Does aerial spraying of *Bacillus thuringiensis* subsp. *kurstaki* (Btk) pose a risk to nontarget soil microarthropods?

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Abstract: This field study was carried out to investigate whether application of an aerial spray containing *Bacillus thuringiensis* subsp. *kurstaki* (Btk) to control the western spruce budworm (*Choristoneura occidentalis* Freeman, 1967) had any measurable effects on aspects of the abundance, distribution, diversity, and feeding behaviour of nontarget soil microarthropods. Although total abundance and species richness (N_0) of Collembola declined significantly in samples taken 3 weeks after spraying, this decline occurred in the control plots as well as in plots sprayed with Btk. Values for the diversity indices N_1 and N_2 were not affected by the treatment. Correspondence analysis did not identify changes in collembolan communities following the aerial application of Btk. Total abundance of mites and populations of different suborders (Prostigmata, Mesostigmata, and Oribatida) were not significantly affected by application of Btk, or by the time of year that the samples were collected. There was no evidence of a selective reduction in the surface-dwelling portions of the collembolan and mite communities following exposure to Btk. Similarly, the experimental spray did not cause a decline in the abundance of the guild of Collembola known to feed on bacteria, and the proportion of bacteria in the diet of these Collembola following application of Btk was also unchanged.

Résumé : Cette étude sur le terrain a été réalisée dans le but de déterminer si un épandage aérien avec *Bacillus thuringiensis* subsp. *kurstaki* (Btk) pour contrôler la tordeuse des bourgeons de l'épinette (*Choristoneura occidentalis* Freeman, 1967) avait ou non des effets mesurables sur l'abondance, la distribution, la diversité et les habitudes alimentaires des microarthropodes du sol qui ne sont pas ciblés. Bien que l'abondance totale et la richesse en espèces (N_0) des collemboles aient diminué de façon significative dans les échantillons prélevés 3 semaines après l'arrosage, cette diminution s'est produite dans les parcelles témoins aussi bien que dans les parcelles arrosées avec Btk. La valeur des indices de diversité N_1 et N_2 n'a pas été affectée par le traitement. L'analyse des correspondances n'a pas détecté de changements dans les communautés de collemboles à la suite de l'application aérienne de Btk. L'abondance totale des acariens et les populations de différents sous-ordres (Prostigmates, Mésostigmates et Oribates) n'ont pas été affectées de façon significative par l'application de Btk, ni par le moment de l'année où les échantillons ont été prélevés. Il n'y avait aucun signe de réduction sélective des portions de communautés d'acariens et de collemboles vivant en surface à la suite d'une exposition à Btk. De la même façon, l'arrosage expérimental n'a pas causé de diminution dans l'abondance de la guilde de collemboles qui se nourrissent de bactéries et la proportion de bactéries dans la diète de ces collemboles est également demeurée inchangée après l'application de Btk.

[Traduit par la Rédaction]

Introduction

The forests of the Interior Douglas-fir Ecosystem (IDF) in British Columbia (B.C.), Canada, are important to local communities, which depend on these forests not only for economic benefits from forestry, rangeland, and tourism activities, but also for year-round supplies of clean water. Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco) is susceptible to attack by the western spruce budworm (*Choristoneura occidentalis* Freeman, 1967), which is controlled by spraying biological control products containing the bacterium *Bacillus thuringiensis* subsp. *kurstaki* (Btk). Although Btk has been used as a biological alternative to chemical pesticides in forestry applications for at least 40 years (van Frankenhuyzen 1995), its use in forest and urban environments is still controversial. The potential impacts of spray programs on nontarget organisms is just one of the concerns expressed by environmentalists and concerned citizens.

The importance of soil invertebrates in decomposition, nutrient cycling, soil formation, and carbon sequestration is well documented in the literature (Seastedt 1984; Coleman and Crossley 1996). Furthermore, because of the diverse interactions of the soil invertebrate community with microbes, which can include dispersing, enhancing, and (or) reducing

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microbial activity (Marshall 1993; Dighton et al. 1997), ensuring its safety should be of special concern during any spray program involving the use of microbial or other pest-control agents.

Even though Btk is commonly found in soil (Martin and Travers 1989), surprisingly little is known about the ecology of this organism in soil (Addison 1993; Jensen et al. 2003). Spores of Btk are able to persist in soil for relatively long periods of time, at least 1 or 2 years in North American forest soils (Smith and Barry 1998). Furthermore, studies have shown that Bt spores resist downward leaching in soil, and remain in the top few centimetres of the soil profile (Hendricksen and Hansen 2002), raising the possibility that Btk may accumulate in the top layers of the soil.

A number of possible explanations for the presence of Bt in soil were put forward by Meadows (1993), including the hypothesis that Bt may have "as yet undiscovered pathogenicity to common soil-inhabiting insects". No soil invertebrates are known to be hosts for Btk, which is generally considered to be safe for nontarget organisms (Flexner et al. 1986). However, to date, only a limited number of soil invertebrate species have been tested.

Addison and Holmes (1995, 1996) found that the soil collembolan *Folsomia candida* Willem, 1902 and the forest earthworm *Dendrobaena octaedra* (Savigny, 1826) were not affected by unformulated preparations of Btk, or by Btk in an aqueous formulation. Although they reported that high concentrations of an oil-based formulation were toxic to both species, the toxicity was shown to be due to the oil-based formulation blank rather than to Btk itself.

In most recent work to determine the persistence and toxicity of Btk in soil, purified toxins, or toxins expressed in transgenic plant material, have been used. Laboratory experiments conducted by Sims and Martin (1997) and Yu et al. (1997) using purified proteins cryIA(b) and cryIA(c) showed that these proteins were not toxic to the collembolans *F. candida* and *Xenylla grisea* Axelson, 1900, or to a species of soil oribatid mite (*Oppia nitens* C.L. Koch, 1835). In a laboratory study using transgenic corn expressing the cryIA(*b*) protein, Saxena and Stotsky (2001) found that the amount of toxin released in root exudates had no effect on a species of earthworm or on the total numbers of nematodes, protozoans, bacteria, or fungi extracted from the experimental microcosms.

