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EXPERIMENTAL ARTICLES

The Antibiotic-Aided Distinguishing of Fungal and Bacterial Substrate-Induced Respiration in Various Soil Ecosystems

E. A. Susyan[1], N. D. Ananyeva, and E. V. Blagodatskaya

Institute of Physicochemical and Biological Problems in Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Fungal and bacterial substrate-induced respiration have been distinguished in gray forest and chestnut soils in various ecosystems (forest, grassland, arable soil, fallow land, and shelterbelt) using the antibiotics cycloheximide and streptomycin. The optimal inhibitory concentrations of the antibiotics, added separately and in combination; the preincubation time of the antibiotics with the soil before glucose addition; and the mass of added inert material (talc) have been determined. The inhibitor additivity ratio (IAR) has been calculated for the antibiotics. With the IAR differing from 1.0 by a value of more than 5%, the fungal and bacterial substrate-induced respiration cannot be distinguished reliably. Respiration measurements show that the microbial communities of natural ecosystems are dominated by fungi (81–95% on average). The smallest amount of fungi (54–59%) is found in the arable soil ecosystem.

Key words: soil, antibiotics, fungi, bacteria.

The microbial biomass of soil is an important parameter in ecological studies. The proportion of fungi to bacteria, which are the major components of the microbial community of soil, depends on the type of soil and the degree of its disturbance. The fungal-to-bacterial biomass ratio can serve as an indicator of the degree of deterioration occurring in natural ecosystems [1, 2]; the pollution of soil with, for instance, SO_2 [3]; the intensity of management of upland grassland [4]; and varying soil pH [5]. The fungi and bacteria present in soil can be distinguished by (1) counts on agar media; (2) direct microscopy; (3) determination of specific cellular components, such as diaminopimelic acid, chitin, adenosine-5'-triphosphate, ergosterol, and fatty acids; and (4) physiological approaches. It should, however, be noted that each of these methods has certain disadvantages. For instance, counting on agar media allows only a small fraction (0.01–1%) of a soil's microbial population to be detected. The specific cellular components of fungi and bacteria may occur outside the cells. Direct microscopy can distinguish fungal and bacterial cells, but fails to detect their metabolic activity. Many of these disadvantages can be avoided through the use of a selective respiratory inhibition method [6, 7], which involves a selective inhibition of the substrate-induced respiration (SIR) of fungi and bacteria by specific inhibitors. The most commonly used inhibitors are streptomycin and cycloheximide, which suppress the synthesis of bacterial (70S) and fungal (80S) ribosomes, respectively [8]. The accuracy of the selective inhibition technique is still the subject of debate [9].

The aim of this work is to optimize the procedure for evaluating fungal and bacterial contributions to the total substrate-induced respiration of soil and to determine the structure (in other words, to estimate the relative amount of fungi and bacteria) of the microbial community for soils of various ecosystems.

MATERIALS AND METHODS

Soil. The experiments were carried out with samples of gray forest, chestnut, and dark chestnut soils collected in various ecosystems from a depth of 0–5 cm. The soils had close pH values but a different content of organic matter and microbial biomass (Table 1). A mixed soil sample from each ecosystem had roots removed, was sieved through a screen with a 2–3 mm mesh size, and was stored in a fridge at 10°C at its natural moisture capacity until further use. Before the experiment, the soil samples were incubated for 3 days at room temperature (22°C) at a 55% water-holding capacity.

Substrate-induced respiration. This parameter (SIR) was estimated from the initial rate of microbial respiration in the soil samples after their enrichment with glucose to provide a source of carbon and energy [10].

Antibiotics. The preliminary experiments were aimed at determining the concentrations of antibiotics (streptomycin and cycloheximide added separately and

[1] Corresponding author; e-mail: susyan@rambler.ru

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Table 1. The organic carbon content (C_{org}), microbial biomass (MB), and pH of the soils under study

