# National Center of Forest Health Management

TECHNOLOGY
TRANSFER

*Non-target Impacts*
*Diflubenzuron*

# Effects of Diflubenzuron on Non-target Organisms in Broadleaf Forested Watersheds in the Northeast

Richard C. Reardon
*Handbook Coordinator*

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## Contributors

Edward M. Barrows
Georgetown University
Department of Biology
Washington, DC 20057

Denise Binion
Forest Health Protection
NA State and Private Forestry
USDA Forest Service
180 Canfield Street
Morgantown, WV 26505

Linda Butler
West Virginia University
P.O. Box 6108
Morgantown, WV 26506-6108

Matt Cochran
WV Dept. of Agriculture
State Capitol Building
Charleston, WV 25305

Dan Downey
James Madison University
Department of Chemistry
Harrisonburg, VA 22807

Tara Dubey
P.O. Box 411
Elizabeth, IL 61028

Pamela J. Edwards
USDA Forest Service
P.O. Box 404 - Nursery Bottom
Parsons, WV 26287

Patricia Gundrum
Forest Health Protection
NA State and Private Forestry
USDA Forest Service
180 Canfield Street
Morgantown, WV 26505

Felton Hastings
North Carolina State University
Department of Entomology
Box 7626
Raleigh, NC 27695

Alan Iskra
Forest Health Management
NA State and Private Forestry
USDA Forest Service
180 Canfield Street
Morgantown, WV 26505

# *Chapter 1. Introduction*

**Richard C. Reardon**

## Introduction

Aerial application of insecticides is one of the control methods used for the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), an important defoliating pest in broadleaf forests. The synthetic arthropod growth regulator diflubenzuron (DFB) is one of the insecticides used in the Federal and State cooperative gypsy moth program. The others are the bacterium *Bacillus thuringiensis* (*Bt*) and the nucleopolyhedrosis virus product Gypchek®. From 1975 when this program began, to 1994, approximately 4 million acres infested with the gypsy moth were treated with DFB (NOTE: DFB wasn't registered until 1976). From 1991 through 1994, approximately 1.1 million acres were treated with DFB, 2.1 million acres with *B.t.*, and 26,000 acres with Gypchek (Machesky 1995). DFB is also used to control a wide variety of other insect pests on agricultural crops (cotton and soybeans), ornamentals in nurseries, and conifers and hardwoods in forests.

Numerous studies, conducted before and after the registration of DFB by the U.S. Environmental Protection Agency (EPA) in 1976, have examined the potential impacts on selected nontarget species (Onken 1995, USDA 1995). One data gap identified by the Appalachian Integrated Pest Management (AIPM) Gypsy Moth Project, however, was potential impacts on nontarget organisms when DFB is applied to an entire broadleaf forested watershed, as can happen when spraying for the gypsy moth. An entire watershed can encompass stream headwaters and adjacent major streams.

## Diflubenzuron

DFB, N-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide], was discovered by scientists at Philip Duphar B.V., Weesp, Holland, and was developed by the Thompson-Hayward Chemical Company for marketing in the United States. It is distributed in the United States by Uniroyal Chemical Company. DFB breaks down into either 2,6-difluorobenzoic acid (DFBA) or 4-chlorophenylurea (CPU). The CPU can further break down into 4-chloroaniline (PCA), which can then degrade to 4-chloroacetanilide (Nimmo and others 1986). DFB is a synthetic arthropod growth regulator that inhibits the deposition of cuticular chitin in many species of chitin-containing invertebrates. Chitin, a nitrogenous polysaccharide, is a major component of arthropod exoskeletons. Because DFB's primary effect follows ingestion and the gypsy moth larva is the only life stage that feeds, the larvae are more susceptible to DFB than other stages. The exoskeleton of a contaminated larva is only partially formed during the molting process, and it subsequently dies from a rupture of its new and delicate malformed cuticle or from starvation.

The most frequently used formulation of DFB for control of the gypsy moth is a water-dispersible powder containing 25 percent DFB by weight, Dimilin® 25-W, which is applied at about 35-70 g AI/ha (0.03-0.06 lb AI/acre) in a total volume of 9.4 l/ha (1.0 gal/acre) for one application during suppression projects or for two applications during eradication projects. Historically, Dimilin has been aerially applied to broadleaf trees after bud burst (e.g., red and white oak foliage is approximately 30-50 percent and 10-30 percent expanded, respectively). This formulation has been used during most of the nontarget evaluations conducted before the studies reported here. Forestry uses were eliminated from the Dimilin 25-W label in 1994 and its use in gypsy moth projects will end as existing supplies (with the forestry use label) are depleted.

Another formulation, Dimilin 4L, was registered by the EPA in 1990. This formulation is an aqueous flowable with a composition of 40.5 percent of DFB by weight (4 lb DFB/gal of formulation). For forestry use it is applied once or twice at 0.08 l/ha (0.03 lbs AI/acre) in a total volume of 4.7 to 9.4 l/ha (0.5 to 1.0 gal/acre).

## The Nontarget Project

The purpose of the nontarget project was to gather data for some nontarget organisms omitted from earlier studies and for those with a probability of impact by DFB in broadleaf forested watersheds typical of the mountainous regions of the eastern United States. This was the first long-term study of nontarget organisms to use the Dimilin 4L formulation.

### *Objectives*

Project objectives were to determine the potential impacts of Dimilin 4L on selected nontarget organisms; determine initial and degradation levels of DFB on tree surfaces, in leaf litter, in soil, and in water; and develop a residue profile for aerially applied DFB (USDA 1992).

Five nontarget groups were monitored: (1) fungi, bacteria, and invertebrates in leaf litter and soil; (2) aquatic macroinvertebrates; (3) canopy arthropods; (4) pollinating insects; and (5) aquatic and terrestrial salamanders. The effects on fish were not studied for several reasons: the stream in each study watershed flows intermittently with isolated pools in summer causing low numbers and heterogeneous distributions of native populations of fish; DFB has a relatively low toxicity to fish (Fischer and Hall 1992); high costs are associated with tissue analysis to detect residues; and concerns about the use of stocked populations of trout (consultation with R. Menendez of the West Virginia Department of Natural Resources).

During the study, gypsy moth populations in the Fernow Experimental Forest were negligible, so the potential additional effects on nontarget organisms due to defoliation were avoided.

### *Study Location*

Although numerous locations were proposed for the study, the choice was limited by study requirements: availability of a site for at least 5 years, application of an insecticide, the need for similar broadleaf forested watersheds in the AIPM Project area, the availability of long-term data on stream water chemistry and flow rate, and a history of stand succession. The Fernow Experimental Forest met all of these criteria, as well as being geographically convenient. The Fernow Experimental Forest is located in north central West Virginia, near the town of Parsons (latitude 39° 2' 30" N and longitude 79° 41' W).

### *Study Design*

All nontarget groups (except native pollinators) were monitored on four study watersheds (2 treated and 2 untreated) before treatment (1989-1991, only 1991 for pollinators) and after treatment (1992-1993). Additional posttreatment monitoring was conducted for salamanders, canopy arthropods, and residue levels in leaf litter and soil on all watersheds in 1994.

Two watersheds were treated in 1992. One application of a tracked lot of Dimilin 4L formulation was applied using a Bell 206 (High Tech Applicators, Elkins, WV) at 0.08 l/ha (0.03 lb AI/acre) in 9.4 l/ha (1 gal/acre). The application was applied using flat fan nozzles with 8003 tips directed straight down at daybreak on May 16 with weather conditions of low wind (<1 mph), a temperature of approximately 23° C, and 72 percent relative humidity. On May 17, 0.1 inch of rain fell, and on May 18 a day long rain fell on the watersheds.

It was not decided until the fall of 1991 to aerially apply the Dimilin 4L formulation (instead of the 25-W formulation); therefore, the operational 25-W formulation was used in some of the laboratory evaluations initiated prior to 1992.

This handbook is a compilation of reports on the studies conducted in support of and on the Fernow Experimental Forest from 1989 through 1994.

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# *Chapter 2. Site Characteristics*

**Pamela J. Edwards**

## Climate

In this mountainous area, precipitation is high and distributed fairly evenly throughout the year. Annual precipitation in the Fernow Experimental Forest from 1982 to 1991 averaged 1458 mm (Table 2-1). Precipitation is acidic (Table 2-2), with the area receiving some of the highest acid loads in the United States. Snow is common from December through March, and earlier and later snowfalls do occur; however, snowpacks rarely last more than about 2 weeks due to temperature increases and rain-on-snow events caused by warm fronts. Fernow Experimental Forest mean annual temperature is 9º C, with lows occasionally falling to -29º C and highs rarely exceeding 32º C. About 145 frost-free days occur each year (USDA Forest Service 1987).

## Study Watersheds

Four watersheds (WS) were selected for study: WS 1, WS 4, WS 7, and WS 13. Dimilin 4L was applied to WS 1 and WS 13, and WS 4 and WS 7 were used as untreated (Fig. 2-1). The watersheds are relatively small, ranging from about 14 to 38 ha. The physical characteristics of these watersheds are typical of the Appalachian Mountains. Topography is rugged, with steep slopes common in at least portions of each watershed. General characteristics of each watershed are given in Table 2-3.

The bedrock and soils on the four watersheds are similar. All are underlain by acidic sandstones and shales of the Hampshire Formation. Most of the watersheds are dominated by Calvin and Berks silt loams. They tend to be fairly shallow, with maximum depths of about 1.5 m. Mineral soil pH is acidic, with pH values typically about 4.5. Upper organic horizons can have pH values as high as 7. Combined, the 01 and 02 layers are thin (2.5 to 5 cm), particularly on the ridgetops due to leaf litter migration downslope. Organic matter mineralization is rapid because of the combination of moderate and moist climate and easily mineralizable leaf tissue (Griffith and Perry 1991). Within a year of leaf fall, most of the leaf litter is humified.

Each of the watersheds has a different treatment history.

> ***Watershed 1*** -- was clearcut (except for cull trees) to a 15-cm diameter at breast height (d.b.h.) from May 1957 through June 1958. Seventy-four percent of the original basal area was cut, with 4.8 $m^3$/ha removed (Kochenderfer and others 1990). At the time of treatment in 1992, the stand was 34 years old and was dominated by yellow-poplar (*Liriodendron tulipifera* L.), chestnut oak (*Quercus prinus* L.), and red maple (*Acer rubrum* L.).
>
> ***Watershed 13*** -- became an active research watershed only in 1984. A light selection cut is believed to have been performed in the 1960's, though the volume of wood or percent basal area removed is not known. The dominant vegetation is yellow-poplar, red oak (*Q. rubra* L.), and sugar maple (*Acer saccharum* Marsh.). Stand age is approximately 65 years.

***Watershed 4*** -- has been used historically as an untreated control so disturbance has been negligible, except for salvage removal of dead American chestnut (*Castanea dentata* [Marsh.] Borkh.) during the 1940's following the chestnut blight. At the time of Dimilin treatment in 1992, the dominant stand was about 90 years old, though scattered residual trees left from turn-of-the-century logging are estimated to be 175 to 200 years old. The dominant overstory species on WS 4 are sugar maple, red maple, and American beech (*Fagus grandifolia* Ehrh.).

***Watershed 7's*** -- stand was harvested in two halves in the 1960's. The upper 12.1 ha, comprising 49 percent of the basal area (6.7 $m^3$/ha), was clearcut from November 1963 through March 1964. It was maintained barren of vegetation with herbicides, principally 2,4,5-T, until October 1969. The lower half of WS 7 was clearcut (6.4 $m^3$/ha) from about October 1966 through March 1967 and also maintained barren until October 1969 (Patric and Reinhart 1971). At the time of the Dimilin study, the overstory vegetation was dominated by black birch (*Betula lenta* L.), red maple, and sugar maple. Because of the herbicide treatment, much of the revegetation on WS 7 is believed to be from seed sources rather than from stump sprouts that typically result from harvesting in this region.

Each of the watersheds contains small first- or second-order headwater streams. The stream channels are fairly well protected with gravel- and cobble-sized materials, so that bed and bank erosion are low. Aquatic bryophytes (mosses and liverworts) dominate in-stream vegetation, and vascular plants are not present within these streams.

Streamflow was measured continuously at the mouth of each catchment. A 120º V-notch weir was used on WS 1, WS 4, and WS 7, and a 0.7 m H-type flume was used on WS 13. In all cases, streamflow was recorded with FW-1 water-level recorders with 7-day charts, and the charts were digitized. Annual discharge summaries for the past 10 years for the watersheds are presented in Table 2-1.

Unlike precipitation, most of the annual flow occurs during the winter. High transpiration rates during the growing season result in low flows, with the streams occasionally drying up during late summer into early fall. In mid-to-late summer, rain events usually must exceed 2.5 cm or must be intense for stream stage increases to exceed more than several hundredths of a meter.

Streamflow and stream chemistry respond rapidly to precipitation and snowmelt during the winter. Typically, during most hydrologic episodes, stream water pH is depressed and the concentration of the other major ions increases. The acid neutralizing capacities of these watersheds are low; however, buffering capabilities remain sufficiently high during storm and melt events so that the lowest stream water pH values remain above about 5.5. Thus, on the basis of solubility and pH relationships, dissolved aluminum concentrations that could stress aquatic organisms are believed to remain low.

Long-term stream chemistry results determined from grab samples at the mouth of the watersheds from 1982 through 1991 for WS 1, WS 4, and WS 7 and from 1989 through 1991 for WS 13 are given in Table 2-2. In stream discharge, the dominant cation is calcium and the dominant anion is sulfate. For this study, stream water was grab sampled each Tuesday from permanently marked sampling locations just upstream from the gauging stations on each watershed. Samples were

processed and analyzed for pH, electrical conductivity, Ca, Cl, K, Mg, Na, $NH_3$, $NO_3$, $SO_4$, and alkalinity using EPA-approved methods (Edwards and Wood 1993). These data were collected to supplement the work of those investigators studying aquatic organisms.

Stream water samples were also collected and analyzed for the presence of DFB using automatic ISCO sequential samplers (ISCO, Inc., Lincoln, NE) and "replicate" grab samples. The latter were taken for quality control purposes. The ISCO sampler is a portable device that collects up to 28 discrete sequential samples from a liquid source. It pumps stream water up through tubing at predetermined times or flow changes into sample bottles contained within its base. For sequential sampling, a portion of the flow from each watershed was diverted through 10-cm diameter PVC pipe into large polyethylene tubs. The tubs were tilted away from the pipe inlets to allow the turnover of water. The uptake line for each ISCO sampler was placed at the pipe inlet in the top of the tub so that "new" inflowing water was sampled.

An ISCO sample and grab sample were collected in all four watersheds just before the aerial application of Dimilin 4L on WS 1 and WS 13, at the time of application and every 15 minutes for the next 2 h, every 30 minutes for the subsequent 2 h, every 60 minutes for another 4 h, and every 120 minutes for another 4 h. ISCO samples were collected at 30- or 60-minute intervals for the next four major storms, which occurred on May 24-25, June 14-15, June 18-19, and August 27, 1992. Other storms were sampled, but they resulted in such small hydrograph responses that they were not considered significant enough to warrant analysis.

The samples collected during application and subsequent storms were analyzed for DFB so that the amount exported from the watersheds in streamflow could be estimated. No inorganic chemical analyses were performed for these samples.

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*Table 2-1.* Streamflow and annual precipitation in the study watersheds (WS), Fernow Experimental Forest.

| Year | Streamflow (mm)[1] WS 1 | WS 4 | WS 7 | WS 13[2] | Precipitation (mm) |
|---|---|---|---|---|---|
| 1982 | 648 | 656 | 910 | — | 1450 |
| 1983 | 692 | 690 | 945 | — | 1523 |
| 1984 | 674 | 690 | 913 | — | 1471 |
| 1985 | 950 | 885 | 1125 | — | 1757 |
| 1986 | 589 | 565 | 782 | — | 1377 |
| 1987 | 403 | 415 | 558 | — | 1175 |
| 1988 | 567 | 626 | 821 | — | 1426 |
| 1989 | 732 | 788 | 995 | 987 | 1588 |
| 1990 | 728 | 770 | 935 | 975 | 1608 |
| 1991 | 416 | 437 | 540 | 553 | 1207 |
| **Mean** | **640** | **652** | **852** | **838** | **1458** |
| ± SD | 152 | 142 | 175 | 202 | 169 |

[1]Depth of water spread over the entire watershed that flowed over the weir in one year.
[2]Streamflow monitoring began in 1989.

*Table 2-2.* Mean annual stream water and precipitation chemistry for the study watersheds (WS), Fernow Experimental Forest, 1982-1991.

| Chemical parameter | Streamwater WS 1 | WS 4 | WS 7 | WS 13[a] | Precipitation |
|---|---|---|---|---|---|
| pH | 6.22 | 5.93 | 6.21 | 6.18 | 4.16 |
| Elect.Cond.[b] | 44.6 | 22.4 | 26.6 | 26.7 | 36.0 |
| Cl[c] | 0.52 | 0.60 | 0.53 | 0.50 | 0.13 |
| $NO_3$ | 8.32 | 3.54 | 5.31 | 2.39 | 1.90 |
| $SO_4$ | 9.01 | 4.32 | 4.45 | 6.78 | 3.29 |
| Ca | 3.35 | 1.56 | 2.07 | 1.86 | 0.37 |
| Mg | 1.49 | 0.77 | 0.85 | 0.88 | 0.04 |
| K | 1.01 | 0.65 | 0.81 | 0.88 | 0.11 |
| Na | 0.91 | 0.44 | 0.44 | 0.61 | 0.18 |
| NH3 | 0.10 | 0.06 | 0.09 | 0.08 | 0.30 |

[a]Data from 1984 to 1991
[b]Electrical conductivity in µS/cm
[c]Ion concentrations in mg/l

*Table 2-3.* Physical characteristics of the study watersheds (WS), Fernow Experimental Forest.

| Characteristic | WS 1 | WS 4 | WS 7 | WS 13 |
|---|---|---|---|---|
| Elevation | | | | |
| Maximum (m) | 792 | 815 | 853 | 860 |
| Minimum (m) | 637 | 713 | 728 | 739 |
| Aspect | NE | NNE | E | ESE |
| Size (ha) | 30.1 | 14.2 | 24.2 | 38.7 |

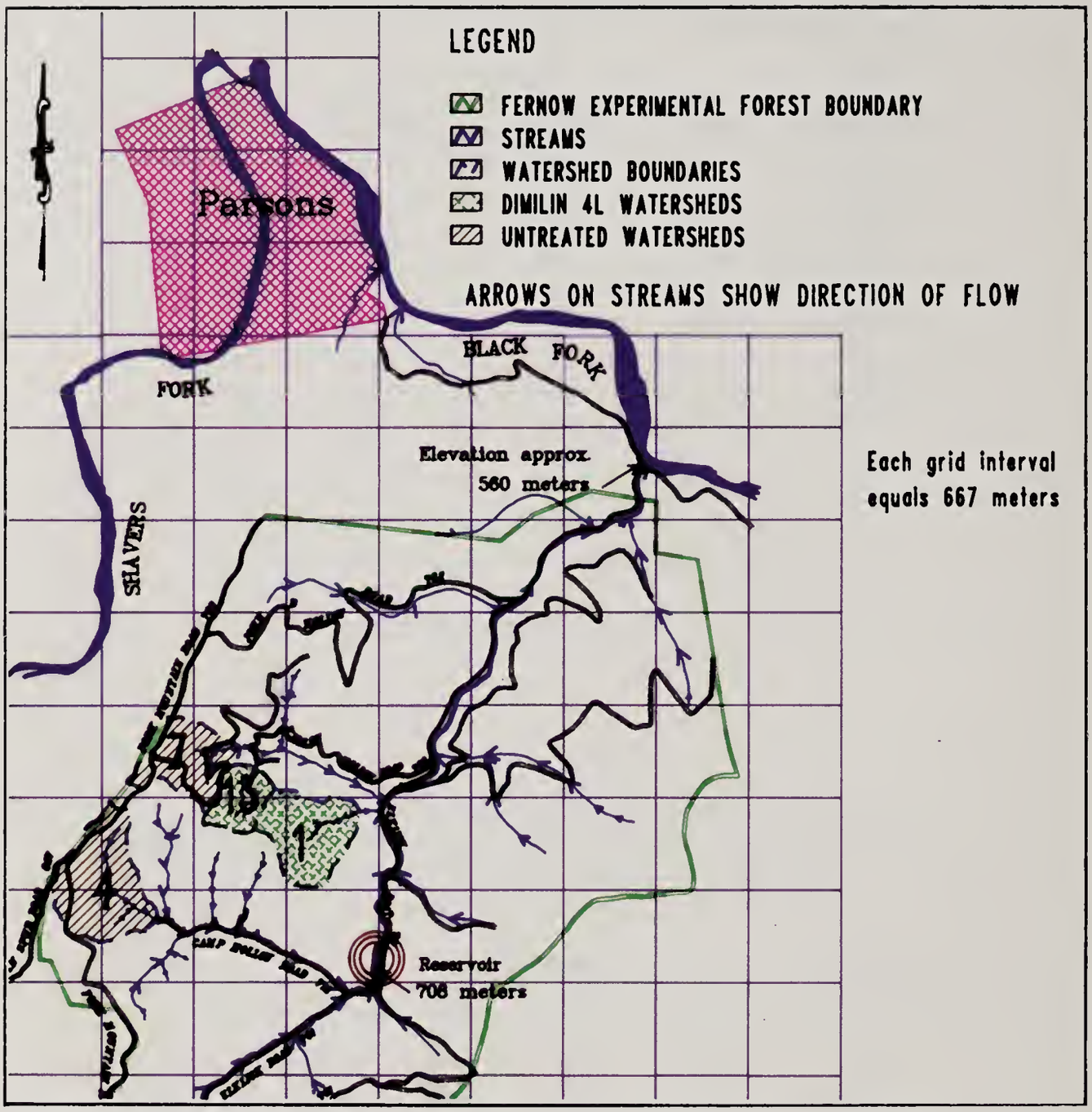


*Figure 2-1.* Study watersheds of the Fernow Experimental Forest near Parsons, West Virginia.

# Part B. Aquatic Organisms

# *Chapter 3. Aquatic Salamanders*

**Thomas K. Pauley**

## Introduction

Salamanders are excellent indicators of environmental chemicals and pollutants because they: (1) have complex life cycles with aquatic and terrestrial stages; (2) compete intensely for vital resources over short periods; (3) have permeable skin, gills, and eggs; and (4) typically hibernate or aestivate in soils (Dunson and others 1992).

While little is known about the effects of insecticides on salamanders, some studies have been conducted that deal with insecticides and anurans (an amphibian group that includes frogs and toads). Cooke (1973a and 1973b) studied the toxicity of DDT (a chlorinated-hydrocarbon insecticide) on tadpoles of the common European frog (*Rana temporaria* L.) in treated pools and ditches, and found that tadpoles either died, showed morphological abnormalities, or were hyperactive. Harri and others (1979) examined the toxicity and retention of DDT in adult *R. temporaria*. Kaplan and Overpeck (1964) determined that adult leopard frogs (*Rana pipiens* Schreber) suffered ill effects such as convulsions and depression of the numbers of red and white blood cell counts when exposed to halogenated hydrocarbon insecticide solutions for 30 days. Guzman and Guardia (1978) examined the effects of parathion (an organophosphorus insecticide) on hibernating and mating toads (*Bufo arenarum* Hensel). The insecticides endrin (a chlorinated-hydrocarbon) and toxaphene had toxic effects on eggs, larvae, and juveniles of the southern leopard frog (*Rana sphenocephala* Cope) (Hall and Swineford 1980). Holcombe and others (1987) examined testing methods to determine LC50 values of several aquatic species including clawed frog, tadpoles, *Xenopus laevis* (Daudin), and found that the tadpoles and midges were among the most sensitive species when exposed to 1, 2, 4-trichlorobenzene and acrolein.

The seal salamander (*Desmognathus monticola* Dunn) (Fig. 3-1) comprises nearly 90 percent of the aquatic salamander species in these four streams, and is common throughout the Allegheny Plateau and Allegheny Mountain Physiographic Provinces in West Virginia (Green and Pauley 1987). It is a robust species that reaches a maximum length of 140 mm. Except for some terrestrial movement in the fall, it spends most of its life in water. In the Fernow Experimental Forest, *D. monticola* deposits eggs in stream banks, and there is a 9- to 10-month larval period (Marcum 1994).

Streams in each watershed are small (first- or second-order), usually dry up in the summer and, therefore, cannot support fish except in isolated pools. As a result, salamanders are the top aquatic predators and are important indicators of food chain and energy flow changes.

## Laboratory Study Methods and Costs

In September and October of each year (1989-1994), 20 adult male and 20 adult female *D. monticola* were collected from under rocks located outside of the 25-m study sections in streams in all watersheds (WS 1 and WS 13 were treated with Dimilin 4L on May 16, 1992 and WS 4 and WS 7 were untreated) and frozen. Stomach contents were examined, and percentages of tail fat and carcass fat, and weight of total fat as well as number and volume of follicles were determined.

To examine food items, stomachs were removed from frozen specimens and fixed in 10 percent formalin for 24 h, bathed in water for 24 h, and stored in ethanol until contents could be studied.

Contents were examined with a dissecting microscope, and each item was identified. Insects and arachnids were identified to order, while other items were identified to phylum or class.

Percentage of fat in the tail was determined from the first 18 mm of the tail (Frazer 1980). Tails were removed, weighed (wet weight), dried in a convection oven (dry weight), bathed in petroleum ether for 8 h, and weighed (lean weight) (Fitzpatric 1973, Kerr and others 1982, Reznick and Braun 1987).

Follicle numbers were determined by removing the ovaries and oviducts and counting all follicles. Follicle volumes were measured by placing follicles in a 10 ml volumetric flask filled with water and measuring the amount of water displaced with a 1.0 ml syringe.

The major cost for the laboratory work is for the petroleum ether, which can be ordered from chemical supply houses for about $30 per liter. The volumetric flask and 1.0 ml syringe can also be purchased from supply houses for about $17.