Despite the lack of demonstrated deleterious effects of Btk (or its toxins) on soil invertebrates, there are studies that have produced contradictory or unexplained results. Paulus et al. (1999) found a short-term decrease in the decomposition rate of litter in mini-containers placed in a plot that had been sprayed with Btk, and Wu et al. (2004) found that Bt-transgenic rice straw altered some biological properties of soil, suggesting a shift in microbial populations or a change in the metabolic activities of the microbial community. A decrease in the decomposition rate of transgenic Bt plants compared with their non-Bt isolines was reported by Flores et al. (2005), who argued that differences in soil microbiota were not responsible for the differential decomposition rates. These studies suggest that there may be as yet unexplained indirect effects of Btk on soil fauna and soil functioning.

Using laboratory tests to predict environmental effects in the field has several limitations. Laboratory tests are typically conducted on a small number of species that are usually not native to the area being sprayed. Experiments in which specific proteins or transgenic plant materials are used do not test the full spectrum of toxins expressed by the bacteria in the commercial spray formulations. Furthermore, interactions with formulation additives, or interactions between different toxins and spores, both factors that have been shown to affect the toxicity of Btk, are not considered in these experiments (Liu et al. 1998; Broderick et al. 2000). Thus, field studies, in which both direct and indirect effects on multispecies assemblages are investigated under realistic conditions over longer periods of time, are a valuable component of evaluating the safety of any pest-control product.

Unfortunately, very few field studies have attempted to investigate effects of Btk on soil invertebrates at the species level. This is not surprising, given that a soil sample from a single location may contain hundreds of species of soil invertebrates (Marshall 1993; Behan-Pelletier and Newton 1999). Yet even within a single taxonomic group there may be a diversity of life histories, feeding behaviours, and ecological requirements (e.g., Rusek 1998), resulting in very different patterns of exposure (and potentially, response) to Btk and its toxins. Thus, studies that are performed only at higher levels of taxonomic resolution (e.g., class, order, family), may miss important environmental impacts. While it is unlikely that any one study can adequately cover all soil invertebrates, a series of studies that focus on specific groups of soil fauna can help to build up a composite picture of the response of the soil community to pest-control products. Thus, the study of Beck et al. (2004) is of particular significance, since the response of enchytraeids, earthworms, and mesostigmatid and oribatid mites to two pesticides (Btk and diflubenzuron) was measured at the species level. Those authors reported that with the exception of a reduction in the dominance rank of a single mite species, the predaceous *Veigaia nemorenis* (C.L. Koch, 1839), application of Btk to the soil had no detectable effects. However, in their study, Collembola (with the exception of one species) were determined only to higher taxonomic groups. In our study we identified Collembola to species and categorized mites and other extracted fauna according to higher taxonomic groupings.

The objectives of this study were to investigate the impact of Btk spray on the abundance of various taxa of soil fauna, and to determine whether aerial spraying of Btk had an effect on the abundance of the soil fauna in the top 2.5 cm of the soil profile, the layer likely to be exposed to the highest concentrations of Btk. Furthermore, at the species level we examined the potential impacts of Btk on different aspects of the ecology of the soil collembolan community, including species diversity and feeding behaviour.

Methods and materials

Study sites

During the summer of 1996, three replicate 50 ha blocks in a Douglas-fir forest near Merritt, B.C., were sprayed with Foray® 48B (active ingredient Btk; 60 billion international units (BIU)/ha in 4.8 L/ha) in an experimental aerial-spray application to determine whether this higher dose and application rate would provide more consistent control of *C. occidentalis*. Three unsprayed blocks of the same forest

Table 1. Characteristics of the study plots.

Treatment plot	Latitude and longitude	UTM grid	Aspect	BGC subzone and variant[a]	Soil type[b]	Soil texture	pH	Elevation (m)
Control 1	50°50′30″N, 120°21′0″W	106855638	SW	IDFdk1	Orthic Gray Luvisol	Sandy loam	5.0–6.0	1200
Control 2	50°50′0″N, 120°21′0″W	106855637	ESE	IDFdk1	Orthic Gray Luvisol	Sandy loam	5.0–6.0	1150–1250
Control 3	50°8′45″N, 120°42′18″W	106610557	ESE	IDFdk2	Eluviated Eutric Brunisol	Loamy sand	5.0–6.0	800
Spray 1	50°52′30″N, 120°21′0″W	106865639	NNE–SSW[c]	IDFdk2 (IDFdk1)	Eluviated Eutric Brunisol (Orthic Gray Luvisol)	Loamy sand	5.5	1100
Spray 2	50°57v30′N, 120°12′30″W	106945649	NNE–SSW[c]	IDFdk2	Eluviated Eutric Brunisol	Sandy loam	5.5	1000
Spray 3	51°1′0″N, 120°11′0″W	106985654	NNE–SSW[c]	IDFdk2	Eluviated Eutric Brunisol	Sandy loam	5.5	1000

[a]The biogeoclimatic subzones are classified according to the B.C. Ministry of Forests' Biogeoclimatic Ecosystem Classification (Meidinger and Pojar 1991), and variants are described in Lloyd et al. (1990).
[b]Soil types follow the classification outlined by the Soil Classification Working Group (1998).
[c]These plots straddled the crest of a ridge that was orientated in a WNW–ESE direction. Approximately one-third of the sampling locations had a NNE aspect, one-third were in the crest area, and the remainder had a SSW aspect.

type, also ~50 ha in area, located ca. 1.5, 4, and 12 km from the treated plots, were used as controls. All the study plots were located in stands of the IDF zone as described in the B.C. Ministry of Forest Biogeoclimatic Ecosystem Classification (Meidinger and Pojar 1991). This area has a continental climate with warm, dry summers, cool winters, a long growing season, and frequent moisture deficits during the summer (Hope et al. 1991). The plots were located on grazing leases and the area was historically subject to frequent wildfires. Details of the physical characteristics of the plots are given in Table 1.

Douglas-fir was the predominant species at control plot 1, with lesser amounts of ponderosa pine (*Pinus ponderosa* Dougl. ex P. Laws. & C. Laws.). Control plot 2 was located on the side of a relatively steep slope and was very open, with scattered Douglas-fir and some Engelmann spruce (*Picea engelmannii* Parry) at higher elevations. The dominant tree species at control plot 3 and the three experimental plots (spray plot 1 – spray plot 3) were Douglas-fir and ponderosa pine.