Soil (0–5 cm)	Ecosystem	Location	Plant	C_{org}, %	pH_{aque}	MB, μg C/g
Gray forest	Forest	Moscow region, Pushchino	Aspen, birch	2.42	6.0	1817
	Grassland		Perennial grasses	1.76	6.6	1354
	Arable soil		Wheat	0.96	5.8	447
Dark chestnut	Shelterbelt	Volgograd region, Netkachevo	Oak	1.55	6.4	1033
	Fallow soil		Grasses	0.49	6.0	229
	Arable soil		Mustard	1.03	6.3	473
Chestnut	Shelterbelt	Volgograd region, Plemkhoz	Elm	1.57	7.7	933
	Fallow soil		Fescue, wormwood	1.99	6.3	961
	Arable soil		Sunflower	0.96	8.2	546

in combination) that provided for the maximum inhibition of the respiration of soil microorganisms. Cycloheximide was added to the soil at different concentrations 0.5, 1, 4, 8, and 16 h before the addition of glucose. Streptomycin was added 0.5 and 1 h before the glucose addition. In the case of a combined addition of the antibiotics to a soil sample, the sample was first incubated with cycloheximide for 4 h and then with streptomycin for 0.5 h, after which glucose was added. As a control, we used soil samples to which only glucose was added. The final moisture capacity of the soil samples was 60–65% of the total. To ensure a better distribution of cycloheximide over the soil samples, the antibiotic was mixed with the inert material talc at a proportion of 1 : 2. The control samples also contained talc in an equivalent amount.

The inhibitor additivity ratio (IAR) was calculated for streptomycin and cycloheximide using the formula

$$IAR = [(A - B) + (A - C)]/(A - D),$$

where A is the respiration with glucose, estimated as the amount of CO_2 evolved from the soil; B is the respiration with glucose and cycloheximide; C is the respiration with glucose and streptomycin; and D is the respiration with glucose, streptomycin, and cycloheximide [11]. IAR = 1 implies that streptomycin and cycloheximide show neither an additive nor antagonistic effect, IAR > 1 implies a high additive effect for streptomycin and cycloheximide, and IAR < 1 implies the occurrence of an antagonism between streptomycin and cycloheximide. In the two last cases, the confidence value for measurements performed by the selective inhibition technique is low.

Fungal and bacterial respiration. The fungal and bacterial components of soil respiration (FR and BR, respectively) were calculated using the following formulas:

$$FR = (A - B)/(A - D) \times 100\%,$$

$$BR = (A - C)/(A - D) \times 100\%,$$

where A, B, C, and D have the same definitions as in the previous paragraph. As was already mentioned, the two last formulas are valid provided that $A - [(A - B) + (A - C)] = D \pm 5\%$ [12].

All the measurements were performed in five replicates. The data are expressed as the average values followed by the standard deviation from the mean.

RESULTS

The concentration of inhibitors. Figure 1 shows the effect of streptomycin (panel a) and cycloheximide (panel b) on the substrate-induced respiration (SIR) of gray forest soil collected in forest and arable soil sites. Streptomycin was added to these soils at concentrations that varied from 10 to 40 and from 10 to 60 mg/g of soil, respectively. The inhibition of SIR by streptomycin was observed at antibiotic concentrations exceeding 10 mg/g (arable soil) and 20 mg/g (forest soil). The inhibition of SIR by cycloheximide was observed at concentrations exceeding 20 mg/g (arable soil) and 30 mg/g (forest soil).

In the case of chestnut soils, the inhibition of SIR by streptomycin was observed at concentrations exceeding 5 (arable soil) and 10 (fallow land and shelterbelt) mg/g of soil (Fig. 2). When streptomycin was added to the soil at higher concentrations (20–30 mg/g of soil), it enhanced the emission of CO_2 from the soil, which can be attributed to the utilization of streptomycin by soil microorganisms resistant to this antibiotic [3, 13]. When cycloheximide was added to the chestnut soil taken from shelterbelt and arable soil at a concentration of 15 mg/g of soil, it inhibited SIR by 49 and 59%, respectively. At lower concentrations (5 and 10 mg/g of soil), cycloheximide inhibited SIR by 4 and 28% (arable soil and shelterbelt, respectively) and by 44 and 35% (arable soil and shelterbelt, respectively).

Thus, as a rule, the inhibitory effect of streptomycin was greater in the case of arable soils, which have a lower microbial biomass than untilled soils. The same pattern, however, was not observed in the case of cycloheximide.

