Effects of long-term fungicide applications on microbial properties in tallgrass prairie soil

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Abstract

Several studies investigating the role of arbuscular mycorrhizal (AM) fungi in plant communities have included manipulations of AM fungal symbiosis using the fungicide benomyl. The objectives of this study were to evaluate the potential non-target effects of benomyl on soil biota and nutrient cycling in tallgrass prairie and to determine how mycorrhizae may influence these belowground properties. To accomplish these objectives, soil samples were collected during the 1996–1997 growing seasons from long-term benomyl-treated plots established on tallgrass prairie (Manhattan, KS) in 1991, and the following measurements were made: total bacterial and fungal biomass; abundance of nematodes; microbial biomass carbon and nitrogen; substrate-induced respiration; and potentially mineralizable C and N. Long-term benomyl applications resulted in an 80% reduction in mycorrhizal root colonization. By reducing root colonization, benomyl applications resulted in significant decreases in total bacterial biomass and abundance of fungal-feeding and predatory nematodes (20, 12 and 33% reductions compared to control, respectively). Total microbial potential activity (i.e., substrate-induced respiration) increased by 10% with benomyl treatment, whereas the relative contribution of fungi to total microbial activity decreased significantly with benomyl applications. In addition, microbial biomass C increased from 1364 (± 51.2 SE) to 1485 (± 51.2 SE) with benomyl treatment, and total carbon increased significantly (~8%) only in annually burned soils treated with benomyl. The magnitude of benomyl effects on soil components and processes were small (<33% change with benomyl) relative to effects on mycorrhizal root colonization (80% decrease with benomyl). These results indicate that rather than having large non-target effects, benomyl applications principally affect mycorrhizal root colonization, thereby indirectly influencing soil biota and nutrient availability. Results also indicate that mycorrhizal fungi play an important role in altering the availability and flow of carbon in prairie soil and may influence the composition and abundance of groups of some soil biota. Changes in soil organisms and nutrient availability associated with altered mycorrhizal symbiosis may influence aboveground plant species responses to mycorrhizal suppression, but further research is needed to understand these potential effects. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Arbuscular mycorrhizal fungi; Benomyl; Microbial biomass; Nematodes; Carbon; Nitrogen

1. Introduction

A number of greenhouse and field studies have shown that arbuscular mycorrhizal (AM) fungi influence plant growth, competition, and species diversity (e.g., Hartnett and Wilson, 1999; Hetrick et al., 1989; Newsham et al., 1995). These studies have examined the role of AM fungi in plant communities by experimentally reducing mycorrhizal root colonization with applications of fungicides. In particular, several studies have utilized benomyl (Benlate®, DuPont) to effectively suppress AM fungal root colonization in the field (e.g., Hartnett and Wilson, 1999; Newsham et al., 1995). Benomyl inhibits fungal sporulation, spore germination, and hyphal growth (Schreiner and Bethlenfalvay, 1997) without significantly influencing the availability...
of soil P (Merryweather and Fitter, 1996) or directly affecting a wide range of plant species (Paul et al., 1989). Recent research indicates that AM fungal species may differ in responsiveness to benomyl treatments (Schreiner and Bethlenfalvay, 1997). Differential tolerances and susceptibilities of AM fungal species to benomyl and, conversely, selectivity of benomyl for certain AM fungi could lead to shifts in AM fungal communities. These changes in AM fungal species composition could potentially affect plant responses and interactions, because host-specific responses to mycorrhizal fungi do exist (e.g., Schreiner et al., 1997).

Besides the direct effects on AM fungal dynamics, benomyl applications can affect other soil organisms (e.g., Jakobsen, 1994), which, in turn, may have indirect effects on plant responses. For example, Newsham et al. (1994) found that improved performance of the mycorrhizal plant species, Vulpia ciliata spp. ambigua, with benomyl applications could be explained by the negative effects of benomyl on plant pathogenic fungi. The non-selectivity of benomyl in this study indicates that the plant responses observed with benomyl-related mycorrhizal suppression may be due partly to net effects on both mycorrhizal and pathogenic fungi. In addition, benomyl may indirectly affect other soil organisms or alter nutrient cycling through its effect on mycorrhizal fungi, which interact with a wide range of soil organisms and may play a critical role in nutrient dynamics (e.g., Linderman, 1988; Miller and Jastrow, 1994). Such alterations could potentially influence the interpretation of experimental results from studies utilizing benomyl applications to determine the role of mycorrhizal fungi in plant communities.

In tallgrass prairie, AM fungi affect plant growth, alter plant competitive interactions, and increase plant species diversity (e.g., Wilson and Hartnett, 1997). Recently, Hartnett and Wilson (1999) conducted a long-term field study to determine the role of mycorrhizal fungi in determining tallgrass prairie plant species diversity. The authors suppressed mycorrhizal root colonization by applying benomyl every two weeks to native prairie plots for five growing seasons. The application of benomyl resulted in significant decreases in cover of highly mycorrhizal-responsive plant species (i.e., obligate mycotrophs). In contrast, several subdominant grass and forb species, which exhibit low and more variable mycorrhizal responsiveness (Wilson and Hartnett, 1998), increased in abundance with mycorrhizal suppression. Because the applications of benomyl resulted in reduced growth of the obligately mycorrhizal grasses rather than an increase in growth as would be expected with suppression of pathogenic fungi, this suggests that benomyl primarily affected root colonization by mycorrhizal fungi and not pathogenic fungi. The authors attributed the observed increase in plant diversity to reduced competition from the dominant mycorrhizal-dependent grass species. However, benomyl-mediated changes in the abundance or activities of other soil organisms (excluding AM fungi or pathogenic fungi) or changes in nutrient availability also may have contributed to plant species responses to long-term benomyl applications.

