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Novel antibiotics as inhibitors for the selective respiratory inhibition method of measuring fungal:bacterial ratios in soil

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Abstract The use of the selective inhibition (SI) method for measuring fungal:bacterial ratios may be limited due to biocide selectivity and the overlap of antibiotic activity. This study evaluated novel pairs of antibiotics for their specificity in soils of different origins and their potential reduction in inhibition of non-target organisms. Four soils selected for this study were from a semi-arid shrub-steppe, a loblolly pine forest and two grassland sites (restored and farmed prairie plots). Three bactericides were tested: oxytetracycline hydrochloride, streptomycin sulphate, and bronopol. Three fungicides were tested: captan, ketoconazole, and nystatin. The inhibitor additivity ratio and fungal:bacterial ratios were calculated from control and treated soils where inhibition was measured as CO₂ respiration reduction with biocides. We were able to minimize non-target inhibition by the antibiotics to <5% and thus calculate reliable fungal:bacterial ratios using captan to inhibit fungi in all four soils, and bronopol to inhibit bacteria in three of the four soils. The most successful bactericide in the restored prairie was oxytetracycline-HCl. Our results demonstrate that application of novel antibiotics is not uniformly successful in soils of different origin and that the SI technique requires more than just optimization of antibiotic concentration; it also requires optimization of antibiotic selection.

Keywords Novel antibiotics · Respiratory inhibition method · Fungal:bacterial ratio · Bactericide · Fungicide

Introduction

The structure of the soil microbial community changes in response to the environment. One level at which this may be monitored is the relative contributions of fungi and bacteria to the soil microbial biomass. High, or increasing, ratios of fungi:bacteria have been proposed as indicators of ecosystem reversion to “natural” states (Bardgett and McAlister 1999). The ratio of fungi:bacteria has also been used to assess the stress placed on a soil by the presence of pollutants such as SO₂ (Bewley and Parkinson 1985) and to monitor changes in intensity of management of upland grassland (Bardgett et al. 1996). Anderson et al. (1981) found that the fungal:bacterial ratio in soil could be changed with ordinary biocide use. Fungicides have also been shown to affect soil microflora, N dynamics and plant growth (Chen and Edwards 2001; Chen et al. 2001). The fungal:bacterial ratio in soils affects litter decomposition and nutrient cycling and can be useful for understanding and studying ecological systems (Bardgett et al. 1996; Yeates et al. 1997; Bardgett and McAlister 1999).

The selective inhibition (SI) procedure for quantifying the relative contributions of fungi and bacteria to soil microbiological activities was first proposed in 1973 (Anderson and Domsch 1973) and later evaluated for agricultural and forest soils (Anderson and Domsch 1975). Since then, this technique has been modified and refined for specific investigations of plant residue decomposition (Neely et al. 1991), N dynamics and soil organic matter decomposition (Landi et al. 1993; Lin and Brookes 1999a) and amended or pesticide-treated soils (Anderson et al. 1981; Heilmann et al. 1995; Lin and Brookes 1999a). The original technique used cycloheximide to inhibit fungal activity and streptomycin sulphate to inhibit bacterial respiration with subsequent determination of the fungal:bacterial ratio ($F:B$) by:

$$F : B = \frac{A - B}{A - C} \quad (1)$$

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where: A =respiration measured (as evolved CO_2) in the absence of inhibitors; B =respiration in the presence of the fungicide; and C =respiration in the presence of the bactericide. This pairing of cycloheximide with streptomycin sulphate has been shown to act on non-target organisms (Landi et al. 1993; Badalucco et al. 1994), furthermore, the hazardous nature of cycloheximide makes it an unattractive antibiotic with which to work.

