Effects of Btk on Aquatic Microbial Activity, Detrital Decomposition, and Invertebrate Communities

NAPIAP Project NA-25

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A Final Report to the USDA Forest Service
in Fullfillment of the NAPIAP grant NA-25
July 1993

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Project NA-25 Final Report

Abstract

Laboratory experiments were conducted under simulated natural conditions to determine the effects of the microbial insecticide Bacillus thuringiensis var. kurstaki (Btk) on respiration, decomposition, and detrital conditioning activity of natural aquatic microbial communities. Applications of Btk (Dipel 64 AF) to leaf disks in recirculating microcosms stimulated microbial respiration, but only at the maximum challenge concentration of 1000X the expected environmental concentration (20,000 IU/mL). This stimulated respiration was determined to be a microbial response to the actual insecticidal components of the Dipel (the bacterial spores and parasporal toxin crystals), not to the formulation materials. Microbial decomposition (mass loss) of leaf material was significantly reduced in microcosms after 9-d exposure to Btk at both the expected environmental concentration, and 1000X that concentration. Exposure of leaf material to Btk did not affect the microbial conditioning of the detrital surfaces, as measured by the palatability of leaf disks to detritivore macroinvertebrates, even at the maximum challenge concentration. A section of forest stream was treated with Btk at 10X the expected environmental concentration to determine effects on the macroinvertebrate community. The Btk application had little affect on the macroinvertebrate community. Invertebrate drift density increased slightly (approximately 2-fold increase over pre-treatment drift densities), but only during the ½-h application and only at the site 10 m below the application point. There were no measurable changes in taxonomic richness of benthic invertebrates after the application. In 11 of 12 benthic taxa for which there were sufficient data to analyze, there were no significant reductions in abundance after the application, in comparison to changes in abundance at the reference site. One taxa, the stonefly Leuctra tenuis, was significantly reduced at the treated site 4 d after the application, and abundance remained considerably lower than at the reference site for at least 18 d. The Btk application had no significant effect on the growth or survival of caged caddisfly larvae. Implications of results from these laboratory and field experiments are discussed.

Introduction

The microbial insecticide *Bacillus thuringiensis* var. *kurstaki* (Btk) has been developed for use in forest pest management programs, and is now widely used for control of forest lepidopteran pests in Canada and the United States (van Frankenhuyzen 1990). This is a relatively narrow-spectrum insecticide, and is generally considered to be a more environmentally acceptable alternative to broad-spectrum chemical insecticides, in terms of safety to non-target organisms (Ennis and Caldwell 1991). Wide-scale use of this insecticide, particularly by aerial application, can result in contamination of forest water bodies, and the safety of Btk to non-target aquatic organisms has not been rigorously investigated. Surgeoner and Farkas (1989) reviewed the literature on effects of Btk in aquatic systems, and although there were no reports of adverse effects on aquatic organisms, most conclusions were based on unpublished field investigations with older formulations of the biological insecticide. Eidt

(1985) conducted static laboratory bioassays with several taxa of macroinvertebrates and concluded that Btk was not toxic to most aquatic insects. More recently, Kreutzweiser *et al.* (1992) (part of NAPIAP project NA-16) measured acute lethal and sublethal effects of Btk on several species of aquatic insects in laboratory and outdoor stream channel experiments, and concurred that Btk contamination of watercourses was unlikely to cause direct adverse effects on macroinvertebrates. The present study expands on our previous work by investigating effects of Btk on indigenous aquatic microbial communities, and by assessing community-level effects on macroinvertebrates in a section of forest stream treated with Btk.

To our knowledge, effects of Btk on natural aquatic microbial communities have not been investigated previously. The microbial community is vital to aquatic environments for such functions as detrital decomposition, nutrient cycling, and conditioning of organic debris for processing by shredding invertebrates. The measurement of microbial activity (e.g. respiration) can provide a sensitive indicator of microbial response to environmental stimuli or perturbation (Boulton and Boon 1991), or an overall measure of system behaviour (Pritchard and Bourquin 1984). We speculated that the addition of high numbers of non-indigenous bacterial spores, or the parasporal toxin crystals, from Btk applications may alter the activity or function of indigenous microbial communities in aquatic substrates. In this regard, we measured microbial respiration and decomposition activity on leaf disks following exposure to Btk, and determined the effects of Btk contamination on the conditioning of leaf surfaces, in terms of palatability to detritivore caddisfly larvae. The application of Btk to a section of forest stream was intended to field-validate results from previous laboratory and stream channel experiments (Kreutzweiser *et al.* 1992).

Methods and Materials

Laboratory Experiments

Microbial Respiration

The effects of Btk on aquatic microbial activity were determined by comparison of microbial respiration in treated and control test units. Respiration of microbial communities on leaf disks was measured as oxygen depletion in darkened chambers of a respirometer, modified from the stream-side incubator of Rosenfeld (1989). This unit consists of a rectangular Plexiglas tank (75 cm long, 25 cm wide, 10 cm high) in which 12 circular Plexiglas chambers (6.5 cm diameter, 7 cm high) are mounted. Each circular chamber contains a magnetic stir bar in a screened cell at the bottom of the chamber to maintain constant water circulation in the chamber. These stir bars are activated by a series of chain-driven magnetic bars mounted and aligned under the Plexiglas tank, and powered by a variable-speed electric motor. The circular chambers are sealed with Plexiglas lids and O-rings, and are held at ambient water temperature by circulation of river water (pumped directly to a head tank in the laboratory) through the rectangular tank containing the circular chambers. For measurements of microbial community respiration, each chamber is fitted with an opaque cover to exclude light. A sealed port on the top of each chamber allows insertion of a dissolved oxygen probe. Because the chambers are sealed and darkened during the experiments, oxygen depletion is considered to be directly proportional to respiration activity, and this activity is expressed as reduction in dissolved oxygen (mg/L).

Microbial communities were colonized on leaf disks (23 mm diameter) held in river water. The disks were cut from yellow birch (*Betula alleghaniensis*) leaves that were collected just before leaf fall, air dried, and held at room temperature. In preparation for each experiment, leaf disks were individually pinned to a wooden rack that was placed in a flow-through trough under 16:8 h light/dark conditions in the laboratory for about 4 d. For each experiment, the respiration chambers were filled with river water, put in motion, sealed and darkened, and pre-treatment dissolved oxygen (DO) levels were monitored. When the pre-treatment DO levels had stabilized (within ½ h after sealing the tops), they were recorded and nine leaf disks were randomly taken from the colonization rack and placed in each of the chambers. Appropriate amounts of test material were added to treated chambers immediately after the nine disks were placed in them. As soon as the leaf disks and test material (in treated units) were added to each chamber, the chamber was re-sealed and the start time was recorded. Subsequent DO measurements were taken at 1, 3, and 5 h after the addition of materials to each chamber. By the 5-h sample time, DO levels in chambers with leaf disks were usually reduced by 30-45%. This was considered potentially stressful to the microbial community, and no further measurements of respiration activity were taken. Water temperature in the respiration chambers was held at 17° C for all experiments.

For each respiration experiment, four replicate chambers served as controls (river water and leaf disks), four replicates were treated with the test material at a single concentration (river water, leaf disks and Btk), and the remaining four chambers were used to monitor changes in DO in river water alone, and/or in river water and Btk. The Btk was applied as the aqueous flowable formulation, Dipel® 64AF. The effects of Btk on microbial respiration were tested at two concentrations: the expected environmental concentration (20 IU/mL) and 1000X that concentration (20,000 IU/mL). The expected environmental concentration (EEC) is calculated as the concentration that would occur in a 15-cm deep body of water that was directly oversprayed at an application rate of 30 BIU/ha. This is the approach used by Canadian regulatory agencies for estimating expected environmental concentrations in hazard evaluations of forest pesticides in aquatic systems. The 1000-fold increase over the EEC is the maximum challenge concentration currently required by Canadian regulatory agencies for conducting first-level screening of microbial pesticides for adverse effects on non-target organisms.