Field sampling

Sampling in both the treated and the control plots was conducted at three times during the summer of 1996, once before spraying (June 19) and twice after spraying (3 weeks later (July 18) and 3 months later (September 29)). Btk takes several days to kill even susceptible insects, so we considered that 3 weeks would allow the short-term effects of the Btk spray to become evident, but would not be long enough for microarthropod populations to effect significant recovery. The sampling at 3 months was designed to allow detection of longer term responses (such as indirect impacts), and signs of recovery from any short-term impacts. Sampling any later in the year would have risked masking any treatment-related effects with those engendered by extreme weather conditions. At each sampling time, a series of soil cores (10 cores, each 5 cm diameter × 10 cm depth) was taken from each of the experimental plots. Each core was divided into four 2.5 cm sections, and microarthropods were extracted from the samples by means of a high-gradient extractor.

Identification of soil arthropods

Extracted soil arthropods were preserved in 70% ethanol and were examined and counted under a dissection microscope. Springtails (Collembola) and mites (Acari) were the most abundant groups collected. Collembola were identified to species, mites to suborder, and the remainder of the fauna to order. Collembola were cleared in lactic acid, examined under phase-contrast microscopy, and identified using the taxonomic keys and descriptions of Christiansen and Bellinger (1998) and Fjellberg (1984, 1985, 1989), plus other taxonomic literature where applicable. In several cases, a species name could not be assigned because the organism differed to some degree from published species descriptions. Voucher specimens of the Collembola collected during this study were deposited at the Pacific Forestry Centre in Victoria, B.C.

Contents of collembolan guts

Gut contents were clearly visible within the bodies of cleared and mounted collembolan specimens, and were ex-

amined using 1000× magnification. The abundance of various ingested items was expressed as a percentage of the total gut content for each individual. Only adult specimens, where gut contents were visible in at least four body segments, were included in the analysis. Ingested materials were categorized as follows: fungal material (hyphae and spores), particulate organic matter (POM), very fine POM (<5 μm diameter), amorphous organic material (AOM) encompassing materials without discernible cellular structure, animal material, exuviae, bacteria, and mineral particles. Bacteria were of special concern in this study and were undoubtedly present in the gut of all species, mixed with other materials, but only individuals with areas of the gut crammed with regularly shaped spherical or rod-shaped particles of a size consistent with bacteria (1–5 μm) were classified as bacterial feeders.

Statistical analysis

One soil-core sample collected from control plot 3 prior to spraying was considered an outlier because it contained nearly 9000 individuals of a single species (*Anurophorus* sp. nr. *septentrionalis* Palissa, 1966). This core was excluded from all statistical analyses that involved Collembola.

Hill's (1973) numbers, N_0, N_1, and N_2, were calculated to express the different aspects of species diversity of the Collembola. The indices were calculated using the composite data from all 10 (9) soil cores of each plot/time combination: N_0 represents species richness (number of species), N_1 represents the number of abundant species ($e^{\text{Shannon's index}}$), and N_2 represents the number of very abundant species (1/Simpson's index).

Two-way repeated-measure ANOVA (RM-ANOVA) and correspondence analysis (CA) were carried out using Minitab® release 14 for Windows (Minitab Inc., State College, Pa., 2003). Two-way RM-ANOVA with time as the repeated measure was used to test for treatment- and time-related effects of Btk spray on the abundance, vertical distribution, and species diversity of different groups of soil fauna. For each RM-ANOVA analysis there were two treatments (control versus sprayed), three replicates per treatment (three control plots and three spray plots), and three sampling times (pre-spray, 3 weeks post spray, and 3 months post spray). To meet the assumptions of the RM-ANOVA, a $\log_{10}(x + 1)$ transformation of the original abundance data was required. Depth-distribution data were expressed as percentages and did not require transformation to meet the assumptions of the RM-ANOVA. Tukey's test at $p \leq 0.05$ was used to detect significant differences among means.

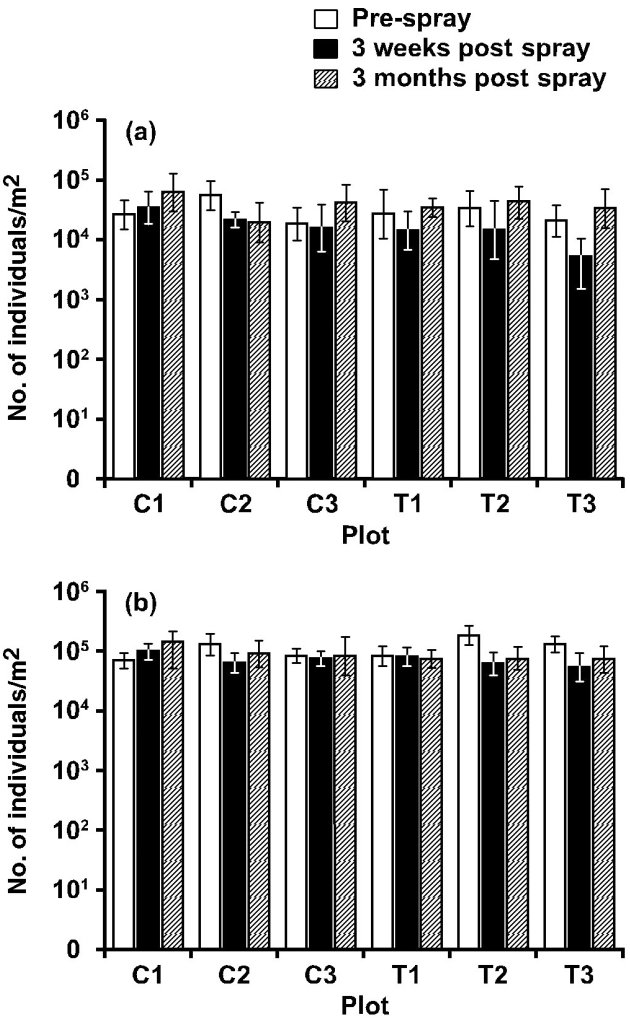
CA was used to provide an ordination of the different plot–time combinations based on the collembolan species composition of the samples. The analyses presented are based on abundance data for all species (except the highly aggregated *A*. sp. nr. *septentrionalis* and *Xenylla humicola* (O. Fabricius, 1789)) for which 30 or more individuals were collected.