## *Field Study Methods and Costs*

Salamanders were studied in two 25-m sections of one stream in each watershed twice per month from May through September, 1989 to 1994. Adults and juveniles were found by searching beneath 100 single rocks (approximately 250 $cm^2$) and 10 rocks (approximately 500 $cm^2$) stacked on top of larger rocks (approximately 900 $cm^2$). Adults were checked for gender and their toes were clipped (with toe nail clippers) to identify individuals for population estimates (Twitty 1966). Because of their small size, juveniles' toes were not clipped.

Juveniles were monitored in refugium bags (approximately 50 $cm^2$) constructed of plastic netting (mesh size 3.2 to 3.8 cm) filled with small rocks, leaves, and moss (Fig. 3-2). To capture juveniles, bags were removed from the stream, immediately placed in white plastic pans (35 cm by 30 cm by 14 cm), and the contents mixed by hand until all salamanders were "chased out." Snout-vent-length (SVL) was measured for each animal using a modified model (Fig. 3-3) of a measuring apparatus by Wise and Buchanan (1992). This apparatus consisted of a three-sided Plexiglas® box (5 cm by 9.5 cm by 14 cm) and a wet sponge. Salamanders were placed between the sponge and the Plexiglas and measured with a vernier caliper from the anterior end of the vent to the tip of the snout.

Plastic netting for the juvenile refugium bags can be purchased from retail stores or ordered from dealers such as forestry supply houses for about $23 (10 m by 10 m). The SVL measuring apparatus (Plexiglas and sponge) costs approximately $2 each.

### *Advantages And Disadvantages*

Techniques described here are the result of several years of experimentation. The three techniques recommended are the use of rocks on rocks as cover objects, the juvenile refugium bags, and the SVL measuring apparatus. Rocks resting on rocks maintain moist, cool spots that appear to be optimum habitat for juvenile and adult salamanders. Juvenile salamanders are difficult to find and capture, but by using refugium bags described above, captures can be

relatively easy. The SVL measuring apparatus is an excellent means for holding a salamander straight to get an accurate measurement. It also reduces the time necessary to handle the salamander which decreases the chances of injury, removal of body moisture (slime), and conduction of the investigator's body heat to the salamander.

All statistical analyses were made using ANOVA with Newman-Keuls multiple comparisons ($P \leq 0.05$).

## Results

### *Laboratory Study*

Major food items of *D. monticola* in the treated watersheds shifted from soft-bodied prey items (e.g. winged hymenopterans) before treatment to hard-bodied prey items (e.g. ants, and coleopteran adults) after treatment but not in the untreated watersheds (Table 3-1). *D. monticola* consumed a significantly greater number of ants in the treated watersheds in 1992 than before and after treatment years and in the untreated watersheds for all years. There was also an increase in the number of lepidopterous larvae and dipterans consumed in both the treated and untreated watersheds during the posttreatment period. There were no significant differences in the percentages of tail fat and carcass fat, or weight of total fat in gravid *D. monticola* females attributable to DFB between the treated and untreated watersheds before or after application of Dimilin 4L (Table 3-2). Follicle numbers and volumes were not significantly different in *D. monticola* in treated and untreated control watersheds.

### *Field Study*

A total of 1,153 *D. monticola* (488 from WS 1 and WS 13; 665 from WS 4 and WS 7, respectively) was observed in the aquatic quadrats from 1989 to 1994. Eighty- nine were females, 167 were males, and 897 were juveniles. The number of recaptures (77 = 3.0 percent) was low. The large number of juveniles observed illustrates the effectiveness of the refugium bags. A total of 577 (64.3 percent) juveniles was collected in the refugium bags compared to 320 (35.7 percent) using conventional methods of turning cover objects. There were no significant differences in snout-vent-lengths (SVL) for *D. monticola* between the treated and untreated control watersheds pretreatment (50 and 51 mm) and after the application of Dimilin 4L (51 and 52 mm). There also was no significant difference in SVL of this species between pre- and posttreatment periods in the treated watersheds.

## Discussion

Salamanders are opportunistic feeders, and their diet generally indicates available prey items. Thus, food analyses most likely reflect common insects in the respective watersheds.

Diflubenzuron was hypothesized to reduce insect fauna in treated watersheds, thereby reducing the percentage of stored fat in salamanders. Because follicle development depends on fat stored in the tail of females, fat reduction will reduce either the follicle numbers or volumes, which ultimately will be reflected in surface abundance of salamanders. The results of this study showed an

increase in the consumption of hard-bodied prey items. The passage of hard-bodied prey items is much slower for thicker chitinous exoskeletons (ants and beetles) than for thinner chitinous exoskeletons (Jaeger and Barnard 1981). The shift in food items to hard-bodied prey in this study did not appear to affect the percentage of tail fat or follicle development.

Because of the high trophic level of aquatic salamanders and their value as bioindicators, understanding the effects of anthropogenic stress on salamanders is critical. The long life span of forest salamanders (20 or more years) and the time necessary to observe negative impacts from ecotoxicological factors mandate that future studies must include at least 5 to 10 years of posttreatment data. Dunson and others (1992) recommended long-term (10-year minimum) studies to examine the relation between amphibian population cycles and anthropogenic changes.

## Recommendations for Future Studies

The impact of defoliation by gypsy moth on salamander populations needs to be studied. Because salamanders are ectothermic, higher soil temperatures and lower surface moisture from greater insolation (which may result from defoliation) may have a greater negative impact than use of insecticides. Also, a detailed study should be conducted to determine the impact of DFB on salamander larvae.

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*Table 3-1.* Distribution of prey items in stomachs of *Desmognathus monticola* from untreated (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds (1989-1994 data pooled).

| | Percent of Stomachs | | | |
|---|---|---|---|---|
| | Pretreatment | | Posttreatment | |
| | WS 4 & 7 (n=85) | WS 1 & 13 (n=82) | WS 4 & 7 (n=237) | WS 1 & 13 (n=200) |
| Hymenoptera - Ants | 11.8 | 7.3 | 9.3 | 10.5 |
| Hymenoptera - Winged | 29.4 | 50.0 | 22.4 | 21.0 |
| Lepidoptera - Adult | 0 | 0 | 1.7 | 0 |
| Lepidoptera - Larva | 0 | 0 | 10.6 | 11.5 |
| Diptera - Adult | 1.2 | 1.2 | 12.7 | 8.0 |
| Diptera - Larva | 0 | 1.2 | 0.8 | 1.5 |
| Coleoptera - Adult | 31.8 | 19.5 | 26.2 | 21.0 |
| Coleoptera - Larva | 1.2 | 0 | 1.7 | 1.0 |
| Chilopoda | 0 | 0 | 0.4 | 0.5 |
| Diplopoda | 3.5 | 0 | 3.0 | 1.0 |
| Annelida | 0 | 1.2 | 0.8 | 0.5 |
| Mollusca | 3.5 | 1.2 | 0.8 | 3.5 |
| Salamander | 2.4 | 1.2 | 0.4 | 1.0 |
| Collembola | 0 | 1.2 | 0 | 0.5 |
| Araneida | 2.4 | 1.2 | 2.5 | 2.5 |
| Plecoptera - Adult | 0 | 0 | 4.6 | 8.5 |
| Plecoptera - Larva | 0 | 0 | 1.7 | 2.0 |
| Trichoptera | 0 | 3.7 | 1.3 | 4.0 |
| Neuroptera | 0 | 1.2 | 0.4 | 0.5 |
| Ephemeroptera | 0 | 0 | 0.4 | 0.5 |
| Acarina | 0 | 0 | 1.7 | 3.0 |
| Homoptera | 0 | 0 | 8.4 | 13.5 |
| Hemiptera | 1.2 | 0 | 1.7 | 4.0 |
| Amphipoda | 0 | 1.2 | 0.8 | 1.0 |
| Nematodes | 0 | 0 | 3.8 | 2.0 |
| Mecoptera | 0 | 0 | 0.8 | 0 |
| Unidentified | 7.1 | 9.8 | 22.4 | 20.5 |
| Debris | 4.7 | 12.2 | 6.8 | 4.5 |
| Empty | 1.2 | 0 | 11.4 | 11.0 |

***Table 3-2.*** Mean percentages of carcass fat and tail fat, and mean weight of total fat of gravid *Desmognathus monticola* females collected in the fall from untreated (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds (1989-1994 data pooled).

| | Pretreatment | | Posttreatment | |
|---|---|---|---|---|
| | 1 & 13 | 4 & 7 | 1 & 13 | 4 & 7 |
| % Carcass Fat | 4.8a[a>] ± 1.9 (n = 25) | 8.3b ± 4.6 (n = 28) | 7.1a ± 4.5 (n = 23) | 9.8a ± 17.8 (n = 45) |
| % Tail Fat | 19.4a ± 8.3 (n = 26) | 19.8a ± 9.3 (n = 26) | 24.9a ± 18.6 (n = 24) | 19.3a ± 7.8 (n = 45) |
| Total Fat (g) | 0.04a ± .03 (n = 26) | 0.06a ± .06 (n = 28) | 0.074a ± .041 (n = 24) | 0.06a ± .03 (n = 46) |

[a>] Mean and standard deviation, unlike letters indicate a significant difference ($P < 0.05$).

***Figure 3-1.*** The seal salamander, *Desmognathus monticola*, a dominant aquatic species in the Fernow Experimental Forest.

***Figure 3-2.*** Refugium bags used to collect juvenile aquatic salamanders.

***Figure 3-3.*** A device used to measure snout-vent-length of salamanders.

# *Chapter 4. Macroinvertebrates*

**Sue A. Perry**

## Introduction

The effects of diflubenzuron (DFB) on nontarget stream macroinvertebrates have been investigated in northeastern hardwood forests by Jones and Kochenderfer (1987) and Swift and others (1988) and reviewed by Fischer and Hall (1992). Hanson and Garton (1982) conducted a laboratory toxicity study and reported that the impact of DFB on some nontarget aquatic insects, such as mayflies and stoneflies, was more severe and longer lasting than on targeted aquatic insects, such as chironomids. Size of the developmental stage, rate of metabolism, frequency of molting during the exposure period, and duration of exposure are biological factors that affect the susceptibility of aquatic organisms to the insecticide (Ivie and Wright 1978, Rodrigues and Kaushik 1986). These factors contribute to the wide range of susceptibilities reported for different aquatic organisms.

Diflubenzuron can enter headwater streams directly at the time of a spray, as washoff from treated foliage, or as treated leaf material during leaf fall. Some insects in small headwater streams rely heavily on leaves for food (Fisher and Likens 1973, Hynes 1963, Ross 1963). For this reason, leaf-shredding aquatic insects may be most adversely affected in the fall after ingestion of treated leaf material. DFB residues toxic to shredders remained on leaves after approximately 4 months incubation in a stream (Swift and others 1988). The rate of loss of DFB from leaves in streams was much slower in December than in summer (Harrahy and others 1993). Because of the mode of action of DFB, adverse effects are delayed until the molting process begins. Long-term observations of stream fauna are therefore necessary to determine if an impact occurs.

Headwater streams are difficult to sample, because discharge is often too low to provide enough flow for conventional macroinvertebrate samplers. During summer and early fall when transpiration rates in the forest are high, these streams may have little or no surface flow, and droughts can compound the difficulties of sampling. We describe two sampling methods that can be used in small headwater streams for assessing the effects of DFB on nontarget aquatic invertebrates. Conventional laboratory methods for testing the toxicity of compounds are also inappropriate for testing the effects of DFB because of the delayed mode of action. We present the methods that we used in laboratory studies to assess the effects of DFB on several species of mayflies and stoneflies.

## Laboratory Study Methods

We conducted two types of laboratory toxicity tests, exposure in water and feeding, to determine DFB effects on nontarget aquatic insects. Standard 96-h toxicity tests do not reflect DFB mode of action, because the observation period is not long enough to observe delayed effects, such as disruption of molting. Therefore, we exposed four species of heptageniid mayflies (*Cynygmula subaequalis* (Banks), *Stenacron interpunctatum* (Say), *Stenonema meririvulanum* (Carle), and *Stenonema femoratum* (Say)) and the stonefly (*Peltoperla arcuata* (Needham)) to Dimilin 25-W (25 percent wettable powder) in water for 96 h and then transferred them to insecticide-free water for an extended observation period of 36 days. Experiments were conducted in Sherer growth chambers at 10° C. Insects were maintained in glass test chambers, because preliminary testing showed that DFB was adsorbed on plastic chambers. A stock solution was prepared immediately before each experiment by mixing 8 mg of Dimilin 25-W with 2 liters of dechlorinated tap water. The mayflies were exposed to 0, 0.6, 5.6, 55.7, and 557.2 ppb and the stonefly to 0, 1.0, 10.2,

101.5, and 1,015 ppb Dimilin 25-W in water. Test concentrations were based on actual measurements of the active ingredient by gas chromatography and mass spectrometry (Wimmer and others 1991).

In the feeding studies, the stoneflies *P. arcuata* and *Pteronarcys proteus* Newman were fed Dimilin 25-W treated yellow-poplar leaves for 24 days and then fed untreated leaves and observed for 60 days (*P. arcuata*) and 90 days (*P. proteus*). Yellow-poplar leaves had been aerially sprayed with Dimilin 25-W in May 1991 and allowed to weather on the tree for 2 months. These leaves were placed in a stream to be conditioned through microbial colonization before they were fed to the insects.

## *Field Study Methods*

Two different quantitative sampling techniques were used to characterize the macroinvertebrate communities of the four study streams, one in each watershed (WS 1 and WS 13 were treated with Dimilin 4L on May 16, 1992, and WS 4 and WS 7 were untreated). Samples were collected once a month from October 1989 through April 1993 using a multilevel artificial substrate basket sampler (Griffith and Perry 1993) (Figure 4-1a, b). Each basket sampler consisted of a 30-cm long section of polyvinyl chloride (PVC) pipe perforated with 1-cm holes, so that 40 percent of the surface area was removed, providing an entry for colonizing organisms. The pipe was inserted vertically into the substrate so that the top was flush with the streambed. Three wire baskets (1.3-cm mesh) containing washed river gravel were stacked on top of each other in the section of PVC pipe. A 250-micron mesh Nitex bag with lines attached along the top edge was inserted at the base of each basket. When collecting the samples, the mesh net bag was drawn up around the baskets by pulling up lines attached to the top of each bag. This prevented loss of invertebrates through the 1.3-cm mesh of the wire baskets.

These samplers provided information about the vertical distribution of invertebrates within the substrata by providing individual top, middle, and bottom basket information. Six basket samplers were placed in each stream, three above the weir and three below it. On sampling dates the contents of the baskets were placed in labeled plastic containers and returned to the laboratory, where the invertebrates and debris were sorted from the river gravel and preserved in a 5 percent formalin solution.

We also sampled with leaf pack colonization samplers (Figure 4-2). Leaf pack sampling was conducted during the fall of 1990 and 1992 to compare communities of insects that feed by shredding leaves before and after the spray in both the treated and untreated control streams. We filled 150 3-mm mesh bags with 5 g of red oak (*Quercus rubra* L.) leaves and 150 bags with 5 g of red maple (*Acer rubrum* L.) leaves. Thirty-five leaf packs of each species were placed in each of the four streams in November. Five packs of each species were removed from each stream after 2 days, 2 weeks, and then every 4 weeks over a 20-week period. The packs were placed in ziplock bags in the field and returned to the laboratory, where invertebrates and debris were separated from the leaf material, then preserved in 5 percent formalin.

In the laboratory, the invertebrates were hand sorted from debris under 10-power magnification and placed in vials containing 75 percent ethanol. Quality control was maintained by a separate examination of sorted samples for accuracy. Invertebrates were then identified to genus (except for

Acari, Oligochaeta, and Chironomidae), counted, and assigned to functional feeding groups (Merritt and Cummins 1984). Calculations were conducted using SAS (SAS 1989).

## Results

### *Laboratory Study*

The heptageniid mayflies were sensitive to DFB in water at concentrations as low as 0.6 ppb (Harrahy and others 1994). The stonefly *P. arcuata* was less sensitive to DFB in water. Survival of *P. arcuata* exposed to measured concentrations of 10.2 and 1,015 ppb DFB in water differed significantly ($P \leq 0.05$) from survival of untreated controls, but survival of those exposed to 101.5 ppb DFB did not differ significantly from untreated controls. In the feeding studies, survival of treated *P. arcuata* was significantly different from the untreated controls at day 60 of the test; mean survival was 60 percent in the treated group versus 82 percent in the untreated control. Survival of treated *P. proteus* did not differ significantly from the untreated controls during the 90-day test period, although the low number of molts that occurred during the test period may have influenced these results.

### *Field study*

The Before-After Control-Impact Pairs (BACIP) analysis (Stewart-Oaten and others 1992) was used with data collected using the multilevel artificial substrate samplers to test for differences in invertebrate densities between treated and untreated streams. Densities of some susceptible species in DFB-treated streams either showed population depressions or failed to increase in density after a drought as compared with those in untreated streams. Species that showed significant differences ($P \leq 0.05$) in densities and decreased in treated streams included the stoneflies *Leuctra* sp. and *Isoperla* sp., the mayfly *Paraleptophlebia* sp., and the crane fly *Hexatoma* sp. Mean densities of *Leuctra* sp. dropped from an average of 58.8 to 47.6 in treated streams, whereas densities in untreated streams nearly doubled (54.2 to 111.4). Densities of *Isoperla* sp. decreased by nearly 50 percent (2.0 to 1.1) in treated streams in the posttreatment period, whereas mean densities in untreated streams nearly doubled (1.9 to 3.7). In treated streams mean densities of *Paraleptophlebia* sp. were reduced by more than 50 percent (from 37.4 to 17.0 per sampler) in the posttreatment period, whereas in untreated streams, densities remained relatively constant (about 26 per sampler) through the pretreatment and posttreatment periods. Mean densities of *Hexatoma* sp. decreased from 2.5 to 1.9 in treated streams, whereas densities increased from 1.4 to 1.9 in untreated streams.

The shredders also exhibited differences at $P \leq 0.05$. Mean shredder densities in treated streams declined from 8.4 to 6.1 in the posttreatment period, whereas untreated densities rose from 7.6 to 12.4. Shredders were the dominant functional feeding group in the four watersheds. In the posttreatment period, populations of roundworms, flatworms, and segmented worms were significantly higher in treated streams compared with those in untreated streams.

## Discussion of the Laboratory Study

The results of our laboratory study agree with the results of a complex laboratory stream experiment, in which organisms of another heptageniid genus (*Epeoris*) were eliminated within 1 month at a concentration of 10.0 ppb DFB (Hansen and Garton 1982). Another heptageniid mayfly (*Stenonema*) was only minimally affected by DFB at 1.0 mg/l over a 30-minute exposure (Rodrigues and Kaushik 1986).

Exposure to DFB in water did not have a highly toxic effect on *P. arcuata* in our study. In a field study, application of Dimilin 25-W at 70 g AI/ha (0.06 lb AI/acre) to a small headwater stream in West Virginia resulted in a spike of 2.1 ppb in water after a heavy rain, but did not adversely affect *Peltoperla* sp. (Jones and Kochenderfer 1987). In a complex laboratory stream, however, *Peltoperla* sp. were eliminated within 1 month at a concentration of 1.0 ppb Dimilin (continuous exposure) (Hanson and Garton 1982).

Results of the feeding studies were inconclusive because of the low number of molts that occurred during the 24-day period when the two species of stoneflies were fed Dimilin-treated leaf material. Survival of treated *P. arcuata* differed significantly from the untreated only toward the end of the 60-day test period. Survival of treated *P. proteus* did not differ significantly from the untreated during the 90-day test period. About half of the *Peltoperla* molted during the 60-day test period, but few *Pteronarcys* molted. Swift and others (1988) observed significantly higher mortality and lower growth in *Tipula abdominalis* (Say) and *Platycentropus radiatus* (Say) fed conditioned, DFB-treated yellow-poplar leaves for 430 and 330 degree days, respectively. *Pteronarcys* used in this experiment were collected in February during the second year of nymphal growth. It is possible that the DFB was cleared from the insect tissues shortly after feeding and that the long period of time between molts allowed the insects to recover. Verloop and Ferrell (1977) reported that DFB may clear rapidly from some insects and that DFB had a half-life of about 1 day.

## Recommendations for Future Studies

Future laboratory studies should use early life stages of aquatic insects that coincide with leaf fall, when treated leaves are introduced to headwater streams. The life histories of many aquatic insects are timed to make maximum use of leaf detritus as a food source, so introduction of Dimilin-treated leaves to headwater streams could adversely affect stream insects in early life stages because they molt more frequently. Different species of aquatic insects have different sensitivities to DFB in water and on their food, so species-specific information is needed.

Two artificial substrate colonization samplers (a rock basket sampler and leaf pack sampler) were used in this study. Artificial substrate samplers reduce the variability of operator efficiency in taking samples and eliminate the subjectivity of choice of sampling locations. In addition, substrate samplers use a nondestructive process of sampling the environment that reduces confounding effects of habitat, such as substrate type. However, a criticism of all artificial substrate samplers is that they are selective for organisms that colonize them (Rosenberg and Resh 1982). Possible adverse effects from the sampling were a concern, so samplers were used that would result in minimal disturbance of the substrate. We were also concerned about oversampling

small streams during a long-term study. The number of samples collected was based on the number needed for statistical certainty, but also took into consideration the possibility of over sampling and the high costs of processing samples when determining the numbers of samples to collect.

The basket sampler is useful in small headwater streams because of its lack of dependence on higher velocities and flow through a net. The multilevel basket sampler was the only possible means of sampling when streamflow was very low and when only subsurface flow was present. The leaf pack samplers were faster to process than the basket samplers, but were selective of the species of insects that colonize natural leaf packs in streams. The data from the leaf pack samplers was not used in analyses of the effects of DFB on stream macroinvertebrates. During processing of the samples, it was evident from the species composition that some of the leaf packs had been dewatered during part of the colonization period (a few were also not wet when retrieved). Therefore, even though leaf pack sampling methods have been widely used to study leaf shredding invertebrates, they may not be a reliable method in first- and second-order streams during low flow periods in early autumn.

The inherent variability and complexity of natural systems, along with problems with availability of streams that are good replicates for a number of environmental variables, make field studies difficult; cost and labor considerations can also make it difficult to sample adequately for statistical reliability. The quantitative basket sampling technique used in this study gave good characterization of stream macroinvertebrate populations, but it is time and labor intensive and may not be feasible for smaller programs. If time and budget are limiting, sampling should be concentrated in those times of the year when Dimilin spraying effects are most likely, that is, right after spraying and 4 to 5 months after leaf fall. To sort out more subtle long-term effects and effects of confounding factors, however, a monthly sampling program begun for at least 1 year before and continued for 1 year after spraying is necessary.

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4a.

4b.

***Figure 4-1a,b.*** Rock basket colonization sampler used to characterize aquatic macroinvertebrate communities.

***Figure 4-2.*** Leaf pack colonization sampler used to characterize aquatic macroinvertebrate communities.

# *Chapter 5. Aquatic Fungi*

**Tara Dubey**

## Introduction

Aquatic hyphomycetes (Imperfect Fungi without an enclosed fruiting structure) dominate the aquatic fungi associated with decaying leaves in lotic (actively moving water) systems (Barlocher and Kendrick 1974, Suberkropp and Klug 1976, Trisca 1970). Decomposition of leaf detritus and its exploitation by other members of stream detrital communities are largely dependent upon the activities of aquatic hyphomycetes, since hyphomycetes have the enzymatic capability to digest the structural polymers that comprise most dead plant tissue (Bjarnov 1972, Monk 1976). Thus, any change in a stream's physiochemical or biological environment will influence aquatic hyphomycete activity. Consequently, concerns exist about how Dimilin 4L might affect aquatic hyphomycetes.

## Field Study Methods

Aquatic hyphomycete distributions in the four watersheds (WS 1 and WS 13 were treated with Dimilin 4L on May 16, 1992 and WS 4 and WS 7 were untreated) were studied by membrane filtration of stream water samples (Iqbal and Webster 1973) and by leaf bag incubation in both streams and weir ponds (Musil and Shearer 1982). For membrane filtration, 250-ml water samples were collected from each stream in 1991 (pretreatment year) and 5 days prior to and 2, 10, 25, and 55 days following Dimilin 4L application in 1992. Ten samples were collected from each stream on the first three sampling dates; because of low water levels, only five samples were collected from each stream on the last two sampling dates. In 1991, sampling was conducted to obtain baseline data on the occurrence and distribution of aquatic hyphomycetes in the four streams.

Samples were filtered through Millipore 0.45-$\mu$m membrane filters in the field at the time of collection. In the laboratory, each filter was treated with 0.1 percent cotton blue and heated in lactic acid at 50-60° C to render it sufficiently transparent for conidial enumeration and identification. Filters were examined microscopically at 100 power, and conidia were counted. Taxa were recorded for 40 noncontiguous fields-of-view (total area 36.2 $mm^2$) on each filter.

For leaf bag incubation, mesh bags containing leaves from red oak (*Quercus rubra* L.) and sugar maple (*Acer saccharum* Marsh.) were prepared in the manner described by Musil and Shearer (1982) and Chamier and others (1984). The leaves were collected in October 1991 shortly after leaf abscission, air-dried, and stored until leaf bags were prepared. Each bag contained approximately 2.0 g of dried leaves of either red oak or sugar maple. One set of four bags of each leaf type was tied to a perforated brick. These were placed in the streams and weir ponds of the four watersheds on May 11, 1992. Bags of each leaf type were retrieved on each of four visits following application of Dimilin 4L (i.e., after 2, 10, 25, and 55 days). Upon retrieval, leaf bags were placed in plastic zipper-lock storage bags, partially filled with stream water, and returned to the laboratory. Leaves were removed from the mesh bags, gently washed in sterile distilled water, and cut into 1- by 1-cm squares. A few squares were examined microscopically to determine the presence of hyphomycetes. The remaining squares were incubated in aerated chambers filled with sterile distilled water, as described by Shearer and Webster (1991). After 24 h, the water was filtered through a membrane filter, and conidia were identified by microscopic examination of the filter paper after treatment with cotton blue and lactic acid, described previously. Fungal

specimens recovered from leaf bags were studied as living material or fixed and stained with cotton blue and mounted in lactophenol. Fungal species were identified using keys and descriptions provided in Ainsworth and others (1973), Barnett and Hunter (1972), Ellis (1971), Ingold (1975), Nilsson (1964), and Subramanian (1983).