Other antibiotic pairs have been used in the SI procedure such as captan (fungicide) and oxytetracycline (bactericide) (Beare et al. 1992). However, recent work has shown that in some soils, the addition of optimized concentrations of a number of antibiotics can cause substantial amounts of non-target inhibition creating activity overlap (Bailey et al. 2002). The inhibitor additivity ratio (IAR) is used to calculate the degree to which the activities of the antibiotics overlap (Beare et al. 1990) and is calculated from:

$$\text{IAR} = \frac{(A - B) + (A - C)}{A - D} \quad (2)$$

where D =respiration measured in the presence of both antibiotics together. An IAR of 1.0 indicates that there is no overlap in the antibiotics' actions on non-target organisms and that there is no antagonistic effect of one antibiotic on the other. The overlap is identified by an $\text{IAR} > 1.0$ and the antagonistic effect by an $\text{IAR} < 1.0$. In some soils, IAR values can greatly exceed 1.0, causing the estimation of the fungal:bacterial ratio to be uncertain. For example, a desert soil with a calculated F:B ratio of 1.97 had an IAR of 1.4, thus the range for the actual F:B ratio would be 1.64–2.29 (Bailey et al. 2002). This range is obtained by calculating the ratio for two extremes. The first extreme assumes that all of the non-target inhibition was caused by the fungicide, causing the ratio to be overestimated and the second extreme underestimates the ratio by attributing all of the non-target inhibition to the bactericide. When the SI procedure was first proposed, a tolerance level of $\pm 5\%$ was proposed (Anderson and Domsch 1975). Velvis (1997) noted this criterion and was able to accommodate it in three of four study soils. In contrast, it has been suggested that the most accurate estimations of F:B are achieved when the concentrations of antibiotics that suppress the maximum amount of respiration are used, even if the IAR significantly exceeds 1.0 (West 1986). In this vein, others who have used SI have reported IAR values as large as 3.12 for the SI procedure using cycloheximide and streptomycin sulphate (Imberger and Chiu 2001). This large IAR decreases the confidence of the reported fungal:bacterial ratio of 1.46.

There are several different types of fungicides available, with varying mechanisms of action. These biocides may function by inhibiting the synthesis of uniquely fungal compounds such as ergosterol, the primary sterol in fungal cell membranes (Heilmann et al. 1995; Heit and Riviere 1995), or by selectively targeting uniquely fungal structures and thereby killing the present population. Others that may have merit as a selective inhibitor in soil include the azole derivatives, such as ketoconazole, which

are broad-spectrum antifungal agents. They inhibit fungal activities by blocking the synthesis of ergosterol (Heit and Riviere 1995; Hart and Brookes 1996). Furthermore, not only is ergosterol production limited, methyl sterols subsequently accumulate in the cell and alter normal cell activity (Heit and Riviere 1995).

There are several bactericides that may be useful in inhibiting bacterial respiration in soils. Two that have received little attention are oxytetracycline hydrochloride and bronopol. The actions of these antibiotics are similar to streptomycin in that they inhibit protein synthesis (Lancini et al. 1995). Bronopol may exhibit some antifungal properties but this has not been reported for oxytetracycline hydrochloride, thus the IAR of these biocides should be different (Lin and Brooks 1999a).

Studies focused on optimizing the concentrations of antibiotics used in the SI procedure have been reported (Alphei et al. 1995). We report here research on optimizing the selection of antibiotics for SI for a wide range of soils. Our goal was to examine novel antibiotics for their potential usefulness in the SI procedure, in an attempt to keep the IAR values near 1.0. Three fungicides were examined: captan, nystatin, and ketoconazole. Three bactericides were examined: oxytetracycline hydrochloride, streptomycin sulphate, and bronopol. Our paper demonstrates that application of novel antibiotics is not uniformly successful in soils of different origin and that the SI procedure requires more than just optimization of antibiotic concentration; it also requires the optimization of antibiotic selection.

Materials and methods

Soils

Four soils from different ecosystems were selected for this study: a semi-arid shrub-steppe soil with little organic C (8.5 mg g^{-1}), a sandy (63% sand) forest soil collected from a loblolly pine plantation, and two grassland soils, one which is currently farmed to corn and neighboring land that was restored to native tallgrass prairie in 1979. At each ecosystem location a number of soil samples (>6) were taken within a 20 by 20 m^2 area. The soils were sampled to a depth of 5 cm, composited, sieved through a 2-mm sieve, thoroughly mixed then packed on ice packs and shipped overnight to the laboratory. Once received at the laboratory the soils were kept at 4°C until analysis. Some soil characteristics are presented in Table 1.

