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Effect of Two Commercial Formulations of *Bacillus thuringiensis* subsp. *kurstaki* (Dipel® 8L and Dipel® 8AF) on the Collembolan Species *Folsomia candida* in a Soil Microcosm Study

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Global use of *Bacillus thuringiensis (B.t.)*-based insecticides is increasing (McDonald 1991). In Canada, only New Brunswick still permits aerial spraying of chemical insecticides for control of forest insects. In all other provinces, *B.t.* subsp. *kurstaki* is the insecticide of choice. Consequently, a corresponding rise in the input of *B.t.* and its toxins into soil systems can be expected, whether by direct spraying, in insect cadavers, or in transgenic plant material or microorganisms. *B. thuringiensis* is generally regarded as a soil bacterium (Delucca et al. 1981, Martin and Travers 1989), but surprisingly little is known about the ecology of this organism in the soil (Lambert and Peferoen 1991). There have been few studies of the effects of *B.t.* on soil invertebrates (Addison 1993), although Atlavinytė et al. (1982) reported that collembolan numbers in soil were decreased after application of Entobacterin® (a formulation of *B.t.* subsp. *galleriae*).

Soils in the northern boreal forest tend to have well developed organic horizons (i.e., "mor" or "moder"type soils), in which the macrofauna is generally reduced, and Collembola and mites are the most abundant representatives of the decomposer arthropod fauna (Petersen and Luxton 1982). In such forest soils, Collembola play important roles in decomposition, and tend to be concentrated in the upper organic horizons, where pesticide impingement is likely to occur.

Proposed Guidelines for the Registration of New Microbial Pest Control Agents in Canada (Anon. 1993) recommend that in addition to non-target test organisms selected on the basis of taxonomic similarities to the host (pest), consideration should be given to species which have the greatest potential for exposure. The Guidelines also state that consideration should be given to species from the ecozone of intended use, particularly species of broad ecological or commercial importance. One of the taxa suggested for non-target testing is the Collembola.

There is an urgent need to develop test protocols for determining long-term lethal and sub-lethal effects of microbial pest control agents in soil because many (proposed) microbial pest control agents, such as *Bacillus thuringiensis* Berliner

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(*B.t.*) and baculoviruses can persist for a long time in the soil (Addison 1993; Thompson et al. 1982). The protocol reported here used simple microcosms to investigate the effects of two commercial products (Dipel®8L and 8AF), both containing *B.t.* subsp. *kurstaki* (*B.t.k.*) as the active ingredient, on the soil collembolan *Folsomia candida* Willem.

METHODS AND MATERIALS

Organic material was collected in the spring of 1991 from the LFH (organic or "duff") layer overlying the mineral soil of a previously uncut and unsprayed coniferous mixedwood forest near Thessalon, Ontario (46°25'N, 83° 33'E). The depth of the organic mat in the sampled areas was approximately 5 cm, and consisted mainly of material of coniferous origin. The moisture content of the LFH layer ranged from 65 to 74%, and the pH(±SD) was 4.9(±0.1). The dominant tree species at the site were balsam fir (*Abies balsamea* (L.) Mill), black spruce (*Picea mariana*, (Mill.) BSP) and white spruce (*P. glauca* (Moench) Voss.), with some aspen (*Populus tremuloides* Michx.). The site was representative of the forest type that is often treated to control the spruce budworm (*Chloristoneura fumiferana* Clem.).

The collected material was air-dried in the laboratory and passed through a 4 mm sieve. The LFH material was then rehydrated, frozen at -15°C for a minimum of 2 weeks, allowed to thaw at room temperature, and left for one week to allow re-establishment of microbial populations before being used in experiments. This treatment was similar to that used by Huhta et al. 1989 and was successful in eliminating the resident populations of Collembola, enchytraeid and lumbricid worms, and dipteran and coleopteran larvae. Although nematodes and the occasional cryptostigmatid mite were able to survive this treatment, predaceous mites were never found in the prepared substrate.