The effect of soil preincubation with the antibiotics. Extending the time of soil preincubation with streptomycin from 0.5 to 1 h before the addition of glucose

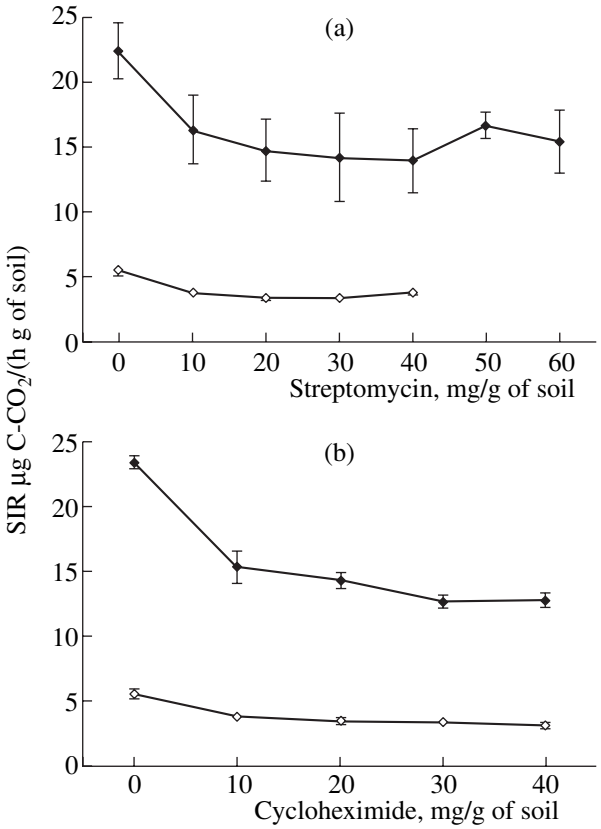

Fig. 1. The effect of different concentrations of (a) streptomycin and (b) cycloheximide on the degree of inhibition of the substrate-induced respiration of gray forest soil in (–◆–) forest and (–◇–) arable field.

was not accompanied by a noticeable increase in the degree of respiration inhibition (Table 2). For this reason, further experiments were performed with the preincubation time equal to 30 min.

Similar experiments with cycloheximide showed that, when this antibiotic was incubated with the soil samples for 0.5 and 1 h, it inhibited SIR by 8 and 16%

Table 2. Inhibition of the substrate-induced respiration (SIR) of soil as a function of the preincubation time with streptomycin

Soil (streptomycin, mg/g)	Ecosystem	SIR inhibition, % ± s.d.	
		0.5 h	1.0 h
Gray forest soil (40)	Forest	46 ± 6	34 ± 1
	Arable soil	25 ± 5	22 ± 7
Chestnut soil (5)	Shelterbelt	28 ± 1	28 ± 3
	Fallow soil	11 ± 3	15 ± 2
	Arable soil	38 (2 mg/g) ± 3	33 ± 4

Note: s.d. stands for "standard deviation".

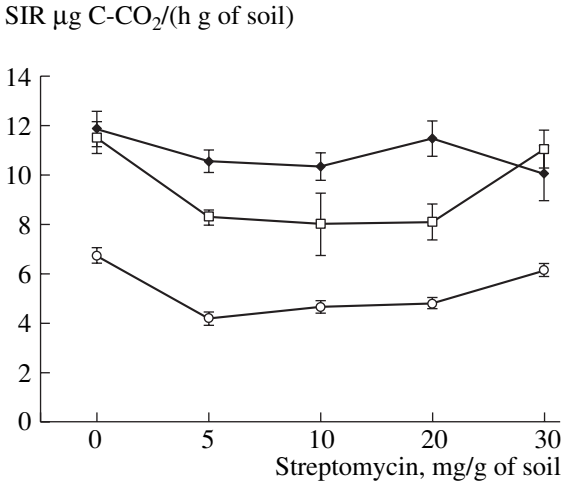

Fig. 2. The effect of different concentrations of streptomycin on the degree of inhibition of the substrate-induced respiration of chestnut soil in (–◆–) fallow field, (–□–) shelterbelt, and (–○–) arable field.

in the case of arable and forest sites of gray forest soils, respectively. Extending the preincubation time to 4 h provided for SIR inhibition of 21 and 46% (arable and forest sites of gray forest soils, respectively). Extending the preincubation time to 8 and 16 h did not result in a noticeable increase in the degree of SIR inhibition (Table 3). The inhibitory effect of cycloheximide correlated with its concentration.