Results

Abundance of soil fauna

Collembola and Acari were the most abundant soil invertebrate taxa found in the samples. Mean densities (based on derived means) prior to spraying ranged from 18 500 to 56 300 Collembola/m^2 and from 70 500 to 186 000 mites/m^2 (Fig. 1).

Fig. 1. Estimated abundances of Collembola (*a*) and total mites (*b*) at six experimental plots. Treated plots (T1–T3) were sprayed with Foray® 48B immediately after the pre-spray samples were taken. C1–C3 are control plots. Derived means and 95% confidence limits are shown.



The two-way RM-ANOVA (Table 2) of collembolan abundance data showed that the treatment effect was not significant. However, the time of year when the samples were collected affected collembolan abundance in both the control and the Btk-treated plots. Application of Tukey's test showed that numbers of Collembola were significantly lower in July samples than in September samples. As the July decline occurred in all plots regardless of whether or not the plot had been sprayed with Btk, we assume that the decline was related to the time of year (midsummer) rather than to the fact that the samples were collected 3 weeks after spraying.

A similar analysis carried out on total mite abundance failed to reveal any significant effect of treatment, time of sampling, or the treatment × time interaction. To determine whether any of the suborders responded differently than the group as a whole, separate RM-ANOVAs were also performed using abundance data for Oribatida, Mesostigmata,

Table 2. Results of two-way repeated-measure ANOVA to determine effects of *Bacillus thuringiensis* subsp. *kurstaki* (Btk) treatment and time on abundance of Collembola and mites.

	Source of variation					
	Treatment		Time		Treatment × time	
Factor (taxon)	*F*	*p*	*F*	*p*	*F*	*p*
Collembola (total)	1.45	0.295	6.63	0.020[a]	1.52	0.276
Mites (total)	0.19	0.695	2.67	0.129	1.90	0.211
Oribatida	0.31	0.592	3.38	0.086	3.34	0.088
Mesostigmata	3.50	0.089	3.72	0.072	0.16	0.856
Prostigmata	0.88	0.375	1.59	0.266	0.99	0.411

[a]Indicates significant effect ($p < 0.05$).

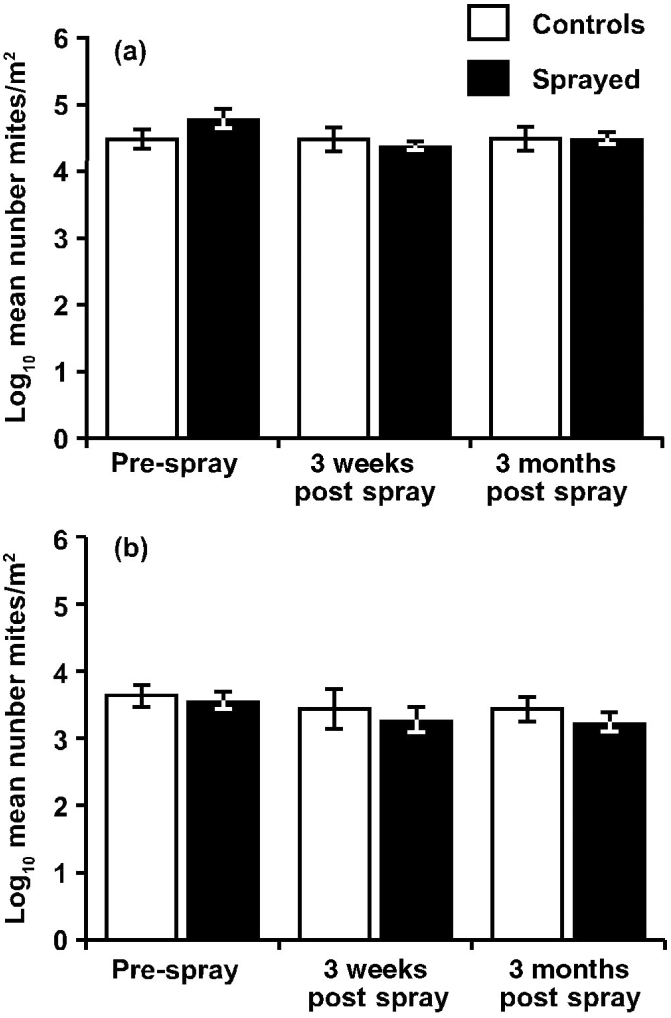
and Prostigmata (Table 2). There were insufficient data on astigmatid mites to conduct statistical analyses. Although none of these analyses showed statistically significant effects, given the low p values for the treatment × time interaction for Oribatida ($p = 0.088$) and the treatment ($p = 0.089$) and time effects ($p = 0.072$) for Mesostigmata, these results were examined more closely (Fig. 2).

Prior to spraying, fewer oribatid mites were collected from the samples taken in the future control plots than in the future sprayed plots, although the difference was not statistically significant. After treatment, however, there was no significant difference between the numbers of oribatids in the control plots and those that had been sprayed with Btk (two-way RM-ANOVA on post-treatment samples only; treatment effect, $p = 0.105$; time effect, $p = 0.262$; treatment × time interaction, $p = 0.350$).

Following spraying, there were fewer mesostigmatid mites on the Btk plots than on the control plots (two-way RM ANOVA on post-treatment samples only; treatment effect, $p = 0.040$; time effect, $p = 0.756$; treatment × time interaction, $p = 0.836$). However, even before the spray was applied, there was a (nonsignificant) tendency for mesostigmatid mites to be more abundant on the future control plots than the future sprayed plots (Fig. 2). When the pre- and post-spray data are considered together (Table 2), the nonsignificant treatment × time interaction indicates that the decline in numbers of mesostigmatid mite occurred over the course of the season, irrespective of whether or not the plots were sprayed.