Despite the potential non-target effects of benomyl applications on experimental outcomes and the common use of fungicide applications in the field and greenhouse, few studies have quantified changes in non-fungal soil organisms or nutrient dynamics associated with fungicide treatments. In addition, few studies have focused on the role of mycorrhizal fungi in ecosystem-level processes, despite evidence from greenhouse research that mycorrhizae may influence soil microbial populations and nutrient cycling and, which, in turn, may be influenced by soil biota (see reviews by Ingham, 1988; Linderman, 1988). Overall, little is known about the role of mycorrhizae in soil biotic interactions and/or nutrient dynamics in the field or the potential effects of these interactions on plant communities.

The purpose of this study was to evaluate potential effects of benomyl applications in the field on several components of the soil biota and soil processes. Specifically, the abundance and activities of major microbial groups, abundance of nematodes by trophic group, and changes in nutrient availability and retention were measured in long-term (7 years) benomyl-treated plots and in untreated control plots. These measurements can provide indirect evidence of the role of mycorrhizae in belowground processes, assuming that changes in soil organisms and nutrient dynamics are related directly to altered AM fungal associations. Because previous work in tallgrass prairie had indicated complex interactions between fire frequency, soil communities and processes, and plant responses, the effects of benomyl applications and the influence of mycorrhizal fungi on soil biota and nutrient cycling were examined in both frequently and infrequently burned sites.

2. Materials and methods

2.1. Study site

Research was conducted on the Konza Prairie Research Natural Area, a 3480 ha tallgrass prairie preserve located in the Flint Hills region of northeastern Kansas, USA (39°05' N, 96°35' W). Konza Prairie is owned by The Nature Conservancy and Kansas State University, Manhattan, KS and is managed for long-term ecological research by the Division of Biology at
Kansas State University. Vegetation of Konza Prairie is dominated by warm-season C₄ grasses *Andropogon gerardii*, *Sorghastrum nutans*, *Andropogon scoparius*, and *Panicum virgatum*. Interspersed within the matrix of warm-season grasses are a number of subdominant warm- and cool-season (C₃) grasses, forbs, and woody species (Freeman, 1998). Average monthly temperature for Konza Prairie ranges from a minimum of ~2.7°C in January to a maximum of 26.6°C in July, and average annual precipitation is 835 mm with the majority (75%) falling during the growing season (Hayden, 1998).

2.2. Experimental design

Four sites at Konza Prairie were selected for this study: two annually burned and two infrequently burned (i.e., every 10 or 20 years). A split-plot experiment with burning as the whole plot treatment and fungicide application as the subplot treatment was established on upland, Florence cherty silt loam soils (Clayey-skeletal, smectitic, mesic Udic Argiustolls) at each site in early 1991 as part of a separate study (see Hartnett and Wilson, 1999). Twenty replicate 2 × 2 m plots were located randomly within a transect located in each site, with plots placed 2 m apart. Benomyl treatments (control and benomyl-treated) were assigned randomly to paired plots for a total of 10 plots per treatment for each site. Treated plots received applications of the fungicide benomyl as a soil drench (7.5 l per plot) at the rate of 1.25 g m⁻² (active ingredient) every 2 weeks throughout each growing season (April–October) from 1991 to 1997. Control plots received an equivalent volume of water only. Annually burned sites were burned in April of each year. One of the infrequently burned sites (10 years) was burned twice during the study period, once in late spring of 1991 (prescribed) and again in 1996 (wild fire), while the other site (20 years) remained unburned during the study period. Soil characteristics of the sites are described in Hartnett and Wilson (1999).

To determine the potential effects of long-term applications of benomyl on soil biota and microbial processes, the following response variables were measured for each of the burn by fungicide treatment combinations: mycorrhizal root colonization, soil bacterial and fungal biomass and respiration rates, abundance of nematodes, microbial biomass C and N, and potentially mineralizable C and N.

2.3. Mycorrhizal root colonization

To evaluate the effectiveness of the benomyl treatment in suppressing mycorrhizal root colonization, soil cores (approx. 2.5 cm diameter × 5 cm deep) were taken for root samples under *A. gerardii* or *S. nutans* plants. In 1996 and 1997, five benomyl-treated and five control plots were selected randomly for sampling from each study site (see Hartnett and Wilson (1999) for details of root sampling methods for 1991–1995 measurements). Root sampling occurred in October when mycorrhizal root colonization has been shown to be high (Bentivenga and Hetrick, 1992). Live roots were extracted from each soil sample, washed free of soil, and stained in trypan blue (Phillips and Hayman, 1970). Roots then were examined microscopically using a petri dish scored in 1 cm squares to determine percent root colonization (Daniels et al., 1981).