The effect of the inert material talc. The inhibitory effect of cycloheximide on SIR increased when the antibiotic was added to the soil samples in the form of a mixture with talc, reaching a maximum at a cycloheximide/talc ratio of 1 : 2. It is known that talc stimulates the respiration of soil by promoting the liberation of CO_2 from the organo-mineral complex of the soil [10]. Our experiments also showed that the respiration of soil was higher in the presence of talc. For this reason, talc was also added to the control soil samples in an amount corresponding to double the mass of the added cycloheximide.

The inhibitor additivity ratio of the antibiotics. The inhibition of the SIR of gray forest soil and the IAR of the antibiotics calculated from the SIR data are presented in Table 5. At IAR < 1, the antibiotics inhibited SIR to a lesser degree than when IAR was within 1 ± 5%. For this reason, the experimental variants for which IAR < 1 were excluded from consideration [7, 11]. For the gray forest soil collected in a forest environment, the appropriate inhibitor combination was found to be either streptomycin at a concentration of 2 mg/g of soil plus cycloheximide at a concentration of 15 mg/g of soil or 8 mg/g streptomycin plus 15 mg/g cycloheximide (the IAR values for these inhibitor combinations are given in Table 5 in bold). The latter combination was the most appropriate because it inhibited SIR to the greatest degree (by 59%).

For arable gray forest soil, three appropriate inhibitor combinations were found: 2 mg/g streptomycin plus 15 mg/g cycloheximide, 4 mg/g streptomycin plus 5 mg/g cycloheximide, or 4 mg/g streptomycin plus 15 mg/g cycloheximide (Table 5). The best inhibitor combinations were the first and the third because they inhibited SIR to the greatest degree (by 60 and 59%, respectively). The combination of 4 mg/g streptomycin plus 5 mg/g cycloheximide, even though its IAR was within the accepted range (1 ± 5%), inhibited SIR by as little as 40%. For this reason, the respective results were excluded from consideration.

Optimization of the combined action of the antibiotics. The optimal combination of the antibiotics is expected to occur when (1) either antibiotic exerts a stable inhibitory effect on SIR; (2) the antibiotic combinations inhibit SIR to the maximum degree; and (3) the IAR of the antibiotics is within the range 1 ± 5%. Table 6 summarizes the data for various types of soil and ecosystems. For instance, when streptomycin and cycloheximide were added separately to the soil sample from fallow land, they inhibited soil respiration by 12 and 51%, respectively. The combined addition of the antibiotics suppressed soil respiration by 60%, which comprised approximately the sum of the inhibitory effects of the individual antibiotics (12 + 51 = 63%). For gray forest soil collected in grassland, the total inhibition of the soil respiration comprised, on average, 88%. The IAR for these experiments was within the accepted range (1 ± 5%).

Proportion of fungal to bacterial contributions to the SIR of various soils and ecosystems. Calculations showed that the fungal contribution to the SIR of gray forest soil collected in grassland and forest locations was 90 and 81%, respectively (Fig. 3). For dark chestnut soil collected in an oak forest and from fallow land, the fungal contribution to SIR was 84 and 95%, respectively, and, for the arable gray forest and dark chestnut soils, 59 and 54%, respectively.

Thus, the measurement of the fungal and bacterial contributions to SIR by a method optimized with respect to the concentrations of the antibiotics and the contact time of a particular antibiotic with the soil samples before the addition of glucose showed that the fungal contribution to the SIR of the soils found in natural ecosystems (forest, shelterbelt, fallow soil, and grassland) varied from 81 to 95% while, in the case of arable soils, this contribution was lower (between 54 and 59%).

Table 3. Inhibition of the substrate-induced respiration of arable chestnut soil as a function of the preincubation time with different concentrations of cycloheximide

Cycloheximide, mg/g	SIR inhibition, % ± s.d.		
	4 h	8 h	16 h
5	4 ± 3	1 ± 1	14 ± 6
10	21 ± 2	3 ± 3	9 ± 4
20	23 ± 5	15 ± 2	17 ± 4
30	21 ± 4	17 ± 1	21 ± 1

Note: s.d. stands for "standard deviation".