Representatives of several other taxa, including tardigrades, proturans, symphylans, pseudoscorpions, and several orders of insects were also extracted from the soil samples. Low abundance of these groups prevented statistical analysis.

Fig. 2. Estimated numbers of oribatid (*a*) and mesostigmatid mites (*b*) per square metre in control and treated plots (expressed as $\log_{10}$ mean ± SD). Each bar represents the mean for three plots.



Depth distribution of Collembola and mites

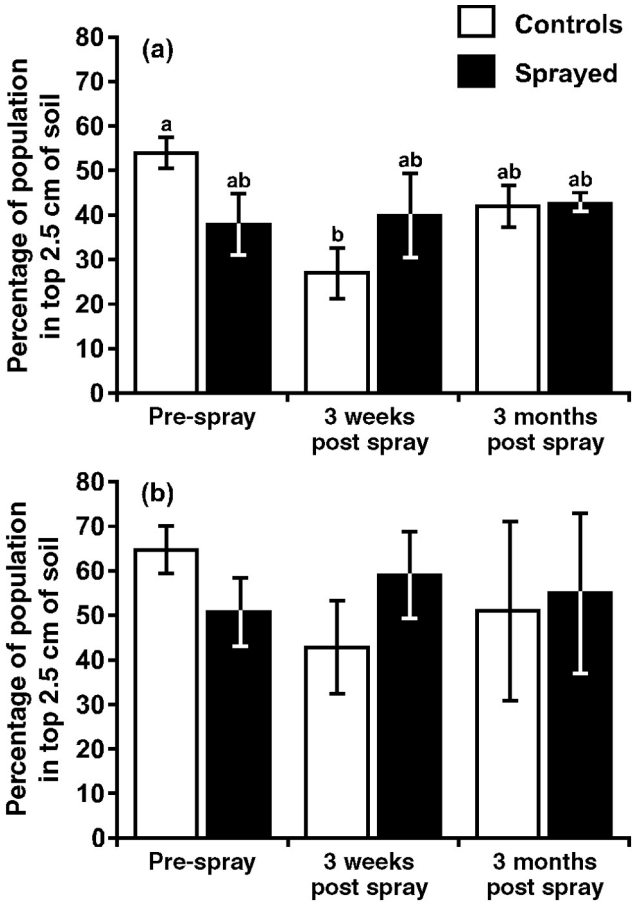
There was no evidence of a selective reduction in microarthropod abundance in the uppermost layer of soil following aerial spraying of Btk. Although two-way RM-ANOVA on Collembola data (Table 3) revealed a significant treatment × time interaction, the reduction in the proportion of the collembolan population in the top 2.5 cm of soil in samples taken 3 weeks after spraying occurred in the control plots and not in the plots sprayed with Btk (Fig. 3; Tukey's test, $p < 0.05$). Similar analyses carried out to detect a selective reduction in mite populations in the upper 2.5 cm of soil (Table 3) showed no significant effects on the total mite population, or on prostigmatid mite densities considered separately. For oribatid mites, RM-ANOVA detected a significant treatment × time interaction, but none of the means differed significantly from one another (Tukey's test, $p \leq 0.05$). Moreover, the percentage of oribatid mites inhabiting the top 2.5 cm of soil 3 weeks after spraying (July samples) actually increased in the sprayed plots compared with the

Table 3. Results of two-way repeated-measure ANOVA to determine effects of treatment and time on the proportion of Collembola and mites inhabiting the uppermost layer of the soil (0–2.5 cm).

	Source of variation					
	Treatment		Time		Treatment × time	
Factor (taxon)	*F*	*p*	*F*	*p*	*F*	*p*
Collembola (total)	0.10	0.760	6.60	0.020[a]	8.48	0.011[a]
Mites (total)	2.10	0.185	0.81	0.477	2.42	0.150
Oribatida	0.27	0.618	0.98	0.416	4.68	0.045[a]
Mesostigmata	2.20	0.176	12.82	0.003[a]	0.84	0.465
Prostigmata	1.03	0.340	1.42	0.296	1.98	0.201

[a]Indicates significant effect ($p < 0.05$).

Fig. 3. Percentages of total collembolan population (*a*) and oribatid mites (*b*) in the upper 2.5 cm of soil. The same letter above the bars indicates no significant difference (Tukey's test, $p < 0.05$). For the data on oribatid mites, application of Tukey's test failed to identify any significant differences among means. Error bars denote SD.



controls. The proportion of the total mesostigmatid mite population in the top 2.5 cm. of soil was significantly lower in the July samples than in either the June or the September samples. However, this relative decline in the proportion of surface-dwelling mesostigmatids 3 weeks after spraying occurred in the control sites as well as the sprayed sites, suggesting again that the decline was due to environmental factors, not Btk.

Species diversity of Collembola

Distribution of collembolan species

More than 24 000 individuals, representing 48 species of Collembola, were identified from the 180 soil cores examined during the course of this investigation. At least two of the species are believed to be new to science, and several others differed in several details from published species descriptions. Appendix A is a complete list of the Collembola species, taxonomic authorities, distribution, and abundances (Table A1).

The most abundant collembolan species collected was *A.* sp. nr. *septentrionalis*, but 90% of the individuals collected (8946 out of 9956 specimens) were extracted from a single sample taken in control plot 3 in June. Eleven species of Tullbergiinae were found in the plots. Very young immatures of several of these species could not be identified with any degree of confidence and constitute the majority of the "unidentified" category in Table A1. The most abundant and widely distributed of the Tullbergiinae species was *Multivesicula columbica* Rusek, 1982. *Mesaphorura krausbaueri* Börner, 1901, although present in high numbers in control plot 3 and spray plots 2 and 3, was virtually absent from the other plots. *Xenylla humicola* and *Folsomia elongata* (MacGillvray, 1896) were relatively abundant at all plots. On the other hand, *Xenyllodes armatus* Axelson, 1903, *Paristotoma notabilis* (Schäffer, 1896), and *Micraphorura absoloni* (Börner, 1910), abundant on control plots 1 and 2, were infrequently encountered on control plot 3 or any of the spray plots. Thus, although there appear to be differences among the collembolan faunas of the various plots, the data presented in Table A1 provide no evidence to support the hypothesis that any species present on a plot before spraying was subsequently significantly reduced or eliminated as a result of the Btk spray.