The recommended application rate for both Dipel®8L (Dipel®176L in Canada) and Dipel®8AF (Dipel®64AF in Canada) for forest use in Canada (1.8L/ha), was used to calculate the Expected Environmental Concentration (EEC). Following the proposed Canadian Guidelines (Anon 1993), the pesticide was assumed to be distributed evenly throughout a 15 cm depth, giving an EEC of 0.0012µL/c.c. organic matter (20.289IU/0.0012µL). The prepared substrate (density 0.4287g/c.c.) was packed into shallow metal trays (325g/tray) and sprayed with 20 mL distilled water (controls), or the test substance applied in distilled water to make a final volume of 20 mL, using a hand-held spray bottle. After spraying, the organic material was gently mixed by hand for 10 minutes to distribute the sprayed material throughout the sample. The final moisture level of the material was 70% (expressed as % wet weight), representative of the moisture level at the time of year when spraying for spruce budworm would occur. Glass shell vials (diameter 2.8 x 6.4mm) were packed with 6.5 g of the treated organic matter, and 30 adult *F. candida* (from a laboratory culture originally isolated from a spruce/fir forest in Québec) were added. The open end was covered with a piece of nylon

mesh (9 holes/mm) and closed with a tight-fitting plastic lid containing two small holes for ventilation. All experiments were carried out at 20°C.

Two experiments, using the following treatments were conducted:

1. Dipel®8L (an oil-based formulation), or Dipel®8AF (an aqueous formulation), at deposits equivalent to 1000X EEC (1.2µL/c.c. organic material). Controls were sprayed with an equivalent volume of distilled water. There were 3 treatments x 5 replicates (vials)/treatment/sampling time, with 4 sampling times (2,4,8,12 weeks after treatment), for a total of 60 vials.

2. The oil-based formulation blank of Dipel®8L, a preparation of *B.t.k.* technical powder (containing the bacteria, crystals and a small amount of broth), or the oil-based formulation blank + the *B.t.k* preparation, each sprayed in 20 mL distilled water, at deposits equivalent to 1000X EEC. The dosage of *B.t.k.* technical powder used in the experiments was calculated on the basis of potency (20,289 I.U./cc organic material, equivalent to 1000X EEC). Controls were sprayed with distilled water. There were 4 treatments x 8 replicate vials /treatment/sampling time x 4 sampling times (2,3,4,6,weeks after treatment) for a total of 128 vials.

At each sampling time, five (Experiment 1) or eight (Experiment 2) vials were randomly chosen from the pool of remaining vials for each treatment, and the Collembola from each vial were extracted into distilled water using modified small Tullgren funnels without heating. A piece of fine nylon mesh was placed over the organic matter in each funnel to prevent the Collembola from escaping during the extraction process. The insects were stored in 70% ethanol and were counted under a dissecting microscope.

During the first three weeks of the experiments, the size difference between adults originally added to the substrate, and juveniles hatching from eggs laid during the experiment was great enough to permit easy separation of the two cohorts and thus the extraction efficiency for the adults could be calculated.

RESULTS AND DISCUSSION

Extraction efficiency of the adult Collembola originally added to each vial was high. In the control vials sampled after 2 weeks, 93% of the original adults were recovered, and in the three week sample, 97% were extracted.

In both the controls and the microcosms treated with the aqueous formulation (Dipel®8AF), populations of the Collembola increased rapidly, reaching a maximum after 4 weeks (Figure 1). They subsequently declined, presumably due to substrate depletion. In contrast, populations in the microcosms treated with 1000X EEC of the oil-based formulation (Dipel®8L) remained extremely low throughout the entire experimental period, although the population never dropped to zero in any of the replicates. In spite of the fact that there were at least a few

adult survivors in every microcosm treated with the oil-based formulation, there was no evidence of a population recovery within the 12 week experimental period.

A two-way analysis of variance (Program 7D, BMDP Statistical Software, Los Angeles, CA) using the Brown-Forsythe statistic revealed highly significant effects of time and treatment, and also a significant interaction between the two (Table 1). *A priori* user-specified contrasts indicated that the collembolan population response was significantly reduced in microcosms containing the oil-based Dipel® 8L ($p<0.001$). There was no significant difference between the numbers of Collembola in the controls and those treated with the aqueous Dipel® 8AF ($p=0.0687$).