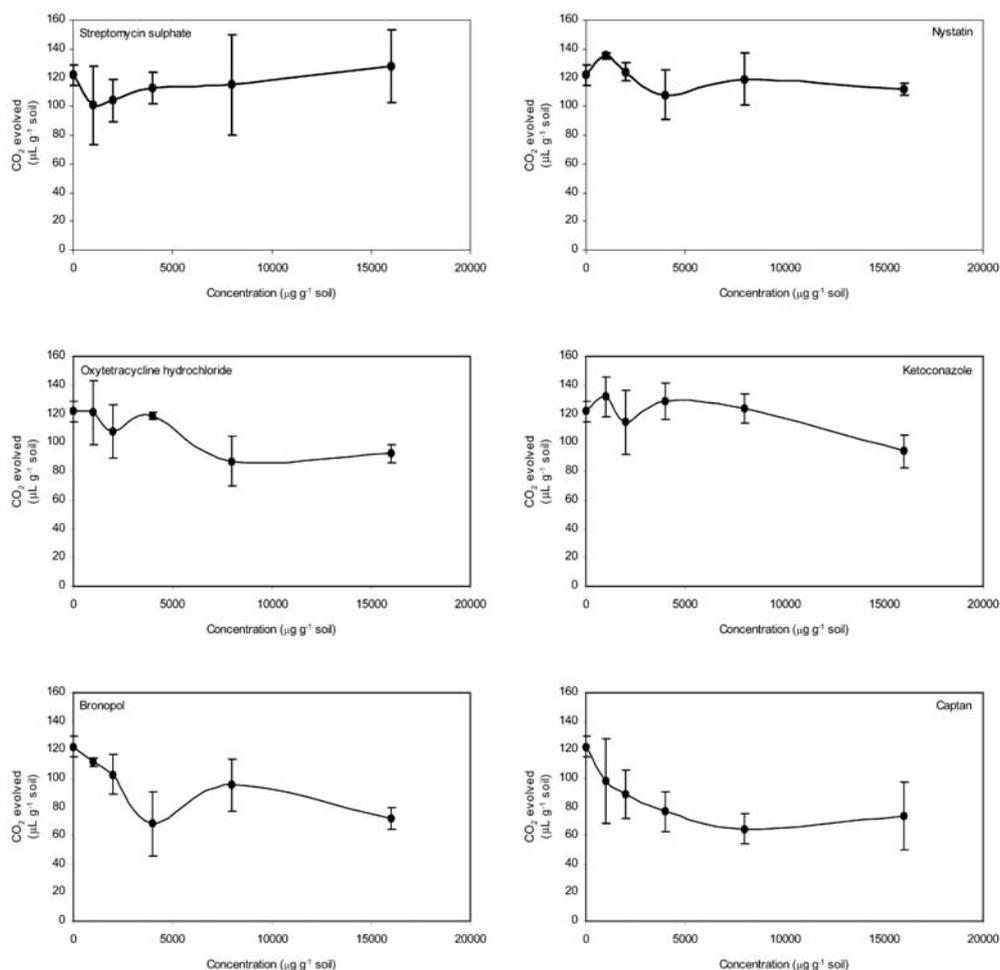
Soil pH was measured on a 1:1 saturated paste with a glass electrode. The texture was analyzed at a university testing laboratory using a continuous flow particle size analyzer. Both total C and N were measured using a combustion GC-infrared analyzer.

Table 1 Selected physical and chemical characteristics of the study soils

Soil	pH	Texture	Total C (mg g^{-1})	Total N (mg g^{-1})
Loblolly pine	5.1	Sandy loam	18.3	0.65
Shrub-steppe	6.9	Loam	8.5	0.85
Prairie farmed	5.6	Clay loam	36.0	3.45
Prairie restored 1979	7.3	Silty loam	49.9	4.59

Table 2 Selective inhibition (SI) treatment parameters and biocide additions for the five study soils, selected from Figs. 1, 2, 3, 4as the optimum pairing and concentrations

