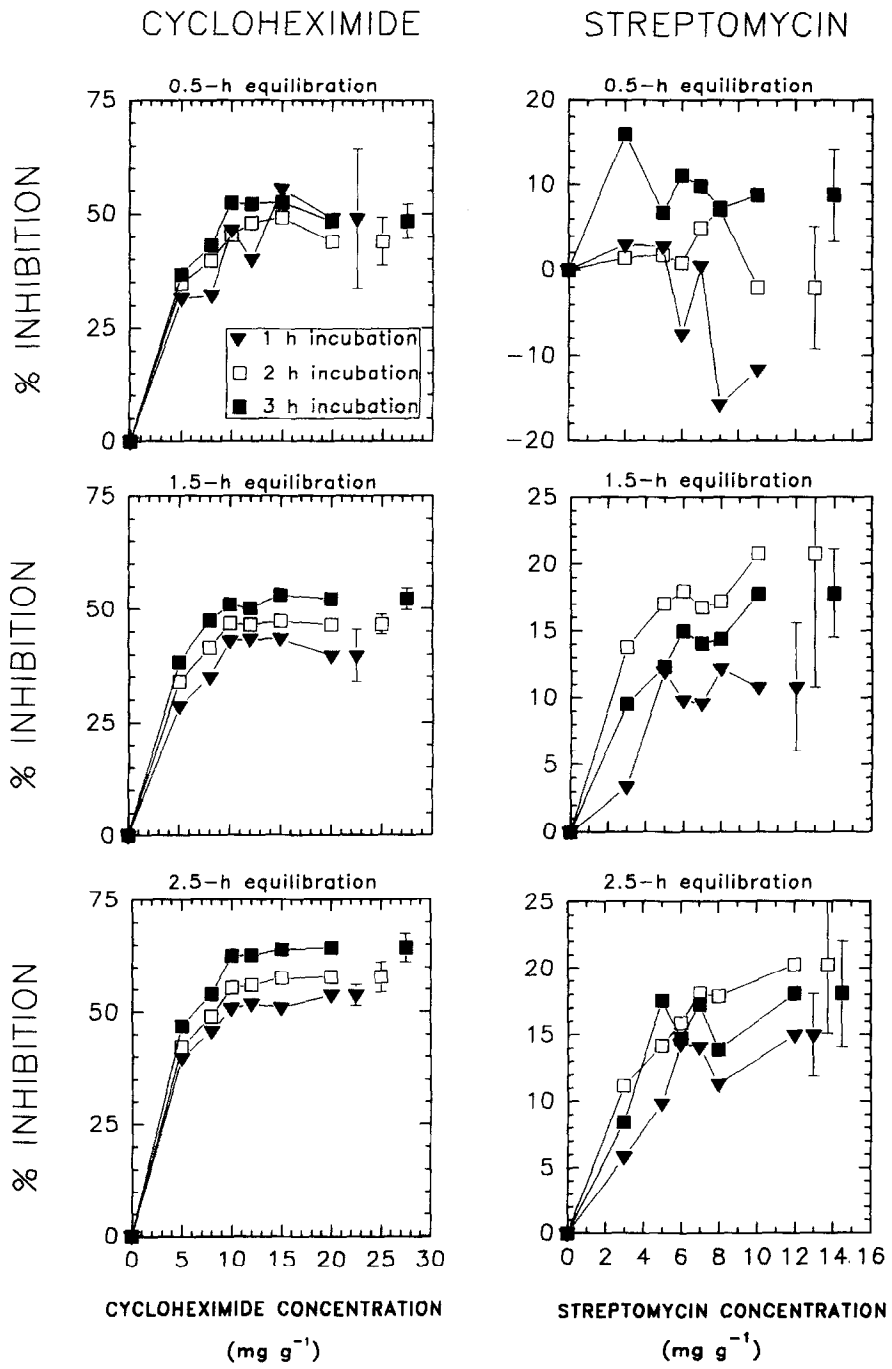

Fig. 2. Effect of varying cycloheximide and streptomycin concentrations on percentage inhibition (the percentage calculated by comparing respiration {mg CO_2 g^{-1} soil} from glucose + inhibitor-amended soil to that from soil amended only with glucose) following different equilibration (0.5, 1.5 and 2.5 h) and incubation (1, 2 and 3 h) times for soil managed under conventional tillage. The error bars represent the largest standard error of the mean for percentage inhibition at each incubation time.