Separate data sets were compiled for aquatic hyphomycetes recorded as conidia filtered from water samples and for those occurring on leaves. The four streams were compared using coefficient of community (CC) indices (Mueller-Dombois and Ellenberg 1974).

## Results

### *Pretreatment 1991*

Thirty-three taxa of aquatic hyphomycetes were recorded from leaf bags placed in the streams. *Flagellospora curvula, Anguillospora crassa, Tetrachaetum elegans, Tricladium* sp. and *Varicosporium* sp. were the most consistently abundant taxa. The mean numbers of taxa recorded from the various types of leaves were 5.4 for red oak and 8.4 for sugar maple. The largest numbers of taxa (20) were recorded from red oak leaves placed in WS 4 and sugar maple leaves (18) in WS 13. In general, the number of taxa present tended to decrease over time for all watersheds except WS 4. Data obtained from filtration of water samples collected from streams indicate that the highest number of spores (618 per 1000 ml of water) for one sample was recorded from WS 4. The highest number of spore types (and thus fungal taxa) for one sample was 37, recorded from WS 1.

### *Treatment 1992*

Water filtration -- Two days after the Dimilin 4L application, the number of conidia in samples from WS 1 was approximately twice that recorded 5 days prior to treatment (May 11) (Table 5-1). However, the number of conidia recorded in samples from the other treated watershed decreased from May 11 to May 18. In both treated watersheds, conidial numbers generally increased on subsequent sampling dates. Conidial numbers recorded from untreated controls WS 4 and WS 7 also declined from May 11 to May 18 and remained below the pretreatment levels throughout the subsequent sampling dates.

Numbers of fungal taxa recorded on May 18 did not differ significantly between the treated and untreated watersheds (Table 5-2). Fewer taxa generally were observed in June and July when compared to the May sampling periods for both treated and untreated watersheds. This decline may be the result of the lower water levels or higher water temperatures common in these headwater streams during the growing season.

Overall, 108 fungal taxa were recorded by means of membrane filtration of water samples from all four watersheds at the five sampling dates. Only 25 taxa were common to all four watersheds.

The CC indices calculated from pooled filtration data for all sampling dates (Table 5-3) indicate that WS 1 and WS 4 were the most similar streams (CC = 0.687), even though WS 1 was treated with Dimilin 4L and WS 4 was not treated. Watersheds 7 and 13 were the least similar (CC = 0.556). The average CC value for all possible combinations of streams was 0.638.

Leaf bag colonization -- Like the filtration data, leaf bag colonization data (Table 5-4) are quite variable. In general, more aquatic hyphomycete taxa were associated with red oak leaves than with sugar maple leaves in both the streams and the weir ponds. In most instances, the highest numbers of taxa for both leaf types were recorded on the first sampling date. The maximum number (28) was recorded for red oak in the weir pond of WS 7 on June 11, whereas the minimum number (4) was recorded for the same leaf type in the weir pond of WS 13, also on June 11. Overall, numbers of taxa recorded from the two types of ecological situations (i.e., weir ponds and streams) were remarkably similar.

A total of 64 fungal taxa colonized red oak leaves. Forty-six of these taxa were freshwater hyphomycetes. Fifteen species of terrestrial geofungi (fungi commonly found in terrestrial soil) and three species of aeroaquatic hyphomycetes (fungi that produce conidia above the water surface) also were recorded. Nine taxa were present only in the treated watersheds, whereas 12 species were restricted to the untreated watersheds.

Colonization of sugar maple leaves was similar to that of red oak leaves. Sixty-five fungal taxa were recorded. Fifty-one of these taxa were fresh water hyphomycetes, 11 were terrestrial geofungi, and 3 were aeroaquatic hyphomycetes. Nineteen taxa were found only in treated watersheds, while seven were restricted to the untreated watersheds. *Anguillospora crassa* and *Flagellospora curvula* were the only taxa recorded from both filtered water samples and leaf bags, with 100 percent consistency.

Coefficient of community indices calculated from leaf bag colonization data are summarized in Table 5-5. The highest CC value was 0.666, which was recorded for pair wise combinations of WS 1 and WS 4, WS 1 and WS 7, and WS 4 and WS 7. Four of these involved red oak and two involved sugar maple. The lowest CC value (0.303) was recorded for sugar maple in WS 1 and WS 13.

## Discussion

Filtration data indicate that conidial numbers in WS 1 increased following the Dimilin 4L application, but a similar increase did not occur in WS 13. In fact, numbers of conidia decreased from May 11 to May 18 for all watersheds except WS 1. Although the decrease in WS 13 was less than that observed in the two untreated watersheds, there is not enough of a difference to suggest that the application of Dimilin 4L caused any sudden increase or decline in fungal occurrence in the treated watersheds.

Generally higher numbers of fungal taxa were observed in leaf bags from treated watersheds than in those from untreated watersheds. This difference was most obvious on May 18 and was more apparent in the weir ponds of treated watersheds than in streams of the same watersheds. DFB may have a greater opportunity to accumulate in weir ponds and thus influence growth and

sporulation of litter-decomposing hyphomycetes. Ongoing investigations on residual analysis of DFB indicate its persistence in litter within the treated area (Wimmer and others 1995).

Overall, numbers of fungal taxa recorded from filtration and leaf bag colonization in treated watersheds on May 18 were comparable to or even higher than those recorded on subsequent sampling dates. This pattern demonstrates the tolerance of these fungi to biochemical change. It seems likely that decreases in fungal occurrence and numbers of conidia during June and July may be related to the low streamflow and resultant elevated temperatures.

In the present study, Dimilin 4L application was not shown to exhibit any clear evidence of a direct influence on conidial production in treated watersheds two days after treatment. Fungal colonization data for red oak and sugar maple leaves suggest that fungal growth and decomposition activities in the treated watersheds were similar to or slightly greater than those occurring in the untreated watersheds. If aquatic hyphomycetes are affected by application of Dimilin 4L, these effects are manifested in an indirect rather than a direct manner.

## *Recommendations for Future Studies*

The possible effects from application of Dimilin 4L on fungal occurrence can be assessed in two ways. The first way is by examining the direct utilization of residual DFB in litter and soil (Wimmer and others 1995). In an earlier study (Dubey and others 1992), five species of aquatic hyphomycetes (*Clavariopsis aquatica, Heliscus lugdunensis, Lemonniera aquatica, Lunulospora curvula* and *Tetracladium marchalianum*) showed increased growth rates with increased Dimilin concentrations. However, direct effects of insecticides, such as Dimilin 4L, on aquatic microorganisms probably are modified by a number of factors, including the extent to which the insecticide is water soluble, the contact time between the insecticide and the fungal mycelium, the nutritional status of the environment in which the fungus interacts with the insecticide, the amount of fungal cell material present, and the initial insecticide concentration applied.

The second way to examine the effects of Dimilin 4L involves monitoring changes that may occur in the biochemical environment of treated watersheds as a result of reduced defoliation as gypsy moth larvae are killed after the application of the insecticide. For example, reduced defoliation may result in increases in the watershed's buffering capacity, pH, and Ca and Mg status, and decreases in streamflow and $NO_3$ and $NH_4$ compared to conditions of greater defoliation (Downey and others 1994). Low streamflow rates also favor the production of coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM), which are basically produced by the enzymatic leaf processing action of fresh water hyphomycetes. CPOM and FPOM serve as major food sources for many aquatic macroinvertebrates. Downey and others (1994) recorded a relatively high density of aquatic macroinvertebrates from mountain streams in Virginia where an abrupt crash in gypsy moth populations due to disease prevented the occurrence of significant defoliation during the 1992-93 growing seasons. This finding illustrates the relationship between aquatic hyphomycetes and DFB -- it kills gypsy moth larvae, thereby minimizing defoliation, permitting CPOM and FPOM production, and contributing to increased macroinvertebrate activity and success.

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***Table 5-1.*** Occurrence of aquatic hyphomycetes as indicated by the presence of conidia filtered from water samples prior to and after the Dimilin 4L application to Watersheds 1 and 13, with Watersheds 4 and 7 serving as untreated. Data are numbers of conidia per 1000 ml of water.

| | Sampling Date (1992) | | | | |
|---|---|---|---|---|---|
| Watershed | May 11 | May 18 | May 27 | June 11 | July 12 |
| 1 | 797 | 1660 | 359 | 304 | 407 |
| 13 | 684 | 485 | 262 | 314 | 347 |
| 4 | 860 | 498 | 414 | 213 | 543 |
| 7 | 1834 | 584 | 231 | 110 | 343 |

***Table 5-2.*** Numbers of aquatic hyphomycete taxa recorded from filtered water samples prior to and after the Dimilin 4L application to Watersheds 1 and 13, with Watersheds 4 and 7 serving as untreated.

| | Sampling Date (1992) | | | | |
|---|---|---|---|---|---|
| Watershed | May 11 | May 18 | May 27 | June 11 | July 12 |
| 1 | 30 | 29 | 19 | 17 | 15 |
| 13 | 18 | 16 | 20 | 13 | 16 |
| 4 | 27 | 24 | 24 | 14 | 19 |
| 7 | 23 | 25 | 14 | 14 | 14 |

***Table 5-3.*** Community coefficient indices calculated from water filtration data.

| Watershed Comparisons | Coefficient of Community Index |
|---|---|
| 1 vs 13 | 0.672 |
| 1 vs 4 | 0.687 |
| 1 vs 7 | 0.631 |
| 4 vs 7 | 0.630 |
| 4 vs 13 | 0.654 |
| 7 vs 13 | 0.556 |

*Table 5-4.* Aquatic hyphomycete colonization of red oak and sugar maple leaf bags, 1992.

**Stream**

| Watershed | Leaf type | May 11[a] | May 18 | May 27 | June 11 | July 12 | Mean |
|---|---|---|---|---|---|---|---|
| | | | *Number of taxa* | | | | |
| 1 | Sugar Maple | | 6 | 8 | 15 | 3 | 8.0 |
| | Red Oak | 19 | 17 | 8 | 6 | 14 | 11.3 |
| 13 | Sugar Maple | | 23 | 12 | 6 | 8 | 12.3 |
| | Red Oak | 14 | 19 | 19 | 12 | 17 | 16.8 |
| 4 | Sugar Maple | | 15 | 10 | 14 | 16 | 13.8 |
| | Red Oak | 13 | 19 | 5 | 10 | 12 | 11.5 |
| 7 | Sugar Maple | | 16 | 13 | 15 | 9 | 13.3 |
| | Red Oak | 14 | 14 | 16 | 21 | 12 | 15.8 |

[a] *Present on natural substrates prior to Dimilin application.*

**Weir Pond**

| Watershed | Leaf type | May 11[a] | May 18 | May 27 | June 11 | July 12 | Mean |
|---|---|---|---|---|---|---|---|
| | | | *Number of taxa* | | | | |
| 1 | Sugar Maple | | 13 | 6 | 15 | 7 | 10.3 |
| | Red Oak | | 16 | 5 | 7 | 8 | 9.0 |
| 13 | Sugar Maple | | 27 | 6 | 11 | 15 | 14.8 |
| | Red Oak | | 21 | 9 | 4 | 18 | 13.0 |
| 4 | Sugar Maple | | 10 | 10 | 10 | 9 | 9.8 |
| | Red Oak | | 12 | 14 | 13 | 15 | 13.5 |
| 7 | Sugar Maple | | 16 | 13 | 11 | 12 | 13.0 |
| | Red Oak | | 15 | 5 | 28 | 9 | 14.3 |

***Table 5-5.*** Coefficient of community (CC) indices calculated from red oak (RO) and sugar maple (SM) leaf bag colonization data obtained on four dates following Dimilin 4L application, 1992.

| Watershed Comparisons | May 18 | | May 27 | | June 11 | | July 12 | |
|---|---|---|---|---|---|---|---|---|
| | RO | SM | RO | SM | RO | SM | RO | SM |
| 1-13 | 0.622 | 0.451 | 0.387 | 0.583 | 0.545 | 0.514 | 0.514 | 0.303 |
| 1-4 | 0.666 | 0.555 | 0.416 | 0.518 | 0.451 | 0.486 | 0.500 | 0.416 |
| 1-7 | 0.666 | 0.611 | 0.666 | 0.551 | 0.381 | 0.500 | 0.600 | 0.384 |
| 4-7 | 0.666 | 0.500 | 0.533 | 0.666 | 0.509 | 0.540 | 0.562 | 0.666 |
| 4-13 | 0.625 | 0.528 | 0.540 | 0.528 | 0.571 | 0.437 | 0.594 | 0.594 |
| 7-13 | 0.577 | 0.490 | 0.540 | 0.592 | 0.434 | 0.514 | 0.564 | 0.410 |
| Average CC Index | 0.637 | 0.522 | 0.513 | 0.573 | 0.481 | 0.498 | 0.555 | 0.462 |

# Part C. Terrestrial Organisms

# *Chapter 6. Terrestrial Salamanders*

**Thomas K. Pauley**

## Introduction

The redback salamander, *Plethodon cinereus* (Green), and the mountain dusky salamander (*Desmognathus ochrophaeus* Cope) are the dominant terrestrial salamander species on the four watersheds (Fig. 6-1a, b). The redback salamander is terrestrial throughout its life cycle; its eggs are deposited on land (usually under rocks), and the larval stage is in the egg. The mountain dusky salamander is terrestrial as an adult, but deposits its eggs in stream banks. The larvae remain in the streams and transform to adults in 1 to 2 weeks (Marcum 1994).

Terrestrial salamanders are important components in trophic cycling in northeastern forests. Salamanders are highly efficient (60 percent) at converting ingested energy into new tissue, and 20 percent of the energy available to birds and mammals passes through salamanders in forests (Burton and Likens 1975b). Salamanders may represent twice the standing biomass of birds and equal that of small mammals (Burton and Likens 1975a). Several studies have demonstrated the large biomass of salamanders including per ha estimates of 5,535 *P. cinereus* (Burger 1935), 2,100 *P. cinereus* (Klein 1960), and 2,324 *P. glutinosus* (Green) and 8,611 *P. jordani* Blatchley (Merchant 1972).

## Laboratory Study Methods

Twenty adult male and 20 adult female *P. cinereus* and *D. ochrophaeus* from each watershed were collected from litter in each watershed but outside the 100-m horizontal transects (used for estimating surface abundance) in September and October each year (1989-1994) and frozen. Stomach contents, percentages of tail fat and carcass fat, and weight of total fat as well as the number and volume of follicles were determined for each species using methods described in the section on aquatic salamanders.

## Field Study Methods and Costs

Terrestrial data were collected along two 100-m horizontal transects located 20 and 40 m upslope from each stream in each watershed from 1989 to 1994 (Fig. 6-2). Seven 25 $m^2$ quadrats were positioned on each transect. In each quadrat, soil temperature, soil moisture, air temperature, and relative humidity were recorded, and salamander surface abundance was determined once a month from May through September.

Surface abundance was determined by capturing specimens under five pine boards (25 cm by 15 cm by 2.5 cm) (Fig. 6-3) placed earlier and five natural objects in each quadrat (Taub 1961). Specimens were measured for snout-vent-length (SVL), their genders determined, and their toes clipped (Twitty 1966).

The only major expense involved in conducting terrestrial salamander studies is the purchase of pine boards. Boards are untreated pine shelving, which can be obtained from retail lumber stores for about $0.40 each.

### *Advantages And Disadvantages*

Horizontal transects with quadrats are excellent means of sampling large areas such as watersheds; however, vertical transects would provide more accurate data on niche breadth and overlap. Changes in niche breadth, niche overlap, or both among species that compete for similar resources such as food, space, and moist spots may be subtle and could be influenced by stress from anthropogenic changes.

More boards should have been used to sample the large quadrats used in this study. Five boards had to be placed too far apart, thus reducing the number of recaptures for population estimates.

Because *P. cinereus* has a small territory, approximately 0.16 $m^2$ (Mathis 1991), a greater number of boards (12 to 18) placed side by side would yield more salamanders and more recaptures for greater accuracy in population estimates. Pauley and others (1995) found that 12 boards in a rectangular design (three across and four down) increased the number of recaptures more than three times. Boards should be no more than 2 cm apart.

All statistical analyses were made using ANOVA with Newman-Keuls multiple comparisons ($P \leq 0.05$).

## Results

### *Laboratory Study*

The major food items of *P. cinereus* before treatment were hymenopterans (ants), coleopterans, dipterans, and mites (Table 6-1). *Desmognathus ochrophaeus* consumed mostly ants, winged hymenopterans, and coleopterans (Table 6-2). Both salamander species showed a shift to hymenopterans (ants) in the treated and untreated watersheds after Dimilin 4L application.

There was a significant difference in the percentage of tail fat for *P. cinereus* gravid females between the treated and untreated watersheds before or after application of Dimilin 4L (Table 6-3). However, the percentage of tail fat for *D. ochrophaeus* gravid females increased significantly in the treated and untreated watersheds after treatment.

While there was a significant increase in carcass fat of *P. cinereus* and *D. ochrophaeus* after the application of Dimilin 4L, the increase was observed in both treated and untreated watersheds (Table 6-3). There was a significant increase in total weight of fat for *D. ochrophaeus* in the treated and untreated watersheds after application of DFB. There was a

significant increase in total weight of fat for *P. cinereus* after treatment in untreated and treated watersheds.

Follicle numbers and volumes were not significantly different in *P. cinereus* and *D. ochrophaeus* in treated and untreated watersheds.

### *Field Study*

Surface abundance of terrestrial salamanders was based on specimens under boards and natural cover objects in each quadrat. Over 2,500 salamanders (Table 6-4) were observed in all watersheds including 824 *P. cinereus* (420 adult females, 307 adult males, and 97 juveniles) and 1,685 *D. ochrophaeus* (602 adult females, 815 adult males, and 268 juveniles). Salamanders were found under boards twice as often as natural cover objects (Table 6-5). There were fewer recaptures using the board design(only 9.4 percent for *P. cinereus* and 5.8 percent for *D. ochrophaeus*) than other designs described by Pauley and others (1995).

There were no significant changes in snout-vent-lengths (SVL) for *P. cinereus* and *D. ochrophaeus* between the treated and untreated watersheds after the application of DFB (36 and 37 mm, and 35 and 34 mm, respectively). There also was no significant difference in SVL of these species between pre- and posttreatment periods in the treated watersheds (36 and 36 mm, and 34 and 35 mm, respectively).

## *Discussion*

Salamanders are opportunistic feeders, and their diet generally indicates available prey items. Thus, food analyses most likely reflect common insects in the respective watersheds.

The size (SVL) of terrestrial salamander species studied in the treated watersheds was not affected by the application of DFB. Because of the longevity of the adult stage of plethodontid salamanders (most live 15 to 20 years), SVL of adults may not be an important parameter to determine effects of DFB. If, however, DFB has a detrimental effect on follicle production and hatching success, juveniles and subadult sizes may comprise a significantly smaller component of the population in the next few years. This has not been determined.

It was hypothesized that if salamander food items are altered by DFB, i.e., if hard bodied insects are more successful after the application of Dimilin 4L, fat reserves in salamanders will be reduced. In particular, any reduction will be manifested in the percentage of fat in the first 18mm of the tail (Fraser 1980). Because females rely on tail fat and carcass fat for follicle development, ovarian follicle size and volumes are related to the percentage of fat reserves. Consequently, reduction in tail and carcass fat would reduce the reproductive success of salamanders exposed to DFB.

Body fat (tail, carcass, and total fat) of all specimens, i.e., males, nongravid females and gravid females, did not show any significant differences between treated and untreated control watersheds that could be contributed to Dimilin 4L application.

The results of this study showed an increase in the consumption of hard-bodied prey. The passage of hard-bodied prey is much slower for thicker exoskeletons (ants and beetles) than thinner exoskeletons (Jaeger and Barnard 1981). The shift in food to hard-bodied prey by both terrestrial species in this study did not appear to affect the percentages of body and tail fat or follicle development.

Because of the great biomass of terrestrial salamanders and their value as bioindicators, understanding the effects of anthropogenic stress on salamanders is critical. The long life span of forest salamanders (15 or more years) and the time necessary to observe negative impacts from ecotoxicological factors, mandate that future studies must include at least 5 to 10 years of posttreatment data. Dunson and others (1992) recommended long-term (10-year minimum) studies to examine the relation between amphibian population cycles and anthropogenic changes.

## Recommendations for Future Studies

As with aquatic salamanders, terrestrial studies of ecotoxicological effects on salamanders must include 5 to 10 years of posttreatment data. Future terrestrial studies should also include a comparable study in a defoliated area. In addition to the potential negative effects of increased insolation on the forest floor from defoliation, terrestrial salamanders need leaf litter on the forest floor for protection from desiccation and predators, and to forage for food. In defoliated areas, the lack of leaf litter could have more adverse effects on terrestrial salamander populations than application of Dimilin.

Because DFB may be more detrimental to juveniles than adults, future research should include detailed studies of juveniles.

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*Table 6-1.* Distribution of prey items in stomachs of *Plethodon cinereus* from untreated control (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds (1989-1994 data pooled).

| | Percent of Stomachs | | | |
|---|---|---|---|---|
| | Pretreatment | | Posttreatment | |
| | WS 4 & 7 (n=159) | WS 1 & 13 (n=177) | WS 4 & 7 (n=370) | WS 1 & 13 (n=278) |
| Hymenoptera - Ants | 37.1 | 29.9 | 52.4 | 75.2 |
| Hymenoptera - Winged | 3.2 | 1.1 | 7.6 | 10.4 |
| Lepidoptera - Adult | 0 | 0 | 1.6 | 1.8 |
| Lepidoptera - Larva | 0 | 0 | 1.9 | 2.2 |
| Diptera - Adult | 9.4 | 5.1 | 2.7 | 5.4 |
| Diptera - Larva | 0 | 0 | 0.3 | 0.4 |
| Coleoptera - Adult | 16.4 | 17.0 | 11.4 | 13.0 |
| Coleoptera - Larva | 0.6 | 0 | 2.4 | 2.5 |
| Chilopoda | 1.3 | 0.6 | 1.4 | 0 |
| Diplopoda | 0 | 1.1 | 2.2 | 5.8 |
| Annelida | 0.6 | 0 | 0 | 0 |
| Mollusca | 1.9 | 1.1 | 4.3 | 5.8 |
| Collembola | 0 | 0.6 | 0.8 | 2.9 |
| Pseudoscorpion | 0.6 | 1.1 | 2.4 | 2.5 |
| Araneida | 1.3 | 1.7 | 2.4 | 3.6 |
| Plecoptera - Adult | 0 | 0 | 0.8 | 0.4 |
| Plecoptera - Larva | 0 | 0 | 0.5 | 0 |
| Trichoptera | 0 | 0.6 | 0 | 0 |
| Neuroptera | 0 | 0 | 0.3 | 0.4 |
| Ephemeroptera | 0 | 0 | 0.3 | 0 |
| Acarina | 10.1 | 10.7 | 25.1 | 29.5 |
| Homoptera | 0 | 0 | 1.1 | 2.2 |
| Hemiptera | 0 | 0 | 1.4 | 2.5 |
| Amphipoda | 0.6 | 1.1 | 0 | 0 |
| Unidentified | 11.3 | 5.7 | 8.4 | 6.1 |
| Debris | 14.5 | 10.2 | 4.3 | 3.2 |
| Empty | 13.2 | 0 | 6.2 | 5.4 |

***Table 6-2.*** Distribution of prey items in stomachs of *Desmognathus ochrophaeus* from untreated control (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds (1989-1994 data pooled).

| | Percent of Stomachs | | | |
|---|---|---|---|---|
| | Pretreatment | | Posttreatment | |
| | WS 4 & 7 (n=116) | WS 1 & 13 (n=126) | WS 4 & 7 (n=315) | WS 1 & 13 (n=342) |
| Hymenoptera - Ants | 6.0 | 4.0 | 16.8 | 17.8 |
| Hymenoptera - Winged | 17.2 | 15.9 | 10.2 | 14.6 |
| Lepidoptera - Adult | 0 | 0 | 1.6 | 1.5 |
| Lepidoptera - Larva | 0 | 0 | 5.1 | 4.7 |
| Diptera - Adult | 2.6 | 0.8 | 8.3 | 6.7 |
| Diptera - Larva | 1.7 | 2.4 | 1.9 | 0.3 |
| Coleoptera - Adult | 8.6 | 4.8 | 8.9 | 11.4 |
| Coleoptera - Larva | 0 | 0 | 2.2 | 1.2 |
| Chilopoda | 0 | 0 | 0.6 | 1.2 |
| Diplopoda | 0.9 | 0 | 2.2 | 0.9 |
| Annelida | 0 | 0 | 0.6 | 0.9 |
| Mollusca | 1.7 | 0.8 | 4.4 | 2.9 |
| Salamander | 0.9 | 0.8 | 0.3 | 0.3 |
| Collembola | 0.9 | 0 | 0.3 | 0.9 |
| Pseudoscorpion | 0 | 0 | 1.6 | 0.9 |
| Araneida | 2.6 | 0 | 3.5 | 5.0 |
| Plecoptera - Adult | 0 | 0 | 1.0 | 1.5 |
| Plecoptera - Larva | 0 | 0 | 1.0 | 0.3 |
| Trichoptera | 0 | 0.8 | 0.3 | 0.3 |
| Neuroptera | 0 | 0 | 0.6 | 0.6 |
| Ephemeroptera | 0 | 0.8 | 0 | 0 |
| Acarina | 5.2 | 0.8 | 8.3 | 8.7 |
| Homoptera | 0 | 0 | 1.0 | 0.9 |
| Hemiptera | 0 | 0 | 0.6 | 0.3 |
| Amphopoda | 0 | 0 | 0.3 | 0.3 |
| Nematodes | 0 | 0 | 1.0 | 0.3 |
| Mecoptera | 0 | 0 | 0.3 | 0 |
| Unidentified | 6.0 | 5.6 | 17.1 | 17.3 |
| Debris | 26.7 | 9.5 | 13.3 | 15.2 |
| Empty | 0 | 0 | 18.7 | 19.0 |

***Table 6-3.*** Mean percentages of carcass fat and tail fat, and mean weight of total fat of gravid *Desmognathus ochrophaeus* and *Plethodon cinereus* females from untreated (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds (1989-1994 data pooled).

| | Pretreatment | | Posttreatment | |
|---|---|---|---|---|
| | 1 & 13 | 4 & 7 | 1 & 13 | 4 & 7 |
| *Desmognathus ochrophaeus* | | | | |
| % Carcass Fat | 9.9a[a>] ± 3.5 (n = 50) | 9.5a ± 2.9 (n = 38) | 15.0b ± 6.3 (n = 76) | 16.7b ± 6.1 (n = 53) |
| % Tail Fat | 26.9a ± 9.4 (n = 41) | 29.4a ± 5.4 (n = 32) | 29.8b ± 19.3 (n = 76) | 28.4a ± 8.4 (n = 54) |
| Total Fat (g) | 0.02a ± .01 (n = 50) | 0.02a ± .01 (n = 38) | 0.03a ± .011 (n = 76) | 0.03c ± .013 (n = 54) |
| *Plethodon cinereus* | | | | |
| % Carcass Fat | 4.5a ± 2.5 (n = 69) | 3.7a ± 1.9 (n = 76) | 13.4b ± 16.2 (n = 34) | 16.6c ± 6.6 (n = 49) |
| % Tail Fat | 15.3a ± 6.1 (n = 75) | 12.8a ± 6.8 (n = 72) | 21.6b ± 20.8 (n = 36) | 21.1b ± 17.5 (n = 52) |
| Total Fat (g) | 0.01a ± .01 (n = 84) | 0.01a ± .01 (n = 77) | 0.03b ± .17 (n = 36) | 0.021b ± .18 (n = 51) |

[a>] Mean and standard deviation, unlike letters indicate a significant difference ($P \leq 0.05$).