Soil	Glucose addition rate ($\mu\text{g g}^{-1}$ soil)	Antibiotics			
		Bactericide	($\mu\text{g g}^{-1}$ soil)	Fungicide	($\mu\text{g g}^{-1}$ soil)
Loblolly pine plantation	1,000	Bronopol	2,000	Captan	4,000
Desert	1,500	Bronopol	1,000	Captan	1,000
Prairie farmed	4,000	Bronopol	1,000	Captan	2,000
Prairie restored 1979	4,000	Oxytet-HCl	8,000	Captan	8,000

Fig. 1 Inhibition of respiration in a loblolly pine forest soil by the bactericides streptomycin sulphate, oxytetracycline-HCl, and bronopol, and by the fungicides nystatin, ketoconazole, and captan. Error bars represent the SD from a mean of three replicates

Antibiotics

A total of six antibiotics were examined for their potential as selective inhibitors (not all data shown). Three bactericides were tested: oxytetracycline hydrochloride (henceforth “oxytet-HCl”), streptomycin sulphate (henceforth “streptomycin”), and bronopol. Three fungicides were tested: captan, ketoconazole, and nystatin. The efficacy of these antibiotics in the soils was tested at 1,000, 2,000, 4,000, 8,000, and 16,000 μg antibiotic g^{-1} soil.

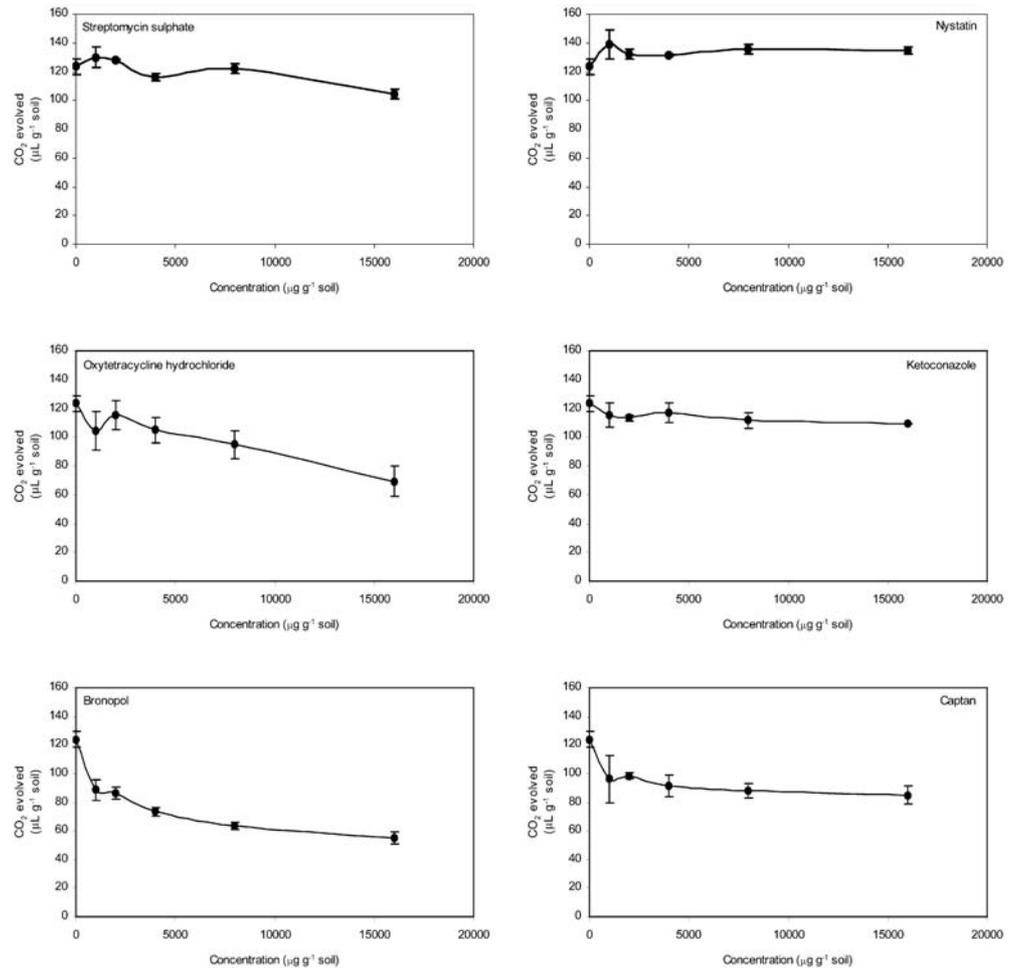
Selective inhibition

Soils (5 g dry weight) were weighed into 50-ml test tubes and pre-incubated at their one-third bar water content 2 weeks prior to the SI incubation. Enough tubes were incubated to account for multiple trials of three replicates in the individual antibiotic concentration