2.4. Nematode abundances

Soil samples were collected in October 1996 and 1997 from five control and five benomyl-treated plots randomly selected from each site. From each plot, two samples (5 cm diameter × 5 cm deep) were taken from the rhizosphere and adjacent bulk soil of *A. gerardii* or *S. nutans* plants, composited, and homogenized. Nematodes were extracted from 100 g subsamples of soil from each plot using a modified Christie–Perry technique as described by Seastedt et al. (1987) and then were assigned to the following trophic or taxonomic groupings (Yeates et al., 1993): root feeders (Hoploaimidae), herbivores, fungal hyphal feeders (Tylenchidae), general fungal feeders (Aphelenchina), bacterial feeders (Cephalobidae), microbivores, and omnivores (Dorylaimida).

2.5. Soil sampling for microbial and nutrient analyses

Soil samples for microbial and nutrient analyses were collected monthly from five control and five benomyl-treated plots randomly selected from each site. From each treatment plot, two to four samples (5 cm diameter × 5 cm deep) were collected from the rhizosphere and bulk soil adjacent to roots of *A. gerardii* or *S. nutans* plants. Samples were composited, passed through a 4 mm sieve and stored at 4°C. Subsamples of soil from each plot were used for determination of total bacterial and fungal biomass, microbial biomass C and N, and substrate-induced respiration (SIR). Gravimetric soil water content of all samples (105°C, 48 h) was determined, and results from microbial analyses are expressed on a per gram oven-dry soil basis.

For determination of potentially mineralizable C and N, intact soil cores were collected from five control and five benomyl-treated plots randomly selected from each site on 2 October 1996. Polyvinyl chloride cores (5.08 cm diameter, 10 cm height) were driven 5 cm into the soil (approximately in the center of each plot) and carefully removed to prevent soil loss. Cores were weighed and stored at 4°C until initiation of incu-
bations. To prevent soil loss during incubations, 43 μm nylon mesh was attached to the bottom of each core.

Additional samples were collected for determination of initial microbial biomass C and N and total C (TC) and N (TN) content. Total C and N was determined by dry combustion with a Carlo Erba C/N analyzer (Carlo Erba, Strumentazione, Milano, Italy). Gravimetric soil water content of all samples (105°C, 48 h) was determined, and results are expressed on a per gram oven-dry soil basis.

2.6. Total bacterial and fungal biomass

Subsamples (10 g) were taken from samples collected in October 1996 (five control and five benomyl-treated per site) and May 1997 (three control and three benomyl-treated per site), and sent to Soil Foodweb Inc. (Corvallis, Oregon) for determination of total bacterial and fungal biomass. Microscopic estimates of total hyphal lengths and total bacterial numbers were obtained using direct count methods described in Ingham and Horton (1987) and converted to estimates of fungal and bacterial biomass.

2.7. Substrate-induced respiration

The relative contributions of bacteria and fungi to total soil respiration were evaluated using the SIR technique (Anderson and Domsch, 1978). Substrate-induced respiration was determined for soils collected during the growing season in 1997 using methods described by West (1986) and Garcia (1992). A specific modification of these methods included substitution of the bacterial inhibitor chloramphenicol for streptomycin sulfate (Sigma Chemical Co.), because the latter did not effectively inhibit respiration of bacteria in these soils (Smith, 1998). Optimum rates of glucose, chloramphenicol, and cycloheximide additions were determined from preliminary experiments (Smith, 1998).

In this study, soil (10 g subsamples) was added to each of four 160 ml serum bottles. These four samples received either: (A) 1 mg g⁻¹ glucose-C; (B) 1 mg g⁻¹ glucose-C + 10 mg g⁻¹ of chloramphenicol; (C) 1 mg g⁻¹ glucose-C + 20 mg g⁻¹ of cycloheximide (Sigma); or (D) 1 mg g⁻¹ glucose-C + 10 mg g⁻¹ of chloramphenicol + 20 mg g⁻¹ of cycloheximide.

Immediately after addition of the solutions, the serum bottles were sealed with rubber septa and aluminum seals and placed on an orbital shaker at 200 rev min⁻¹ at approximately 22°C. During the 4 h incubation period, CO₂-C concentration in the headspace of the serum bottles was sampled (0.5 ml gas samples) four times using a Shimadzu GC-8A gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD). The relative contribution of CO₂ evolution by bacteria (BACT) was calculated as the difference between the rate of CO₂-C evolution for glucose only (A) and glucose plus chloramphenicol (B) divided by the rate of CO₂-C evolution for glucose only (A) and glucose plus both inhibitors (D). The relative contribution by fungi (FUNG) was calculated as the difference between the rate of CO₂-C evolution for glucose only (A) and glucose plus cycloheximide (C) divided by the rate of CO₂-C evolution for glucose only (A) and glucose plus both inhibitors (D). The ratio of bacterial to fungal respiration (BFratio = BACT/FUNG) was calculated to provide an index of the relative activity of bacteria to that of fungi.