Microbial Decomposition

For determining the effects of Btk on microbial decomposition activity, the circular respiration chambers described above, served as individual microcosms in which the microbial decomposition of leaf material was measured. A series of air-dried leaf disks were randomly allocated to 12 groups of 9 disks, and batch-weighed to determine pre-treatment weights of the leaf material. The 12 groups of disks were incubated in river water, as above, for 4 d, then transferred to the 12 circular chambers containing river water, and the treatments were initiated. Four randomly-selected chambers were treated with Btk (Dipel 64AF) at the EEC, four were treated at 1000X the EEC, and four contained only river water, to serve as controls. During this experiment, the chambers were set in motion to provide constant water circulation, but were left open on top to avoid DO depletion. The test material in treated units was renewed every 48 h, and the leaf disks were held in the chambers for 9 d. At the end of the 9-day period, the leaf disks were removed from the chambers, air-dried at room temperature for 48 h, and batch-

weighed to determine mass loss as a measure of microbial decomposition. The chambers were held at 18° C for the 9-d period.

Microbial Conditioning of Leaf Surfaces

In this experiment, the effects of Btk treatments on the palatability or acceptability of leaf material to detritivore macroinvertebrates were determined by measuring the preference for, or avoidance of, treated leaf disks in a flow-through preference test. Leaf disks were incubated in river water for 4 d, treated with Btk (Dipel 64AF) in the circular chambers for 2 d, as above, and placed in 4 replicate flow-through units (20 X 20 cm, 8 cm high). Each flow through unit contained 18 leaf disks, 6 treated at the EEC, 6 treated at 1000X the EEC, and 6 that were incubated in river water alone, systematically arranged in a 3 X 6 matrix with each treatment level occurring in every third cell. The disks were attached to each cell location with a stainless steel pin. When the leaf disks were arranged and attached, 6 caddisfly larvae (*Hydatophylax argus*) were placed in the centre of each flow-through unit. After 24 h, the numbers and treatment levels of leaf disks that were consumed to some extent, as well as estimates of the amount of each disk consumed, were recorded. Ambient temperatures in the flow-through units ranged from 10 to 12° C during this experiment.

Stream Treatment

Site Description

The treatment was conducted in a second-order stream in the Icewater Creek Research Area, north of Sault Ste. Marie, Ontario. The stream flows through a mixed forest type consisting primarily of hard maple (*Acer saccharum*), soft maple (*Acer rubrum*), yellow birch (*Betula alleghaniensis*), white spruce (*Picea glauca*), balsam fir (*Abies balsamea*), and speckled alder (*Alnus rugosa*). The insecticide application point was 70 m upstream from the confluence with a larger tributary. All sampling to determine effects of the application on the macroinvertebrate community was conducted within the 70-m treated section. A site immediately upstream of the application point served as a spatial reference for biological sampling. The average width of this section of stream was approximately 3 m, and mid-stream depths at the time of application ranged from 10 to 50 cm. The average gradient over the treated and reference sections was 2.2 m/ 100 m, with a bottom type consisting of coarse gravel, rubble, and boulders. Between May and September 1992, daily maximum water temperatures typically ranged from 10 to 18° C. Dissolved oxygen ranged from 10.4 to 11.2 mg/L, pH was 6.3 to 6.9, and conductivity was 0.017 to 0.034 MS/cm.

Insecticide Application

The Btk was applied directly to the stream as a point-source application of Dipel 64AF, at a nominal concentration of 200 IU/mL. Prior to the application, discharge measurements were taken to determine the amount of Dipel required to achieve the desired nominal concentration of Btk. This treatment approach was not intended to simulate an aerial overspray of the stream. Experiments conducted to simulate aerial applications (i.e. direct applications to stream surfaces with hand-held sprayers) result in widely variable aqueous concentrations

because of variations in stream depths and water velocities. The intent of this treatment was to expose the stream biota to initial concentrations of 10 times the EEC. Because our previous applications of Btk in laboratory and stream channel experiments indicated that there was little likelihood of inducing lethal effects or drift responses in aquatic insects, even at concentrations near 600 IU/mL (Kreutzweiser *et al.* 1992), we treated the stream at 10 X the EEC (200 IU/mL) to determine if there was a ten-fold margin of safety.

The Dipel was dripped into the stream from a Mariotte bottle at a point of natural turbulence. Instream baffles were also installed at the application point to ensure complete mixing within a few metres. The test material was dripped into the stream for 30 minutes. A preliminary treatment with a Rhodamine dye was conducted several days before the Btk application to determine the rate at which the test material would move through the treated section. Based on these observations, the 30-min Btk treatment was expected to ensure that all or most of the treated section was exposed to the nominal test concentration, assuming no degradation or dissipation of the active material during the applications. There was no attempt to quantify the actual concentration of Btk spores and toxin crystals in the stream water. The Btk treatment was applied between 1115 and 1145 h on 8 June 1992.

Biological Sampling

Macroinvertebrate drift. Drifting invertebrates were collected in replicate drift nets at the reference site, and at 10 m (T1) and 60 m (T2) below the application point. At each sample time, the drift nets at all sites were simultaneously placed in the stream and held for 30 minutes. At each site, 3 drift nets (32 cm wide and 47 cm high with a mesh size of 363 µm) were placed side by side at a marked location. Drift collections were made just after dark on each of 2 d prior to the application, on the day of the application, and 1, 8, and 15 d after the application. These nightfall drift collections were considered to be the best indicators of diurnal drift for comparative purposes among sites (Allan and Russek 1985). Additional drift samples were taken on the application day at 40 min before, during, and at 10 min, 40 min, 80 min and 3.5 h after the application. All samples were preserved in the field and retained for subsequent sorting, identification, and enumeration.

Benthos. Benthic invertebrates were sampled from the stream bottom at the reference site, and at a treated site 40 to 50 m below the application point. Benthos samples were taken using a modified Surber-type sampler. This consisted of a 0.5-m wide collection net (363 µm mesh) with rubber side panels extending 1 m upstream from both sides of the collection net, enclosing a 0.5 m^2 area on three sides, with the upstream end open. Benthic material within the enclosed area was rigorously agitated to dislodge benthic invertebrates. Benthos samples were collected from reference and treated sites at 3 d before, and 4, 11, 18, 31, 52 d and 4 mos after the application. Six replicate samples were randomly collected from each of the two sites at each sample time. Samples were preserved in the field and retained for subsequent sorting, identification, and enumeration.

Caged invertebrates. Groups of caged *Pycnopsyche guttifer* larvae were placed at the reference site and in the treated section at 50 m below the application point to determine the effects of the Btk treatment on survival and growth of these detritivore caddisfly larvae. All specimens were collected from a nearby stream 5 d before the application. Four groups of 15

larvae were randomly drawn from the total collection, killed in hot water, oven dried for 2 d at 60° C, and batch-weighed to determine initial weights of the caddisfly larvae. The remaining specimens were randomly allocated to four cages in groups of 15 larvae per cage, and placed in the appropriate locations (four cages at the reference site, and four at the treated site) 4 d before the application. The cages were removed from the stream 17 d after the application, the number of surviving larvae was recorded, and the larvae were killed and weighed as above to determine post-treatment weights.

Results and Discussion

Laboratory Experiments

Microbial Respiration

In all respiration chambers with leaf disks, there were measurable declines in dissolved oxygen (DO) by the 1-h sample times. There were no measurable changes in DO over the 5-h respiration experiments in chambers that contained river water alone, or river water and Btk. Because the respiration chambers were sealed and darkened during each experiment, and because there was no measurable microbial activity in the volume of river water used, or in the test material, reductions in DO in test units were considered direct measures of respiration activity of microbial communities on leaf disks.