incubation as well as the combined inhibition incubation to estimate the IAR (Eq. 2) using the optimized antibiotics.

SI was conducted according to a modification of the technique described by (Anderson and Domsch 1975). Preliminary experiments indicated that in general maximum inhibition was achieved in a soil when the antibiotics were added 1 h before the addition of glucose. All antibiotics were added with talc (Anderson and Domsch 1973); the total mass of antibiotic added to each soil sample was brought to a concentration of 0.02 g insoluble material g^{-1} soil. One hour following antibiotic addition glucose was added as 0.1 ml aqueous solution in a concentration to achieve maximal respiration (over 6 h) for each soil (1,000–4,000 μg glucose g^{-1} soil; Table 2). Tubes were sealed with air-tight valves immediately following glucose addition, and placed in the dark at 22°C ($\pm 1^\circ$) for 6 h. At the end of the 6-h incubation, headspace CO_2 was withdrawn through the Mininert valve with a gastight syringe and measured on a Hewlett Packard 5890 series II gas chromatograph

Fig. 2 Inhibition of respiration in a semiarid shrub-steppe (desert) soil by the bactericides streptomycin sulphate, oxytetracycline-HCl, and bronopol, and by the fungicides nystatin, ketoconazole, and captan. *Error bars* represent the SD from a mean of three replicates



equipped with a 4.5-m carboxen column. The injection temperature was 150°C , and the detector temperature was 300°C . The initial oven temperature was 150°C , and ramped up to 210°C in the first 2 min. The total run time was 4.5 min and CO_2 elution had an R_t of 3.9 min.

The combined SI was conducted as described above with the exception that both inhibitors were added to a set of six tubes at the beginning of the incubation to determine the effect of the antibiotic combination on respiratory activity.

Results and discussion

To select which bactericide and fungicide were most appropriate for applying the SI procedure to a soil, plots of the respiration response of the soil to a range of concentrations of each antibiotic were examined (Figs. 1, 2, 3, 4). From these, we eliminated antibiotics that did not significantly depress respiration at any concentration. When the concentration of antibiotic that caused the greatest suppression of respiration was used in the procedure, the IARs were often much greater than 1.0. If non-target inhibition was occurring, it should happen in the presence of the concentrations of antibiotics that cause maximum inhibition of soil respiration when added individually. Thus, to minimize the risk of inhibiting

non-target organisms, optimal concentrations were selected to be those either just less than the concentration causing maximal inhibition or the concentration where the depressional plateau in the curve just begins. Trials using antibiotics at concentrations that cause maximum inhibition led to IARs >1 for all soils (data not shown). For example, when captan and bronopol were both applied to the farmed prairie soil at $8,000 \mu\text{g g}^{-1}$ soil, an IAR of 1.60 was obtained. We reduced these concentrations stepwise, one antibiotic at a time until an IAR of ~ 1.0 was obtained. The concentrations at which this occurred were $1,000 \mu\text{g g}^{-1}$ soil for bronopol and $2,000 \mu\text{g g}^{-1}$ soil for captan.