2.8. Microbial biomass

Microbial biomass C and N (MBC and MBN) were determined by the fumigation–incubation method (Jenkinson and Powelson, 1976). Microbial biomass C and N were calculated as the difference in CO₂-C (MC) and N mineralized (MN) between fumigated and unfumigated samples (Voroney and Paul, 1984)

\[
MBC = MC/0.41 \quad MBN = MN/kN
\]

where \( k_N = -0.014(C_f/N_f) + 0.39 \), \( C_f = \text{CO}_2\text{-C evolved from fumigated sample and } N_f = \text{NH}_4^+-\text{N+NO}_3^-\text{N mineralized from the fumigated sample. Short-term N mineralization (Nmin) was calculated as the difference between mineralized N (\text{NH}_4^+-\text{N+NO}_3^-\text{N}) of unfumigated soil after 10 days and unincubated control soil (0 days) as determined by KCl extraction.}

2.9. Potentially mineralizable C and N

Pools of potentially mineralizable C and N were determined from long-term laboratory incubations of undisturbed soil samples using methods described by Cabrera and Kissel (1988) and Garcia (1992). Intact cores were leached periodically for mineralized NH₄⁺-N and NO₃⁻-N with 500 ml of 0.01 M CaCl₂ applied in increments of 50 ml. Cores were leached weekly (month 1), biweekly (months 2–4), and monthly (>4 months) to 326 days. Leachate was brought to 500 ml volume and stored at 4°C until colorimetric analysis for NH₄⁺-N and NO₃⁻-N as described above. Immediately following leaching, 50 ml of N-free nutrient solution (Garcia, 1992) was added to cores to replenish nutrients (Ca, Mg, S, P, and K) potentially lost in leaching. After samples drained to field capacity (6 h@0.03 MPa), cores were placed in 940 ml mason jars, which were sealed with lids fitted with a rubber septum and incubated at 35°C.

Between leachings, the headspace of the mason jars was sampled (0.5 ml gas samples), and CO₂-C concentration was determined as described above for mi-
After CO₂ measurements, jars were opened, cores were allowed to equilibrate with the atmosphere, and then jars were resealed. Concentrations of CO₂–C evolved were measured prior to each leaching and every 2–4 days for the first 60 days, twice per week the next 60 days, and once per week thereafter.

### 2.10. Statistical analyses

Each response variable was analyzed as a split-plot design in which the whole plot corresponded to burning treatments and the subplot to fungicide treatments. Measurements of mycorrhizal root colonization, total bacterial and fungal biomass, and total C and N were analyzed separately using a mixed-model ANOVA (PROC GLM; SAS Institute, Inc., 1997). Monthly measurements of substrate-induced respiration and microbial biomass C and N were analyzed using repeated measures mixed-model ANOVA with a heterogeneous compound symmetric (microbial biomass C and N, SIR) or compound symmetric (bacterial or fungal respiration) covariance structures (PROC MIXED; SAS Institute, Inc., 1997). Yearly measurements of nematode abundances were analyzed using a repeated measures mixed-model ANOVA. Nematode data were log transformed to conform to assumptions of normality and equal variances. When treatment interactions were not significant, differences between treatments were determined using LSMEANS tests for unequal samples and LSD tests for equal samples.

Potentially mineralizable pools of C and N and mineralization rates of these pools were estimated using a kinetic analysis approach. Cumulative CO₂–C evolved and N mineralized (NH₄⁺-N + NO₃⁻-N) from undisturbed cores were fitted to one-pool, two-pool, and mixed-order models (Brunner and Focht, 1984; Molina et al., 1980) using a nonlinear curve-fitting program (PROC NLIN, SAS Institute, Inc., 1997). Carbon mineralization data were described best by the following one-pool model fitted to each undisturbed sample:

\[
C_m = \text{PMC} \times [1 - \exp(-k_0 t)]
\]

- \(C_m\) = mineralized C in mg CO₂–C g⁻¹ at time \(t\)
- \(\text{PMC}\) = potentially mineralizable C in g CO₂–C kg⁻¹
- \(k_0\) = rate constant of mineralization in day⁻¹
- \(t\) = time in days.

Nitrogen mineralization curves for each undisturbed sample were fitted to one-pool and mixed-order models, but SSE errors were minimized, and greater convergence occurred with the following mixed-order model:

\[
N_m = \text{PMN} \times [1 - \exp(-k_1 t - k_2 t^2/2)]
\]

- \(N_m\) = mineralized N in μg N g⁻¹ at time \(t\)
- \(\text{PMN}\) = potentially mineralizable N in mg N kg⁻¹
- \(k_1\) = rate constant of mineralization in day⁻¹
- \(k_2\) = rate constant of mineralization in day⁻¹
- \(t\) = time in days.

Estimates of potentially mineralizable C and N and mineralization rates also were analyzed using a mixed-model ANOVA, and differences between treatments were tested using LSD tests, when treatment interactions were not significant. In addition, the short-term (30 days) mineralization rates for each core was calculated as the slope of cumulative CO₂–C over time using linear regression analysis. Treatment effects on rates of mineralization were analyzed using mixed-model ANOVA, and differences between treatments were tested using LSD tests.