***Table 6-4.*** Numbers of *Desmognathus ochrophaeus* and *Plethodon cinereus* observed in the treated (WS 1 and WS 13) and untreated (WS 4 and WS 7) watersheds (1989-1994 pooled data).

| | Watershed | Numbers |
|---|---|---|
| *Desmognathus ochrophaeus* | | |
| | 1 | 531 |
| | 4 | 421 |
| | 7 | 173 |
| | 13 | 560 |
| | **Total** | 1685 |
| *Plethodon cinereus* | | |
| | 1 | 143 |
| | 4 | 242 |
| | 7 | 260 |
| | 13 | 179 |
| | **Total** | 824 |

***Table 6-5.*** Numbers and percentages of individuals observed under experimental boards versus natural cover objects for two common terrestrial species of salamanders in the Fernow Experimental Forest, 1989-1994.

| | Boards | Natural cover | Total |
|---|---|---|---|
| *Plethodon cinereus* | 613<br>(71.7 percent) | 242<br>(28.3 percent) | 855 |
| *Desmognathus ochrophaeus* | 1226<br>(68.6 percent) | 561<br>(31.4 percent) | 1787 |
| **Total** | 1839<br>(69.6 percent) | 803<br>(30.4 percent) | 2642 |

**6a.**

**6b.**

***Figures 6-1a,b.*** The redback salamander, *Plethodon cinereus*, and mountain dusky salamander, *Desmognathus ochrophaeus*, dominant terrestrial salamander species on the Fernow Experimental Forest.

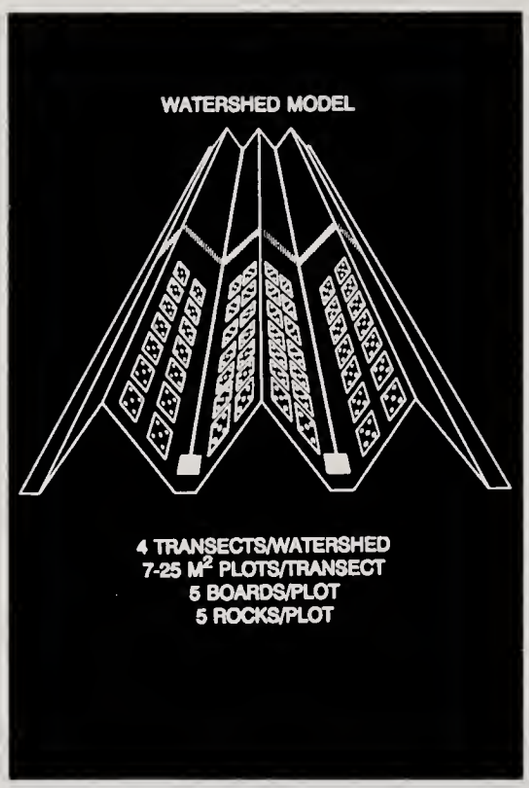


***Figure 6-2.*** Transects of pine boards used to estimate surface abundance of salamanders.

***Figure 6-3.*** Pine boards (25 cm by 25 cm by 2.5 cm) used as resting locations by terrestrial salamanders.

# *Chapter 7. Canopy Arthropods*

**Linda Butler**

## Introduction

Insects and their arthropod relatives comprise a large percentage of the animal biomass in forest communities. Insects act as decomposers, herbivores, predators, parasitoids and as prey for other arthropods and many vertebrates. They are a critical component of the diet of many animals.

In recent years considerable interest has been shown regarding arthropod communities of forest canopies. Trees are ideal habitats for comparative community studies. The arboreal habitat is easily defined and clearly delimited. Individuals in the canopy are more or less trophically interlinked because of their dependence on a tree. Trees are structurally complex, providing great niche diversification. They are also stable resources. These attributes taken together account for the extraordinary species richness of arboreal communities (Moran and Southwood 1982).

While numerous studies have been conducted on diflubenzuron (DFB) impact on nontarget aquatic arthropods, little information is available on impact in the forest canopy. Martinat and others (1988) evaluated the impact of DFB on arthropods on foliage of chestnut oak (*Quercus prinus* L.), red oak (*Q. rubra* L.), and red maple (*Acer rubrum* L.). DFB reduced abundance and species richness in mandibulate herbivores, especially macrolepidopterous larvae.

In a study conducted in North Carolina, arthropod number and richness in an untreated forest canopy were significantly greater than in a Dimilin treated canopy (Blanton 1989). Among feeding guilds showing reduction were chewing herbivores and predatory arthropods including Phalangida (harvestmen) and Vespidae (yellowjackets and hornets).

The effects of an operational application of Dimilin on nontarget insects were evaluated in eastern West Virginia. Insects were collected by light traps at seven pairs of Dimilin-treated and untreated control sites. Lepidoptera experienced the greatest impact, showing reduced abundance and species richness at treated sites (Sample and others 1993).

Due to the great diversity of life history traits and habitats exploited by insects, no single method is efficient for capturing all insects (Julliet 1963). Every collecting method has some associated biases and provides reliable population estimates for only a limited number of taxa (Cooper and Whitmore 1990, Kunz 1988). Southwood (1978) gives an excellent presentation of collecting methods for insects and other arthropods.

Several methods that can be used to evaluate impact of DFB on nontarget canopy arthropods are described below.

***Light traps.*** Many studies have been conducted on the use of light traps in evaluating the macrolepidoptera community at specific locations (Butler and Kondo 1991). Placement of light traps greatly influences moth catch (Hausmann 1990, Waring 1989, Williams and others 1955). Because light traps interfere with an insect's sensory orientation and movement, population estimates based solely on light traps may be biased (Kunz 1988). Other factors that may affect catches and should be considered when using light traps include ambient weather conditions (Morton and others 1981), intensity and wavelength of light source (Kunz 1988), and amount and intensity of moonlight (Bowden 1982).

Recommendations (Butler and Kondo 1991) for more effective use of light traps in evaluating DFB effects on nontarget organisms are: (1) use all traps of the same type, power output, light-intensity, and operate for the same amount of time; (2) keep records of weather conditions at light trap sites during each night of trapping; (3) simultaneously operate pairs of traps at treated and untreated control sites; and 4) run as many traps at as many plots as is possible; replication will help to reduce the effects of the variability of catches.

***Foliage sampling.*** Direct sampling of arthropods in the forest canopy has been conducted in a variety of ways. Ticehurst and Yendol (1989) studied gypsy moth larvae in the canopy by the felling of co-dominant oaks. Southwood and others (1982) compared canopy arthropods among six tree species in Great Britain and South Africa by pyrethrum knock-down on 15 1-$m^2$ sheets placed under each tree.

Pruning foliage may be accomplished by using insect nets with closeable plastic bags (Showalter and others 1981), pole pruners equipped with metal hoops and drawstring bags (Ohmart and others 1983), and pole pruners with collecting baskets (Paul 1979). Martinat and others (1988) cut branches on trees and allowed them to fall onto white sheets on the ground. In sampling from Garry oak (*Quercus garryana* Dougl.) in Oregon, Miller (1990) shook oak foliage over a sheet.

Blanton (1990) compared a collapsible bag sampler and a knockdown insecticide fog for assessing canopy arthropod community structure in deciduous forests in North Carolina. She concluded that the collapsible bag was more reliable and provided a better estimate of total resident arthropod biomass on vegetation. Fogging yielded more arthropods per person-day and greater species richness, particularly for winged parasitoids. Fogging sampled a greater portion of canopy but was more susceptible to mechanical failure and inclement weather.

***Malaise traps.*** Malaise traps are a passive collecting system that collects a wide variety of flying insects (Steyskal 1981, Townes 1962). Malaise trap catches depend on many variables, such as the type of trap (e.g., Townes versus Cornell type), trap color, mesh size, and any modification of the trap design (Darling and Packer 1988, Matthews and Matthews 1970). Daily weather, particularly temperature and precipitation, exercise a strong influence upon catch size (Matthews and Matthews 1983). Height of trap and placement of the trap with respect to sunlight and surrounding vegetation and other structural features are also important.

Cooksey and Barton (1981) compared Malaise trap catches between a woodland and an old field community and found that the woodland produced about 65 percent of the total catch. The orders Diptera (57 percent), Lepidoptera (17 percent), Hymenoptera (15 percent), and Homoptera (8 percent) made up 97 percent of the total catch.

***Tree bands.*** Burlap or canvas bands on trees have long been used to sample for gypsy moth larvae and their parasitoids (Tigner 1974, Weseloh 1974). Bands also collect numerous other arthropod taxa. Butler and Kondo (1993) recorded 41 species of native macrolepidopterous larvae under canvas tree bands in West Virginia.

## *Laboratory Study Methods*

In the laboratory, light-trap samples were sorted and all macrolepidoptera were identified to species.

Foliage samples were examined leaf by leaf and all arthropods removed, stored in ethanol, and identified to family with the exception of macrolepidopterous larvae, which were identified to species. As foliage samples were examined, all leaves were removed, placed in paper bags, dried in an oven, and weighed. The number of arthropods was quantified as number per taxon expressed per 50 g dry leaf weight. Some larval Lepidoptera that were not immediately identified to species were reared on appropriate foliage for positive identification of adults. The rearing methods successfully employed by Butler (1992) are summarized below. Larvae are placed in 150- by-25 mm plastic Petri dishes, and fresh foliage was supplied over 1 to 2 days (Fig. 7-1). Small plastic freezer cartons also provide suitable rearing containers. At prepupation, insects were placed in a 5-cm deep layer of moist vermiculite in 1 qt canning jars and held at 24º C for pupation and eclosion. For those species requiring a cold period, jars with pupae were held at 4º C in a walk-in cooler for a minimum of 90 days. Upon emergence, all adults were identified to species.

In the laboratory all macrolepidopterous larvae taken from burlap bands were identified to species. All other arthropods were preserved and stored in ethanol, and identified to family. Arthropod abundance was expressed as number per tree diameter at breast height (d.b.h.).

Mean richness and abundance of arthropods and macrolepidopteran larvae were compared between treated and untreated control watersheds by analysis of variance.

## *Field Study Methods and Costs*

Each year of the study (1989-1994), samples were collected from early May through mid-August by blacklight traps, foliage pruning, and burlap banding.

One 8-watt battery-operated blacklight trap (Model 2851K, BioQuip Products, Gardena, CA 90248) was operated in each watershed on the same night, one night each week (Fig. 7-2). Traps were placed near the center of each watershed at a height of 3 to 4 m above the ground.

Foliage samples were collected once each week during the field season. On each collecting day, one sample of 25 branch tips each was taken from black birch (*Betula lenta* L.), black cherry (*Prunus serotina* Ehrh.), mixed maple, and mixed oak on two sites of each watershed, giving a total of eight samples (bags of foliage) per watershed or 32 for the study each week. Samples were taken by pole pruners from low to mid canopy (3-5 m) (Fig. 7-3). Branch tips of one sample dropped into a large plastic catch bag attached to a metal ring below the pruner knife. All bags were appropriately labeled and were held overnight in a cooler (4º C) until the samples could be processed the next day.

Burlap bands (about 46 cm wide) were stapled at the middle of each band around the circumference of each tree at breast height (Fig. 7-4). The upper half of the band was folded

down. On each watershed, equal numbers of black birch, black cherry, mixed maples, and mixed oaks were banded. A total of 40 trees were banded per watershed, or 160 for the study. Bands were installed in early May and were examined once each week during the season and all arthropods were removed and carried to the laboratory.

Small commercial 10-watt blacklight traps are available with photoelectric eye and adapted for DC use for about $170. A sealed gel-cell battery recommended for use with this trap costs about $127 each. Pole pruners comparable to those used by Butler (1992) can be purchased and modified with brackets and catch-bag rings for about $180. Plastic catch bags are about $0.25 each. A 100-m roll of plain burlap, 1-m wide, costs about $50. At least 200 trees can be banded per roll.

### *Advantages and Disadvantages*

Light traps are particularly useful for collecting nocturnal Lepidoptera, although species of many other orders are taken as well. Blacklight traps are active attractants of mobile insects. It is possible to attract and catch insects (particularly some moths) which originated outside small treated plots. Light trap catches are also highly variable because of changing weather conditions night to night, and season to season. Placement of light traps is critical. Care should be taken to match trap sites as closely as possible with respect to vegetation type, understory vegetation, and natural barriers and openings in the forest.

Because DFB affects immature insects during molting, it is not likely to affect adult Lepidoptera that are collected in light traps. Since most moths are univoltine (one generation per year), larvae exposed to Dimilin spray during one season cannot be monitored as adults until the following season. Therefore, if enough light traps are used to detect a treatment effect, that effect may be seen only in the year after treatment. Counts of total insects or Lepidoptera in light traps will be of little use in DFB impact studies. Effort must be made to identify taxa of interest to at least family, if not species.

Foliage samples may be taken from any of the common trees within a Dimilin treatment area, but care should be taken to compare arthropod abundance and richness from treated foliage with equal quantities (dry weight, for example) of foliage of the same species from similar untreated control sites. Different arthropod species are found on different species of foliage.

Foliage sampling can show treatment differences within a few days of Dimilin application to the forest canopy. Rapid effects can be seen on abundance and richness, particularly of macrolepidopterous larvae. Since DFB may persist on foliage throughout the treatment year, impact may continue to be seen in susceptible species until leaf fall. Foliage samples should be taken well within the borders of a treated or untreated control block to avoid problems caused by poor spray coverage or drift.

Tree bands take advantage of the tendency of some arthropods to move vertically on tree trunks. Some species typically shelter under bark flaps or move daily from the canopy to the ground. Larvae of some species of Lepidoptera (for example, lymantriids or some noctuid larvae such as *Catocala* spp.) are sampled more commonly from bands than from foliage. Other arthropods commonly found under bands include some beetle, cricket, millipede, phalangid, and spider species.

Canvas or burlap bands are generally attached around the circumference of the tree at breast height by means of staples or string. While oak trees have been banded most commonly in gypsy moth studies, other hardwood trees, such as maples, black cherry, and birches, yield equally high or higher richness and abundance of nontarget caterpillars (Butler and Kondo 1993).

## Results

Light traps were used only during 1990 to 1994. Innate differences in the watersheds with respect to size, understory vegetation, and canopy makeup probably resulted in marked differences in macrolepidoptera catch, particularly in abundance. Considerably greater abundance of moths was trapped on WS 4 and WS 7 (untreated watersheds) than WS 1 and WS 13 for all years, regardless of Dimilin 4L application. Total macrolepidoptera species richness for the study was 376 species, and total abundance was 58,463 (Butler and others 1995a).

During the 6-year study (1989-1994), a total of 47,788 arthropods representing 188 families was identified under burlap bands (Butler and others 1995c). While overall abundance of arthropods was not reduced on treated watersheds in 1992, there was a significant ($P \leq 0.05$) reduction in arthropod richness in the treatment year. Some recovery in richness occurred in 1993. Treatment effects were seen in the Carabidae (ground beetles), Gryllacrididae (camel crickets), and Noctuidae and Geometridae (caterpillars), among the more abundant of band arthropods. No treatment effects were noted for the Phalangida, Formicidae (ants), Agelenidae (funnel-web spiders), and Diplopoda (millipedes).

The most dramatic decline of arthropods under bands on treated watersheds was seen in the macrolepidoptera during the treatment year. Only one moth species appeared unaffected under bands on treated sites, that being the Noctuidae *Abagrotis alternata* (Grt.), a species that overwinters as the late instar larva and therefore had completed most of its development before Dimilin 4L was applied. Overall macrolepidoptera richness and abundance of caterpillars under bands significantly declined ($P \leq 0.05$) on treated watersheds in the treatment year.

During the entire study, total foliage arthropod richness (determined using pole pruners) was 225 families, and arthropod abundance was 115,551 (Butler and others 1995c). Significant ($P \leq 0.05$) decline occurred in taxa richness on treated watersheds in 1993, the year after treatment, and in arthropod abundance during 1992 and 1994. Among the 16 most abundant families on foliage, 12 declined on treated plots: the haustellate late sap-feeders Aphididae, Miridae, Membracidae, and Eriosomatidae; the thrips Phlaeothripidae, the Psocoptera, Psocidae and Polypsocidae; the Coleoptera Curculionidae and Chrysomelidae; the spiders Araneidae and Dictynidae; and the microlepidopteran Gelechiidae (Table 7-1). Included here are juice feeders, leaf feeders, lichen feeders, and predators. The families that did not decline were Cicadellidae (leafhoppers), Theridiidae (comb-footed spiders), Sciaridae (fungus gnats), and Formicidae (ants).

Among macrolepidopterous larvae collected from foliage, total richness for the study was 111 species and total abundance was 7,277 (Butler and others 1995b). Caterpillar richness declined on treated plots in the treatment and posttreatment years. Caterpillar abundance declined in 1992, the treatment year, and remained significantly reduced ($P \leq 0.05$) through the end of the study in the

fall of 1994. Macrolepidoptera abundance was 71 percent lower on the treated watersheds in the treatment year.

In the Geometridae, the most abundant family of nontarget macrolepidoptera, there were 65 percent fewer larvae on the treated plots in 1992, 57 percent fewer in 1993, and 43 percent fewer in 1994. The most abundant macrolepidopterous larvae collected during the study were the geometrids *Lomographa glomerata* (Grt.), *Erannis tiliaria* (Harr.), *L. vestaliata* (Gn.), *Melanolophia canadaria* (Gn.), *Alsophila pometaria* (Harr.), and *Itame pustularia* (Gn.), and the noctuids *Orthosia hibisci* (Gn.) and *Polia latex* (Gn.).

In summary, Dimilin 4L reduced richness and abundance of nontarget arthropods (Table 7-1). The most affected group was the macrolepidopterous larvae. Recovery had not occurred when the study was terminated 27 months after the Dimilin 4L application.

## Discussion

The sampling methods of burlap banding and foliage pruning provided adequate data with which to monitor effects of Dimilin 4L application. Foliage sampling was the superior method in that it targeted more of the sensitive taxa. While arthropod overall richness and abundance declined with the treatment, certain taxa were more affected than others and the treatment effect remained evident longer for some taxa.

Macrolepidoptera, particularly larvae of the two most abundant families Geometridae and Noctuidae, declined on the treated watersheds in the treatment year and did not recover by the end of the study. Other chewing herbivores such as sawflies (Hymenoptera Symphyta) remained suppressed into the second posttreatment season and Coleoptera (including weevils and leaf beetles) remained suppressed through the third posttreatment season. A treatment effect was shown for certain juice-feeding herbivores (Hemiptera) for all 3 years. The predatory thrips (Thysanoptera) and bark lice (Psocoptera) declined in the treatment year, but showed considerable recovery by 1993. Some families of spiders (Araneae) declined for 2 years, whereas others showed little treatment effect. Treatment effects on the most abundant family of Orthoptera, Gryllacrididae, were noted during the treatment year, but were more evident in the post-treatment year. These hump-backed crickets associate with leaf litter and apparently showed a delayed Dimilin effect, which increased after fall of treated leaves in October 1992.

The Dimilin impact we see in this study may reflect both indirect and direct effects. Direct effects are particularly noted for taxa that are herbivores during their immature stages on the foliage. Direct effects include disruption of molting after ingestion of Dimilin and egg inviability of some exposed arthropods. Indirect effects of Dimilin may explain some of the impact noted for predatory or parasitic families that suffer from a reduction of host arthropods.

## Recommendations for Future Studies

In planning DFB impact studies for canopy arthropods, several points should be considered. Because biases are innate in each collecting method, several methods should be used

contemporaneously if possible. Be aware of the types of arthropods favored (or excluded) by each collecting method and choose a method most likely to show impacts if they exist.

For example, blacklight traps select for nocturnally flying insects, particularly moths. It is simple, although expensive, to purchase and deploy light traps. It takes no expertise and minimal time to run several traps and store samples in a freezer. Nothing will be gained, however, unless these samples are processed with moths (particularly) being identified to family, or preferably, species. Identification is time consuming and requires considerable experience. If light traps are used, operate matched pairs simultaneously on similar treated and untreated control sites. Use as many traps as are reasonable. Because of natural variability among sites, nothing useful will be gained by operating a single trap each on a treated and untreated control site. Also remember that impacts shown by a well designed light-trap study likely will not appear until the year after treatment.

Pole pruning of foliage is an effective method for showing DFB impact. While various foliage arthropods may be affected, lepidopterous larvae are probably most sensitive and can be readily observed in foliage samples. While it is desirable to identify caterpillars to species to define effects better, few people have the required expertise. Alternatively, total caterpillar counts (emphasizing macrolepidopterous larvae, not leaf rollers) from similar quantities of foliage from treated and untreated control sites can be useful. Impact might be shown through foliage pruning in the treatment year and if significant decline has occurred, reduced abundance of caterpillars may be noted in the sites for several years after treatment.

Burlap (or canvas) bands are time consuming to install, but many dozens can be examined for arthropods in a short period. While bands are particularly successful for sampling crickets, spiders, phalangids, millipedes, and some beetles, sufficient numbers of caterpillars are often present to show a possible treatment effect.

For future DFB impact studies, several needs are evident. Large study plots of several hundred acres are essential. Small plots can be rapidly recolonized by flying adults and ballooning larvae so that impacts are obscured the year after treatment. Long-term studies of several years are needed to allow for sampling and establishing baseline data during pretreatment years, a treatment year, and sampling for more than one posttreatment year. Further, large randomized blocks that are treated with Dimilin, *Bacillus thuringiensis* (*B.t.*), and Gypchek and blocks left untreated to allow for gypsy moth defoliation, should be compared. The study should be of sufficient length to allow spray application to designated plots on a schedule that is similar to common use patterns -- every 1 to 2 years for *B.t.* and every 4 years for Dimilin.

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*Table 7-1.* Summary of Dimilin impact on canopy arthropods as sampled from burlap bands and foliage, 1989-1994. A = no apparent difference in mean abundance or richness between treated and untreated watersheds. B = reduced on treated watershed, but not significant at P = 0.05; C = significant reduction (P = 0.05) on treated watersheds.

| | Year | | | | | |
|---|---|---|---|---|---|---|
| | 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
| **Bands** | | | | | | |
| Family Richness | A | A | A | C | A | A |
| Total Abundance | A | A | A | A | A | A |
| Macrolepidoptera Richness | A | A | A | C | B | A |
| Macrolepidoptera Abundance | A | A | A | C | A | A |
| Gryllarididae | A | A | A | A | B | A |
| Carabidae | A | A | A | A | B | B |
| Agelenidae | A | A | A | A | A | A |
| **Foliage** | | | | | | |
| Family Richness | A | A | A | B | C | A |
| Total Abundance | A | A | A | C | B | C |
| Macrolepidoptera Richness | A | A | A | B | B | A |
| Macrolepidoptera Abundance | A | A | A | B | C | C |
| Phlaeothripidae | A | A | A | B | A | A |
| Psocoptera | A | A | A | B | A | A |
| Coleoptera | A | A | A | C | B | B |
| Haustellate Sap-feeders | A | A | A | B | A | A |

*Figure 7-1.* Rearing containers for field collected larvae, Fernow Experimental Forest.

***Figure 7-2.*** Blacklight trap used to collect nocturnal insects, especially moths, Fernow Experimental Forest.

***Figure 7-3.*** Pole pruner equipped with a metal ring and plastic catch bag used to collect foliage samples from low-to-mid canopy, Fernow Experimental Forest.

***Figure 7-4.*** Burlap band stapled around sample tree provides a resting niche to collect larvae, Fernow Experimental Forest.