Aquatic microbial respiration was significantly increased in the presence of Btk as the aqueous flowable formulation Dipel 64AF, but only at the maximum challenge concentration of 1000X the EEC (20,000 IU/mL). At the maximum challenge concentration, microbial respiration in treated chambers was substantially higher than in control chambers by 5 h after the treatment (Figure 1A). The interaction term in a repeated-measures analysis of variance (RM-ANOVA, within factor = time, grouping factor = treatment) of DO reductions was highly significant ($p<0.0001$), indicating that the change in respiration activity over time differed significantly between treated and control units. In this, and all other respiration experiments, the time factor in RM-ANOVA was always significant ($p<0.001$), indicating that irrespective of treatment group, DO levels declined significantly over the 5-h periods. At the EEC (20 IU/mL), changes in respiration over time did not differ from controls (RM-ANOVA, interaction term $p=0.0878$, treatment factor $p=0.8353$) (Figure 1B).

Because the Btk treatments had significant effects on microbial respiration at 1000X the EEC, we conducted further tests to determine if this was a microbial response to the formulation components, and/or to the actual Btk spores and toxin protein crystals. A sample of the Dipel 64AF formulation blank was obtained from the manufacturer, and this was tested at 1000X the EEC in the respiration chambers. The ingredients of the formulation blank are proprietary information, but presumably contain the Dipel 64AF components without the Btk spores, crystals, and fermentation broth. This formulation blank had no measurable effects on aquatic microbial respiration (RM-ANOVA, interaction term $p=0.9999$, treatment factor $p=0.8619$) (Figure 1C).

To determine if the aquatic microbial communities were responding to the insecticidal components of the Btk formulation, a spore-crystal preparation of the NRD-12 strain of Btk was obtained from the Bacterial Pathogens Project of the Forest Pest Management Institute, and was tested in the respiration chambers at 1000X the EEC. This washed preparation contained no

detectable ß-exotoxin and little or no fermentation broth, and although this was not the same strain of Btk as is present in Dipel 64AF (HD-1), this was the only strain available in washed concentrate form at the time of the experiments. Van Frankenhuyzen *et al.* (1992) showed no differences between the two strains in terms of toxicity to target Lepidoptera, or in the number of *cryIA* toxin genes present in the two isolates.

The presence of the spore-crystal preparation in treated units caused immediate, significant increases in microbial respiration (Figure 1D). In this case, the changes in respiration between 1 and 5 h were similar in treated and control chambers (RM-ANOVA, interaction term $p=0.8817$), but the treatment factor was significant ($p=0.0244$), indicating that respiration activity was significantly higher in the treated chambers than in the controls by the first sample time, and that this significant difference was sustained over the 5 h.

Direct comparisons of microbial respiration activity among experiments is not possible because different microbial communities (incubated at different times in natural river water) were tested in each experiment. This was evidenced by differential respiration activities of microbial communities in the control chambers in the different experiments. In two instances, microbial respiration in control chambers was continuous over the 5-h duration, resulting in linear reductions in DO (Figure 1B and 1D), while in the other two experiments, microbial respiration was not continuous (Figure 1A and 1C). For this reason, treatment effects were identified on the basis of significantly different ($p<0.05$) respiration activity in treated and control chambers in each experiment.

The reasons for significant increases in aquatic microbial respiration in the presence of Btk at 1000X the EEC are unclear, and further study is required to elucidate this microbial response. It is unlikely that the increased respiration resulted from germination and activity of Btk spores. We were unable to detect microbial respiration over 5 h in river water spiked with Dipel 64AF at 1000X the EEC, suggesting that the river water in microcosms did not provide appropriate conditions for germination. However, the conditions and requirements for successful germination of Btk spores are uncertain (Addison 1993), and Bt (not necessarily *kurstaki*) has been found to readily colonize on the roots of aquatic plants (Manonmani *et al.* 1991). The increased respiration may indicate opportunistic utilization of protein in the form of spores and toxin crystals, by indigenous microbials, particularly protozoans. Protozoa can be prolific on substrates in flowing water (Bott and Kaplan 1989), and are known to graze heavily on aquatic bacteria (Bott and Kaplan 1990).

Microbial Decomposition

Microbial decomposition (mass loss) of leaf material was significantly less in circular chambers treated with Btk (Dipel 64AF) at both 1000X the EEC and the EEC, than in control chambers by the end of the 9-d exposure period (Table 1). RM-ANOVAs were performed separately on each treated group versus the control group, to determine if mass loss in treated groups was significantly different from the control group. This did not facilitate comparison between the two treated groups, but increased the power of the general F-test in RM-ANOVA. At 1000X the EEC, mass loss of leaf material was significantly less than in controls over the 9-day period (RM-ANOVA interaction term $p=0.0188$), while at the EEC, significant differences in mass loss between treated and control chambers were marginal (interaction term $p=0.0579$). This probability level was considered sufficient evidence to reject the null hypothesis that mass loss of leaf material in control and EEC chambers did not differ.

Reduced microbial decomposition of leaf material in the presence of Btk was not expected. Experiments in the aquatic respiration chambers had indicated that Btk increased microbial respiration, and this was expected to be a reflection of increased decomposition activity as well. One explanation for this may be that over the short term (5 h in our experiments), aquatic microbial activity was stimulated by exposure to Btk, but after longer term exposures (9 d in our decomposition experiment), microbial activity was decreased. On the other hand, reduced decomposition of leaf material may have resulted from preferential utilization by the microbial community of the available nutrients in the Btk formulation, and/or the spores and toxin crystals themselves. Further testing is required to account for this significant reduction in microbial decomposition of leaf material, and to determine the biotic components of the microbial community that exhibited this response to the Btk treatments.

Microbial Conditioning of Leaf Surfaces

Exposure to Btk at both 1000X the EEC and the EEC had no effect on the palatability or acceptability of leaf disks to detritivore caddisfly larvae (*Hydatophylax argus*). Data from the four replicate flow-through units were tested for heterogeneity among replicates with chi-square analyses of 2 X 3 contingency tables (Zar 1984). The tests for heterogeneity were non significant ($p>0.05$), and the replicates were combined to increase the power of the general chi-square test. After 24 h, there were no differences among the three treatment groups (control, EEC, 1000X EEC) in the numbers of leaf disks that were fed upon (chi-square $p=0.9416$), or in the extent to which leaf disks were consumed (chi-square $p=0.8354$) (Table 2).

Stream Treatment

Macroinvertebrate Drift

The application of Btk to a section of forest stream resulted in a small, but statistically significant, increase in macroinvertebrate drift, but only at the site closest to the application point, and only during the application (Figure 2). A two-way analysis of variance (2-way ANOVA), with the main factors of site (two treated, one reference) and time, was performed on the drift data from samples taken immediately before, and for several hours after, the application. There was neither a significant interaction between factors ($p=0.2917$) nor a significant effect of time ($p=0.3309$), but there was a significant difference in total drift among sites (2-way ANOVA, site factor, $p=0.0079$). Pairwise t-tests, adjusted for experiment-wise error rate at $\alpha=0.05$ by the Bonferroni method, indicated that during the treatment, the total drift abundance at T1 (10 m below the application site) was significantly greater than drift abundance at the reference site ($p<0.05$). None of the other drift samples from the treated sites were significantly different in total abundance from drift collections at the reference site ($p>0.05$). To increase the power of the multiple comparisons procedure, pairwise t-tests were restricted to comparison of both treated sites to the reference site at each of the six sample times; a total of 12 comparisons. These drift data satisfied Levine's test for homogeneity of variances ($p>0.01$) (Dixon 1990), and no data transformations were necessary prior to the ANOVAs.

Taxonomic richness (number of taxonomic units, usually genus or species) of the drift samples varied over the six sample times on the application date, but was generally low, with mean richness in drift collections ranging from 2.4 to 8.0 taxonomic groups (Table 3). This

reflects the typically low drift rates of most macroinvertebrates during daylight hours (Allen and Russek 1985). At T1, the significant increase in drift abundance during the Btk application (1115-1145 h) was accompanied by an increase in richness (Table 3), but overall there were no significant differences in richness among sites over the 5-h period (2-way ANOVA, interaction p=0.5286, time factor p=0.0571, site factor p=0.0929). The increase in richness at T1 during the application does indicate that the short-term drift response by macroinvertebrates to the Btk treatment occurred as small increases among several taxa, and does not suggest a particular sensitivity of one or two groups. The major components of the drift at T1 during the application were chironomid larvae (32%), *Taeniopteryx nivalis* (19%), *Simulium* sp. (16%), *Baetis* spp. (mainly *B. flavistriga*) (11%), and *Leuctra tenuis* (9%). All of these groups showed some increase in drift during the Btk application (Table 4).