Table 4. The effect of different concentrations of cycloheximide and talc on the degree of inhibition of the substrate-induced respiration of gray forest soil (the preincubation time was 4 h)

Ecosystem	Cycloheximide, mg/g	SIR inhibition, % of the control	Cycloheximide + talc (mg/g of soil)	SIR inhibition, % of the control
Forest	5	ND	5 + 10	0
	10	35	10 + 20	37
	15	30	15 + 30	50
Arable soil	5	ND	5 + 10	39
	10	31	10 + 20	44
	15	37	15 + 30	57

Note: ND stands for "not determined".

DISCUSSION

It is known that the concentrations of antibiotics needed to inhibit the respiration of soil fungi and bacteria depend on the properties of the soil in question. For instance, the inhibitory concentrations of the antibiotics for arable and forest soils were found to be 0.5–0.6 mg/g and up to 16 mg/g of soil, respectively [7, 14]. The effect of the antibiotics when added to the soil at high concentrations may be mitigated due to their adsorption by humus. Nevertheless, the autochthonous microbial community of forest soil can adapt to the utilization of complex plant detritus and humus components [14]. Our experiments also showed that fertile soils rich in organic matter and biomass should receive elevated concentrations of antibiotics.

Most of the experiments aimed at distinguishing the fungal and bacterial components of SIR in arable soil [6, 7], forest soil [15], grassland soil [16, 17], and plant debris [18] have shown that the microbial soil community is dominated by fungi, which agrees with our data.

It should be noted that researchers who have comparatively evaluated the methods of selective respiratory inhibition and direct microscopic examination came to different conclusions. In particular, Lin and Brookes [12] and Beare *et al.* [18] concluded that the two methods give comparable results, but West [16] and Velvis [19] disagreed with this conclusion. West found that the proportion of fungi (F) to bacteria (B) in two grassland soils when estimated by the selective respiratory inhibition and direct microscopy techniques was F : B = 1 : 1 and 1 : 2, respectively. In the case of arable soils, direct microscopy revealed three times as much

Table 5. The effect of different concentrations of cycloheximide and streptomycin, added separately and in various combinations, on the degree of inhibition of the substrate-induced respiration of gray forest soil (forest and arable soil) and the inhibitor additivity ratio (IAR) of the antibiotics

Antibiotics	Antibiotic concentration, mg/g of soil	SIR inhibition, % of the control ± s.d.		IAR	
		Forest	Arable soil	Forest	Arable soil
Cycloheximide	5	0	39 ± 1	–	–
	10	37 ± 4	44 ± 4	–	–
	15	50 ± 4	57 ± 8	–	–
Streptomycin	2	5 ± 1	8 ± 1	–	–
	4	6 ± 1	1 ± 1	–	–
	8	14 ± 4	23 ± 1	–	–
Streptomycin + cycloheximide	2 + 5	13 ± 7	35 ± 8	0.04	1.31
	2 + 10	52 ± 4	46 ± 3	0.79	1.10
	2 + 15	54 ± 3	60 ± 2	**0.98**	**1.04**
	4 + 5	15 ± 10	40 ± 1	0.01	**1.01**
	4 + 10	47 ± 6	52 ± 1	0.85	0.87
	4 + 15	56 ± 9	59 ± 4	0.92	**0.98**
	8 + 5	21 ± 7	43 ± 7	0.52	1.39
	8 + 10	42 ± 2	38 ± 15	1.18	1.67
	8 + 15	59 ± 3	27 ± 4	**1.03**	2.69

Notes: s.d. stands for "standard deviation". IAR is only calculated when the antibiotics are added in combination.

Table 6. The effect of different concentrations of cycloheximide and streptomycin, added separately and in various combinations, on the degree of inhibition of the substrate-induced respiration of soils in various ecosystems and the inhibitor additivity ratio of the antibiotics