# *Chapter 8. Pollinating Insects -- Native Species*

**Edward M. Barrows**

## Introduction

Hundreds of species of insect pollinators inhabit eastern broadleaf forests, including bees, bombyliid flies, syrphid flies, and some beetle, butterfly, moth, other fly, and sawfly species. The first three groups are probably the more important in forest pollination, but no comprehensive, quantitative studies have determined the overall importance of forest pollinators, nor the exact roles of most known and presumed pollinating species. In fact, the pollination biologies of most of these insect species are unknown.

Pollinators are significant in forest ecosystems beyond their pollination activities. Many bee, beetle, and wasp species aerate soil by their burrowing. Yellowjackets and other large wasps and many flower-fly species consume arthropod herbivores. Bee flies feed on grasshopper eggs and immature Coleoptera, Diptera, Hymenoptera and Neuroptera (Borror and others 1989). All types of pollinators are food for other invertebrate and vertebrate wildlife.

Studies of diflubenzuron (DFB) sprayed on a forest to control the Douglas-fir tussock moth, *Orgyia pseudosugata* (McD.), in the Northwest suggested short-term depressions in populations of native bees (major pollinators) during the year of application and a large decrease in yellowjackets (probably minor pollinators) during the year of application and following years (Robinson and Johansen 1978, Roush and Akre 1978). Diflubenzuron sprayed on a forest to control the sawfly *Pristiphora abietina* (Christ) also killed honey bee broods in hives in Germany (Egger 1977).

Samples of adult pollinators were collected in 1991 (the year before application), 1992 (the year of application), and 1993 (the year after application) in the Fernow Experimental Forest. Twenty Townes-style Malaise traps were used to collect insects for quantitative studies (Fig. 8-1). The traps were emptied every 10 days from late April through late September of each year, starting on Julian day 120 and ending on Julian day 270. In surveying pollinator species in the forest, the 20 Townes-style Malaise traps were also operated from early through late April and from late September through late October 2 of the 3 years. Also, one Cornell-style Malaise trap (Fig. 8-2) was positioned in an opening near forest edges in each watershed in 1991 and 1992. Cornell-style Malaise traps were used to collect insects for qualitative studies.

The study was designed to yield a statistical sample in the most efficient way and at the lowest cost. This method was based on consultation with William E. Potts (Statistical Consulting Services, USDA, Beltsville, MD) and the experienced Malaise-trap user David R. Smith (USDA, Washington, DC), and past experience with such traps, pollinators, and experimental design (Barrows 1986, Smith and Barrows 1987).

## Field Study Methods

Each Townes-style Malaise trap (Townes 1972) used in this study was 1.2 m wide, 1.7 m long, 2 m tall at its front, and 1 m tall at its back and made of nylon gauze with 1-mm mesh and metal and wooden supports (Golden Owl Publishers, 182 Chestnut Rd., Lexington Park, MD, 20653). The top of each trap was white, and the lower front, back, sides, and central baffle were black. The collecting head was made of a translucent white plastic, 0.48-l (1-pt) jar, and a glass 0.95-l (1-qt)

jar that contained ethanol (initially at 95 percent). To conserve alcohol, about 0.6 l alcohol per jar was used in April and September, and 0.8 l alcohol per jar in May through August. To make the traps last as long as possible, tops were sprayed with glossy enamel paint and other gauze parts with glossy black enamel once per summer. This paint reduced damage to the gauze by UV light and other factors. No insect bait was used intentionally in traps; however, the ethanol may have attracted some taxa.

To keep the traps erected on steep mountain slopes and during periods of high wind, the supports were securely tethered to logs, rocks, woody plants, and pegs driven into the ground. Each year, the Townes-style traps were initially set up in April. This yielded early-season qualitative information on pollinators, enough time to perfect trap tethering before quantitative sampling in May, and time for large mammals (e.g., white-tailed deer and black bears) to become accustomed to traps and walk around them.

Each Cornell-style Malaise trap was 1.2 m wide by 1.2 m long by 2.3 m tall, made of nylon gauze with 1-mm mesh sprayed black, and with a central metal support and a plastic, cylindrical capture head placed on its peak (BioQuip Products, Gardena, CA 90248-3602). Potassium cyanide was used to kill insects in the trap head and continuously used Cornell- and Townes-style Malaise traps 24 h per day for 10 days between collection times.

Like other traps, Malaise traps have their advantages and disadvantages. They can collect large samples of many species in all pollinator groups, during all hours of the day and night. They are unlikely to obtain taxa in the exact proportions that they occur in nature (e.g., yellowjackets, Barrows 1986). These traps do capture bumble bees and large carpenter bees; however, some individuals of these taxa and others turn around and crawl out of these traps. Some insects (e.g., *Andrena* sp.) fly into trap baffles and then fly away from them without being trapped. Nonetheless, pollinator taxa can be trapped in large numbers, and this enables investigation of their relative numbers between study sites and sampling periods. Because trap design affects the numbers and kinds of pollinators captured (Darling and Packer 1988, Matthews and Matthews 1983), using all the same style of trap in a study avoids trap characteristics as confounding variables.

Pollinators were sampled in four watersheds in the Fernow Experimental Forest. In each watershed, we used a transect of five Townes-style Malaise traps that ran approximately perpendicular to the stream draining the watershed (Fig. 8-3). At transect sites, each stream runs easterly. In a watershed, one trap was placed from 0 to 6 m from a stream's edge depending on terrain, two traps on its northerly facing bank, and two traps on its southerly facing bank. The traps were placed randomly from 10 to 35 m apart and on the forest floor, not in openings or on roads, and at least 20 m within deep forest. In each watershed, the highest trap was on or below a ridge. The front of each trap (which has a collecting-head) faced downstream, and its longitudinal axis was approximately parallel to the stream. Each trap was placed in the same spot and orientation in each of the 3 years, except for one trap in 1993 which was placed about 3 m from its original position due to a large fallen tree. Each trap location was marked during the non-sampling period by its wooden supports, which we left standing.

To extract specimens from a collection, they were strained using a tea strainer; placed on a 25-cm diameter white plate; covered with a 2-cm deep layer of fresh ethanol to facilitate specimen recognition; and moved across the plate two to three times while removing target individuals. When needed, magnification of 5 to 10 power was used to examine smaller specimens.

Extracted specimens were sorted into labeled Petri dishes, vials, jars, or Whirl-paks (Forestry Suppliers, Inc., Jackson, MS) and air dried in dishes for 1 to 2 days before closing the dishes.

Samples from Cornell-style Malaise traps were emptied into labeled plastic bags in the field. They were kept cold, or frozen, until they could be placed in labeled Petri dishes where they were air dried for 1 to 2 days, before the dishes were closed. Dishes of dried specimens were stored in boxes in the laboratory.

To determine whether DFB decreases pollinator population sizes, it at first may seem logical to test experimental hypotheses that directly compare treated and untreated watersheds within years, or treated watersheds between years.

Testing these hypotheses is inappropriate for three reasons: (1) treated watersheds may have more pollinators than untreated watersheds in any of the study years regardless of Dimilin 4L application, because the former are better pollinator habitats; (2) treated watersheds may have fewer pollinators of certain taxa in cooler, rainier years than in warmer, drier years; and (3) pollinator populations in any habitat may naturally fluctuate between years (Owen 1991). Therefore, a method is needed that can find a DFB effect (if one exists) regardless of the differences in pollinator-habitat quality between treated and untreated watersheds and regardless of weather differences between years.

A split-plot analysis was used to look for possible DFB and trap-site effects on the number of captured yellowjacket workers (SAS Institute, Inc. 1992). The whole-plot factors are treated and untreated watersheds and the subplot factors are trap sites. The grand mean of the trap means of workers from 15 sampling periods per watershed per flight season was calculated. These grand means and the remainders between the 1991 and 1992 grand means and the 1991 and 1993 grand means were analyzed. To test for a possible DFB effect, we tested the $H_o$: the treated watershed grand mean of worker numbers equals the untreated watershed grand mean of worker numbers. To test for a possible trap-location effect, we tested the $H_o$: the mean number of pollinators in trap-1 equals the mean number in trap-2 equals the mean number in trap-3 equals the mean number in trap-4 equals the mean number in trap-5.

## *Results*

The 20 Townes-style Malaise traps captured 6,661 yellowjacket males, queens, and workers in all four study watersheds, 1991-1993 (Fig. 8-4). These wasps represented 10 species with workers distributed among nine species (Table 8-1). A total of 2933 workers was captured in the treated (WS1 and WS13) and 1857 workers in the untreated (WS4 and WS7) watersheds from 1991 to 1993 (Table 8-2). These wasps flew throughout the monitoring periods showing yearly fluctuations of queen and worker numbers similar to those seen in other yellowjacket species (Fig. 8-5). In 1991 and 1993, workers showed two main abundant peaks. The early (July) peak is workers of species with short life-cycles, and the late peak (August or September) was caused by workers of species with long life-cycles.

A plot of number of workers per trap site for all 3 years combined suggests that trap-site-2 was the least desirable for trapping these insects (Fig. 8-6). A split-plot analysis, however revealed no trap-site effect on worker sample sizes for any of the 3 study years ( $P \geq 0.05$).

The split-plot analysis found that DFB lowered worker numbers in the year of DFB application (1992), but not in the year after application (1993) ($P = 0.009$ and 0.627, respectively) (Table 8-3). This decrease means that there was a greater decrease in mean worker numbers in the treated watersheds from 1991 to 1992 than in the untreated watersheds. This decrease may have resulted from a DFB-caused fertility reduction in queens, a DFB-caused reduction in yellowjacket prey numbers (Butler 1993), weather, or a combination of these factors.

Preliminary analyses indicated that DFB also affected other pollinators. It decreased the number of a pompilid-wasp species in 1993 (Tedesco and Barrows, in prep.), but it did not decrease numbers of bumble bee workers (Barrows and Batra, in prep.) and milesiine syrphids in either 1992 or 1993 (Cicero and others, in prep).

## Discussion

### *Methodological changes*

Future pollinator studies could be improved by: (1) installing a barbed wire fence around traps that are prone to damage by mammals, (2) getting local help to maintain and empty traps to reduce commuting time and costs, (3) developing and using cyanide-head traps that produce samples of size and quality comparable to alcohol-head traps, and (4) modifying traps so they are easier to erect and maintain in the field as described below.

### *Trap location*

No trap-location effect on the number of yellowjacket workers caught when all nine species were analyzed simultaneously was found (Barrows and others 1994). Bumble bees, a geometrid moth (not a known pollinator) and a pomplilid wasp showed trap-site effects (Barrows and Batra, in prep.; Barrows and Ieng, in prep.; Tedesco and Barrows, in prep.) In studies of major insect orders, small differences in trap positions can significantly change their collection sizes (Matthews and Matthews 1983). These findings indicate that a mean of the collection sizes from a transect of simultaneously run traps, rather than the collection size from a single trap, is a more accurate reflection of focal insect populations in a particular location.

### *Trap modifications*

To collect pollinators with Malaise traps it is desirable to be as efficient, economical, and as safe as possible yet obtain adequate collection sizes. Knowledge about how Malaise-trap characteristics affect collection compositions and sizes is limited because only a few quantitative investigations have been made on this subject (Matthews and Matthews 1983, Darling and Packer 1988). Trap characteristics include size, style (Cornell or Townes style), color (including the netting, frame, and head), gauze coarseness, the compass direction of Malaise traps (longitudinal axis of Townes-style trap or corner of a Cornell-style trap), and head design.

Traps with ethanol heads have the advantage of obtaining large collection sizes of museum-quality specimens of many taxa, because dead specimens suffer little or no damage from arthropods crawling among them; salamanders entering heads and crawling through specimens;

mice entering heads, walking over specimens, and eating them, or a combination of these things. The disadvantages of working with ethanol compared with cyanide include the danger of transporting it, its greater cost per sample obtained, the inconvenience and extra time needed in working with wet specimens, the expense of storage jars and flammable-liquid cabinets, and the space that these take in a laboratory.

Compared with traps with ethanol heads, traps with cyanide heads have the advantages of posing less danger in transporting the chemical, a lower cost per sample obtained, and the convenience and time savings in working with dry specimens in plastic Petri dishes. The disadvantages of working with cyanide heads include obtaining many specimens that are not museum quality, samples that are too small, and mice and salamanders damaging or eating specimens, or both. Storing dry specimens has the disadvantage that they are eaten by skin beetles, booklice, and other arthropods, unless they are kept under insect-proof conditions, e.g., they are in insect-tight containers or protected by naphthalene. Cyanide is likely to be more dangerous to use where there is a possibility that a child would remove it from a trap head and ingest it. To reduce this possibility, the trap heads should be appropriately labeled and made childproof.

The kind of cyanide head that biological supply companies sell is cylindrical and has an inverted "funnel" in its base through which arthropods enter. This kind of head generally yields high-quality samples, but usually excludes large insects such as bumble bees, cicadas, papilionid butterflies, and saturniid moths.

Improvements that make traps easier to erect and maintain in the field include using heavier gauze, fortifying the gauze near trap heads with a layer of muslin, or other fabric, and making trap ties and tethers of plastic cord (e.g., clothesline rope), rather than lighter nylon, cotton, cord, or twine that can fray and break in one season or be chewed apart by rodents.

## *Recommendations for Future Studies*

Although this 3-year study provides information on how DFB impacts pollinators, it involved only a small sprayed area, not the large acreage treated in many gypsy moth suppression efforts. In this study, pollinators might have quickly reentered treated areas from untreated ones. In large suppression efforts, vast numbers of pollinators could be killed and not fully recolonize a treated area for years. Therefore, biologists should perform long-term, e.g., 10-year studies in which large forested areas are treated with gypsy moth insecticides (e.g., *Bacillus thuringiensis*, diflubenzuron, and Gypchek) in more treated and untreated plots, to determine how these insecticides affect pollinators in an actual gypsy moth suppression situation.

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***Table 8-1.*** Numbers of yellowjacket workers (by species) captured in all four watersheds, 1991-1993.

| Species | No. workers |
|---|---|
| *Dolichovespula adulterina* (Buyson) | 0 |
| *D. arenaria* (F.) | 360 |
| *D. maculata* (L.) | 541 |
| *D. norvegicoides* (L.) | 1 |
| *Vespula acadica* (Sladen) | 1 |
| *V. consobrina* (Saussure) | 165 |
| *V. flavopilosa* Jacobson | 2,664 |
| *V. maculifrons* (Bupson) | 743 |
| *V. vidua* (Saussure) | 1 |
| *V. vulgaris* (L.) | 314 |

***Table 8-2.*** Numbers of yellowjacket workers captured in treated (WS1 and WS13) and untreated (WS4 and WS7) watersheds from 1991 to 1993 in the Fernow Experimental Forest.

| Year | Watershed Type | No. Workers |
|---|---|---|
| *1991* | untreated | 1,336 |
| | treated | 1,967 |
| *1992* | untreated | 165 |
| | treated | 106 |
| *1993* | untreated | 356 |
| | treated | 860 |
| *Total* | untreated | 1857 |
| | treated | 2933 |

Table 8-3. Split-plot analyses of possible diflubenzuron effects on numbers of yellowjacket workers in untreated (WS 4 and WS 7) and treated (WS 1 and WS 13) watersheds, Fernow Experimental Forest.

| Treatments | Year | Mean[a>] ± SEM | P |
|---|---|---|---|
| pre (WS 1 and 13) | 1991 | 8.9 ± 1.11 | — |
| pre (WS 4 and 7) | | 13.1 ± 1.11 | |
| Dimilin 4L (WS 1 and 13) | 1991 minus 1992 | 12.4 ± 0.94 | 0.009 |
| untreated (WS 4 and 7) | | 7.8 ± 0.94 | |
| Dimilin 4L (WS 1 and 13) | 1991 minus 1993 | 7.2 ± 0.95 | 0.627 |
| untreated (WS 4 and 7) | | 6.5 ± 0.95 | |

[a>] Grand mean is the mean of the number of workers per trap per collecting period per watershed.

*Figure 8-1.* Townes-style Malaise trap used to collect adult insects, Fernow Experimental Forest.

*Figure 8-2.* Cornell-style Malaise trap used to collect adult insects, Fernow Experimental Forest.

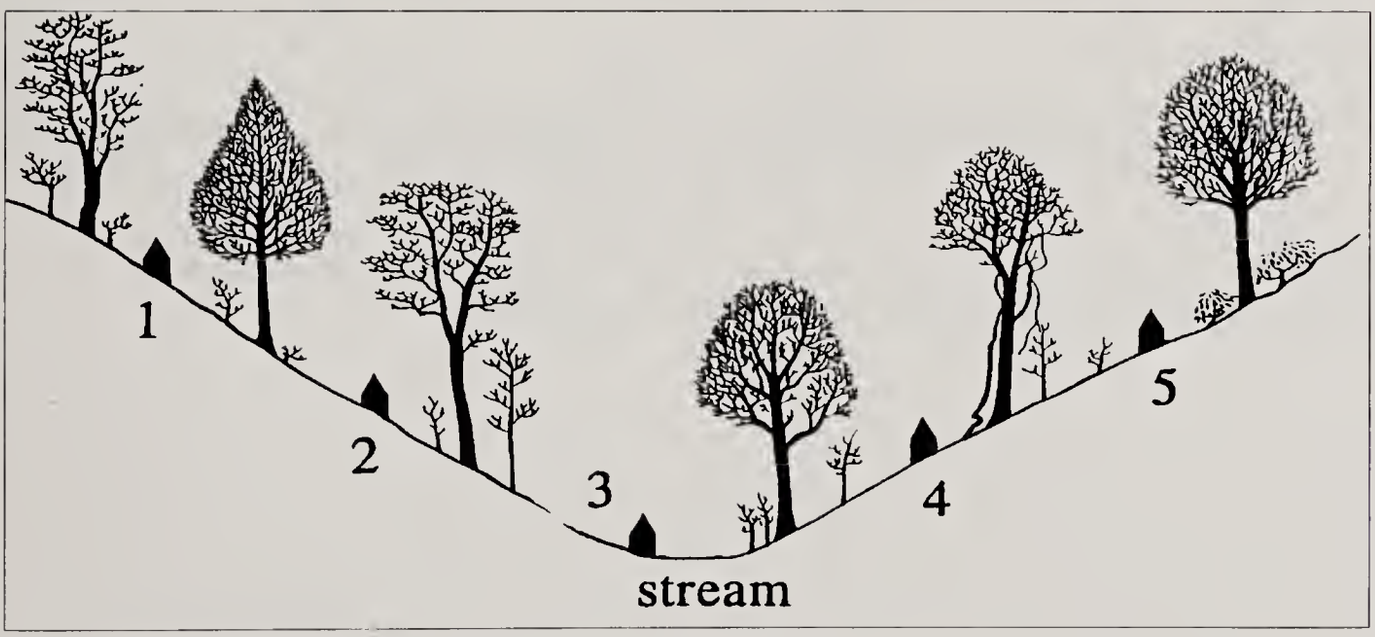


*Figure 8-3.* Positions of traps in a study watershed (diagram not to scale).

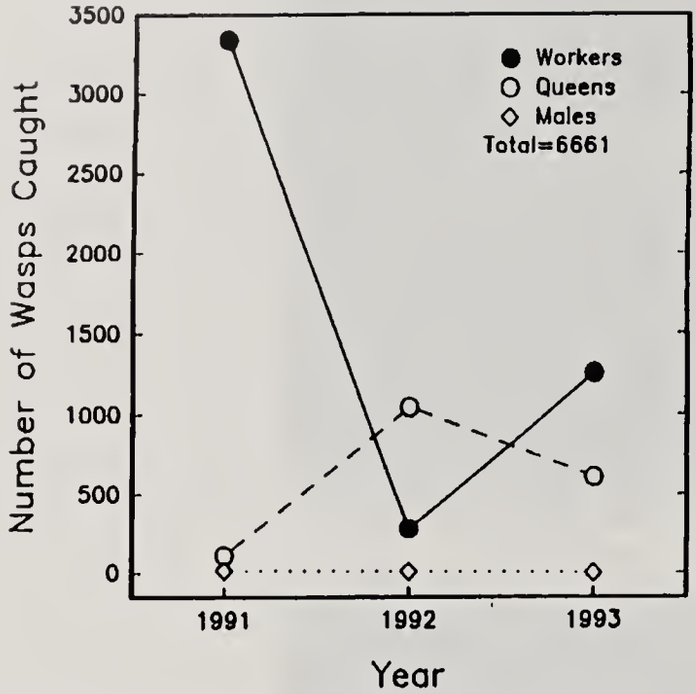


*Figure 8-4.* Numbers of yellowjackets captured in all four study watersheds, 1991-1993.

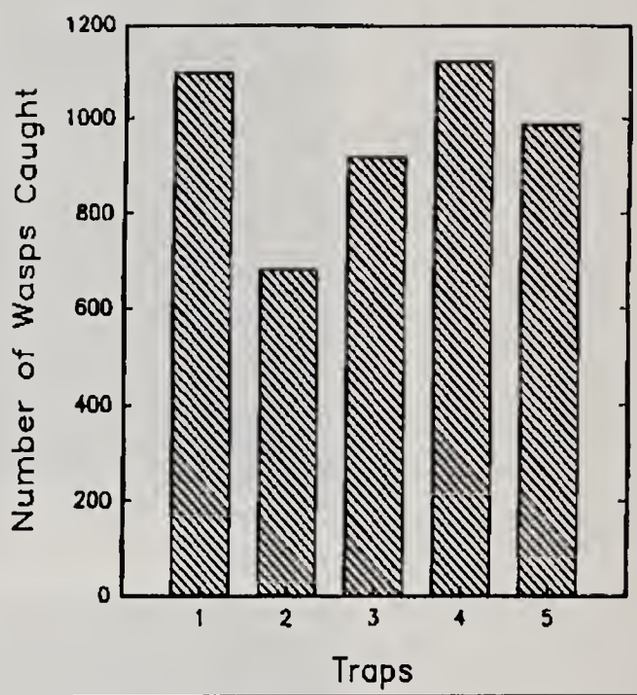


*Figure 8-6.* Numbers of yellowjackets captured by trap positions shown in figure 8-3, in all four watersheds, 1991-1993.

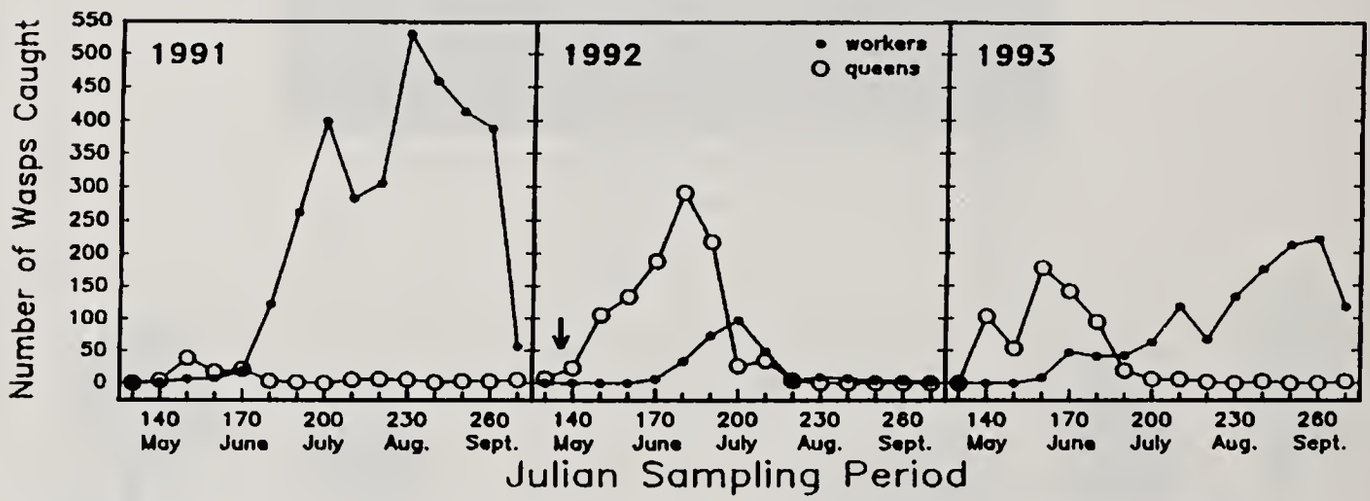


*Figure 8-5.* Numbers of yellowjackets captured in each 10-day sample in all four watersheds, 1991-1993. The arrow indicates the time of diflubenzuron application.

# *Chapter 9. Pollinating Insects -- Honey Bees*

**Matt Cochran and Paul Poling**

## Introduction

Honey bees (*Apis mellifera* L.) are important pollinators of more than 50 common agricultural crops and native plants. The U.S. Department of Agriculture has estimated that 80 percent of the fruit, seed and vegetable crops in the United States yield more abundantly when an adequate number of honey bees are available during the blooming season. Honey bees are also important honey and wax producers. The increased use of insecticides to control agricultural crop pests has caused honey bee colony poisoning and purity of honey to become critical broad-based issues.

Barker and Taber (1977), Barker and Waller (1978), and Emmett and Archer (1980) documented only minor effects on honey bees from induced feeding of high concentrations of diflubenzuron (DFB) in artificial feed and direct contact sprays.

The relative importance of honey bees as pollinators in forest ecosystems is unknown although most of the honey crops produced in the eastern United States comes from forest trees. Yellow-poplar (*Liriodendron tulipifera* L.) blooms in April through June and is a primary source of nectar in broadleaf forests. The flowers are in an upright position and diflubenzuron (DFB) spray applied to control gypsy moth could enter into the open flower and contaminate the nectar. The yellow-poplar flowers secrete nectar so abundantly that honey bees and native pollinators often do not remove it as fast as it appears. At the height of the blooming period, drops of nectar fall on leaves from the flowers. This deposit can increase the chance of pollinators coming into contact (e.g., may also gather the nectar from the contaminated leaves) with DFB. Honey bees also forage on the flowers of basswood (*Tilia americana* L.), sourwood (*Oxydenrum arboreum* (L.)), and many other species of forest plants.