The Btk application had little or no effect on the diurnal drift of macroinvertebrates at the treated sites, as measured in nightfall drift collections (Figure 3). For the nightfall drift samples, a square-root transformation was required to normalize the data and increase homogeneity of variance. A 2-way ANOVA indicated no significant interaction (p=0.4708) between site and date factors, but there were significant differences among sites (p=0.0008). Differences among sites, however, did not appear to indicate treatment effects. Pairwise t-tests (adjusted for experiment-wise α=0.05 using the Bonferroni method restricted to two comparisons on each of six sample dates) indicated that there were no significant differences between treated and reference sites on any given sample date, including the increased drift at T2 on 23 June (Figure 3). Drift abundance at T2 tended to be higher than at the reference site on pre-treatment sample dates as well. There were no significant differences among sites or over time in taxonomic richness of nightfall drift collections (Table 5) (2-way ANOVA, interaction p=0.1413, site p=0.3498, date p=0.3580).

The short-term drift response exhibited by several taxa during the actual application of Btk was very limited (approximately 2X increase in drift density for ½ h), and was much less, and of shorter duration than drift increases following operational applications of chemical forest insecticides (Eidt 1975, Symons 1977, Courtemanch and Gibbs 1980), and considerably less than drift increases resulting from natural spates (Anderson and Lehmkuhl 1968). The drift increase during the application was about 3X less than the natural diurnal peak measured at the three drift sites at nightfall before the application. This level and duration of drift increase during the application probably reflect an avoidance response to the Btk or its formulation by a very small proportion of the benthos at the treatment site. For example, the most abundant taxon in the drift collection at T1 (10 m below the application site) during the application was Chironomidae, and the drift catch contained a total of 21 chironomids in 3 drift nets. Based on estimates from benthos samples at the reference site (approximately 20 m above the application point) 3 d before the application, there were roughly 1100 chironomids/m^2. By conservative estimate, the area within 5 m upstream of the drift nets contained 5500 chironomids. The drift catch during the application represents a drift response by about 0.4% of the chironomids present. While this may be a rough approximation, there is little doubt that the drift increase during the application represents a minor behavioural response by a small fraction of the benthic invertebrate community.

Benthos

The Btk treatment did not alter the taxonomic structure of the macroinvertebrate community, as measured by taxonomic richness (Table 6). In a comparative study of several indices for measuring community structure of benthos, Camargo (1992) found that richness was highly correlated with the most sensitive diversity and biotic indices. In our stream experiment, there were significant differences in taxonomic richness over time (2-way ANOVA, $p<0.0001$), but there were no differences between treated and reference sites (2-way ANOVA, interaction $p=0.4359$, site $p=0.2356$).

Benthos samples were analyzed to determine changes in abundance of macroinvertebrates at the treated site in relation to changes in abundance at the reference site. In all, 49 taxa (mostly genera and species, subfamily and tribes for Chironomidae) were found in benthos samples, but many of these occurred rarely or in small numbers. Exploratory data analysis was performed to identify numerically dominant taxa, those for which there were sufficient data for statistical analysis, and those that exhibited aberrant trends in abundance at the treated site relative to the reference site. This was done at the order, family, and genus/species levels. Groups that were identified through this process were analyzed by 2-way ANOVA to determine significant differences between sites, over time, and the interaction between these two factors. A significant interaction term ($p<0.05$) indicated that changes in abundance over time differed between sites, and pairwise t-tests were applied to determine if densities at given sample dates were different between treated and reference sites (adjusted for experiment-wise $\alpha=0.05$ using the Bonferroni method restricted to one comparison on each of seven sample dates). Prior to the ANOVA procedures, all data sets were subjected to Levine's test for homogeneity of variance, and when this was significant ($p<0.01$) or near the significance level, appropriate transformations were applied according to Box-Cox diagnostic plots (Dixon 1990). For the benthos data sets, acceptable homogeneity of variance was achieved by square root or log transformation.

Following exploratory data analysis, abundance data were analyzed by 2-way ANOVA for total benthos, four orders, and 12 lower taxonomic groups. Table 7 gives mean abundance at each sample time and results of ANOVA for each group. There were no significant differences in abundance of total benthos between treated and reference sites before or after the application, indicating that the Btk treatment had little overall effect on benthos abundance. Analysis at the ordinal level, however, indicated that changes in abundance of Plecoptera at the treated site differed significantly from the reference site (2-way ANOVA interaction $p=0.0370$). Abundance of plecopterans at the reference site increased over a 3-wk period after the application, while abundance at the treated site did not (Figure 4A). Pairwise t-tests (adjusted for 7 comparisons) indicated that there was no difference in abundance of Plecoptera between the two sites ($p>0.05$) prior to the application, but that there were significantly fewer stoneflies ($p<0.01$) at the treated site 4 d after the application. Numbers of Plecoptera were significantly less ($p<0.05$) at the treated site by 18 d after the application as well. At 11 d after the application, Plecoptera abundance was considerably less than at the reference site (Figure 4A), but this difference was not significant ($p>0.05$). By 31 d after the application, Plecoptera abundance declined at both sites (probably from emergence of adults), and there were no differences between sites (pairwise t-tests $p>0.05$) to the end of the season. The Btk application had no significant adverse effects on abundance of the other three major orders (Table 7).

Analysis of the 12 lower taxonomic groups indicated that for 7 taxa, changes in abundance over time did not differ between treated and reference sites (2-way ANOVA interaction terms $p>0.05$), indicating that the Btk treatment had no measurable effect on abundance of those groups (Table 7). In two groups, *Heptagenia* spp. (mostly *H. flavescens*) and *Taeniopteryx nivalis*, interaction terms were not significant ($p>0.05$), but there were significant differences between sites (site terms $p<0.05$). In both cases, lower abundance at the treated site after the application (the only significant one at $p<0.05$ in pairwise comparisons was *T. nivalis* at 11 d) cannot be attributed to treatment effects because abundance of these taxa was also lower at the treated site before the application (Table 7).

Significant site-date interaction ($p<0.05$) was found in analysis of the mayflies *Baetis* spp. (mostly *B. flavistriga*), and the stoneflies *Dolophilodes distinctus* and *Leuctra tenuis* (Table 7), indicating that changes in abundance of these taxa differed between treated and reference sites. Although there were no significant differences between the treated and reference sites (ANOVA site term $p=0.2189$) in abundance of *Baetis* spp., mean abundance was higher at the reference site on the first two sample dates, and higher at the treated site thereafter. This resulted in non-parallel trends giving significant interaction, but does not indicate adverse effects of the Btk treatment on abundance of *Baetis* spp. Much the same pattern occurred in abundance of *D. distinctus*, with higher numbers at the reference site before and shortly after the application, and higher abundance (although not statistically significant) at the treated site by 18 d (Table 7). This again resulted in significant site-date interaction, but does not suggest adverse treatment effects on *D. distinctus*.

The density of *Leuctra tenuis* was clearly reduced at the treated site within 4 d after the Btk application, while density increased at the reference site (Figure 4B). *L. tenuis* was by far the most abundant species within Plecoptera, and the changes in abundance of this species closely follow the pattern exhibited by total plecopterans (Figure 4A). Mean abundance of *L. tenuis* was the same at treated and reference sites 3 d before the application (pairwise t-tests $p>0.05$), but was significantly less at the treated site by 4 d after the application ($p<0.01$). None of the other post-application densities at the treated site were significantly different from those at the reference site on same sample dates (pairwise t-tests $p>0.05$), but the mean abundance of *L. tenuis* at the treated site at 11 and 18 d after the application tended to be less than at the reference site (Figure 4B). As with total Plecoptera, abundance of *L. tenuis* declined at both sites by 31 d after the application, and there were no differences between sites to the end of the season.