Soil	SIR, μg C-CO_2/(h g of soil); (SIR inhibition, % of the control); mean ± standard deviation				
	Without antibiotics	Streptomycin (10; 2; 10)*	Cycloheximide (10; 10; 15)*	Streptomycin + cycloheximide (10 + 10; 4 + 4; 10 + 15)*	IAR
Fallow chestnut soil	9.76 ± 0.56 (0)	8.59 ± 0.8 (12 ± 8)	4.79 ± 0.48 (51 ± 7)	3.87 ± 0.64 (60 ± 5)	1.04
Arable dark chestnut soil	5.84 ± 0.18 (0)	4.14 ± 0.43 (29 ± 7)	4.02 ± 0.3 (31 ± 5)	2.45 ± 0.33 (58 ± 6)	1.04
Gray forest soil in grassland	16.17 ± 0.61 (0)	14.95 ± 0.33 (11 ± 2)	3.55 ± 0.79 (79 ± 5)	2.01 ± 0.49 (88 ± 3)	1.02

* The concentrations of streptomycin, cycloheximide, and their combinations are shown in mg/g of soil for fallow chestnut soil, arable dark chestnut soil, and gray forest soil in grassland, respectively.

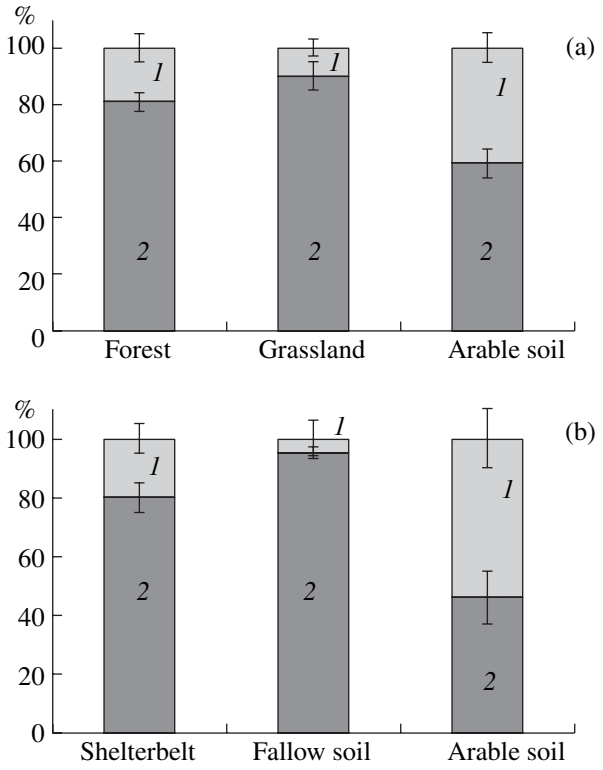

Fig. 3. Diagram showing the proportion of (*2*) fungi and (*1*) bacteria in gray forest (a), fallow chestnut, and dark chestnut (shelterbelt and arable soil) (b) soils.

soil fungi as bacteria; however, the selective respiratory inhibition method failed to distinguish fungi and bacteria [16]. In the arable soils of Denmark, the F : B ratio was found to be 0.5–0.6 with the selective respiratory inhibition method and 0.19–0.46 using direct microscopy [19]. In some soil horizons, both methods have given comparable results [20]. We believe that the results obtained by the selective respiratory inhibition method commonly correlate with the results obtained by direct microscopic examination.

It is noteworthy that the degree of SIR inhibition and the calculated values of IAR indicate the high accuracy of the selective respiratory inhibition method. For instance, at IAR = 1.4, the F : B ratio varies from 1.64 to 2.29, averaging 1.97 [11]. Our experiments showed that high IAR values correspond to low values of SIR inhibition.

It should be emphasized that the selective respiratory inhibition method is based on the assumptions that, first, the F : B ratio is the same in microbial components sensitive and insensitive to antibiotics [16] and that, second, the fungal and bacterial components of a microbial biomass respond to glucose in a similar way [17]. The inhibitors of fungal and bacterial respiration (recall that they are evaluated by the evolution of CO_2 from soil) cannot provide for their complete suppression. In our experiments, the degree of respiratory inhibition varied from 60 to 90%. The complete inhibition of soil respiration cannot be achieved for the following reasons: (1) glucose is metabolized in soil into CO_2 due to the functioning of constitutive enzymes; (2) these enzymes undergo degradation, as is evident from the fact that the evolution of CO_2 tends to decline 2 h after the addition of antibiotics and plateaus between 4 and 9 h, whereas new enzymes are not synthesized because of the inhibitory action of the antibiotics; (3) extending the incubation time does not increase the inhibition of soil respiration, presumably because of the growth of antibiotic-resistant microorganisms.

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