Honey bees carry nectar in their stomachs to the hive and place it in cells where moisture evaporates. In the process of removing the water from the nectar, solutes are concentrated. Eventually, the nectar becomes honey, usually when its water content is below 86 percent.

In 1992, several local residents near the Fernow Experimental Forest expressed concern about the purity of honey and direct impact on brood in hives from aerial application of Dimilin 4L; therefore, we studied possible effects of DFB on honey bees.

## Field Study Methods

Initially, 10 colonies of honey bees were to be placed within each of the treated watersheds to obtain optimum exposure levels and facilitate sampling throughout 1992. Since there was a lack of pre-treatment data (1989-1991) and concern about competition between the honey bees and native pollinators, it was necessary to maintain the apiary of 10 hives at its original location approximately 0.6 km from the treated watersheds. This site has supported a healthy apiary for many years. This was a less effective experimental design because nectar, pollen and water were available to the honey bees within a short foraging distance from the apiary site; therefore, the bees might not have foraged in the treated watersheds.

All honey bee colonies were sampled at biweekly intervals during pretreatment, spray and posttreatment periods (primarily May 1 through June 28, 1992). Samples primarily consisted of 1 liter per week of adult honey bees, bee larvae, beeswax, honey, and pollen. Adults were collected from the outer frames of the hives by scraping the bees off using a 1-liter jar. Ether was used to kill the bees. Larvae were removed from the cells using forceps and placed into a 1-liter jar. Frame tops and bottoms were cleaned prior to the wax sampling by scraping. Bees often build what is commonly known as a burr comb between the frames and this area was then used to collect the wax. The wax was removed from the bars and placed into a 1-liter jar. Nectar was collected by the bees and placed in boxes called honey supers which were placed on the top of each hive. The frames in the honey supers had drawn combs in which the nectar was stored. Frames were removed from the supers and placed in a stainless steel extractor and the nectar was removed by centrifugal force. The nectar was then placed in a 1-liter jar. Pollen traps were used to collect the pollen. As the bees passed through a series of screens, the pollen was scraped from their legs and fell into a drawer in the bottom of the trap where it was removed and placed into a 1-liter jar. Diflubenzuron was extracted from samples by the Wimmer laboratory (Department of Biochemistry, West Virginia University) and analyzed using the mass spectrometric method (Wimmer and others 1991).

## *Methods for Diflubenzuron Extraction and Analysis*

***Honey***

1. Each honey sample was placed in lukewarm water and stirred to homogeneity before accurately measuring (by water displacement) duplicate 120-130 ml samples for extraction and analysis.

2. Each sample was diluted to a volume of 800 ml with distilled water, 50 µl of 500 ppm deuterated DFB was added as internal standard, and the sample was mixed well.

3. The sample was extracted with 3 x 48 ml of methylene chloride, and the solvent was evaporated to dryness after passage over anhydrous sodium sulfate to remove any residual water.

4. The sample was taken up in 800 µl acetonitrile. For partial purification to improve the signal-to-noise ratio during the GC and mass spectrometric analyses, 300 µl (2 x 150 µl) was passed through a C-18 HPLC column in 70:30 methanol:water and the DFB peak cut. After evaporation of the methanol, and a quick methylene chloride extraction from the residual water, the sample was dried down in a pear-shaped flask, taken up in 50 µl of acetonitrile, and stored at 4° C prior to BC and mass spectrometric analysis.

5. A standard of deuterated DFB was run the day of each analysis to correct for background peaks. The detectability limit was 1.0-10 ppb, depending on sample size and instrument sensitivity (sensitivity is highest just after cleaning).

***Pollen***

1. Thirty to seventy-five g (most) of each well-mixed pollen sample was placed in a Waring blender and ground to a homogeneous powder. Duplicate subsamples, 9 to 12 g each, were weighed and extracted by shaking in acetone to remove the DFB, similar to the leaf extraction method. After the first acetone addition, 50 µl of 500 ppm deuterated DFB was added as an internal standard. Each sample received three acetone washes.

2. The acetone washes were pooled and the acetone removed by rotary evaporation.

3. The sample was taken up in two washes of 600 µl each of acetonitrile, and the HPLC partial purification (as in Step 4, for honey) was performed before GC and mass spectrometric analysis. The detectability limit was 10-100 ppb.

***Bees***

The only difference in bee methods from those for the pollen samples was that any water residue left after acetone evaporation was transferred, with water rinses of the flask, into a conical centrifuge tube. The DFB was extracted with 3 ml of methylene chloride, using a vortex to mix the sample. After bench-top centrifugation to separate the layers, the methylene chloride layer was removed, passed through anhydrous sodium sulfate, and dried. The final sample was taken up in two washes of 600 µl each of acetonitrile for HPLC, before GC and mass spectrometric analysis. The detectability limit was 10-100 ppb.

## Results

There were no detectable amounts of DFB in any of the honey, pollen or bee samples. Extraction of the larvae and wax samples was attempted, but problems arose due to the high concentration of fats, lipids and waxes in the samples. These results are preliminary because of problems associated with the extractions from certain samples, and because the colonies could not be located in the watersheds.

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# *Chapter 10. Invertebrates In Leaf Litter And Soil*

**William B. Perry**

## Introduction

Previous studies in West Virginia on effects of forest application of diflubenzuron (DFB) on nontarget arthropods in the canopy reported reductions in the populations of canopy-dwelling larval macrolepidoptera and herbivorous Hymenoptera (Martinat and others 1988) and differences in Lepidoptera populations at the family level, which significantly affected species richness (Sample and others 1993). No impacts on populations of larval microlepidoptera, sucking herbivorous arthropods, or predaceous arthropods were observed. A study by Martinat and others (1993) reported impacts to spider and orthopteroid populations based on short-term pitfall trap data. Because of the potential for exposure to DFB from direct spray, washoff, and particularly autumn leaf fall, the objective of our study was to evaluate the effects of Dimilin 4L on invertebrates in soil and leaf litter.

Soil and leaf litter support invertebrates that are important in forest ecosystems (Edwards and others 1970, Seastedt and Crossley 1984). Invertebrates contribute to forest functions by restructuring soil organic matter, ingesting microbes that are involved in coarse organic matter decomposition and by disseminating fungal spores. Biodiversity of invertebrates in leaf litter and soil may be very high and may be one of the highest diversities in any habitat (Spurr and Barnes 1973).

## Field Study Methods

### *Soil Organisms*

Common soil and leaf litter organisms include arthropods (e.g., mites, springtails, and insects), annelids (segmented worms), and nematodes. Characterization of communities in soil and leaf litter requires extraction or removal of the animals from the soil matrix to permit identification and enumeration.

### *Soil Core Samples*

Soil samples were taken from a 15- by 30- m plot established in each of the four watersheds in the Fernow Experimental Forest. We used a coring device, 5 cm in diameter, that was pushed into the soil through the leaf litter layer to a depth of 10 cm. Soil cores were taken randomly (determined by a random number table) and placed in plastic bags in an insulated container for transport to the laboratory. Cores were weighed fresh (wet) and placed into Tullgren separation funnels for 48 h to extract invertebrates. A 25-watt light with a voltage controller was placed just over the funnel to provide heat. As a soil sample dried, its organisms migrated downward until they fell into a labeled collection vial below the funnel. To permit organisms to move to the collection vial, care was taken to prevent drying too quickly by slowly decreasing the voltage.

After extraction, we processed the soil material to determine several environmental variables and to express the data as number of organisms per dry weight of sample. Samples were oven-dried for 48 h at 45º C and reweighed to determine moisture content. Samples were passed through several sieves which separated material into rock and coarse organic matter (> 12.5 mm), and soil (< 12.5 mm), and weighed.

### *Litter Bag Samples*

Colonization samplers consisted of polyethylene 5-mm mesh bags containing leaf litter. Each bag contained 2 g each of red maple (*Acer rubrum* L.), yellow-poplar (*Liriodendron tulipifera* L.), and red oak (*Quercus rubra* L.), collected just after leaf fall from the study sites. Leaf bags were placed at study sites and left for at least 3 months before collection to allow organisms to colonize them. Bags were carefully removed and placed in plastic bags for transport to the laboratory, where invertebrates were extracted in a manner similar to that described for core samples.

### *Identification And Data Types*

Invertebrates extracted from samples were placed in 70 percent ethanol and examined at 20 power for routine identification and enumeration, and at 40-100 power for taxonomic purposes. Invertebrates were put in general taxonomic categories and trophic groups (Table 10-1). For identification to general groups, references included Dindal (1990), Borror and others (1989), Barnes (1980), and Brusca and Brusca (1990).

Mites and springtails, the most frequently encountered group (90 percent of all organisms), were identified to genus and species. Identification to the genus level required use of references such as Dindal (1990), Christiansen and Bellinger (1980), and Krantz (1978).

Invertebrates were also placed in the following trophic groups based on feeding behavior determined from either the literature or on the basis of mouth structure. Herbivores feed on algae, bacteria, roots, or freshly fallen leaves. Carnivores prey on smaller invertebrates. Omnivores feed on detritus, algae, bacteria, and other invertebrates. Fungivores are assumed to feed primarily on soil fungi. Detritivores feed on dead organic matter within the litter and soil. Literature sources that identify trophic categories include Dindal (1990) and Borror and others (1989). Use of trophic categories may provide information concerning soil arthropod functional groups that may be affected by treatment.

Invertebrate density may be expressed on the basis of sample area (number per square meter), sample weight (number per kilogram dry sample), or both (number per square meter per kilogram dry sample). When expressed on the basis of sample weight, rock weight was excluded. Invertebrate density was expressed in terms of total number, by order, group, trophic category, or species. From the data, species richness (number of species per square meter), diversity, and indices of association may be calculated. Table 10-2 illustrates the difference in collection frequencies of microarthropods between soil cores (sample weight) and leaf bags (area). Organisms that tend to inhabit leaf litter were collected more frequently. In particular, spiders were found six times more frequently in leaf bag samples than in core samples. Earthworms were found four times more frequently, which is probably a result of using a coring device with a 5-cm diameter. Variance of data from the two samplers was similar in 1992, but leaf bag samples showed a higher variance compared with that of core samples in 1993 (Table 10-3).

Environmental variables, such as percent soil moisture, soil temperature, rainfall, and forest stand composition were also measured, because these factors can affect invertebrate density and composition.

### *Assessment Of Effects: Study Design*

A common problem in impact assessment is to determine whether a particular population or community has changed in response to application of a toxicant. Controlled experimentation with replication and randomly assigned treatments for 'before-after' assessment are often not possible, particularly under the formidable constraints imposed by forest application testing. Further, discriminating between treatment effects and normal variation is complicated because of high sample variability and changes associated with organisms such as microarthropods that have high turnover rates. Based on between-sample variance for core samples in this study, a sample size of 28 would be needed to be 95 percent certain to detect a 25 percent change in total density (number per square meter) and 56 samples to detect a 15 percent change. Additional replication would be needed to identify time-treatment interactions.

Processing soil and leaf litter samples is time consuming, and the scope of a study (number of samples) was effectively limited by the time and effort required for processing. An average of 3 h per sample is needed for collection and processing to database analysis.

Because the opportunity for replicating sites was limited and because of limitations imposed by sample processing time and expense, an alternative to the before-after design is the Before-After Control-Impact Pairs (BACIP) design (Stewart-Oaten and others 1986, 1992). Using this approach, samples are collected at both treated and untreated sites simultaneously (same day) for a period pretreatment and posttreatment. Replication comes from collecting such paired samples a number of times before and after treatment. Each observed difference between the treated and untreated sites before treatment is taken as an estimate of the mean difference that would have existed in the posttreatment period. The observed difference between the treated and untreated sites pretreatment is compared with observed differences posttreatment.

Using this design, we sampled watersheds by taking soil cores the last week of each month for 3 years of baseline and 1 year of posttreatment data. Sampling in the last week of each month was done to maintain consistency and reduce possible differences due to circadian rhythms in invertebrate populations. In each of four watersheds, 10 cores per month were taken in the pretreatment and posttreatment period from 1990 through 1993. Two watersheds were treated (WS 1 and WS 13) and two watersheds were untreated (WS 4 and WS 7).

We also used litter bag colonization samplers placed in the four watersheds 3 months before treatment. Ten litter bags per watershed were collected 8 and 4 weeks before treatment. Ten litter bags per watershed were collected 24 h, 48 h, 72 h, 2 weeks, and then monthly for 1 year after treatment.

We used analysis of variance to partition treatment, time, watershed, and sample variation. Calculations were conducted using SAS (SAS 1989). The BACIP test can be accomplished with the general linear model used by SAS, as an ANOVA Type III calculation. Alternatively, mean differences pretreatment and posttreatment may be tested with SAS using a modified t-test (Stewart-Oaten and others 1992). An ANOVA was also used for testing for differences in litter bag invertebrate densities between treated and untreated watersheds.

## Results

Over 39,800 organisms in 17 orders were identified during the study. Overall, samples were dominated by mites (49 percent) and springtails (28 percent). We observed 47 species of mites, 41 species of springtails, and 14 species of spiders. Arthropod densities were highly variable, both temporally and spatially in soil and leaf litter. Seasonal cycles were evident, with densities generally higher in spring and fall. Significant annual differences also existed; mean annual invertebrate densities (total) were lowest in 1990, increased 30 percent in 1991, and another 32 percent in 1992 and 1993. Differences also existed between watersheds. Watershed 4, an untreated watershed which had not been logged since the early 1900's, had significantly higher densities than the other watersheds, which had been logged more recently. There were also differences with aspect; northerly-facing slopes (cooler, damper) tended to have lower mite densities, and southerly-facing slopes (warmer, drier) tended to have lower springtail densities.

In terms of trophic categories, herbivores were collected the most frequently (40-55 percent), followed by carnivores (20-30 percent), detritivores (10-15 percent), fungivores (5-10 percent), and omnivores (5-10 percent). The mean monthly total for species richness varied from 24 to 36, for mite species richness varied from 11 to 15, and for springtail species richness varied from 8 to 12. Mean monthly spider species richness varied from 2 to 5. Measures of richness were underestimates, since not all taxa were identified to the species level.

## Core Samples

***Soil and leaf litter arthropods*** -- Although a total of 143 taxa were counted from soil core samples, only 19 were present consistently enough to be used for analysis of DFB effects (Table 10-4). Based on total densities within major groups, there was a significant difference observed for the order Araneae (spiders), but no significant differences for other groups. The $H_o$ for springtails and oligochaete worms (Enchytraeida) was narrowly accepted ($P = 0.0766$ and 0.0624, respectively). The difference in spider densities between untreated and treated watersheds increased from 0.3 per kg of sample (pretreatment mean) to 1.1 per kg sample (posttreatment mean). Although spider densities increased after treatment in all watersheds, including those treated with Dimilin 4L, the increase in untreated watersheds was more than three times higher than in treated watersheds.

Within major groups, we observed differences for only two species of mites (*Cheiroseius* sp. and *Gamasellus concinnus*). No differences in frequency of occurrence of trophic groups, orders, or lower taxonomic units were observed after treatment.

## Bag Samples

***Leaf litter bag arthropods*** -- In leaf litter bags, only 19 taxa were present consistently enough to be used for analysis of DFB effects (Table 10-5). Since no long-term leaf litter bag data was available before treatment, analysis is limited to comparisons between posttreatment leaf litter bag populations. Based on total numbers of organisms per sample weight, differences between untreated and treated watersheds were not significant. Significant negative differences (a decline in treated watersheds) were observed for only one species of springtail (*Sminthurus purpurescens*). A significant positive difference was also observed for one species of mite (*Oribatella* sp. A) as a

result of an increase in density after treatment. We also compared leaf litter degradation rates from litter bag data; there was no significant difference in mass loss rates during the 1-year posttreatment period between treated and untreated watersheds.

## Discussion and Recommendations

Based on 12 months of posttreatment data (core samples), taxa that showed significant decline ($P \leq 0.05$) included the spiders and two species of mites. Spiders may be a biological indicator of spray effects in forest floor invertebrates. Changes in spider density may be a secondary effect of Dimilin 4L application if prey are affected. Several mite species may have potential to be indicators; however, it is not known if these species occur in many other locations.

A shorter time than 3 years for pretreatment assessment seems to be acceptable; however, sampling at least 12 months before and after treatment is needed due to the large variability within invertebrate communities as well as the persistence of DFB in the ground litter (greater than 16 months after spray). Sampling 12 months before and after treatment, using two treated and two untreated sites results 12 pairs of untreated treated comparisons for the BACIP design.

Leaf litter bag sampling is a faster method of collecting data on invertebrate communities than is core sampling. Core samples need to be weighed and sieved whereas bags need only to be extracted for data. Soil cores may be more accurate in terms of richness and numbers, however, due to the fact that cores include litter and soil layers. In general, core sampling yielded more organisms per unit area and also appeared to be more consistent in terms of variance over years than was leaf litter bag sampling.

Extraction efficiency can vary depending on laboratory conditions, and leaf litter and soil conditions. Immature invertebrates do not migrate well vertically; large numbers of immature stages will bias results if extraction is too rapid. Because the analysis examines relative differences between sites, however, extraction bias is reduced or compensated by similar sample treatment and simultaneous extraction and processing of samples from a sample date.

Although differences may be detected, interpretation of significance relies on an assessment of magnitude of differences and biological significance of invertebrates. Few guidelines exist, and interpretation relies on judgment. There is a critical need for more data on longer-term effects, roles of microarthropods, detrital processing effects, and interactions of invertebrates with microbial biomass within forest systems. Effects of DFB on arthropod communities may not occur until years after treatment.

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***Table 10-1.*** Summarized data on trophic categories, invertebrate orders and taxonomic groups represented in soil core extracts taken from four watersheds in the Fernow Experimental Forest, May-December 1989-1992.

Part A. Trophic Categories

| Trophic Category | Code | Count Frequency | Percent | Cumulative Frequency | Cumulative Percent |
|---|---|---|---|---|---|
| Herbivore | H | 431 | 45.5 | 431 | 45.5 |
| Carnivore | C | 268 | 28.3 | 699 | 73.8 |
| Detritivore | D | 95 | 10.0 | 794 | 83.9 |
| Fungivore | F | 91 | 9.6 | 885 | 93.5 |
| Omnivore | O | 61 | 6.5 | 946 | 100.0 |

Part B. Orders

| Order | Code | Count Frequency | Percent | Cumulative Frequency | Cumulative Percent |
|---|---|---|---|---|---|
| Acari | A | 592.7 | 62.7 | 592.7 | 62.7 |
| Collembola | C | 256.5 | 27.1 | 849.2 | 89.8 |
| Protura | P | 16.1 | 1.7 | 865.3 | 91.5 |
| Chelonethida | CH | 11.7 | 1.2 | 877.1 | 92.7 |
| Coleoptera | CO | 11.4 | 1.2 | 888.6 | 93.9 |
| Araneida | AR | 8.9 | 0.9 | 897.5 | 94.9 |
| Symphyta | SY | 7.0 | 0.7 | 904.6 | 95.6 |
| Hymenoptera | HY | 6.4 | 0.7 | 911.0 | 96.3 |
| Lumbricidae | OL | 5.5 | 0.6 | 916.6 | 96.9 |
| Chilopoda | CI | 5.4 | 0.6 | 922.0 | 97.5 |
| Psocoptera | PS | 5.3 | 0.6 | 927.4 | 98.0 |
| Diptera | F | 5.2 | 0.6 | 932.6 | 98.6 |
| Diploda | DI | 3.7 | 0.4 | 936.3 | 99.0 |
| Nematoda | N | 3.6 | 0.4 | 940.0 | 99.4 |
| Euthytraeida | O | 1.4 | 0.1 | 941.4 | 99.5 |
| Gastropoda | G | 0.9 | 0.1 | 942.4 | 99.6 |
| Thysanoptera | TH | 0.9 | 0.1 | 943.3 | 99.7 |
| Phalangida | OP | 0.7 | 0.1 | 944.8 | 99.9 |
| Hemiptera | H | 0.6 | 0.1 | 945.4 | 99.9 |
| Lepidoptera | L | 0.2 | 0.0 | 945.7 | 100.0 |
| Diplura | D | 0.1 | 0.0 | 945.8 | 100.0 |
| Isopoda | I | 0.1 | 0.0 | 946.0 | 100.0 |

*Table 10-1.* Continued

Part C. Taxonomic Groups

| Taxon | Code | Frequency | Percent | Cumulative Frequency | Cumulative Percent |
|---|---|---|---|---|---|
| Mite | M | 594.4 | 62.8 | 594.4 | 62.8 |
| Springtail | SP | 256.4 | 27.1 | 850.8 | 89.9 |
| Proturan | PR | 16.1 | 1.7 | 866.9 | 91.6 |
| Pseudoscorpion | P | 11.7 | 1.2 | 878.7 | 92.9 |
| Beetle | B | 11.6 | 1.2 | 890.3 | 94.1 |
| Spider | S | 9.0 | 1.0 | 899.4 | 95.1 |
| Symphylan | SY | 7.0 | 0.7 | 906.4 | 95.8 |
| Round worm | W | 6.8 | 0.7 | 913.3 | 96.5 |
| Ant | AN | 6.4 | 0.7 | 919.7 | 97.2 |
| Centipede | C | 5.4 | 0.6 | 925.2 | 97.8 |
| Bark louse | BL | 5.3 | 0.6 | 930.5 | 98.4 |
| Thrips | T | 5.3 | 0.6 | 935.8 | 98.9 |
| Millipede | D | 3.8 | 0.4 | 939.6 | 99.3 |
| Nematode | N | 3.6 | 0.4 | 943.3 | 99.7 |
| Snail | SN | 1.0 | 0.1 | 944.3 | 99.8 |
| Harvestman | H | 0.7 | 0.1 | 945.1 | 99.9 |

***Table 10-2.*** Frequency of occurrence as percent composition of organisms from soil core and leaf bag extracts taken from four watersheds in the Fernow Experimental Forest. Soil core data are from May - December 1989-1992, and leaf bag data are from May - December 1992.

| Group | Soil core | Leaf bag |
|---|---|---|
| | *Percent Composition* | |
| Trophic category: | | |
| Herbivore | 45.5 | 39.4 |
| Carnivore | 28.3 | 31.2 |
| Detritivore | 10.0 | 11.0 |
| Fungivore | 9.6 | 11.7 |
| Omnivore | 6.5 | 6.7 |
| Order: | | |
| Acari | 62.7 | 54.8 |
| Collembola | 27.1 | 22.1 |
| Protura | 1.7 | 0.5 |
| Chelonethida | 1.2 | 2.1 |
| Coleoptera | 1.2 | 1.9 |
| Araneida | 0.9 | 6.2 |
| Hymenoptera | 0.7 | 0.8 |
| Symphyla | 0.7 | 0.1 |
| Lumbricidae | 0.6 | 2.7 |
| Chilopoda | 0.6 | 2.1 |
| Psocoptera | 0.6 | 2.5 |
| Diptera | 0.6 | 0.0 |
| Diploda | 0.4 | 1.5 |
| Nematoda | 0.4 | 1.1 |
| Gastropoda | 0.1 | 0.8 |
| Thysanoptera | 0.1 | 0.4 |
| Phalangida | 0.1 | 0.8 |
| Hemiptera | 0.1 | 0.4 |

***Table 10-3.*** Mean numbers of organisms from soil core and leaf bag extracts taken from four watersheds in the Fernow Experimental Forest, 1989-1993.

| Year | Litter Bag | | | Soil Core | | |
|---|---|---|---|---|---|---|
| | N | Mean | Variance | N | Mean | Variance |
| 1989 | | | | 263 | 15 | 278 |
| 1990 | | | | 440 | 14 | 248 |
| 1991 | | | | 400 | 26 | 471 |
| 1992 | 556 | 21 | 628 | 634 | 23 | 594 |
| 1993 | 192 | 43 | 1,097 | 200 | 24 | 374 |

*Table 10-4.* Results of BACIP tests for treatment effects based on taxa found in soil and litter core samples.

| Orders | Family or Species | df | P |
|---|---|---|---|
| Total number of organisms | | 34 | 0.4820 |
| Acari | | | |
| | All | 34 | 0.1756 |
| | *Ceratoppia* sp. A | 31 | 0.4731 |
| | *Ceratoppia* sp. C | 16 | 0.1030 |
| | *Oribatella* sp. A | 20 | 0.3536 |
| | *Diarthrohallus* sp. A | 24 | 0.0833 |
| | *Rhodacarus roseus* | 30 | 0.1979 |
| | *Heminothrus* sp. A | 20 | 0.4667 |
| | *Maerkelotritia gibbera* | 30 | 0.4165 |
| | *Cheiroseius* sp. A | 9 | 0.0451* |
| | *Rhodacurus* sp. A | 30 | 0.1979 |
| | *Gamasellus concinnus* | 29 | 0.0353* |
| | *Tenuilala nuda* | 17 | 0.3324 |
| Araneae | | | |
| | All | 21 | 0.0452* |
| Collembola | | | |
| | All | 34 | 0.0766 |
| | *Onychiurus encarpatus* | 30 | 0.2469 |
| | *Isotomurus palustroides* | 8 | 0.0838 |
| | *Isotomurus japonica* | 24 | 0.1920 |
| | *Triacanthella copelandi* | 14 | 0.1239 |
| | *Hypoquastura brevispina* | 18 | 0.1003 |
| | *Isotomurus tricolor* | 6 | 0.4606 |
| Enchytraeida | | | |
| | All | 13 | 0.0624 |

*changes between pre- and post-treatment densities significant at $P \leq 0.05$.