### 3. Results

#### 3.1. Mycorrhizal root colonization

Applications of benomyl significantly reduced mycorrhizal root colonization of the dominant grasses (\(P < 0.001\)) to less than 25% of that in mycorrhizal

Fig. 1. Reduction in mycorrhizal root colonization (% ± SE) with long-term (1991–1997) benomyl applications to tallgrass prairie (Konza Prairie Research Natural Area, Manhattan, KS, USA). Data from 1991 to 1995 are taken from Hartnett and Wilson (1999). Asterisks indicate significant (\(P < 0.05\)) differences between control (black bars) and benomyl treatment (open bars).
control plots in each season since initiation of benomyl treatments in 1991 (Fig. 1; Hartnett and Wilson, 1999). In 1991, levels of mycorrhizal root colonization did not differ significantly between the control and benomyl-treated plots (Fig. 1). During the present study, mycorrhizal root colonization was maintained at 5.9% in benomyl-treated compared to 21.2% for control plots in 1996 and at 3.5% for benomyl-treated compared to 21.3% for control plots in 1997 (Fig. 1).

3.2. Microbial populations

Bacterial and fungal biomass as estimated from direct counts were not affected significantly by burning or fungicide treatments in October 1996. For soil collected in May 1997, bacterial biomass was affected significantly by an interaction between burning and fungicide treatments (P < 0.05; Fig. 2). Benomyl applications did not affect bacterial biomass in burned plots but resulted in significant reductions in total bacterial biomass in infrequently burned plots (Fig. 2). Overall, total bacterial biomass was lower in benomyl-treated plots (184.6 ± 16.6 SE µg g⁻¹ soil) compared to control plots (228.7 ± 13.6 SE µg g⁻¹ soil). In contrast, fungal biomass in Spring 1997 soil communities was not affected significantly by long-term benomyl applications (Fig. 2).

Mean total bacterial to fungal ratios did not differ significantly with burning or mycorrhizal suppression, but did differ temporally (data not shown). Bacterial to fungal ratios < 1 for soils collected in October indicated that the tallgrass microbial community was dominated by fungi (Fig. 2). For May 1997 samples, these ratios were >1 indicating that bacteria dominated the community at the beginning of the growing season (Fig. 2). Benomyl treatments tended to increase bacterial to fungal ratios compared to the control treatments.

3.3. Responses of nematodes

Benomyl treatments significantly affected nematode abundances and composition in tallgrass prairie (Fig. 3). Benomyl applications tended to increase abundance of root feeders and herbivores but this increase was not significant. In contrast, abundances of fungal hyphal feeders (Tylenchidae) and omnivores (Dorylaimida) were reduced significantly (P < 0.05 and P < 0.10, respectively) with benomyl treatment.

3.4. Substrate-induced respiration

Substrate-induced respiration (SIR) is an index of potential microbial activity and can be used to estimate the size of the microbial biomass pool (Anderson and Domsch, 1978). Substrate-induced respiration varied significantly with date and was significantly affected by benomyl applications (Table 1). Substrate-induced respiration was highest in April and August, which corresponded to times of highest soil moisture (~0.31 and 0.28 g H₂O g⁻¹, respectively). Across all sampling dates, potential microbial activity was significantly higher with benomyl treatment compared to the control (SIR=21.3 and 19.1 ± 0.58 SE mg kg⁻¹ h⁻¹, respectively; Table 1, Fig. 4). The relative contribution of bacteria to total respiration (BACT) was affected significantly by date of sampling but not by burning or

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**Fig. 2.** Responses of bacterial and fungal biomass to long-term benomyl applications to tallgrass prairie in October 1996 and May 1997. Different letters indicate significant (P < 0.05) differences between burning (BA = burned annually, BI = burned infrequently) and benomyl treatment combinations (black bars = control, open bars = benomyl-treated).

**Fig. 3.** Responses of nematode groups to long-term benomyl applications to tallgrass prairie. Nematode groups: Ho, Hoploaimidae; He, herbivores; Ty, Tylenchidae; Ap, Aphelechina; Ce, Cephalobidae; Mi, Microbivores; Do, Dorylaimida. Asterisks indicate significant (P < 0.05) differences between control (black bars) and benomyl treatment (open bars).
Table 1
Summary of repeated measures mixed-model analysis of variance for substrate-induced respiration (SIR), the contribution of bacteria (BACT) or fungi (FUNG) to total respiration, and the ratio of bacteria to fungal respiration (BFratio) in tallgrass prairie.

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<tr>
<td>Burn (B)</td>
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<td>Fungicide (F)</td>
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<td>B × F</td>
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<td>Burn × Fung</td>
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* †, *, **, and *** indicate significance at $P < 0.10$, 0.05, 0.01, and 0.001 level, respectively. All other values are not significant at $P > 0.10$ level.

3.5. Microbial biomass C and N

Microbial biomass C (MBC) varied significantly with date of sampling (Table 2). Averaged across all treatments MBC was highest in May and declined throughout the growing season by a total of ~40% (Fig. 4). Although burning significantly interacted with sampling date, the burn treatment did not significantly affect MBC response to benomyl treatments (Table 2). As observed with SIR, benomyl treatments resulted in a significant increase in MBC across all burn treatments and sampling dates (Fig. 4). Microbial biomass C increased by > 8% in benomyl-treated (1485 ± 51.2 SE mg kg$^{-1}$ soil) compared to control prairie soils (1364 ± 51.3 SE mg kg$^{-1}$ soil).