These results demonstrated that the direct application of Btk at 10X the maximum expected environmental concentration to a section of forest stream had no measurable effects on the macroinvertebrate community composition or abundance, except for the stonefly *Leuctra tenuis*. The apparent treatment effects on *L. tenuis* were not expected. From previous laboratory bioassays of Btk on aquatic insects, including *Leuctra* spp., Eidt (1985) concluded there were no toxic effects of Btk on *Leuctra* spp. at concentrations ranging from 4.3 to 430 IU/mL, although delayed mortality of *Leuctra* (12 d) was much higher at all concentrations than in the controls. In previous laboratory and stream channel experiments of our own (Kreutzweiser *et al.* 1992), we concluded from maximum-hazard tests that there was little likelihood of lethal or behavioural response by aquatic insects to Btk, even at concentrations as high as 600 IU/mL, although we did not test *L. tenuis*. The significant reduction of *L. tenuis* after the stream application was not an immediate toxicological response to the Btk or its formulation. *L. tenuis* was among the taxa that demonstrated drift response to the Btk

application (discussed above) but the drift increase was very short-lived (½ h) and was well below the drift increases required to induce measurable reductions of benthos as determined in previous stream treatments (Eidt 1975, Kreutzweiser and Sibley 1991). The significant decline in *L. tenuis* abundance by 4 d after the application suggests mortality of the stoneflies resulting from ingestion of Btk. *L. tenuis* is a detritivore, and the route of exposure to Btk spores or crystals is likely to be through consumption of organic material contaminated by Btk. Further experiments are required to determine if Btk on detrital material is lethal to *L. tenuis*.

Caged Invertebrates

The Btk application did not appear to affect the survival of the caddisfly larvae, *Pycnopsyche guttifer*, held in cages in the stream. There was higher mortality of larvae in the treated section by 15 d after the application (Table 8), but this was not significantly different from the mortality in the reference section (t-test on number alive, $p=0.1289$). Growth of the larvae appeared to be significantly reduced by handling and caging effects, but not by the Btk application. The mean weights of larvae at both the treated and reference sites were significantly less than the mean pre-treatment weight (Table 8), but there was no difference between post-treatment weights at the two sites (ANOVA $p=0.0005$, Scheffe's multiple comparisons test at $\alpha=0.05$).

Conclusions

The laboratory experiments demonstrated that natural aquatic microbial communities colonized on leaf disks were affected by the Btk treatments, and that this appeared to be a microbial response to the actual insecticidal components of Btk, the spores and toxin crystals. At short-term exposures (5 h), this response was measured in terms of stimulated respiration, but only at the maximum challenge concentration of 1000X the expected environmental concentration (20,000 IU/mL), and may simply indicate bio-degradation of the Btk by the natural microbial community.

While the Btk treatments did not inhibit the microbial conditioning of leaf surfaces in terms of palatability to detritivore macroinvertebrates, reduced microbial decomposition of leaf material in Btk treatments occurred at the expected environmental concentration (EEC). This may be of ecological significance if it is indicative of preferential utilization of Btk components by the microbial communities on detrital surfaces, or inhibition of microbial decomposition activity following longer-term exposure to Btk. In natural systems, Btk is not likely to persist in aquatic environments at concentrations near the EEC, because of natural depuration processes, and the likelihood of adverse effects on decomposition activity of indigenous microbial communities may be reduced. However, little is known about the fate of Btk in aquatic environments, particularly with regard to the fate of the toxin crystals and the potential for germination of the spores. This, and the ecological implications of reduced microbial decomposition of organic matter, should be investigated further.

The direct application of Btk at 10X the EEC to a section of forest stream had little effect on the macroinvertebrate community. The slight, short-term increase in drifting invertebrates was considered inconsequential in terms of reduction of benthos. The abundance of one invertebrate taxon, the stonefly *Leuctra tenuis*, was significantly reduced following the application, and because this detritivore was not particularly abundant in drift, the reduced

abundance in benthos samples suggested a delayed mortality due to ingestion and toxicity of Btk on organic material. If this stonefly is susceptible to Btk, this may be of interest to molecular biologists in determining the cellular or molecular basis for toxicity of Btk to insect larvae.

The minor impact of the Btk application on the stream invertebrate community lends support to the contention that this narrow-spectrum biological insecticide poses little risk of adverse effects in aquatic environments (Surgeoner and Farkas 1989), and agrees with indications from previous laboratory and stream channel experiments that contamination of water courses with Btk will not adversely affect most macroinvertebrates (Eidt 1985, Kreutzweiser *et al.* 1992). The level of exposure to macroinvertebrates in this direct stream application (200 IU/mL) was much higher than would normally occur in forest water bodies contaminated by aerial application of Btk.

While these laboratory and field experiments have contributed to a hazard evaluation for Btk contamination of water bodies, several potentially important issues have been raised which should be resolved in future research projects, to more fully investigate the environmental safety of Btk in aquatic systems. These include:

1. characterizing the stimulated aquatic microbial respiration after exposure to Btk, and determining the duration of this effect,
2. determining the fate of Btk spores and crystals in aquatic environments,
3. determining the mechanisms or organisms responsible for reduced microbial decomposition of organic material, and assessing the ecological importance of this in natural systems, and
4. determining the toxicity of Btk-contaminated organic material to the stonefly *Leuctra tenuis*.

Acknowledgments

The technical assistance of Julia Fogg, John Graham, and Aaron MacGillivary is greatly appreciated. The aquatic respiration chambers were constructed by Jim Beveridge. Abbott Laboratories, Chicago, supplied the Dipel 64 AF and the formulation blank. This project was supported in part by a NAPIAP grant NA-25 provided by the Northeastern Area State and Private Forestry, U.S. Department of Agriculture Forest Service, under a USDA Forest Service - Forestry Canada cooperative agreement.

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Table 1. Decomposition (mass loss) of leaf disks after 9 d in recirculating microcosms treated with Btk (Dipel 64AF) at 1000X the expected environmental concentration (EEC) and at the EEC, and in control microcosms containing river water only. Weights are means (± 1 SE) of batch-weighed leaf disks in 4 replicate chambers of each treatment.

treatment	initial dry weight (mg)	final dry weight (mg)	mass loss (mg)
1000X EEC	180.1(3.5)	170.8(4.3)	9.27(0.9)
EEC	182.8(3.1)	165.7(2.7)	17.08(1.9)
control	179.2(4.8)	155.1(6.9)	21.78(1.6)

Table 2. Consumption of leaf disks by *Hydatophylax argus* in 24-h preference tests containing naturally-incubated leaf disks (controls), and disks treated with Btk (Dipel 64AF) at the EEC and 1000X the EEC. Results are combined from four replicates, following nonsignificant tests for heterogeneity among replicate preference tests ($p>0.05$).

measurements	controls	EEC	1000X EEC
total number of leaf disks	24	24	24
number of disks fed upon	15	15	16
number of disks left intact	9	9	8
number of disks consumed ≤ 20%	6	5	7
number of disks consumed > 20%	9	10	9

Table 3. Taxanomic richness (mean no. of taxa and 1 SE, n=3) of drift samples from treated (T1 and T2) and reference sites taken immediately before, during, and for several hours after the Btk application. The application occurred at 1115-1145 h on 8 June 1993.

	time of drift collections					
site	1025-1055 h	1115-1145 h	1155-1225 h	1235-1305 h	1315-1345 h	1530-1600 h
T1						
mean	4.0	8.0	7.3	5.3	4.7	5.0
SE	1.15	0.58	0.33	0.33	0.88	0.58
T2						
mean	5.0	5.0	6.3	4.7	3.7	4.7
SE	1.15	1.15	0.88	0.33	0.67	1.20
reference						
mean	4.0	3.0	6.3	4.7	4.0	4.3
SE	2.00	1.15	1.20	1.45	0	0.88

Table 4. Mean number per 10 m^3 (n=3) and 1 SE of most abundant taxa in drift collections from the treated site T1 immediately before and during the Btk application. The application occurred at 1115-1145 h on 8 June 1993.