For the loblolly pine soil, streptomycin and nystatin were both ineffective at suppressing the soil respiration at any point on the concentration curve (Fig. 1). Further examination found that of the remaining two fungicides and two bactericides, the best pairing was bronopol and captan, at $2,000$ and $4,000 \mu\text{g g}^{-1}$ soil, respectively. Other combinations were attempted using these antibiotics in combinations with oxytet-HCl and ketoconazole; however, the pairing of bronopol with captan at these concentrations combined to generate an IAR very close to 1.0 (1.03). When added together this combination of biocides inhibited the greatest proportion of the glucose-stimulated

Fig. 3 Inhibition of respiration in a farmed prairie soil by the bactericides streptomycin sulphate, oxytetracycline-HCl, and bronopol, and by the fungicides nystatin, ketoconazole, and captan. Error bars represent the SD from a mean of three replicates

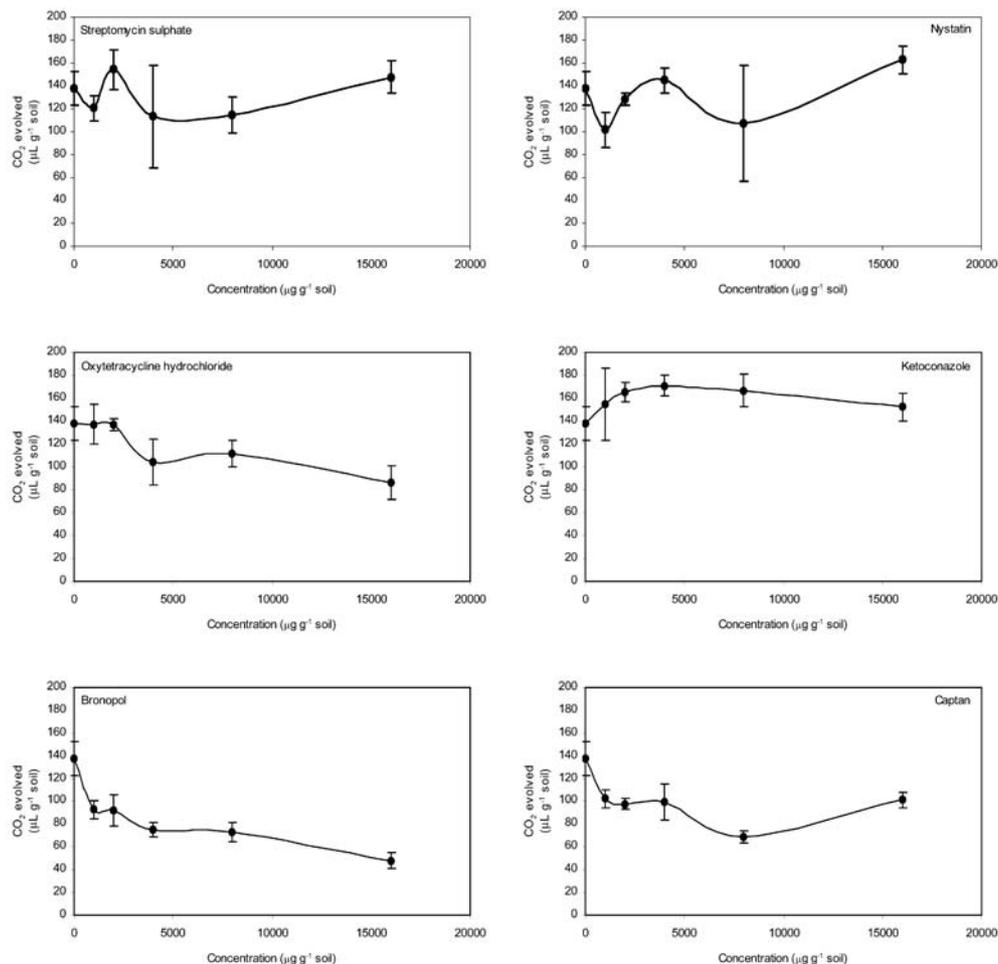


Table 3 Fungal:bacterial ratios (*F:B*) and inhibitor additivity ratios (*IAR*) for study soils subjected to S1 with inhibitors listed in Table 2 (means of three replicates)

Soil	Bacterial respiration ($\mu\text{L g}^{-1}\text{ soil}$)	Fungal respiration ($\mu\text{L g}^{-1}\text{ soil}$)	F:B	IAR	% Inhibition
Loblolly pine plantation	26.9	21.4	0.80	1.04	39.3
Desert	28.9	21.4	0.74	1.01	42.5
Prairie farmed	35.8	29.1	0.81	0.99	50.2
Prairie restored 1979	10.4	132.9	12.76	1.00	59.2

respiration in the absence of antibiotics (%inhibition=39%; Table 3). An equation derived from Eq. 2, $A-D/A \times 100$, was used to calculate the percent inhibition. Using Eq. 1 the fungal:bacterial ratio was estimated to be 0.79 (Table 3). Earlier work with this soil using oxytet-HCl and captan produced an IAR of 1.13. Based on this overlap, it was expected that the F:B ratio would fall between 0.85 and 1.43 (Bailey et al. 2002).