***Table 10-5.*** Results of ANOVA tests on invertebrates from litter bags. For treatment effects (untreated vs treated watersheds) df = 1; for treatment effects among months (treatment * months) df = 10. Differences in densities between treated and untreated watersheds that are considered significant at $P \leq 0.05$ are indicated with an *.

| Orders | Family or Species | Effects (P-values) | |
|---|---|---|---|
| | | Treatment | Treatment*Month |
| Total number of organisms | | 0.5924 | 0.9594 |
| Acari | | | |
| | All | 0.2813 | 0.7886 |
| | *Ceratoppia* sp. C | 0.3848 | 0.9983 |
| | *Ceratoppia* sp. A | 0.0869 | 0.1917 |
| | *Diarthrohallus* sp. A | 0.7285 | 0.9774 |
| | *Oribatella* sp. A | 0.0246* | 0.4098 |
| | *Cheiroseius* sp. A | 0.2741 | 0.9917 |
| | *Heminothrus* sp. A | 0.5998 | 0.8407 |
| | *Maerkelotritia gibbera* | 0.0912 | 0.9958 |
| | *Gamasellus concinnus* | 0.1741 | 0.9352 |
| Araneae | | | |
| | All | 0.8845 | 0.7613 |
| Collembola | | | |
| | All | 0.0730 | 0.9958 |
| | *Onychiurus encarpatus* | 0.2193 | 0.9988 |
| | *Triacanthella copelandi* | 0.5605 | 0.4710 |
| | *Sminthurus purpurescens* | 0.0043* | 0.1060 |

# *Chapter 11. Soil Bacteria And Fungi*

**John C. Landolt and Steven L. Stephenson**

## Introduction

Soil communities are diverse, consisting of complex assemblages of bacteria, fungi, algae, nematodes, insects, and many other organisms (Brady 1974). Cellular slime molds exist in many soil communities and function as predators on bacteria and as food resources for other soil dwelling microorganisms; thus cellular slime molds commonly are closely integrated with the soil microcosm (Cavender and Raper 1965b, 1965c, Kuserk 1980, Raper 1984). A life cycle of the cellular slime mold *Dictyostelium discoideum* is depicted in Figure 11-1.

Procedures to isolate, identify, and quantify most soil microorganisms are generally tedious, time consuming, expensive, and sometimes imprecise or even technologically impractical or impossible. Standard, accepted procedures do exist to isolate, identify, and enumerate cellular slime molds and representative aerobic, heterotrophic soil bacteria, which are likely food resources for cellular slime molds. Thus, cellular slime molds are useful "indicator" organisms for monitoring soil-related ecosystem parameters (e.g., successional recovery after disturbance), including those that may be subject to disturbance by Dimilin application. Procedures for monitoring cellular slime molds are relatively simple and inexpensive (Cavender and Raper 1965a, Landolt and Stephenson 1986, Landolt and Stephenson 1990, Stephenson and Landolt, 1987).

## Laboratory Study Methods

Laboratory experiments concerning effects of diflubenzuron (Dimilin 25-W) upon broth cultures of two species of bacteria and upon three species of cellular slime molds were conducted. Bacteria and slime molds were exposed to ten-fold dilutions (0.001, 0.01, 0.1, 1.0 grams per liter) of DFB during their life cycles. The Dimilin 25-W used in exposures to bacteria and slime mold cultures was not autoclaved prior to use. Effects upon bacterial reproduction were measured by spectrophotometric analysis, while effects upon cellular slime mold growth and development were measured by direct microscopic counts and measurements.

## Field Study Methods

Ten samples of soil leaf litter were collected for cellular slime mold analysis from each of the four study watersheds (WS 1 and WS 13 were treated and WS 4 and WS 7 were untreated) on eight occasions in May to June (the most likely period for application of Dimilin) and five times in September to October (the best period for cellular slime mold activity and leaf fall), from 1989 to 1992. The watersheds were sampled once in spring 1993. A total of 13 periods was sampled for a total of 520 samples. For each sample, 10-50 g of soil-litter was scraped into a sterile collecting bag. On each sampling date, samples were collected at 10-m intervals along transect lines. Samples were processed by the dilution-culture plate procedure and clones of cellular slime molds were identified and quantified by methods given by Cavender and Raper (1965a).

Grab samples of leaf litter from the watersheds were taken for bacterial counts in May and September to October 1991 and 1992. In 1991, three samples from each watershed were taken on each of three sampling dates, while in 1992, 10 samples were collected from each watershed on each of four sampling dates. Ten samples were examined from each of the four watersheds in May

1993. The October 1992 samples were of freshly fallen leaves. On occasions when 10 leaf litter samples were collected, the samples were taken at 10-m points along a transect. Samples were processed by a dilution-drop plate procedure similar to that of Miles and Mishra (1938) using brain-heart agar as the culture medium with incubation at 37° C. Bacterial densities were estimated from the numbers of bacterial colonies that appeared in the culture plates.

On each sampling date, five bulk samples of soil were collected from each of the four watersheds to provide data on soil moisture content by comparison of wet weight to oven dry weight.

## Results

### *Laboratory study*

Tables 11-1 a, b, and c summarize the results of laboratory studies conducted to determine effects of direct application of Dimilin 25-W on the life cycles of three of the most common cellular slime molds found in the watershed soils and leaf litter. These data indicate that, at the exposures used, DFB had no significant negative effect on the number of cells or number of fruiting bodies (sporocarps) that developed in *D. discoideum* Raper, *D. minutum* Raper, or *Polysphondylium violaceum* Brefeld or the number of migrating aggregates (pseudoplasmodia) that developed in *D. discoideum* cultures. Figures 11-2, 11-3, and 11-4 depict the growth curves of two species of common bacteria exposed to different concentrations of DFB (Dimilin 25-W). Calculated F-values indicate that significant differences ($P \leq 0.05$) existed for bacterial growth when control groups were compared to those exposed to 1.0 g/l of DFB. Growth curves for bacteria exposed to 10-fold and 100-fold dilutions of DFB were not significantly different from controls.

### *Field study*

A total of at least 12 species of cellular slime molds, from all three recognized genera, was recovered from the Fernow watersheds during the 5-year study (Table 11-2). All species were representative of upland deciduous forests of North America (Raper 1984). The most commonly recovered forms were *Dictyostelium mucoroides* Brefeld, *D. minutum*, *D. discoideum* and *Polysphondylium violaceum*, with the last seeming to be more common later than earlier in the growing season. Densities of slime mold clones were similar among the four watersheds in each of the 5 years (Table 11-3) and indicate that densities tend to be somewhat lower in Watershed 7 than in the other three, and that densities determined during 1991 were somewhat lower in the watersheds than the 5-year average.

Average species richness for the watersheds (Table 11-4) shows that the watersheds are similar in this respect from year to year, with watershed 7 usually having the lowest number of different slime mold species. These observations might correlate to: (1) the fact that Watershed 7 had been clearcut and herbicided in the past, and (2) in 1991 there was a fairly severe summer drought.

Results of the soil-litter bacterial plate counts are given in Tables 11-5. These data indicate that the four watersheds were similar in the densities of recoverable bacteria on any one sampling date. In 1992 and 1993, when 10 different samples were analyzed in each watershed on each sampling date, there was a large variation in bacterial density within each watershed as

indicated by the relatively large standard deviations of the mean numbers. Such within-watershed variation was not as pronounced in the 1991 data based upon a smaller number of samples. Also in 1992, there seemed to have been an increase in bacterial densities from May to September, which is not seen during drought conditions in 1991. The generally low bacterial densities for the October 1992 sampling reflect the fact that the material used consisted of freshly fallen leaves. In general, bacterial densities before the October 1992 sampling were higher in the watersheds that were to be treated (WS 1 and WS 13) than in the untreated watersheds (WS 4 and WS 7). In the October 1992 data, using leaf material most likely to have been exposed to Dimilin 4L application in May, bacterial densities were lower in the treated watersheds than in the untreated. In spring 1993, densities of recoverable bacteria were relatively low in all watersheds and differed little among watersheds.

Table 11-6 provides values of soil moisture for samples from the four watersheds as an average for each year's spring and fall collections. These data show that all four of the watersheds were similar from year to year in soil moisture, an edaphic factor that has a direct effect upon both bacteria and cellular slime molds. While the average soil moisture values for 1991 were not very different from other years, it should be noted that fairly high average moisture values for May samples (46.5 percent) obscured a lower average soil moisture of 31.9 percent for the September samples, which reflected the relatively dry conditions of the Fernow Forest during much of 1991.

## Discussion

The four watersheds (with some exceptions for Watershed 7) appear to be similar in terms of cellular slime mold and soil bacteria microfloral makeup and soil moisture features during the four years of the study. Few, if any, effects of DFB can be supported by the results of this examination of soil microflora nontarget organisms, at least in the short, acute term. Detection of possible longer term influences, either direct or indirect, upon the soil microflora of the treated watersheds, however, would require additional sampling and analysis beyond Spring 1993.

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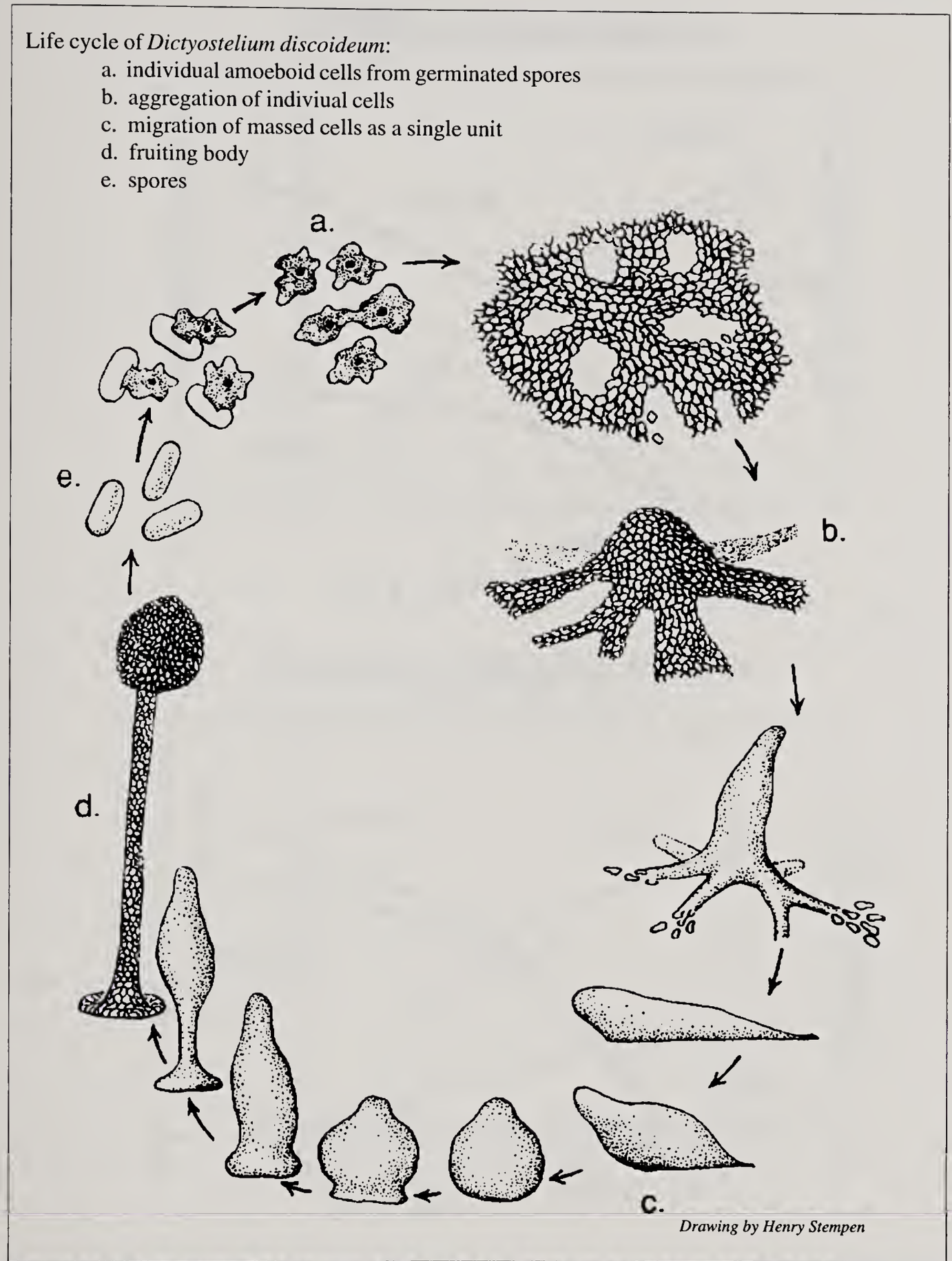
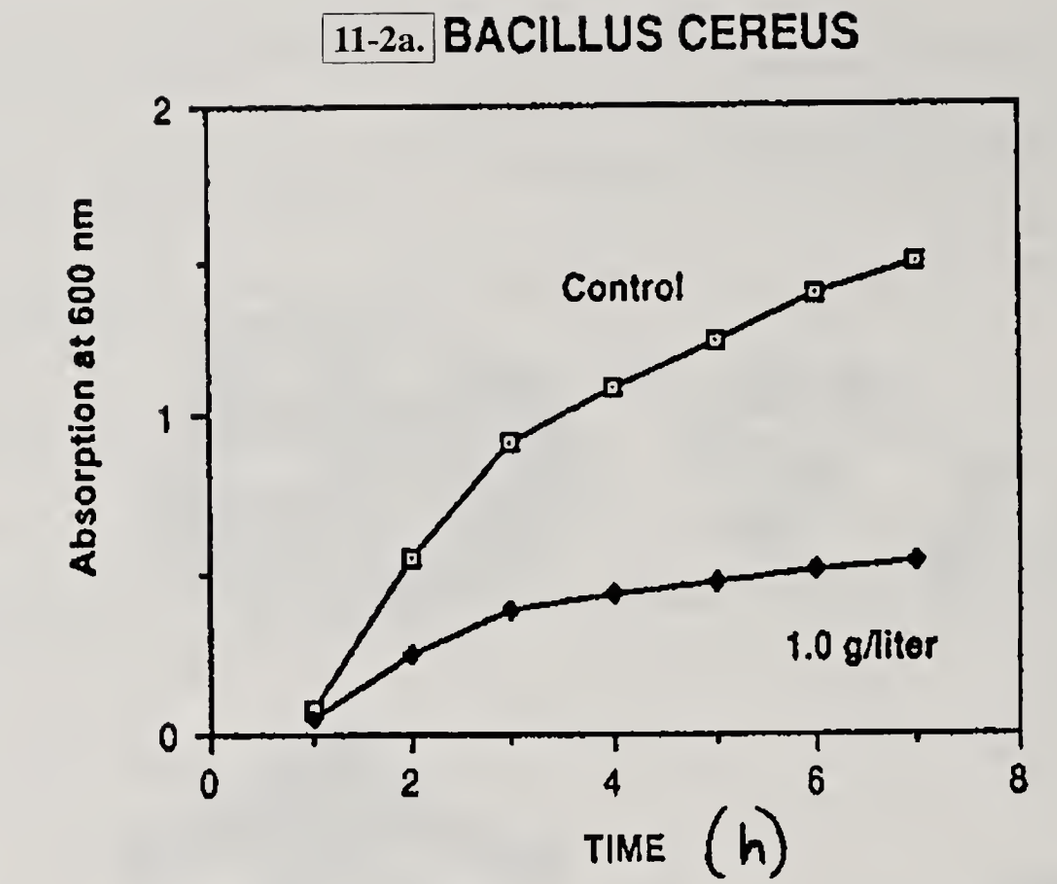
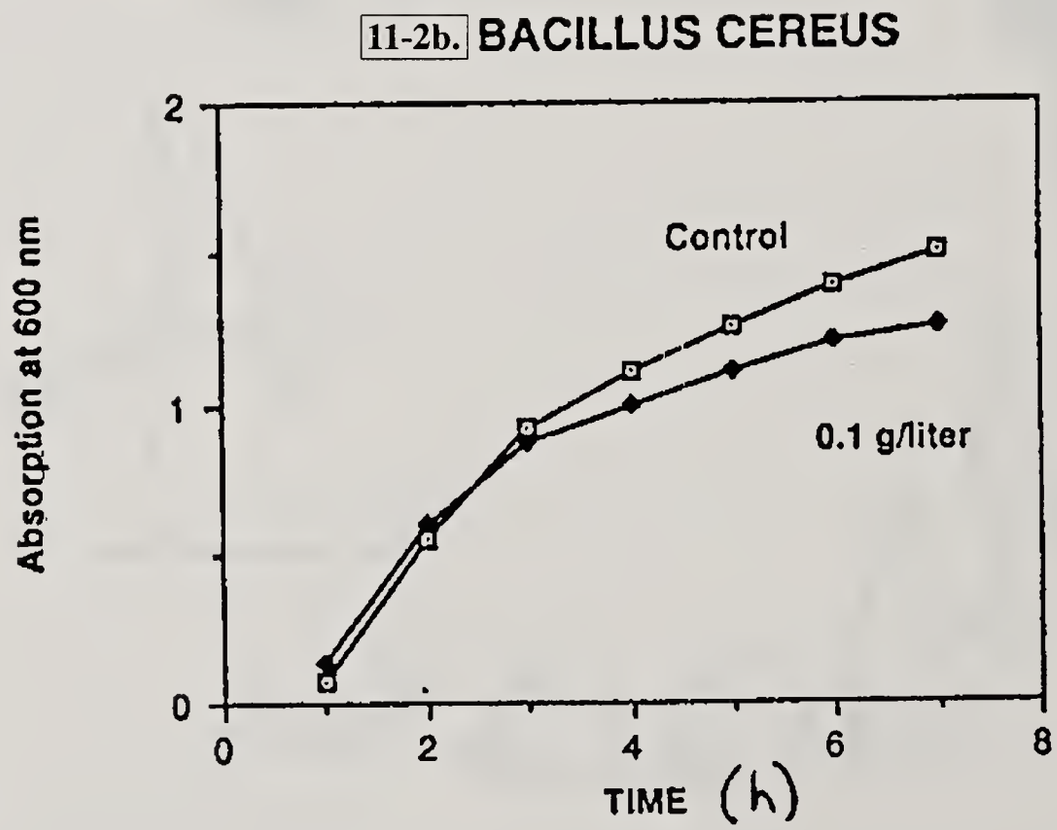
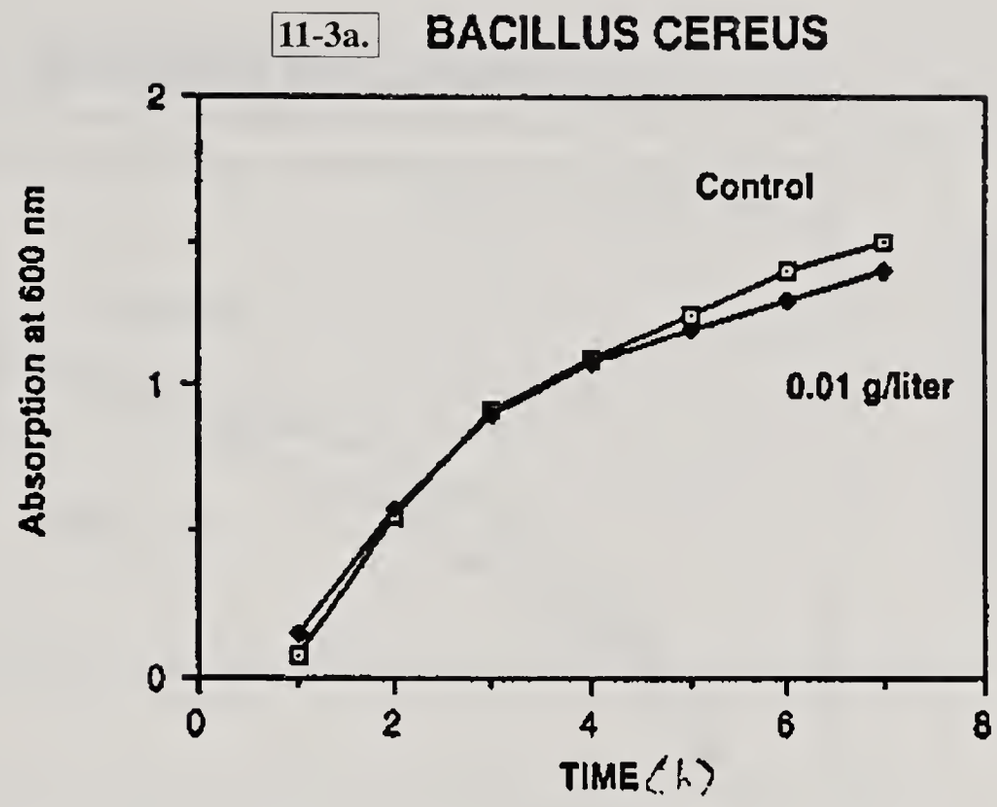