Table 2. SIR response of conventional and no-till soils to paired concentrations of streptomycin and cycloheximide

Tillage	mg streptomycin g^{-1} soil[a]					
	0.5	1.0	1.5	2.0	3.0	4.0
	Inhibitor additivity ratio					
CT	0.99 ± .07	1.00 ± .08	1.08 ± .06	1.07 ± .01	1.13 ± .01	1.23 ± .00
NT	0.98 ± .11	1.02 ± .06	0.98 ± .07	1.08 ± .02	1.09 ± .10	1.17 ± .15
	Total combined inhibition (%)					
CT	51.0 ± 3.0	50.6 ± 1.8	51.2 ± 2.7	54.0 ± 0.3	54.4 ± 0.1	55.0 ± 0.2
NT	46.1 ± 0.6	45.8 ± 0.6	47.4 ± 1.7	48.6 ± 1.8	50.3 ± 0.0	50.2 ± 2.3

[a]In combination with 15.0 mg cycloheximide g^{-1} soil (equilibration time of 1.5 h). Values shown for 0.5, 1.0 and 1.5 mg streptomycin g^{-1} soil are means of four samples. Values corresponding to 2, 3 and 4 mg streptomycin g^{-1} soil are means of duplicate samples.

DISCUSSION

Accurate determination of fungal-to-bacterial ratios using SIRIN requires a procedure which results in consistent and reproducible rates of CO_2 evolution over short incubation periods. We evaluated variations in conditioning, equilibration and incubation times, the timing of water and inhibitor additions, and the quantity of water and inhibitors added to soil. The appropriate application of each of these factors resulted in stable CO_2 evolution rates required for SIRIN. For the procedure to be effective, it should be specifically tailored for a given soil type by determining: (1) the glucose concentration maximizing microbial respiration, and (2) the inhibitor concentrations yielding maximum inhibition and optimal additivity.

The SIR method requires substrate concentrations that maximize respiration rates. Because streptomycin and cycloheximide function by inhibiting protein synthesis, microorganisms must be actively growing in order for these antibiotics to be effective. However, respiratory response must be assessed following short incubation periods (during lag phase growth), prior to the onset of logarithmic microbial growth. For the soil under study, we found glucose concentrations of 3.0 mg g^{-1} dry soil to be optimal. This did not change with varying equilibration or incubation times. The C respiratory response curves produced slopes approximately equal to zero during the 3-h incubation, suggesting that soil microbial populations were in the lag phase of growth. These findings are consistent with those of Beare *et al.* (1990) for decomposing plant residues.

While incubation time must be of sufficient duration to allow microbial response to added substrate and inhibitors, incubations longer than 8 h may result in interference from resistant populations, antibiotic degradation and non-target antibiotic effects (Anderson and Domsch, 1975; Stamatiadis *et al.*, 1990). We found the shortest total incubation period yielding stable CO_2 readings and optimal additivity was 3.5 h (1.5-h equilibration plus 2-h incubation). Beare *et al.* (1990) used 2.5 h (30-min equilibration plus 2-h incubation) for SIRIN studies of plant residues. Possibly, lower microbial accessibility to substrate in soil, as compared with plant residue, prolongs microbial response to substrate.

The addition of water to dry soil samples not only enhances respiratory response but also permits the addition of substrate and inhibitors in solution, resulting in more complete soil incorporation with improved accessibility to microorganisms (West and Sparling, 1986). Stamatiadis *et al.* (1990) analyzed soil samples adjusted to 60% WFP, the volume-based water content believed to maximize aerobic microbial activity (Linn and Doran, 1984). However, respiration rates plateau between 52 and 60% WFP and decline rapidly when WFP exceeds 60% (unpubl. data from our lab); therefore, we chose 55% WFP for our study.