Microbial biomass N (MBN) also was affected by date of sampling, but was not affected by burning and benomyl treatments (Table 2). In general, MBN dynamics mirrored MBC dynamics, except MBN declined more sharply near the end of the growing season (Fig. 5). Short-term N mineralization (Nmin) was affected significantly by an interaction between sampling date and benomyl treatments (Table 2). The amount of N mineralized was higher in benomyl-treated compared with control soils throughout much of the growing season (Fig. 5). Second-order interactions between sampling date and burning or benomyl treatments significantly affected microbial biomass C:N ratios (MBC:MBN) (Table 2). When averaged across all sampling dates, MBC:MBN was 3.22 (± 0.15 SE) in benomyl-treated compared to 3.02 (± 0.15 SE) in control plots.

3.6. Carbon and nitrogen mineralization

Short-term (30 days) C mineralization rates and predicted values of mineralizable C and N and mineralizable N and mineralizable C to N (MBC:MBN) in tallgrass prairie soils.

Table 2
Summary of repeated measures mixed-model analysis of variance for microbial biomass C (MBC), microbial biomass N (MBN), short-term (10 days) N mineralization (Nmin), and the ratio of microbial C to N (MBC:MBN) in tallgrass prairie soils.

<table>
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<th>Source</th>
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<tr>
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<td>M × B × F</td>
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* †, *, **, and *** indicate significance at $P < 0.10$, 0.05, 0.01, and 0.001 level, respectively. All other values are not significant at $P > 0.10$ level.
ation rates from kinetic analyses are summarized in Table 3. Short-term mineralization rates were significantly higher for benomyl-treated than for control soil cores. Long-term C mineralization (potentially mineralizable C, PMC) increased with benomyl treatment, but mineralization rates ($k_0$) were unaffected. Potentially mineralizable N (PMN) also tended to be lower with benomyl applications but not significantly, and the first and second rate constants ($k_1$ and $k_2$) for N mineralization did not differ significantly between benomyl-treated and control cores.

Burning and benomyl applications interacted to significantly ($P < 0.05$) alter total C (TC). Benomyl resulted in a significant increase in TC in annually burned sites (control = 47.6 ± 0.13 SE g C kg$^{-1}$; benomyl-treated = 51.3 ± 0.14 SE g C kg$^{-1}$) but had no effect in infrequently burned sites (control = 49.5 ± 0.20 SE g C kg$^{-1}$; benomyl-treated = 47.0 ± 0.20 SE g C kg$^{-1}$). Although TN was not affected significantly by benomyl applications, total C:N was significantly higher for benomyl-treated (13.1 ± 0.31 SE) compared to control (12.4 ± 0.32 SE) plots.

4. Discussion

Long-term applications of benomyl in the field strongly suppressed AM fungal root colonization, but effects on non-target soil biota and nutrient cycling were mixed. Results from direct counts, SIR, and microbial biomass measurements indicated that bacteria and fungi responded differently. The reduction in total bacterial biomass occurred only in infrequently burned prairie for one sampling date, probably due to the greater bacterial biomass in these soils. Interestingly, fungal biomass was not affected by benomyl treatments. These results were surprising, because mycorrhizal root colonization had been suppressed (average of ~80% reduction) for 6 years, but this reduction was

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fungicide</th>
<th>30 days$^a$ (mg C kg$^{-1}$ d$^{-1}$)</th>
<th>PMC (g C kg$^{-1}$)</th>
<th>$k_0$ (day$^{-1}$)</th>
<th>PMN (mg N kg$^{-1}$)</th>
<th>$k_1$ (day$^{-1}$)</th>
<th>$k_2$ (day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burned annually</td>
<td>Control</td>
<td>49.1 (10.1)$^b$</td>
<td>10.7 (2.4)</td>
<td>0.004 (0.0003)</td>
<td>618.8 (51.3)</td>
<td>0.003 (0.0003)</td>
<td>1.0 × 10$^{-5}$ (3.0 × 10$^{-6}$)</td>
</tr>
<tr>
<td></td>
<td>Benomyl</td>
<td>59.0 (19.6)</td>
<td>12.6 (2.1)</td>
<td>0.004 (0.0003)</td>
<td>557.3 (54.2)</td>
<td>0.004 (0.0003)</td>
<td>1.0 × 10$^{-5}$ (4.0 × 10$^{-6}$)</td>
</tr>
<tr>
<td>Burned infrequently</td>
<td>Control</td>
<td>49.0 (13.0)</td>
<td>15.3 (1.5)</td>
<td>0.003 (0.0003)</td>
<td>782.4 (61.6)</td>
<td>0.003 (0.0003)</td>
<td>3.0 × 10$^{-6}$ (3.0 × 10$^{-6}$)</td>
</tr>
<tr>
<td></td>
<td>Benomyl</td>
<td>56.3 (11.7)</td>
<td>18.1 (1.4)</td>
<td>0.003 (0.0002)</td>
<td>747.7 (54.3)</td>
<td>0.003 (0.0003)</td>
<td>6.0 × 10$^{-6}$ (4.0 × 10$^{-6}$)</td>
</tr>
</tbody>
</table>

Source df | Mixed-model ANOVA$^c$
---|---
Burn (B) | 1 | 0.1 | 8.6$^†$ | 28.0$^*$ | 3.5 | 1.0 | 3.2 |
Fungicide (F) | 1 | 4.0$^†$ | 2.9$^†$ | 0.3 | 0.8 | 2.3 | 0.1 |
B × F | 1 | 0.1 | 0.1 | 2.0 | 0.1 | 0.1 | 0.1 |

$^a$ Calculated as the slope of the linear regression between cumulative CO$_2$–C and time (days).