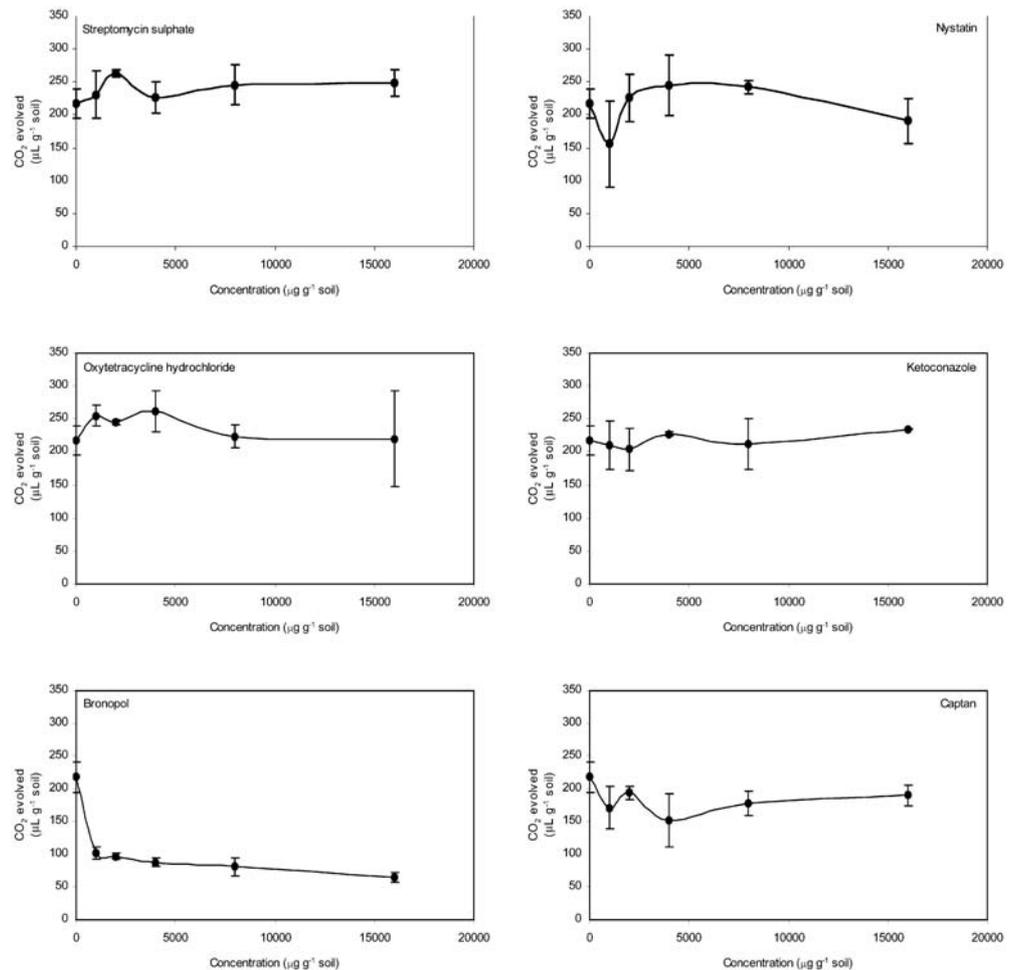
Only oxytet-HCl, bronopol, and captan depressed respiration in the desert soil (Fig. 2). The best pairing of antibiotic concentrations was captan ($1,000 \mu\text{g g}^{-1}$) with bronopol ($1,000 \mu\text{g g}^{-1}$), which combined to produce an IAR of 1.01 and an F:B of 0.74. These antibiotics were able to inhibit 42% of the respiration when added together to the desert soil (Table 3).