Diversity indices

As all three diversity indices used in the study (N_0, N_1, and N_2) are expressed in the same units (number of species), the results are easily interpreted and compared (Table 4).

The mean number of collembolan species per plot (N_0) was higher in the control plots than in the treated plots (Table 5). However, there were more species on the control plots than on the treated plots, even before the latter were sprayed with Btk. The mean number of collembolan species dropped significantly in July (Tukey's test, $p < 0.05$), but this decline in species richness occurred in both the control

Table 4. Values of three diversity indices for collembolan species, calculated from samples collected prior to spraying and on two sampling dates after spraying.

	Before spraying			3 weeks after spraying			3 months after spraying		
Treatment	N_0	N_1	N_2	N_0	N_1	N_2	N_0	N_1	N_2
Control plots	25.7	9.2	6.1	22.3	7.8	4.7	25.3	6.4	3.8
Sprayed plots	23.0	9.1	6.3	19.0	6.9	5.2	22.7	7.8	4.9

Note: N_0 is the total number of species, N_1 is the number of abundant species ($e^{\text{Shannon's index}}$), and N_2 is the number of very abundant species (1/Simpson's index).

Table 5. Results of two-way repeated-measure ANOVA to determine effects of Btk treatment and time on three measures of collembolan species diversity.

	Source of variation					
	Treatment		Time		Treatment × time	
Factor	F	p	F	p	F	p
N_0	7.01	0.029[a]	4.60	0.047[a]	0.04	0.964
N_1	0.04	0.842	2.60	0.135	0.72	0.520
N_2	0.75	0.102	2.65	0.131	0.06	0.854

[a]Indicates significant effect ($p < 0.05$).

and the sprayed plots. Since the treatment × time interaction was not significant, there was no evidence to suggest that species richness responded in a different manner in sprayed plots compared with control plots. Values of diversity indices N_1 and N_2 were not significantly affected by Btk treatment (Table 5).

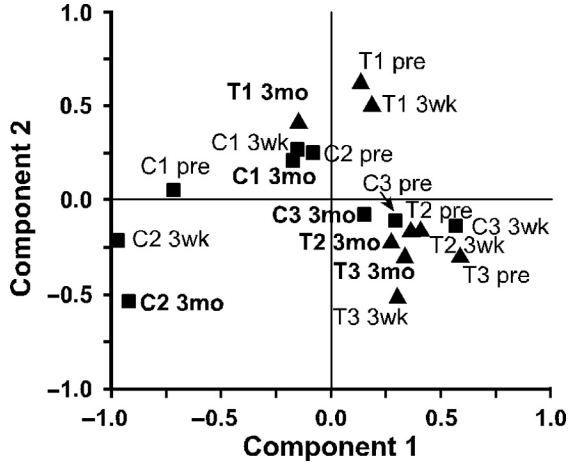
Collembolan communities

CA indicated that, based on the composition of their collembolan faunas, control plot 3 and spray plots 2 and 3 were all very similar throughout the sampling period (Fig. 4). In particular, the samples taken 3 months after spraying at spray plots 2 and 3 were virtually indistinguishable from samples taken at the same time of year at the unsprayed control plot 3 (Fig. 4). Control plots 1 and 2 and spray plot 1 formed a more diffuse grouping, but again there was no indication that application of Btk influenced the ordination of the plot/time combinations. Three weeks after spraying, the collembolan fauna of spray plot 1 was still virtually identical with the species composition at the same location before spraying, and after 3 months the collembolan fauna of the sprayed site closely resembled that of the unsprayed control plot 2 (Fig. 4).

Feeding biology of collembolan species

The present study focuses on a group of Collembola that were found to have a significant (i.e., >5%) component of bacteria in the gut contents. These were *Hymenaphorura* sp. *subtenuis* grp. (Folsom, 1917), *A.* sp. nr. *septentrionalis*, *Multivesicula columbica* Rusek, 1982, *Multivesicula puncata* Rusek, 1982, *Mesaphorura ruseki* Christiansen and Bellinger, 1980, *M. krausbaueri*, and *Willemia intermedia* Mills, 1934. Of these species, two were excluded from analyses: *H.* sp. *subtenuis* group because it did not occur on the treated plots even before spraying, and *A.* sp. nr. *septentrionalis* because of its extremely patchy distribution. Abundances of the other species in this group were combined to provide a single estimate of "bacterial feeders" for each plot

Fig. 4. Distribution of plot–time combinations based on correspondence analysis of collembolan species. The analysis included all species with >30 individuals, except *A.* sp. nr. *septentrionalis* and *X. humicola*. C1–C3 are control plots, T1–T3 are treated (sprayed) plots. "Pre" denotes pre-spray samples and "3wk" and "3mo" denote samples taken 3 weeks and 3 months after spraying, respectively (data from plots 3 months after spraying are in boldface type).



at each sampling time, and two-way RM ANOVA was used to test for time and treatment effects on population numbers. The analysis showed no significant effects of either treatment ($F = 0.10$, $p = 0.937$) time ($F = 1.49$, $p = 0.282$) or the treatment × time interaction ($F = 0.45$, $p = 0.625$).

Gut contents of three of these species extracted from sprayed samples were compared with gut contents of the same species in control samples taken at the same time of year (i.e., 3 weeks and 3 months after spraying). Bacteria formed a relatively small proportion of the gut contents for all these species, even after the plots had been sprayed with Btk (Table 6). None of the results suggested that the proportion of bacteria in the diet increased after spraying with Btk. Although sample numbers were low and results were variable, for each species considered in this analysis, the most abundant category of ingested material (excluding mineral particles) was the same in specimens collected from control plots as in specimens from soil samples collected from plots sprayed with Btk.

Discussion

Analyses of abundance data for Collembola and the different suborders of mites in sprayed and control plots did not identify any treatment-related differences in abundance. Although there were some instances of significant declines

Table 6. Percent composition of total gut contents of three species of Collembola.