Soil amendment with increasing concentrations of cycloheximide and streptomycin produced respiratory inhibition similar to that described by Beare *et al.* (1990) for plant residues. In method development trials, total inhibition approached 60% following addition of both antibiotics, but centered near 50% when additivity was optimal. These levels of inhibition are consistent with those shown by Anderson and Domsch (1975), West (1986) and Wardle and Parkinson (1990b). Beare *et al.* (1990) demonstrated higher total inhibition in plant residues (near 70%), possibly due to greater contact between microbes and applied inhibitors than can occur in soils.

We found that cycloheximide concentration had little effect upon additivity when amounts producing maximum inhibition were applied (> 10 mg g^{-1} soil). Apparently, there were no non-target effects, i.e. suppressed bacterial respiration, with cycloheximide. However, streptomycin clearly affected non-target eukaryotic organisms (i.e. fungal respiration) adversely. Although inhibition exceeding 20% was obtained when high concentrations of streptomycin (10 mg g^{-1}) were applied to soil, additivity was optimal with much lower concentrations (0.5 and 1.0 mg g^{-1}); this resulted in respiratory inhibitions of 3% for NT and 6% for CT. Difficulty in measuring inhibition produced by the small streptomycin–responsive biomass present in these soils may have decreased method sensitivity.

Anderson and Domsch (1973) cautioned against both antagonistic (additivity ratio greater than 1.00) and synergistic (additivity ratio less than 1.00) reactions to inhibitors. Consistent with Beare *et al.* (1990), we found no evidence of synergism (Table 2).

For some samples, the inhibition response due to streptomycin amendment fell within the range of error for measurable CO_2 concentrations by g.c., resulting in negative inhibition. Wardle and Parkinson (1990a) described this phenomenon when applying SIRIN to soil samples having 15% gravimetric moisture content.

Consistent with the studies of Anderson and Domsch (1975), we found a predominance of fungi in our soils. While the ratios reported here, 29:1 for conventional tillage and 15:1 for no-till, are higher than were found by Anderson and Domsch (1975), Wardle and Parkinson (1990a) reported even higher ratios (49:1) when applying SIRIN to a non-moistened field soil. These variations may be due to climatic and soil management differences. Northeast Colorado is a semi-arid region with low amounts of organic matter in soil, as compared to the soils tested by Anderson and Domsch (1975). In addition, the wheat–fallow system used in this region includes shallow (surface 5–8 cm) sweep–plow tillage, with crop residues only partly incorporated into typically-dry surface soils. The filamentous nature of fungi, allowing for translocation of nutrients between soil and surface residues (Holland and Coleman, 1987), may make them better suited to utilizing these dry surface residues than are bacteria. Furthermore, fungal tolerance for low water potentials, combined with population increases following *N* application and during the summer months (Holland and Coleman, 1987), may have contributed to fungal dominance in the soil under study.

This method yielded consistent and reproducible results for freshly-collected soils from a semi-arid region having gravimetric moisture contents ranging between 7.5 and 23.2%. However, method sensitivity was not sufficient to detect variations in fungal-to-bacterial ratios due to management practices for the soil we studied. Measured fungal-to-bacterial ratios of 29:1 and 15:1, for conventionally and no-till managed soil, were not significantly different at a probability level of 5%.

Substrate-induced respiration measures only that biomass capable of utilizing the substrate applied, in this case glucose. Moreover, only a fraction of the glucose–responsive biomass is measured when adding inhibitors; less than 50% of the biomass in the soils tested was affected by amendment with cyclohexamide and streptomycin. The incomplete inhibition occurring with this method suggests that SIRIN provides, at best, only an estimate of the relative contributions of fungi and bacteria to glucose-induced respiration.

Acknowledgements—We thank Michael Beare for constructive suggestions in this research. The technical assistance of Kurt Reule and Carolyn Brandon is also gratefully acknowledged.

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