$^b$ Mean (±1SE) of $n = 10$ replicates per treatment combination.

$^c$ $^†$ and $^*$ $P < 0.10$ and 0.05 level, respectively. All other values are not significant at $P > 0.10$ level.
not reflected in measures of total fungal biomass. Little correlation occurs between AM fungal colonization and abundance of extra-radical hyphae; therefore, changes in root colonization are probably not equivalent to changes in the external hyphal phase (Schreiner and Bethlenfalvay, 1997). Instead, different groups of fungi, such as saprophytic fungi, may have replaced those that were negatively affected by benomyl applications resulting in no change in total fungal biomass.

Although total bacterial biomass was reduced with benomyl treatments in infrequently burned prairie for one sampling date, bacterial activity as determined by SIR was unaffected by benomyl or burn treatments. In contrast, the relative contribution of fungi to potential microbial activity was reduced with benomyl application independent of burn treatment. This indicates that with mycorrhalzal suppression, the total fungal biomass may be comprised of a greater proportion of inactive and dead hyphae compared to untreated controls. However, the decrease in fungal activity with benomyl does not necessarily indicate the AM fungal activity was reduced with benomyl application. Measurements of SIR did not include mycorrhizal hyphae, because soils were highly disturbed (i.e., sieved), and mycorrhizal fungi, once disrupted, do not remain active (Read and Birch, 1988).

Alterations in ratios of dead to live fungal hyphae may affect nematode abundances. For example,ingham (1988) found that nematicide applications resulted in large increases in active mycorrhizal root colonization, which implies that nematodes negatively affect colonization of plant roots. Therefore, a decrease in mycorrhizal root colonization could negatively affect abundances of nematodes that depend on mycorrhizal fungi as a food source (i.e., Tylenchidae). In the present study, nematode abundances were significantly affected by benomyl treatments. Specifically, fungal hyphal-feeders (Tylrenchidae) and omnivores (Dorylaimida) were both significantly reduced (by 12 and 33%, respectively) with benomyl treatments. This decrease in fungal hyphal-feeders indicates that this nematode group may be responding to changes in the amount of active fungal hyphae or alterations to the fungal community with benomyl treatments. Despite decreases in abundance of these two groups, the majority of nematode groups did not respond to benomyl applications.

To our knowledge, this is the first multi-year field study to demonstrate that benomyl applications affect microbial activities and nematode populations in undisturbed soils. Other fungicides have been shown to influence microbial populations in laboratory studies (Colinas et al., 1994), whereas several studies have shown that benomyl applications may not affect microbial populations (Hart and Brookes, 1996). Only a few studies have assessed the impacts of benomyl applications in the field on microbial populations (e.g., Hart and Brookes, 1996; Shukla and Mishra, 1996). In those studies, bacteria and fungi were either reduced in agricultural soils by benomyl applications (Shukla and Mishra, 1996) or were not affected by long-term benomyl applications to tilled soils (Hart and Brookes, 1996). In a laboratory study designed to evaluate the effectiveness of a substrate-induced respiration technique for pesticide-treated soils, Harden et al. (1993) found that total microbial biomass (i.e., fumigation-extraction method) increased with benomyl application after 20 days, but substrate-induced microbial activity was unaffected. The authors suggested that benomyl applications did not alter the proportion of organisms that were able to mineralize glucose.

In tallgrass prairie soils, microbial populations and activities, as well as nematode abundances, were most likely affected indirectly by benomyl, due to direct effects on mycorrhizal root colonization, which may have resulted in both quantitative and qualitative changes in other microbial populations. Arbuscular mycorrhizal fungi may directly affect rhizosphere microorganisms through exudation and by competing with microbes for available C (i.e., mycorrhizosphere effect; Linderman, 1988). In turn, colonization of plant roots by mycorrhizal fungi may alter root exudation patterns or other aspects of host plant physiology and, therefore, indirectly affect microbial growth in the rhizosphere (e.g., Ames et al., 1984; Azaizeh et al., 1995). Root exudates can account for up to 40% of dry matter produced by plants (Lynch and Whipps, 1990), and microbes probably metabolize most of these exudates. Because up to 20% of fixed carbon in plants is estimated to be exuded by roots (Barber and Lynch, 1977), changes in root exudation quantity and quality with mycorrhizal root colonization have implications for host-plant C-allocation patterns, as well as microbial populations.

The complexity of soil communities in the field makes it difficult to separate direct and indirect effects of AM fungi on microbial populations. Changes in labile and total pools of C provide evidence for both direct and indirect effects of reduced AM fungal root colonization on bacterial numbers and fungal activities in tallgrass prairie. With mycorrhizal suppression, MBC and PMC increased, indicating that a greater amount of labile C was available for microbial utilization. Ratios of potentially mineralizable C:N also tended to be higher, indicating either lower rates of turnover or higher rates of inputs of C to this pool. However, since rate constants of mineralization for PMC were not affected by benomyl treatments, this indicates that turnover rates were similar. Thus, greater pools of C probably resulted from greater inputs of C to the soil.