	Treatment	*n*	Fungal material	POM	Animal material	AOM	POMvf	Bacteria	Mineral material
Multivesicula columbica	Control	15	12.67	2.67	4.67	17.30	14.67	15.33	32.77
	Btk	19	1.05	1.05	0.53	37.89	26.84	10.00	22.63
Multivesicula punctata	Controls	6	10.00	1.67	13.33	18.33	6.67	11.67	38.33
	Btk	13	5.45	1.82	0.00	36.36	27.27	10.00	20.00
Mespahorura krausbaueri	Controls	9	8.89	0.00	8.89	48.89	2.22	11.11	17.78
	Btk	28	10.36	1.43	13.93	39.64	12.14	5.00	17.50

Note: Data are from individuals collected after spraying with Btk (July and September samples); *n* is the total number of individuals with gut contents, POM is particulate organic matter; POMvf is very fine POM (<5 μm diameter), and AOM is amorphous organic material.

in numbers of individuals in the July samples (3 weeks after spraying), these declines were seen in both the control plots and in those exposed to aerial deposition of Btk. Consequently, it is likely that the observed declines were due to environmental factors common to all plots rather than to Btk. Similarly, although the vertical distribution of Collembola and the different groups of mites changed over the sampling period, there was no evidence of a reduction in the surface-dwelling collembolan and mite populations following exposure to Btk.

The results of the CA showed that the collembolan faunas at the control sites were not as homogeneous as we had expected. In particular, the collembolan community at control plot 2 seemed to differ from that at other sites, especially later in the season. The control sites were chosen to encompass the range of conditions at the spray sites (pH, soil type and texture, elevation, and Biogeoclimatic Ecosystem Classification subzone), and within these criteria, were chosen to be as similar as possible. However, greater variability in the control plots makes it more difficult to detect significant treatment effects against the background noise in the data. We attempted to mitigate this limitation by closely examining ANOVA effects at a higher level of significance, but even then we could find no evidence that Btk had affected the abundance or vertical distribution of the microarthropods.

Diversity indices have been used in the past to detect the response of communities to stressors (e.g., Cárcamo and Parkinson 2001). In this study we hypothesized that any impact of spraying activity on the collembolan community would be reflected in changes to the values of N_0, N_1, or N_2. This was not seen. Although species richness (N_0) declined significantly in the samples collected 3 weeks after spraying (July samples), this decline was seen in the control plots as well as in those treated with Btk.

Although it is intuitively appealing to use diversity indices to detect changes in community structure as a result of land-management practices, they have not always proved useful (Siepel and van de Bund 1988). A major shortcoming of diversity indices is that they do not identify which species are contributing to the diversity (Magurran 1988). Consequently, plots can yield similar values for diversity indices, yet have very different species compositions. Soil ecologists and ecotoxicologists have therefore suggested that the aspects of community structure of soil organisms in the field may be a better indicator of the impacts of activities such as pesticide use and management practices on the soil system (Siepel and van de Bund 1988; van Straalen 1998). Multivariate analyses, such as the CA presented in this study, are one method by which changes in the species composition of communities in response to pesticide application can be assessed.

This study did not produce any evidence to support the idea that application of Btk caused detectable changes in the collembolan community. On the other hand, the analysis clearly showed that the collembolan communities of control plots 1 and 2, and to a lesser extent spray plot 1, were distinct from those of control plot 3 and the other two spray plots through the entire sampling period. Control plots 1 and 2 were located at a slightly higher elevation than the other plots (Table 1). Moreover, these plots were located in a slightly different variant of the same subzone (IDFdk1 for controls plots 1 and 2; IDFdk2 for control plot 3 and spray plots 2 and 3). Spray plot 1 occupied an intermediate position on the elevation gradient and although classed as IDFdk2, also exhibited characteristics of the IDFdk1 variant (Table1). Thus, CA was able to distinguish subtle differences between the collembolan communities of closely related variants of the same subzone, but did not detect any difference between treated and control plots within the same subzone (Fig. 4).

Determining the natural diet of Collembola is difficult. In the laboratory, individuals will eat a number of items that are not part of their natural environment (including filter paper, charcoal, and baker's yeast), so extrapolation from the laboratory to the field situation becomes problematic. Broza et al. (2001) fed several different strains of Bt (including Btk) to *F. candida* in laboratory tests where the collembolans were not offered any food except the proffered bacteria. Although *F. candida* showed high rates of survival during the experiment, the growth rate and fecundity of collembolans fed on a diet of Bt was much lower than in the controls, which were fed on yeast. This result could be interpreted to mean that ingestion of Bt had a negative impact on growth and fecundity, although it is more likely that the Bt was not an adequate diet for *F. candida*. While this experiment showed that *F. candida* can ingest Bt, it does not answer the question of whether Bt would be likely to form a significant portion of the collembolan diet in the field, where the insects are less restricted in their choice of food items.

If, as has been hypothesized (Lambert and Peferoen 1992; Meadows 1993), toxin production in Btk evolved as a mechanism to limit predation by bacteria-feeding invertebrates, then it is possible that collembolan species that typically ingest bacteria might also be susceptible to the toxins. This hypothesis was supported by studies of Borgonie et al.

(1995, 1996) which showed that spores of Bt (identified only by strain number) germinated in the gut of the bacteriophagous nematode *Panagrellus redivivus* L., 1767, colonizing the entire nematode within 24 h, and furthermore that the toxicity of spores and crystals of Bt to different species of nematodes was related their trophic group. However, another study (Mozgovaya et al. 2002) showed no link between nematicidal activity of five different strains of Bt and the trophic group of the exposed nematodes.

We hypothesized that those species that normally ingested bacteria would be more likely to show impacts of Btk than those feeding on other foods. None of the collembolan species examined can be considered true bacteriovores, although bacteria were clearly visible in the gut contents of several species. Our study produced no evidence that population numbers of those species that normally include bacteria in their diet were affected in any way by the Btk spray. Collembola might respond to aerial deposition of Btk by increasing the amount of bacteria in their diet, but the present study yielded no evidence of increased bacterial consumption by Collembola collected from plots sprayed with Btk.

Conclusions

This study yielded no evidence that aerial spaying of Btk as Foray® 48B (60 BIU/ha in 4.8 L/ha) had any significant effect on the overall abundance of soil Collembola and mites, or on aspects of the community ecology, species diversity, or feeding biology of Collembola.