	1025-1055 h		1115-1145 h	
taxa	mean	1SE	mean	1SE
Baetis spp.	1.36	1.13	1.51	0.70
Plecoptera (early instar)	0	0	2.55	1.03
Leuctra tenuis	0.4	0.4	1.20	0.60
Chironomidae	1.85	0.51	4.06	1.64
Simulium sp.	1.59	0.43	2.20	0.44

Table 5. Taxanomic richness (mean no. of taxa and 1 SE, n=3) of drift samples from treated (T1 and T2) and reference sites taken at nightfall before and after the Btk application. The application occurred at 1115-1145 h on 8 June 1993.

	evening sample dates					
site	6 June	7 June	8 June	9 June	16 June	23 June
T1						
mean	8.3	11.0	12.3	11.0	9.3	7.3
SE	1.20	1.53	0.88	1.00	0.88	0.88
T2						
mean	9.7	12.0	9.3	12.3	8.0	6.7
SE	0.33	0.58	0.88	0.88	2.62	1.33
reference						
mean	10.0	5.7	9.0	8.3	9.0	9.7
SE	1.00	3.67	2.00	2.19	1.00	1.20

Table 6. Taxanomic richness (mean no. of taxa and 1 SE, n=6) of benthos samples from treated and reference sites taken before and after the Btk application.

	days before and after application						
site	-3	4	11	18	31	52	122
treated							
mean	21.7	19.8	23.0	27.0	23.8	27.5	27.3
SE	1.2	1.2	1.3	1.4	1.5	1.1	1.7
reference							
mean	24.7	20.2	24.5	28.2	23.5	25	30.3
SE	1.2	0.7	1.7	1.5	1.1	1.9	1.0

Table 7. Mean number per 0.5 m² (± 1 SE in parentheses) of macroinvertebrates in benthos samples (n=6) from treated and reference sites before and after the Btk application.

taxanomic group	days before and after application							2-way ANOVA probabilities		
	-3	4	11	18	31	52	122	site	date	interaction
total macroinvertebrates										
treated	937(215)	521(99)	732(113)	1309(329)	747(135)	1907(409)	1770(266)	p=0.9496	p=0.0002	p=0.6284
reference	1106(153)	899(180)	1110(155)	1245(184)	624(124)	1688(533)	1314(311)			
Ephemeroptera										
treated	159.0(26.2)	120.5(23.3)	218.7(53.3)	218.8(29.0)	126.2(25.7)	216.0(13.9)	150.2(25.9)	p=0.7110	p=0.0001	p=0.0733
reference	246.7(37.4)	200.7(48.4)	260.5(20.5)	208.7(24.1)	105.2(32.2)	168.7(24.8)	91.5(18.8)			
Plecoptera										
treated	149.7(61.1)	70.0(11.6)	155.5(34.7)	153.3(25.6)	80.3(19.8)	79.7(19.0)	162.8(12.4)	p=0.0003	p<0.0001	p=0.0370
reference	179.0(28.7)	249.7(49.4)	283.3(74.6)	317.7(44.9)	74.8(23.6)	69.8(14.3)	205.2(23.4)			
Trichoptera										
treated	20.0(6.5)	14.8(4.0)	25.3(12.9)	138.7(72.0)	27.8(7.0)	207.8(67.4)	205.0(26.9)	p=0.5996	p<0.0001	p=0.1061
reference	45.3(10.9)	26.5(9.4)	64.2(23.1)	63.7(25.9)	22.5(5.0)	122.2(44.3)	147(42.6)			
Diptera										
treated	599.6(126.1)	315.8(74.7)	330.0(49.8)	796.7(222.8)	546.8(94.5)	1400.3(348.7)	1251.1(249.0)	p=0.7543	p<0.0001	p=0.3370
reference	636.5(90.2)	422.8(85.3)	500.8(77.6)	655.8(118.2)	418.8(88.2)	1324.8(452.4)	866.5(244.7)			
Baetis spp.										
treated	106.8(19.2)	74.2(15.0)	159.0(47.0)	139.5(21.3)	73.7(16.1)	140.0(20.8)	63.8(12.9)	p=0.2189	p<0.0001	p=0.0253
reference	162.2(18.2)	131.5(34.9)	127.5(18.4)	121.7(15.0)	34.5(7.0)	100.5(15.1)	34.8(11.2)			
Eurylophella spp.										
treated	6.8(2.5)	16.5(3.7)	11.2(3.6)	21.3(8.4)	5.8(1.4)	10.7(3.6)	7.5(3.7)	p=0.0646	p=0.0422	p=0.1192
reference	5.8(2.5)	4.8(2.5)	38.8(17.2)	8.2(3.9)	10.3(6.7)	2.7(1.4)	5.7(2.2)			
Epeorus vitrea										
treated	8.2(1.4)	3.8(2.3)	2.2(0.9)	10.7(3.4)	2.5(0.4)	4.0(2.0)	46.3(12.0)	p=0.7811	p<0.0001	p=0.4386
reference	32.0(14.9)	11.8(5.1)	6.3(2.8)	10.8(3.3)	2.0(1.4)	2.8(1.2)	22.0(6.3)			
Heptagenia spp.										
treated	8.2(1.5)	14.7(5.9)	22.2(8.4)	38.2(10.1)	19.2(5.6)	40.8(5.3)	30.8(8.4)	p=0.0140	p=0.0125	p=0.1795
reference	31.8(9.6)	41.3(10.0)	46.5(15.2)	45.3(9.9)	13.2(3.6)	51.5(14.8)	27.3(9.9)			
Leuctra tenuis										
treated	143.0(62.0)	39.9(3.2)	110.3(24.3)	95.0(16.3)	75.8(18.9)	24.3(7.1)	35.2(11.1)	p=0.0027	p<0.0001	p=0.0400
reference	159.8(24.0)	175.3(37.5)	194.3(69.0)	239.7(41.1)	65.0(18.8)	17.2(2.5)	45.0(12.4)			
Taeniopteryx nivalis										
treated	0.8(0.8)	30.0(8.4)	34.7(13.2)	41.0(14.6)	0	1.7(1.1)	30.2(10.6)	p=0.0405	p<0.0001	p=0.0620
reference	9.2(4.5)	62.8(14.1)	72.0(15.0)	46.5(10.8)	0	2.3(1.5)	15.8(2.2)			
Dolophilodes distinctus										
treated	5.3(4.0)	3.0(1.9)	18.7(11.8)	86.0(41.3)	10.2(4.1)	38.3(11.4)	83.8(15.4)	p=0.1432	p=0.0092	p=0.0019
reference	14.3(4.9)	19.0(9.0)	33.2(19.1)	24.3(17.6)	2.0(0.6)	18.7(13.7)	28.8(15.0)			
Natarsia sp.										
treated	71.3(30.5)	32.7(10.3)	47.2(10.5)	53.0(6.2)	22.2(5.3)	19.0(8.9)	25.0(9.7)	p=0.0650	p<0.0001	p=0.5488
reference	50.0(7.4)	40.2(5.5)	78.3(10.4)	80.2(18.1)	33.5(8.7)	30.8(7.3)	21.5(6.1)			

Tanytarsini										
treated	204.2(44.0)	86.0(26.9)	92.2(17.9)	315.3(90.1)	281.8(58.2)	907.3(220.4)	669.2(166.6)	p=0.5887	p<0.0001	p=0.5070
reference	165.3(20.6)	111.8(26.4)	128.7(28.8)	273.5(69.3)	216.8(52.4)	1008.3(394.7)	454.2(188.1)			
chironomid group A[1]										
treated	26.8(10.8)	7.0(4.1)	5.7(2.6)	0	0	0	0	p=0.0171	p<0.0001	p=0.6537
reference	66.3(26.1)	17.7(7.3)	20.0(9.8)	0.3(0.3)	0	0.2(0.2)	1.2(1.2)			
other chironomids										
treated	247.8(61.1)	168.5(40.1)	135.7(17.5)	356.7(118.9)	192.2(48.3)	394.0(132.5)	175.2(31.7)	p=0.4728	p=0.1259	p=0.4522
reference	276.8(64.8)	196.0(43.9)	214.8(28.3)	231.0(37.3)	195.2(72.0)	223.2(53.9)	158.2(40.0)			
Simulium sp.										
treated	16.3(6.5)	5.8(3.8)	4.3(1.6)	17.7(8.8)	10.0(7.7)	23.3(9.8)	3.7(3.3)	p=0.2588	p=0.0527	p=0.1324
reference	34.5(12.7)	30.2(14.4)	5.8(2.7)	17.5(6.6)	3.0(1.6)	6.7(3.3)	18.8(15.5)			