Collembolan populations in the control microcosms and those sprayed with *B.t.k.* increased rapidly, achieving levels approaching 1000 individuals/vial by weeks 4 and 6 (Figure 2). In contrast, numbers remained low in both treatments containing the oil-based formulation blank.

Table 1. Effect of Dipel®8AF and Dipel®8L on population growth of *F. candida* over 12 weeks. (Two-way ANOVA using the Brown-Forsythe statistic on $\log_{10}$ transformed counts)

Source of Variation	D.F.	F statistic	P
Time	3,21	51.72	<0.001
Treatment	2,21	240.27	<0.001
Time x Treatment	6,21	14.83	<0.001

Table 2. Effect of *B.t.k.*, the oil-based formulation blank of Dipel®8L, and the oil-based formulation blank + *B.t.k.* on population growth of *F. candida* over 6 weeks. (Two-way ANOVA using the Brown-Forsythe statistic on $\log_{10}$ transformed counts)

Source of Variation	D.F.	F statistic	P
Time	3,66	12.5	<0.001
Treatment	3,66	134.22	<0.001
Time x Treatment	9,66	28.85	<0.001

Two way analysis of variance (Program 7D, BMDP Statistical Software, Los Angeles, CA) using the Brown-Forsythe statistic revealed highly significant effects of time and treatment, and also a significant interaction between the two

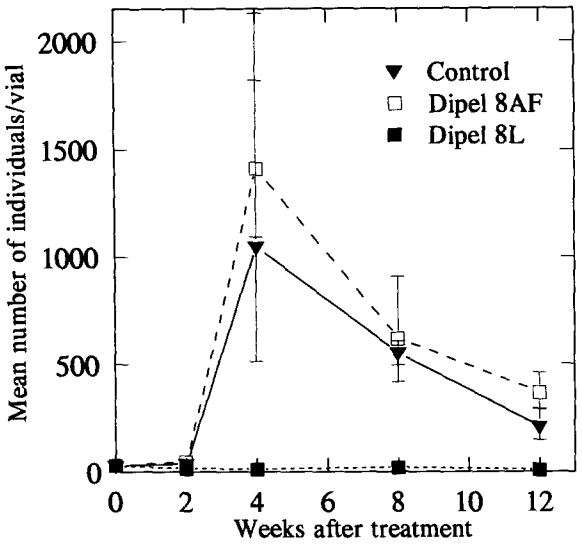

Figure 1. Effect of Dipel® 8L and Dipel® 8AF on population growth of *F. candida*. Error bars are 95% confidence limits on geometric means (n=5).

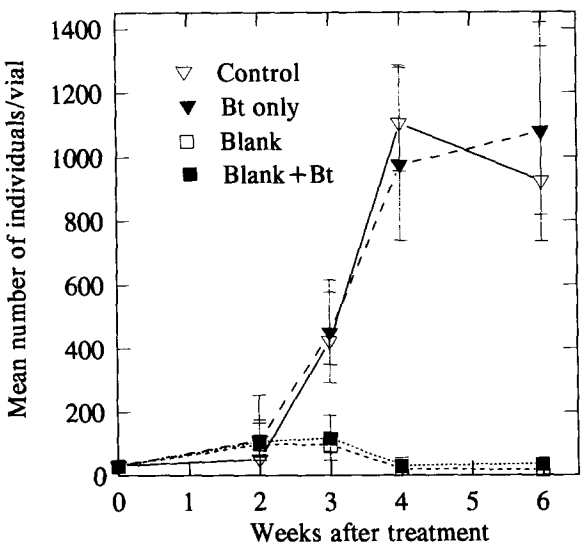

Figure 2. Effect of *B.t.k.* and the oil-based formulation blank of Dipel®8L on population growth of *F. candida*. Error bars are 95% confidence limits on geometric means (n=8).