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Appendix A

Appendix appears on the following page.

Table A1. Abundances (total numbers) and distribution of collembolan species.

	Before spraying						3 weeks after spraying						3 month after spraying					
	Control plots			Sprayed plots			Control plots			Sprayed plots			Control plots			Sprayed plots		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Anurophorus sp. nr. *septentrionalis* Palissa, 1966	0	7	9017	660	0	0	4	0	160	100	0	1	2	0	2	2	0	1
Multivesicula columbica Rusek, 1982	7	45	24	21	46	3	236	4	33	56	19	7	509	5	427	240	211	35
Mesaphorura krausbaueri Börner, 1901	0	1	119	0	112	250	0	13	141	0	110	79	0	8	289	0	158	389
Xenylla humicola (O. Fabricius, 1780)	84	485	9	72	42	13	127	2	15	57	5	693	8	1	0	12	0	19
Folsomia elongata (MacGillvray, 1896)	13	250	101	91	275	130	78	3	23	54	65	20	69	3	59	69	186	126
Xenyllodes armatus Axelson, 1903	122	68	0	1	0	5	122	167	0	0	29	5	559	327	0	8	21	10
Willemia sp. (*similis* grp. Mills, 1934)	25	113	0	40	141	1	54	6	4	7	47	4	79	14	68	61	82	53
Willemia intermedia Mills, 1934	55	165	9	76	83	22	52	5	1	3	9	4	117	13	21	9	35	25
Multivesicula punctata Rusek, 1982	1	30	5	8	8	3	118	4	4	21	16	2	332	0	45	91	75	24
Chaetaphorura mala (Christiansen and Bellinger, 1980)	1	8	8	31	9	66	0	6	61	26	12	4	5	1	42	58	29	70
Willemia arida Fjellberg, 1991	14	15	12	8	23	19	10	7	0	18	5	0	16	24	44	16	93	98
Mesaphorura ruseki (Christiansen and Bellinger, 1980)	151	3	3	0	0	0	14	71	1	22	0	0	0	3	102	41	0	0
Hymenaphorura cocklei (Folsom, 1908)	0	4	2	149	20	41	6	0	4	26	70	5	1	0	2	25	12	0
Deuteraphorura sp. ? *lusa* (Christiansen and Bellinger, 1980)	54	21	1	0	19	14	6	1	2	1	13	1	22	3	1	2	28	46
Folsomia bisetosa Gisin, 1953	28	33	4	3	24	0	9	7	2	2	10	2	10	20	8	5	6	17
Mesaphorura macrochaeta Rusek, 1976	0	0	2	32	0	0	0	27	0	0	0	0	12	21	0	88	0	0
Hymenaphorura sp. (*subtenuis* grp. (Folsom, 1917))	2	25	16	17	0	1	17	0	20	7	0	0	10	0	0	1	0	0
Parisotoma notabilis (Schäffer, 1896)	29	12	0	0	0	0	2	23	0	1	0	1	4	36	1	1	0	0
Folsomia sp. (*nivalis* grp. Packard, 1873)	0	24	1	26	2	1	7	0	0	7	3	0	18	0	2	8	6	0
Chaetaphorura bella Fjellberg, 1988	1	14	0	0	5	7	16	0	2	0	1	0	26	0	1	3	4	13
Mesaphorura pacifica Rusek, 1976	0	44	0	0	0	8	0	0	2	0	0	0	0	0	3	0	0	0
Micraphorura absoloni (Börner, 1910)	24	16	0	3	0	0	3	5	0	0	0	0	0	4	0	0	0	0
Onychiurus sp. ?*reluctus* Christiansen, 1961	2	0	4	0	0	0	2	3	0	0	0	1	18	12	7	0	0	0
Micranurida pygmaea Börner, 1901	1	3	2	4	18	1	2	0	1	0	2	0	0	1	4	0	2	1
Hymenaphorura similis (Folsom, 1917)	9	4	0	0	1	0	2	17	0	0	0	0	0	6	0	2	0	0
Pseudisitoma sensibilis (Tullberg, 1876)	0	4	2	2	4	9	6	0	1	1	1	0	1	0	1	1	0	4
Entomobrya triangularis Schött, 1896	1	2	2	10	0	0	0	1	3	2	0	0	2	2	4	6	1	0
Entomobrya comparata Folsom, 1919	0	0	4	0	2	12	0	0	4	0	1	2	0	7	0	0	2	0
Schaefferia duodecimocellata Bonet, 1945	0	0	0	0	0	0	0	4	0	0	0	0	0	25	0	0	1	0
Unidentified	17	77	15	18	64	25	3	70	120	10	121	48	3	33	29	1	163	62
Total no. of Collembola extracted	657	1484	9373	1278	913	647	908	453	605	424	551	880	1829	608	1172	758	1140	1003

Note: Data from all 10 replicate cores per plot taken on each sampling date are combined. Only species with a total abundance of >30 individuals are shown. Scientific names follow Bellinger et al. (1996–2005). The following additional rare species (<30 individuals) were collected at the sites: *Willemia denisi* Mills, 1932, Tullberginae sp. A (sp. nov.?), *Ceratophysella* sp. (*denticulata* grp. Bagnall, 1941), *Pseudachorutes* sp. (cf. *indiana* Christiansen and Bellinger, 1980), *Tullbergia* sp. B, *Isotomodes productus* (Axelson, 1903), *Mesaphorura yosii* Rusek, 1967, *Sminthurinus quadrimaculatus* (Ryder, 1879), *Friesea cera* Christiansen and Bellinger, 1973, *Isotomiella minor* (Schäffer, 1896), *Pogonognathellus flavescens* (Tullberg, 1871), *Willemia granulata* Fjellberg, 1985, *Desoria* sp. 1, *Xenylla canadensis* Hammer, 1953, *Vertagopus arboreus* Ågren, 1903, *Mackenziella psocoides* Hammer, 1953, *Pseudosinella octopunctata* Börner, 1901, *Megalothorax minimus* Willem, 1900, and *Isotoma viridis* Bourlet, 1839.