[1] identification unknown, chironomid group based on consistent morphological characteristic

Table 8. Growth (change in weight) and survival of *Pycnopsyche guttifer* larvae held in cages (n=4, 15 larvae per cage) in treated and reference sections of the stream from 4 d before to 15 d after the Btk application. Pre-treatment weight is the average of 4 groups of 15 individuals taken randomly from larval collections.

site	mean (± 1 SE) pre-treatment weight (mg)	mean (± 1 SE) post-treatment weight (mg)	mean (± 1 SE) % mortality
treated	29.4(1.3)	21.7(0.2)	18.2(6.8)
reference	29.4(1.3)	23.1(0.9)	5.2(3.3)

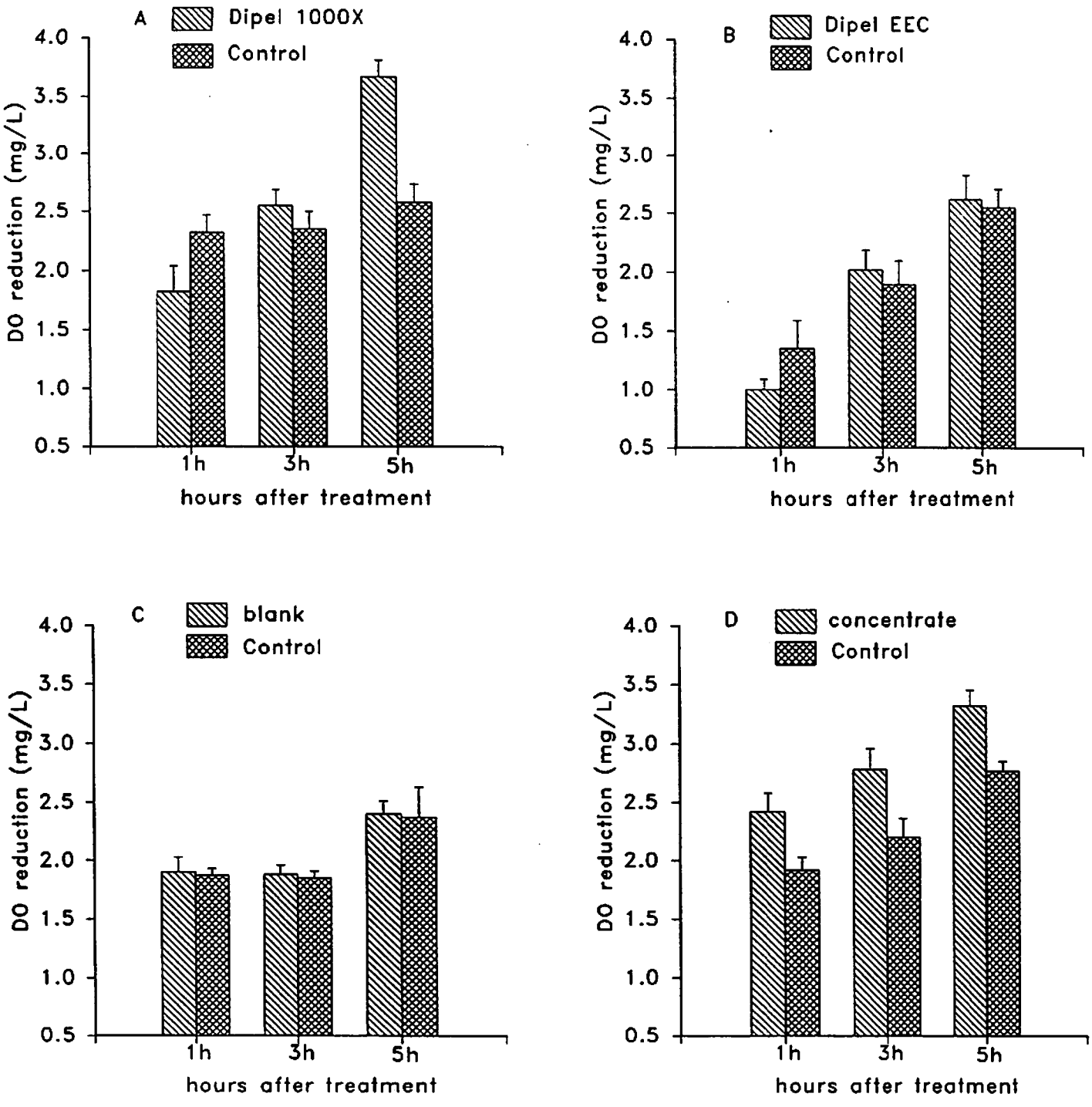

Figure 1. Microbial respiration on leaf disks (expressed as mean + 1S.E. reduction in dissolved oxygen from pre-treatment concentrations, n=4) in darkened respiration chambers treated with A) Dipel 64AF at 1000X the expected environmental concentration (EEC), B) Dipel 64AF at the EEC, C) the formulation blank at 1000X EEC, and D) a spore-crystal concentrate at 1000X EEC, and in control chambers.

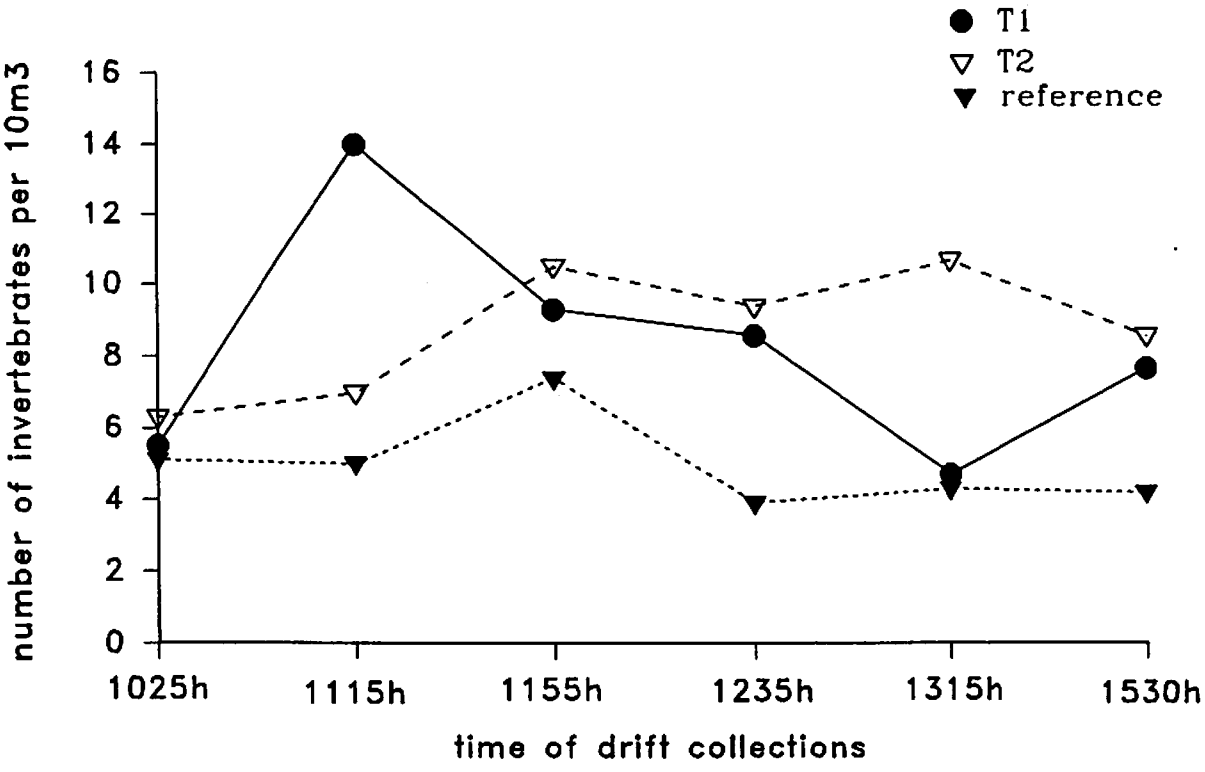

Figure 2. Invertebrate drift (mean number, n=3) at treated (T1 and T2) and reference sites immediately before, during, and up to 4 h after the Btk application. The application occurred at 1115-1145 h on 8 June 1992.