(Table 2). A series of comparisons was carried out using Bonferroni t-tests (which adjust the α value for the specified number of comparisons) to identify, within each sampling time, if any of the treatments differed from the control, and whether the addition of *B.t.k.* to the oil-based blank caused a different response than exposure to the blank alone. Thus for each of the 4 time periods there were four planned comparisons.

These tests (Table 3) indicated that the population response of *F. candida* to *B.t.k.* alone was not significantly different than in the controls at any of the sampling times, but that the Collembola were significantly affected by all treatments containing the formulation blank from week 3 on to the end of the experiment. Populations of *F. candida* in microcosms containing both the active ingredient *B.t k.*, and the oil-based blank were not significantly different than they were in microcosms containing the oil-based blank only.

Table 3. Results of planned comparisons (t-tests) designed to test effects of *B.t.k.*, the oil-based formulation blank, and a combination of the two on population growth in *F. candida*. Using the Bonferroni adjustments, a single comparison must have a p value of <0.003125 to be significant at the α=0.05 level when comparing 16 pairs of means (0.05/16).

Contrast	Week 2	Week 3	Week 4	Week 6
Control vs. *B.t.k.*	0.0085ns	0.8447ns	0.6800ns	0.6100ns
Control vs. oil-based formulation blank	0.0262ns	<0.001	<0.001	<0.001
Control vs. oil-based formulation blank+ *B.t.k.*	0.0118ns	<0.001	<0.001	<0.001
Oil-based formulation blank vs. *B.t.k.*+ formulation blank	0.7060ns	0.4979ns	0.0869ns	0.0259ns

Proposed Canadian Registration Guidelines (Anon. 1993) assume that the pesticide is distributed evenly throughout a 15 cm layer of substrate. However, both laboratory (Krieg 1983) and field studies (Delisle et al. 1991) have reported that *B.t.* is not readily leached in soil. Thus the assumption that the pesticide is evenly distributed within a 15 cm layer of soil probably underestimates the true concentration to which soil organisms would be exposed. More realistically, the *B.t.* would remain concentrated within the organic horizons of forest soil, probably within 5 cm of the surface. Thus the 1000X EEC concentration used in the above experiments probably represents a level ~333 times the amount impinging on the soil, assuming no interception in the canopy. This is considerably higher than would occur in the field in operational spray programs.

Since the collembolan populations were not adversely affected by the unformulated preparation of *B.t.k.* or the aqueous formulation (Dipel® 8AF), but

were decreased only in the presence of preparations containing the oil-based formulation blank, we concluded that it was the oil-based formulation blank, rather than the *B.t.k.* itself which was responsible for the population reductions observed in the presence of Dipel® 8L. There are several mechanisms whereby an oil-based formulation might adversely affect collembolan populations, including direct toxicity or suffocation (Collembola rely on cutaneous respiration), or indirectly by affecting the microbial community on which *F. candida* feeds. Studies on the effects of Dipel®8L (1000X EEC) on soil microflora (Visser et al. 1994), using organic material collected at the same site as in the present study, indicated that this pesticide had significant effects on microbial processes.

The addition of the *B.t.k.* preparation to organic matter already sprayed with the oil formulation blank did not change the population response from what could be attributed to the oil-based formulation blank alone (Figure 2; Table 3). The results of this experiment do not support the hypothesis (Addison 1993) of a possible synergistic interaction between *B.t.* and ingredients of formulation (e.g. oil) that may increase susceptibility of non-target invertebrates to the bacterium.

The ecotoxicological test procedure described in this paper allows for long-term (2 months) testing of indigenous members of the soil fauna in substrates taken from the ecozone where the pesticide is to be used. The method is easy to perform, yet was sensitive enough to detect subtle differences in sub-lethal effects under experimental conditions that can be related to the field situation. The experiments showed that *F. candida* populations were not affected by *B.t.k. per se*, or in an aqueous formulation. However the oil-based formulation blank used in Dipel®8L suppressed populations of Collembola at 1000X EEC. Further experiments are in progress to determine the dose-response of *F. candida* to Dipel®8L.

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