Increased labile and total pools of C with suppression of mycorrhizal fungi may result from several po-
tential sources of C. Although benomyl applications inhibited hyphal growth, hyphal re-growth could have occurred between the applications every 14 days. Decreased hyphal growth and subsequent regrowth between applications of benomyl could artificially inflate hyphal turnover rates. This potential increase in senesced hyphae with mycorrhizal suppression could result in greater C mineralization (Azcon-Aguilar and Barea, 1992; Olssen et al., 1996), given that fungal biomass can make up a large portion of total microbial biomass in tallgrass prairie soils (Miller et al., 1995). In addition, increased root growth with mycorrhizal suppression may also increase C inputs (Miller et al., 1995; Olssen et al., 1996), but only if phosphorus is not limiting and mycorrhizal-suppressed plants are able to acquire sufficient P for growth. The increase in TC observed in annually burned relative to infrequently burned prairie soils provides indirect support for this mechanism, because root biomass is generally greater in frequently burned prairie due to increased rates of production and P probably does not limit root growth (Rice et al., 1998). Reduced mycorrhizal root colonization may result in even greater root production from higher C allocation, because as much as 40–50% of a plant’s net production may be allocated to mycorrhizal fungi (Harris and Paul, 1987). With long-term suppression of mycorrhizae, increased root growth in conjunction with increased hyphal turnover, as well as increased root exudation could contribute to greater C pools. However, exudation rates have not been quantified in tallgrass prairie soils. Benomyl applications and reduced mycorrhizal root colonization probably did not affect or increase decomposition rates, because microbial activity was higher and total fungal biomass did not increase with fungicide treatments. Therefore, increased soil C pools could have resulted from a combination of the mechanisms described above. Increased labile and total pools of C provide evidence for the role of mycorrhizal fungi in nutrient cycling. Furthermore, the increase in labile pools with mycorrhizal suppression indicates that mycorrhizal fungi may mediate the flow of labile C from plants to soil microorganisms in tallgrass prairie (e.g., Rygiewicz and Andersen, 1994).

Changes in plant community composition with benomyl applications could also alter C availability. Conversely, changes in C availability to soil microbes could potentially affect plant growth, which could lead to changes in plant community composition. Plant species diversity and total plant cover did not differ between control and benomyl-treated plots used in this study prior to benomyl applications in 1991 (Hartnett and Wilson, 1999). However, Hartnett and Wilson (1999) observed an increase in plant species diversity after 3 and 5 years of benomyl applications. They attributed this increase in diversity to a decrease in percent aerial cover of the dominant C₄ grasses, which are highly dependent on mycorrhizal fungi for growth, and a concomitant increase in percent cover of the subdominant grass and forb species, which vary in their dependence upon mycorrhizal fungi for growth (Wilson and Hartnett, 1998). The dominant grasses create an environment in tallgrass prairie in which N availability is low and N supply rates are slow because of high C:N ratios of their litter (Miller and Jastrow, 1994). Therefore, reductions in cover of these grasses and increases in cover of forbs species could potentially alter C availability because forb species generally have lower C:N ratios than the dominant grasses and exudation patterns may differ considerably between grass and forb species. Furthermore, the subdominant species in tallgrass prairie cannot compete as well under the N-limiting conditions. Consequently, if greater C availability with mycorrhizal suppression results in higher N supply rates, this could potentially contribute to observed increases in cover of the subdominant species with benomyl applications. In addition, the greater C:N ratios of labile and active fractions observed with mycorrhizal suppression could result in greater N immobilization, thereby decreasing N availability. However, this study provided no clear evidence for greater immobilization. Increased labile C with mycorrhizal suppression did appear to be related to higher N mineralized during short-term incubations, which indicates that N availability to plants may be increased and, therefore, may have important implications for aboveground processes. These potential effects require further study to determine whether these are occurring due to benomyl applications.

Results from this study indicate that the main effect of long-term benomyl applications was on colonization by mycorrhizal fungi rather than on non-target soil biota or nutrient cycling. The magnitude of benomyl effects on soil biota and processes was small (e.g., 12–33% reduction in several nematode groups) relative to the intended target effects of benomyl on mycorrhizal fungi (e.g., average of 80% reduction in mycorrhizal root colonization). This indicates that the principle effect of benomyl in tallgrass prairie soils is suppression of mycorrhizal fungi, and that benomyl applications remain the most useful tool for experimentally manipulating mycorrhizal symbiosis in the field. However, by altering mycorrhizal development, benomyl applications may indirectly affect soil biota and nutrient cycling in tallgrass prairie. These changes in nutrient supply and retention in tallgrass prairie soils potentially could alter aboveground responses. In turn, changes in plant communities associated with reduced mycorrhizal root colonization could also influence microbial communities (Bever et al., 1997). Although this study suggests that benomyl applications are a viable tool for manipulating mycorrhizal fungi in the field, it
is difficult to make generalization about the use of benomyl in field studies because many of the effects of benomyl applications and reductions of mycorrhizal root colonization are likely to vary between different plant communities. Further studies are needed that focus on interactions between mycorrhizae and soil biota and the role of mycorrhizal fungi in ecosystems processes to better understand these complex interactions.

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