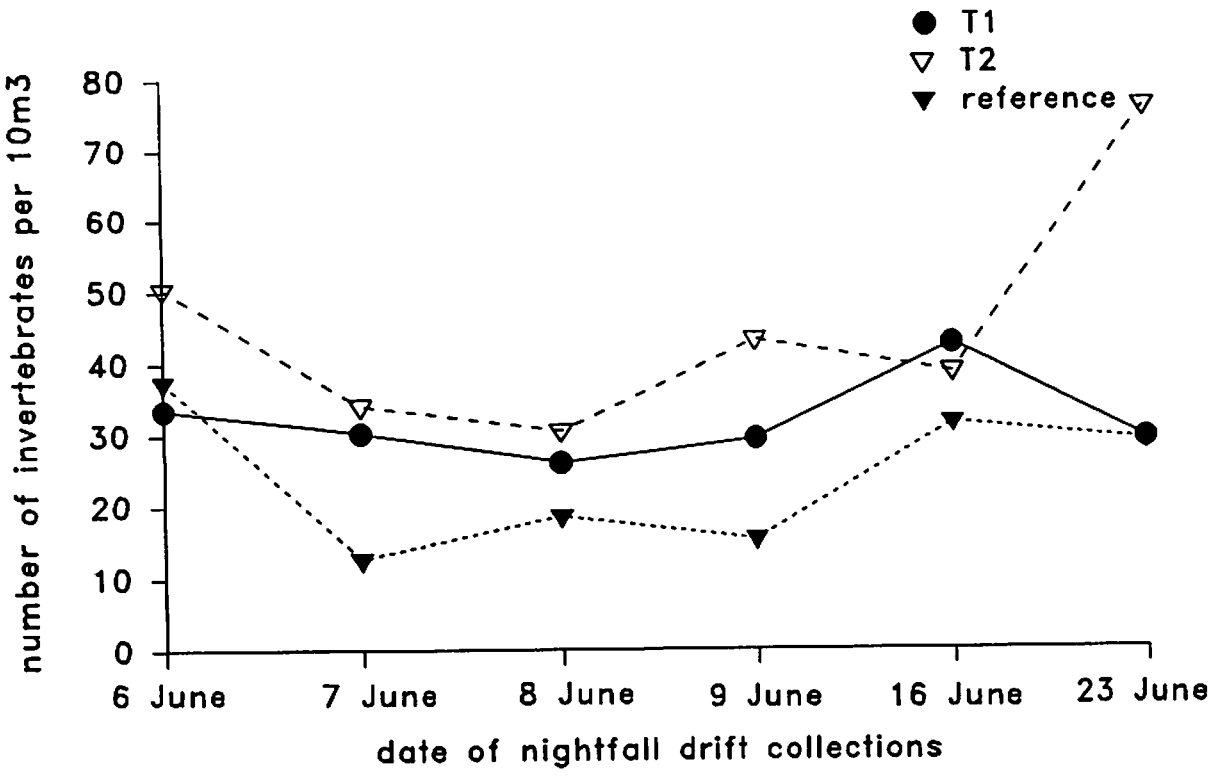

Figure 3. Invertebrate drift (mean number, n=3) at treated (T1 and T2) and reference sites in nightfall drift collections before and after the Btk application. The application occurred at 1115-1145 h on 8 June 1992.

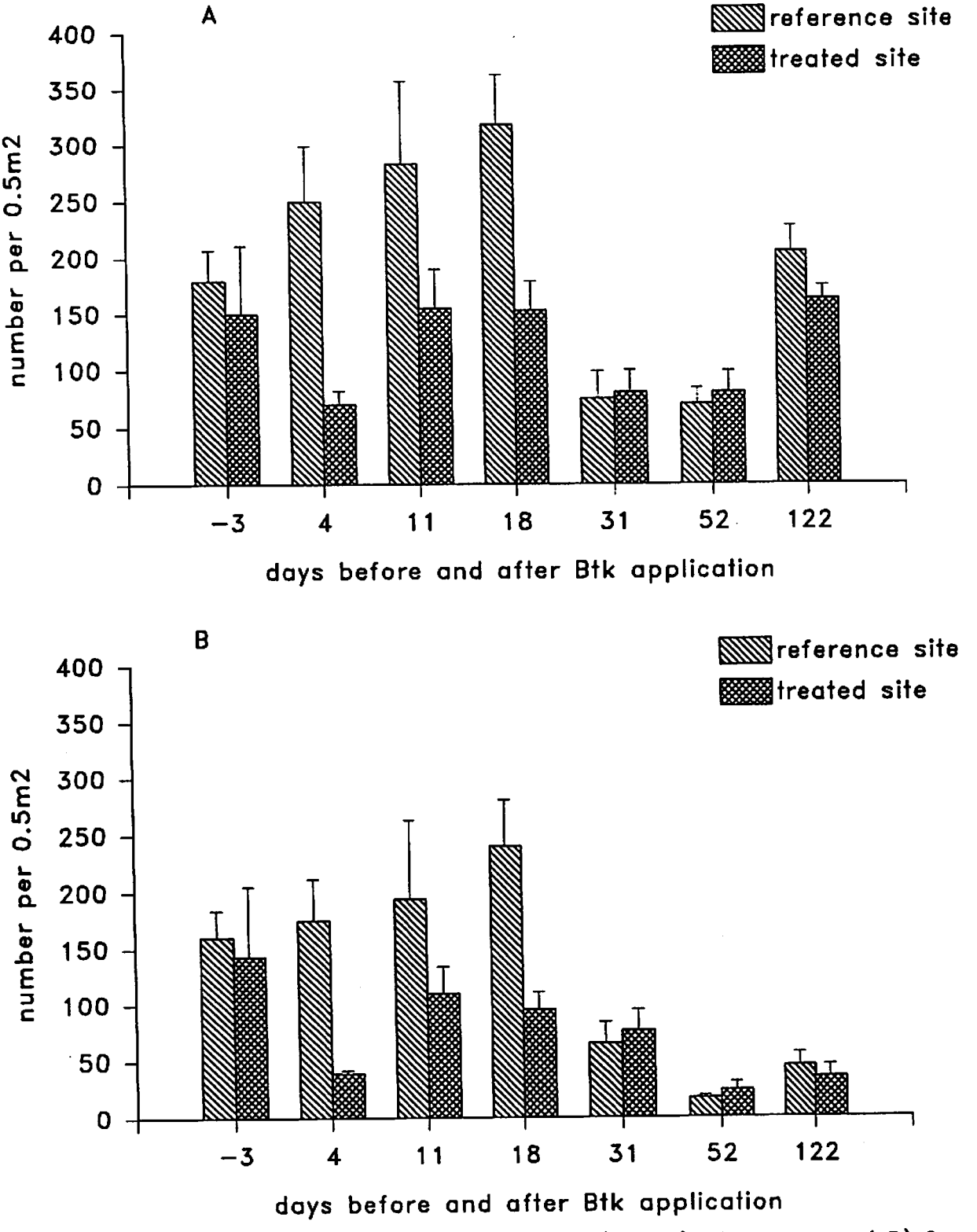

Figure 4. Abundance (mean + 1 S.E., n=6) of A) Plecoptera and B) Leuctra tenuis, at treated and reference sites before and after the Btk application.