In the farmed prairie soil, ketoconazole did not inhibit soil respiration at any concentration, and at some

concentrations respiration was increased above the respiration of the control (Fig. 3). This suggests that ketoconazole may have been used as a substrate. The nystatin and streptomycin were rejected as possible antibiotics because of the large amounts of variation in the amount of respiration inhibited by the different concentrations (Fig. 3). The best pairing identified was bronopol ($1,000 \mu\text{g g}^{-1}\text{ soil}$) with captan ($2,000 \mu\text{g g}^{-1}\text{ soil}$). The bronopol inhibition curve decreased steadily as the concentration increased, thus the chosen concentration of bronopol and captan was the initial concentration observed to depress respiration. Using this pair of antibiotics, which had an IAR of 0.98 resulted in a calculated F:B ratio of 0.81 and together the antibiotics inhibited 50% of soil respiration.

The restored prairie soil was the most difficult to optimize for antibiotic pairing and concentrations. Strep-

Fig. 4 Inhibition of respiration in a restored prairie soil by the bactericides streptomycin sulphate, oxytetracycline-HCl, and bronopol, and by the fungicides nystatin, ketoconazole, and captan. *Error bars* represent the SD from a mean of three replicates



tomycin, nystatin, and ketoconazole showed little ability to depress respiration (Fig. 4). Of the bactericides, bronopol was the most effective inhibitor; however, all combinations using bronopol at $1,000 \mu\text{g g}^{-1}$ soil, with captan as the fungicide, resulted in IARs of 2–4.5, indicative of large overlaps. It was concluded that microorganisms in this soil were very sensitive to bronopol, and that these included non-target microorganisms. Consequently, the best pair of antibiotics was oxytet-HCl ($8,000 \mu\text{g g}^{-1}$ soil) and captan ($8,000 \mu\text{g g}^{-1}$ soil). Together, they inhibited 59% of the soil respiration and produced an IAR of 1.00. The F:B ratio calculated from this pairing was 12.8.

Similar soils and litter can behave in a consistent fashion when subjected to increasing levels of glucose (Smith et al. 1985; Scheu and Parkinson 1994), however, the response can vary dramatically for soils from different ecosystems (Lin and Brookes 1999b). This can also be true for the biocides used for SI using the substrate-induced respiration method. Due to these conditions a suite of different biocides, and biocides with different modes of action, are advantageous for optimizing the inhibition of respiration and the IAR. For example, Scheu and Parkinson (1994) found that their soils did not respond to glucose. Thus a protein inhibitor would be of

little use in the inhibition method unless it also interfered with enzyme synthesis of the non-growing population. In this case an alternative biocide may have been more useful. In addition, a suite of biocides may be useful for sites that have suffered heavy metal contamination or for bioremediation sites where the condition or treatment favors one group of organisms over another group.

In conclusion, in this study a series of fungicides and bactericides were examined to find antibiotic pairs to serve as alternatives and improvements to cycloheximide. Of the fungicides we tested, the best option for fungicidal activity was captan, a fungicide traditionally used for the SI procedure. Streptomycin, which is the traditional bactericide for the SI procedure was the least useful bactericide in the four soils examined. Oxytetracycline was effective in at least one of the four soils and may have been acceptable in some of the others. In a novel application, the bactericide bronopol was the best option for three of the four soils. However, it was also the antibiotic most likely to suppress non-target organisms if used in excessive concentrations.

We conclude that novel antibiotics may improve the usefulness of the SI procedure for assessing fungal and bacterial contributions to microbial activity in different soils; no single pair of antibiotics is likely to be

universally applicable to all soils, even those of similar geographic origin. Care must be taken when selecting antibiotics and their concentrations to ensure that enough antibiotic is used to affect the population, but that the antibiotic does not inhibit non-target organisms. The SI procedure yields information that is useful for a wide variety of soil ecological purposes, however, one should not expect that its simple underlying principles be reflected by a quick and simple incubation. Refinement of this procedure for the best concentrations of antibiotics and now for the best selection of antibiotics can be time-consuming as the target IAR of 1.0 is sought.

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