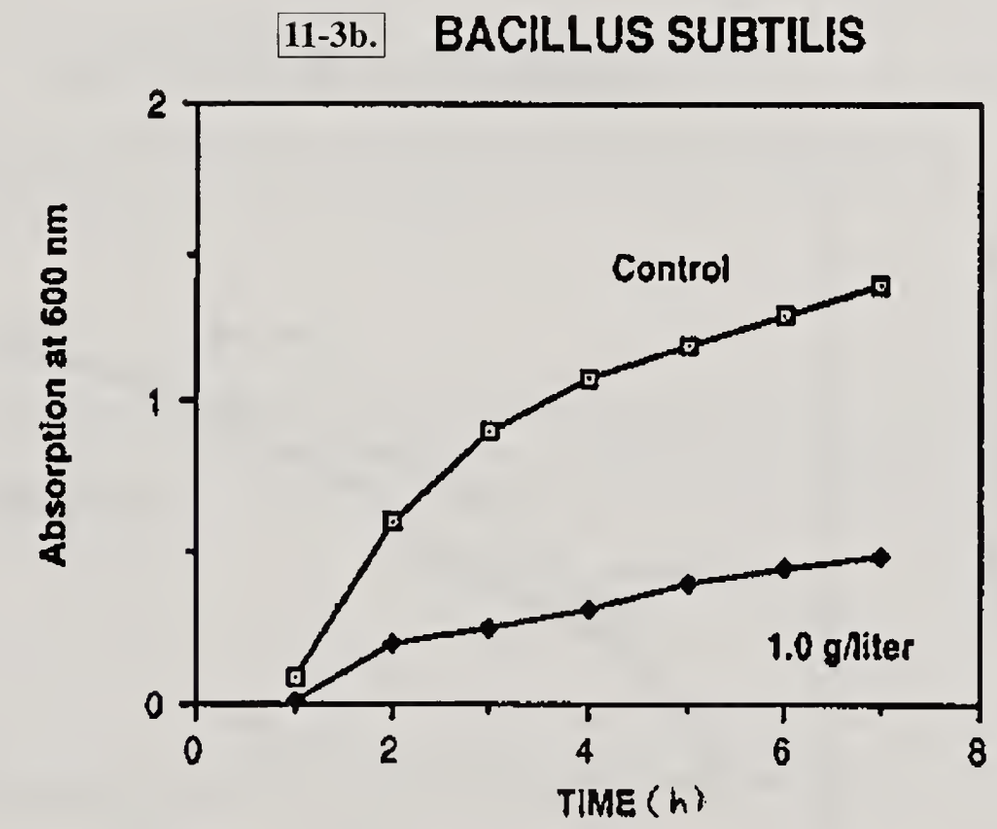

***Figure 11-1.*** Life cycle of the cellular slime mold *Dictyostelium discoideum*, found in soil.



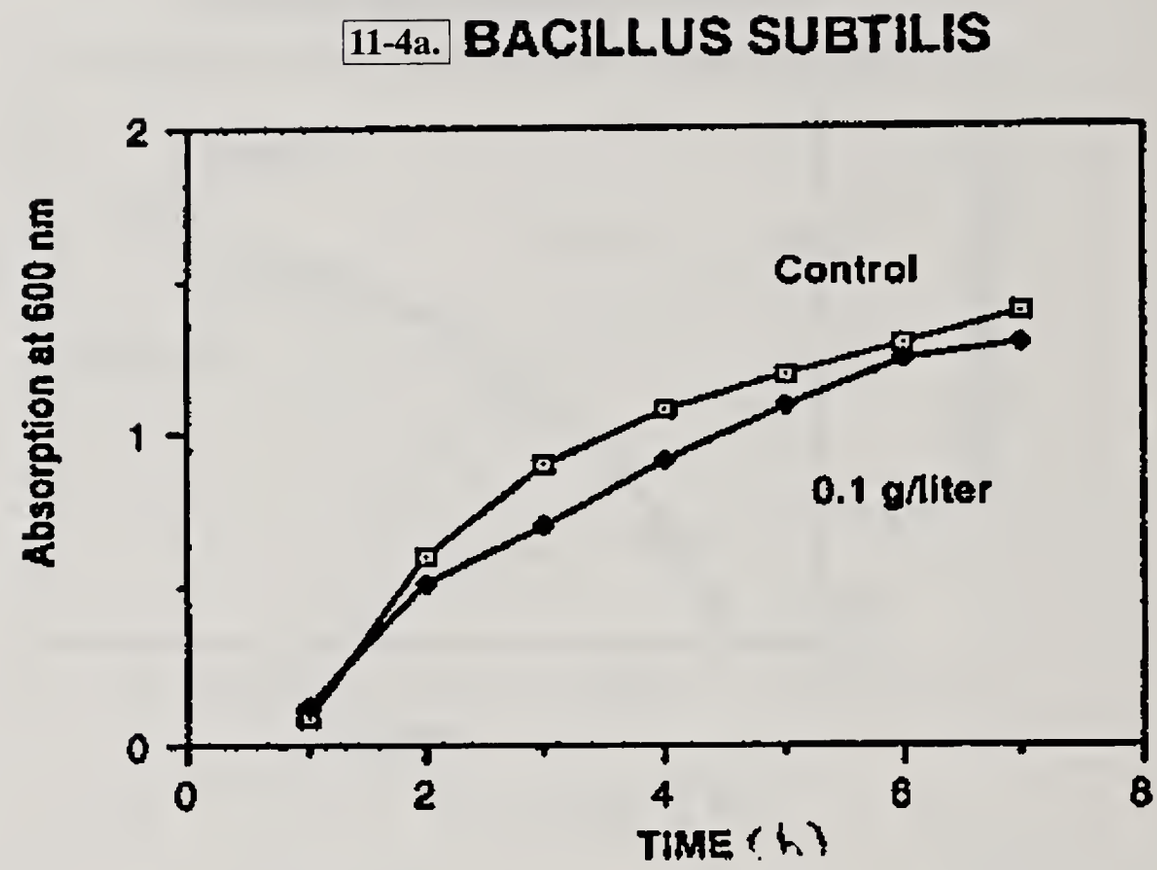



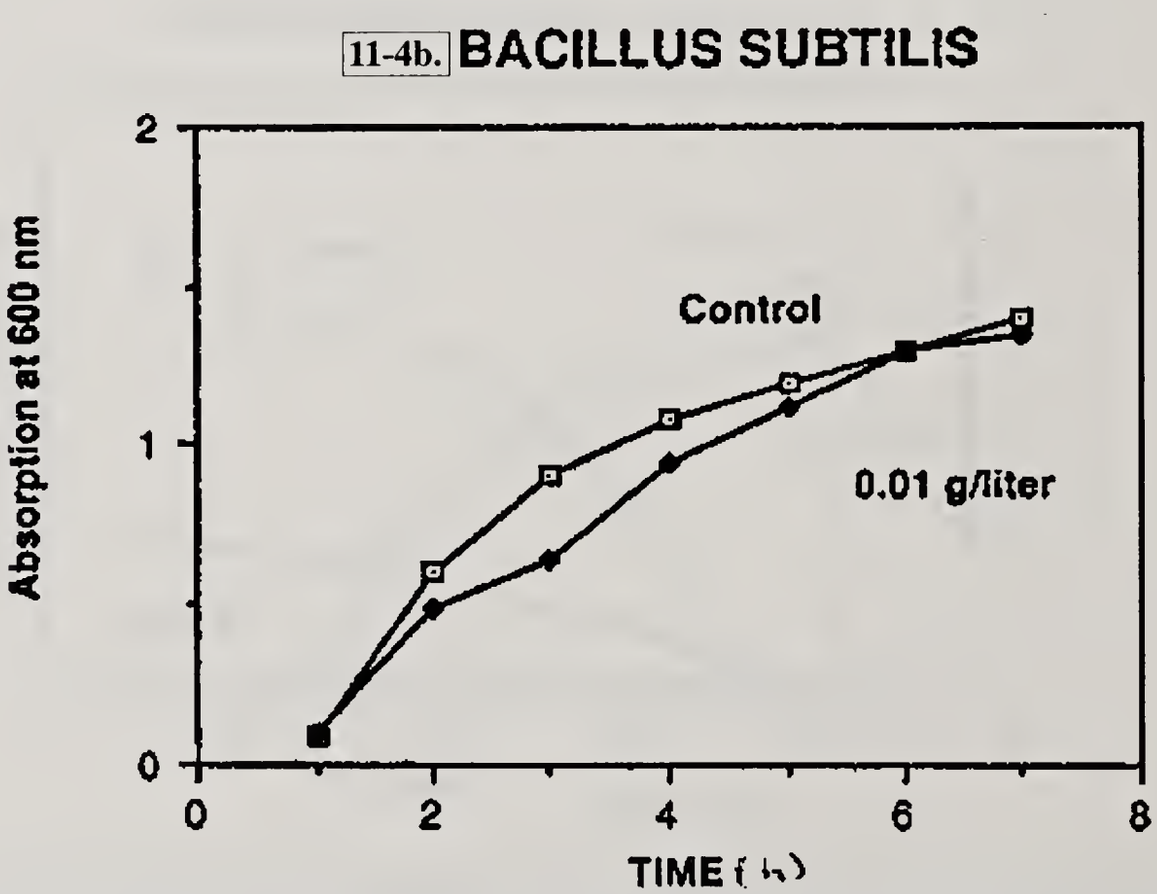

*Figures 11-2a, b.* Growth rate (each numbered time interval represents 8 hours) of *Bacillus cereus* in a nutrient broth with Dimilin present (a) at a concentration of 1.0 g per liter and (b) at a concentration of 0.1 g per liter. Growth rate of the same bacterium in the control flask is given for comparison.




*Figures 11-3a, b.* Growth rate (each numbered time interval represents 8 hours) of (a) *Bacillus cereus* in a nutrient broth with Dimilin present at a concentration of 0.01 g per liter and (b) *Bacillus subtilis* in a nutrient broth with Dimilin present at a concentration of 1.0 g per liter. Growth rate of the same bacteria in the control flask is given for comparison.




Figures 11-4a, b. Growth rate (each numbered time interval represents 8 hours) of *Bacillus subtilis* in a nutrient broth with Dimilin present (a) at a concentration of 0.1 g per liter and (b) at a concentration of 0.01 g per liter. Growth rate of the same bacterium in the control flask is given for comparison.

***Table 11-1a.*** Growth characteristics of *Dictyostelium discoideum* when subjected to different concentrations of Dimilin. Data given are sample means. Initial spore concentrations were 3000 spores per plate. Differences in means between treatment groups and control are not significant.

| Characteristic | Sample | Dimilin concentration (g/l) 1.0 | | 0.1 | | 0.01 | | 0.001 | | H2O control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD |
| Cells/grid | 6 | 43.8 | 35.3 | 19.7 | 8.1 | 36.7 | 21.4 | 19.0 | 16.3 | 22.7 | 19.0 |
| Sporocarps/cm2 | 9 | 14.7 | 9.9 | 15.9 | 7.0 | 10.8 | 5.1 | 12.2 | 8.3 | 13.9 | 8.1 |
| Pseudoplasmodium length (units) | 20 | 20.9 | 9.0 | 15.5 | 6.6 | 21.3 | 7.6 | 19.8 | 5.3 | 17.6 | 5.7 |

***Table 11-1b.*** Growth characteristics of *Dictyostelium minutum* when subjected to different concentrations of Dimilin. Data given are sample means. Initial spore concentrations were 15000 spores/plate.

| Characteristic | Sample | Dimilin concentration (g/l) 1.0 | | 0.1 | | 0.01 | | 0.001 | | H2O control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD |
| Cells/grid | 9 | 56.2 | 50.1 | 22.0 | 23.1 | 52.2* | 46.0 | 40.3* | 34.1 | 10.0 | 14.2 |
| Sporocarps/cm2 | 9 | 120.0 | 65.6 | 106.0 | 53.0 | 89.4 | 37.1 | 87.2 | 31.7 | 97.8 | 44.3 |

*Student's t-test indicates a significant difference exists when this value is compared to the corresponding value obtained for the control ($P \leq 0.05$).

***Table 11-1c.*** Growth characteristics of *Polysphondylium violaceum* when subjected to different concentrations of Dimilin. Data given are sample means. Initial spore concentrations were 16000 spores/plate. Differences in means between treatment groups and control are not significant.

| Characteristic | Sample | Dimilin concentration (g/l) 1.0 | | 0.1 | | 0.01 | | 0.001 | | H2O control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD | $\bar{x}$ | SD |
| Cells/grid | 6 | 45.8 | 17.8 | 27.8 | 16.9 | 45.0 | 33.0 | 21.7 | 20.8 | 32.3 | 40.9 |
| Sporocarps/cm2 | 9 | 13.9 | 5.2 | 13.2 | 5.6 | 14.7 | 5.2 | 10.8 | 4.1 | 12.0 | 5.5 |

*Table 11-2.* Species of cellular slime molds isolated from soil-litter samples collected from watersheds of the Fernow Experimental Forest, 1989-1993.

Acytostelium leptosomum Raper
Dictyostelium aureo-stipes Cavender, Raper and Norberg
Dictyostelium discoideum Raper
Dictyostelium giganteum Singh
Dictyostelium lacteum van Tieghem
Dictyostelium minutum Raper
Dictyostelium mucoroides Brefeld
Dictyostelium sphaerocephalum (Oud.) Sacc. and March.
Polysphondylium candidum Hagiwara
Polysphondylium pallidum Olive
Polysphondylium tenuissimum Hagiwara
Polysphondylium violaceum Brefeld

*Table 11-3.* Mean values for numbers of cellular slime molds (clones per gram wet soil) in the Fernow Experimental Forest, 1989-93.

| | Watershed | | | |
|---|---|---|---|---|
| Year | 1 | 4 | 7 | 13 |
| 1989 (2)[a] | 457 | 612 | 164 | 368 |
| 1990 (3) | 352 | 262 | 237 | 562 |
| 1991 (3) | 269 | 281 | 205 | 309 |
| 1992 (4) | 309 | 308 | 275 | 390 |
| 1993 (1, spring only) | 243 | 215 | 82 | 267 |
| Mean | 326 | 336 | 193 | 379 |

[a] number of sampling dates

*Table 11-4.* Average species richness for cellular slime molds based on sets of samples collected in the Fernow Experimental Forest, 1989-1993.

| Year | Watershed 1 | 4 | 7 | 13 |
|---|---|---|---|---|
| 1989 (2)[a] | 6.0 | 8.0 | 6.0 | 6.5 |
| 1990 (3) | 7.0 | 6.7 | 5.7 | 7.3 |
| 1991 (3) | 6.7 | 8.0 | 6.3 | 7.3 |
| 1992 (4) | 6.8 | 8.0 | 6.8 | 7.5 |
| 1993 (1, spring only) | 6.0 | 10.0 | 6.0 | 8.0 |
| Mean and SD | 6.5 ± 1.3 | 7.9 ± 1.9 | 6.2 ± 1.4 | 7.5 ± 1.6 |

[a] number of sampling dates

*Table 11-5.* Mean values (± SD) for numbers (N x 105) of bacteria recorded from samples of leaf litter collected from the Fernow Experimental Forest, 1991-1993.

| Date | Watershed 1 | 4 | 7 | 13 |
|---|---|---|---|---|
| May 7, 1991 | 9.0 ± 3.5 | 5.3 ± 2.4 | 6.7 ± 2.4 | 8.8 ± 6.6 |
| May 14, 1991 | 21.4 ± 2.3 | 9.1 ± 3.8 | 6.9 ± 1.4 | 12.2 ± 6.7 |
| Sept 12, 1991 | 4.1 ± 4.9 | 4.5 ± 3.5 | 7.4 ± 3.7 | 6.8 ± 2.1 |
| May 10, 1992 | 6.4 ± 5.3 | 7.4 ± 9.5 | 1.2 ± 1.8 | 5.2 ± 6.3 |
| May 19, 1992 | 15.0 ± 16.0 | 1.9 ± 2.2 | 8.4 ± 20.0 | 5.5 ± 10.0 |
| Sept 12, 1992 | 31.5 ± 47.0 | 14.4 ± 17.9 | 13.8 ± 13.6 | 15.0 ± 22.3 |
| Oct 31, 1992 | 3.3 ± 8.1 | 4.2 ± 7.1 | 17.1 ± 35.5 | 0.9 ± 1.6 |
| May 5, 1993 | 2.0 ± 1.2 | 0.9 ± 0.8 | 1.0 ± 1.2 | 1.2 ± 1.1 |

*Table 11-6.* Levels of soil moisture (percent dry weight) based on samples collected from the Fernow Experimental Forest, 1989-1993.

| Year | Watershed 1 | 4 | 7 | 13 |
|---|---|---|---|---|
| 1989 (2)[a] | 39.0 | 39.7 | 42.2 | 38.0 |
| 1990 (3) | 44.9 | 46.5 | 48.5 | 46.8 |
| 1991 (3) | 37.8 | 45.4 | 40.8 | 42.7 |
| 1992 (4) | 42.4 | 49.8 | 44.4 | 42.7 |
| 1993 (1, spring only) | 41.9 | 47.3 | 40.6 | 40.4 |

[a] number of sampling dates

# *Chapter 12. Decomposer And Ectomycorrhizal Fungi*

**Alan Iskra**
**Denise Binion**
**Patricia Gundrum**
**Steven L. Stephenson**

## Introduction

Saprophytic fungi utilize nutrients from plant debris for their growth and reproduction until the substrates are depleted. The fungal growth stage is composed entirely of thread-like elements (hyphae) that branch throughout the substrate. Extracellular digestive enzymes produced by fungi breakdown complex debris to simple carbohydrates that are absorbable by the fungus. When conditions are favorable, the fungus forms a fruiting body, wind-blown spores are carried to new substrates, and the process begins anew.

The dependence of forest trees on another type of fungus called a symbiotic or mycorrhizal fungus has long been known. These fungi are usually unable to degrade enzymatically the complex carbohydrates of most organic debris, and instead rely on living trees for their energy. A complex and permanent union becomes established between tree roots and hyphae. The tree supplies the fungus directly with simple photosynthate sugars; in return, the fungus efficiently absorbs nutrients and water from the soil and translocates these to the tree. Mycorrhizae extend the root systems of their hosts because of the growth and branching habit of hyphae.

Diflubenzuron (DFB) might affect the associations between saprophytic fungi and their food, forest trees and their associated mycorrhizal fungi, or both. Similar to the arthropod exoskeleton, the main component of the fungal hyphal wall is chitin. Deposition of Dimilin, as throughfall onto plant debris during typical operational spray applications, might affect saprophytic and mycorrhizal fungi if this growth regulator disrupts fungal chitin formation.

The purpose of this study was to obtain data on composition, richness, diversity, equitability, and abundance of plant debris decomposers and mycorrhizal macrofungi before and after Dimilin 4L application. Dimilin 4L was applied to watersheds 1 and 13 (WS 1 and WS 13) on May 16, 1992, while two other watersheds (WS 4 and WS 7) served as untreated. Macrofungi belong to the class Basidiomycetes and form large fruiting bodies or sporocarps (0.25 to 10.0 cm) above ground. Primary emphasis was on quantifying the productivity and relative dominance of species of macrofungi known or suspected to be plant debris decomposers or to form ectomycorrhizae.

## Field Study Methods

Previous studies of macrofungal community structure (e.g., Bills and others 1986) have used sporocarp density and biomass to quantify productivity. This same approach was used in the present study. Five permanent 100 $m^2$ study plots were established in each of the four watersheds on the Fernow Experimental Forest during June of 1989. These study plots, representing a total sampling area of 2,000 square meters, were visited at 14 $\pm$ 1-day intervals during July 1 - October 8 from 1989 through 1992. On each visit, all sporocarps belonging to those families of Basidiomycetes known to form ectomycorrhizae (mostly species of *Amanita*, *Russula*, and *Scleroderma* in the families Amanitaceae, Russulaceae, and Sclerodermataceae, respectively), and those that are debris saprophytes (species of *Collybia*, *Marasmius*, and *Mycena*, all in the family *Tricholomataceae*), were collected and tallied.

Additional information on fruiting body occurrences for 1993 and 1994 on each of the watersheds also is provided. This information was taken from a survey conducted by Stephenson and others (1994). These data were included to assess any additional effects of DFB on established debris decomposers from the residual amounts of DFB found on newly fallen canopy leaves. These data also provide insight into any long-range effects of DFB on fungal colonization that possibly might occur well beyond the 1992 growing season. The numbers of fungi collected do not, however, represent fruiting body quantities as would be found within the 5 individual study plots within each watershed. Rather, fungi were collected throughout each of the watersheds. Even though the numbers of fungi cannot be compared directly between both surveys, the numbers in either case provide some indication of their persistence at the family level following Dimilin 4L application.

The occurrence of fungi in the four watersheds is described in two ways. First, numbers of individual fruiting bodies representing a particular family are directly compared during the years 1989 through 1992. Incorporation of the 1993 and 1994 data is done using a coefficient of community (CC) index (Mueller-Dombois and Ellenberg 1974) whereby fungal families associated with the different watersheds in different years can be compared

$$\text{coefficient of community (CC) index} = \frac{2c}{a+b}$$

where a = the total number of families in the first community (i.e., a particular watershed in a given year), b = the total number of families in the second community, and c = the number of families common to both communities. The value of CC indices range from 0 (when no families are present in both communities) to 1.0 (when all families are present in both communities).

To evaluate seasonal variation in site moisture conditions, a gypsum block was placed in the soil (at a depth of 10 cm) near the center of each study plot in each of the four watersheds. Readings of soil moisture (made with the use of a Bouyoucos soil moisture meter) were taken in all study plots on each visit.

The woody vegetation of each study plot was surveyed during September and October 1989. Diameters at breast height (d.b.h.) of all live stems of trees $\geq 2.5$ cm were recorded by species. From these data, values for relative basal area and relative density were calculated and then used to derive importance values. For each study plot, the importance value for a particular woody plant species was calculated as one-half the sum of relative density and relative basal area for that species (Table 12-1).

## *Results and Discussion*

A total of 2,659 fruiting bodies of macrofungi were collected from the treated and untreated watersheds from 1989-1992. Table 12-2 presents data on numbers of fruiting bodies (identified to fungal families) collected 3 years before and the year of Dimilin 4L application to the two treated watersheds. Table 12-3 provides similar data for the untreated watersheds. Data representing fungi collected in 1993 and 1994 are also provided in Tables 12-2 and 12-3. Twenty-two families of fungi were identified. Since the vegetation on all four watersheds is represented by varying ages, densities, and sizes of trees, as well as by different types and spatial characteristics of plant debris, considerable differences in fruiting body types and numbers were expected. The different

species of trees found on the watersheds also directly affect the type of mycorrhizae encountered, i.e., ectomycorrhizae versus endomycorrhizae (Table 12-4). In addition, other factors (e.g., aspect, soil structure, and soil moisture) affect the numbers and species composition of fruiting bodies. The major species of ectomycorrhizal fungi and debris-decomposing fungi are recorded in Tables 12-5 and 12-6, respectively. Additional information on the fungi of the general study area is provided by Stephenson and others (1994).

Coefficient of community indices are listed in Table 12-7. Values were calculated for pairwise combinations of watershed data both prior to and after Dimilin 4L application. A comparison of the coefficient of community indices calculated using data from watershed 1 in the periods of 1989-1991 and 1992-1994 shows little evidence of any change. In fact, the mean values for the two time periods were exactly the same (0.631). A similar situation occurred on the second treatment watershed (WS 13), where the average value was 0.642 before Dimilin 4L application and 0.632 after application. Community of coefficient indices for the two untreated watersheds also were rather similar. Watershed 4 had an average value of 0.698 for 1989-1991 and a value of 0.694 for 1992-1994. The values for WS 7 were almost identical, with an average of 0.709 for 1989-1991, and 0.701 for 1992-1994.

The average coefficients of community indices on the treated watersheds, before and after Dimilin 4L application, were 0.636 and 0.631, respectively. Average coefficients on both untreated watersheds were 0.703 for years prior to 1992 and 0.701 for 1992-1994.

It is apparent from these data that little or no change occurred for fungi at the family level both within each watershed and also between treated and untreated watersheds as a consequence of Dimilin 4L application.

Selected fruiting bodies occurring on the study watersheds are described below by family origin.

***Hygrophoraceae***

The Hygrophoraceae are a group of often highly colorful fungi with gills that are usually thick and waxy in appearance. Unlike many of the fungi that live deeper within soil or woody debris, many of these fungi colonize leaves and a few are mycorrhizal. As such, an effect of DFB might be expected. Most of the Dimilin 4L application in May 1992 that reached the ground was deposited on the layer of old decaying leaves on the forest floor. Presumably, fungi directly associated with these leaves would be most noticeably affected. The numbers of fruiting bodies in this family actually increased on both WS 1 and WS 13 (Table 12-2). A similar increase occurred on the two untreated watersheds and was most noticeable on WS 7. DFB apparently does not affect this ecologically important family of plant decomposing fungi.

***Lycoperdaceae***

Similar to the Hygrophoraceae, the Lycoperdaceae inhabit leaf debris and humus and wood, and thus are more likely to be immediately affected by Dimilin 4L application than other groups of fungi. The member of this family that was most often found on the watersheds is a small, white, spiny stalked puffball (*Lycoperdon perlatum*). A dramatic increase of this fungus was noted on WS 1, but a slight decrease occurred in WS 13. Relatively moderate increases were observed on the untreated watersheds (Table 12-3).

*Russulaceae*

The Russulaceae are "typical mushrooms" and their fruiting bodies are sometimes very colorful and often large (0.5 to 2 cm diameter). They were found in all the watersheds and are important because of their ectomycorrhizal relationships with many tree species found on the watersheds. Several species within this family were collected. Both WS 1 and WS 13 showed increases in numbers of fruiting bodies of this fungus following Dimilin 4L application, with numbers comparable to former collection years (Table 12-2). Numbers on WS 4 were both high and consistent, whereas on WS 7 numbers of this family decreased after 1990. The reasons for this trend are unknown.

*Tricholomataceae*

The diverse Tricholomataceae is the single largest taxonomic family of fungi collected and consists of many species including leaf litter decomposers and ectomycorrhizal fungi. Common genera on the watersheds were *Marasmius*, *Mycena*, and *Collybia*. As such, any decreases noted for members of this family on the treated watersheds were considered to be of particular importance. Fruiting bodies were found on both treated watersheds in 1992. On WS 1 in 1992, fruiting body numbers were similar to those recorded in 1989 and 1991 (allowing for the drought conditions present that year) but were much lower than the numbers recorded in 1990 (Table 12-2). Counts in 1992 were comparable with those in all years except 1989 on WS 13, where the numbers were higher (Table 12-2). Numbers of fruiting bodies collected on WS 7, however, were much higher in 1992 than in previous years (Table 12-3). Higher numbers of fruiting bodies for this family might also be expected on the treated watersheds based on the data from the untreated watersheds. In comparison, numbers did not substantially increase on WS 1 or WS 13, but they did not decrease either.

## Conclusions

The most noticeable effect of DFB on fungal fruiting body production was expected to occur during the 1992 growing season. Decomposition of plant debris by fungi begins early in the growing season, and possibly could be affected by the throughfall of DFB during the insecticide application. Further, a substantial amount (upwards to 80 percent, Wimmer and others 1993) of DFB deposited on the canopy leaf surface eventually becomes lost due to rain, wind, and abrasion to plant debris on the forest floor throughout the 1992 growing season. An additional DFB deposition onto decomposing debris can occur during the weathering of newly fallen canopy leaves. This weathering begins in late fall, extends through winter, and continues into the 1993 growing season.

The pooled data for all the study plots within a watershed contained at least some species of trees known to form ectomycorrhizae and the relative abundance of such species ranged from 35 to 59 percent in the four watersheds. As a general observation, species of *Amanita* or *Russula* would seem to be the most abundant ectomycorrhizal fungi present in the four watersheds, whereas species of *Collybia* and *Marasmius* in the family Tricholomataceae are among the most important fungi associated with decomposing leaf litter.

On the basis of total mycorrhizal fruiting bodies produced, only WS 1 had equal numbers (24) of ectomycorrhizal fruiting bodies in 1990 as in 1992 (both years having similar moisture conditions). In contrast, WS 13 had nearly twice as many fruiting bodies in 1990 (60) than in 1992 (34) (Table 12-2). The elevated numbers in 1990, however, are due solely to an unusual increase in the

*Russula* spp. fruiting bodies the same year on this watershed. We saw another unusual *Russula* spp. trend developing in 1989 on WS 7 where twice as many *Russula* spp. (40) were observed, then sharp decreases (23, 5, and 11, respectively) occurred in following years. This observation once again emphasizes the variability associated with fruiting body occurrences on the watersheds. A general trend, however, became apparent when more *Russula* spp. fruiting bodies occurred on WS 13 after Dimilin 4L application (21) than in either 1989 (14) or 1991 (1). In fact, moisture and not Dimilin 4L application appears to be the single limiting factor related to fruiting body occurrence on this watershed for the study period.

The largest number of fungi on all the study sites and in all years were in the family Tricholomataceae, the important plant debris decomposers. In both treated and untreated watersheds, fruiting bodies in this family were more numerous following Dimilin 4L application than in the previous drought year (Tables 12-2 and 12-3). Despite the large number of fruiting bodies (138) found in 1990 on WS 1 the number (72) found in 1992, the treatment year, was similar to that (88) in 1989 (Table 12-2). On WS 13 a similar situation occurred, but elevated numbers of fruiting bodies (119) were found in 1989 instead of 1990. On this watershed, however, the second highest number of Tricholomataceae fruiting bodies (49) occurred after Dimilin 4L application (81) (Table 12-2).

Despite the variability of fungal fruiting, the numbers of fruiting bodies in general indicate: (1) fruiting body production in both ectomycorrhizal and plant decomposing fungi occurred after Dimilin 4L application; (2) changes at the family level did not occur on any treated or untreated watershed; (3) fungi fruiting after Dimilin 4L application does occur within a frequency range that was comparable to one or more of the pretreatment years; and (4) families with low or high numbers of fruiting bodies before Dimilin 4L application had comparable levels of abundance after Dimilin 4L application. The only exception was the wood debris colonizing family Lycoperdaceae whose numbers increased dramatically after Dimilin 4L application, but only on WS 1 (Table 12-2).

General trends at the fungal family level appear to indicate that Dimilin 4L had no effect on mycorrhizal or debris decomposing fungi based on fruiting body counts.

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*Table 12-1.* Composition of the woody vegetation (stems ≥ 2.5 cm DBH) for the four study watersheds, the Fernow Experimental Forest. Values are importance value indices based on 100.

| | Watershed | | | |
|---|---|---|---|---|
| Species | 1 | 4 | 7 | 13 |
| Acer saccharum | 31.9 | 17.3 | 14.7 | 31.4 |
| Tilia americana✓ | 29.9 | 7.4 | - | 1.4 |
| Fagus grandifolia✓ | 9.0 | 30.9 | 0.5 | 1.9 |
| Robinia pseudoacacia | 5.4 | - | 2.9 | - |
| Magnolia acuminata | 0.8 | 4.1 | - | 3.9 |
| Quercus rubra✓ | 3.6 | 20.3 | 9.2 | 28.9 |
| Juglans cinerea✓ | 1.6 | - | - | - |
| Betula lutea✓ | 2.4 | - | - | - |
| Fraxinus americana✓ | - | - | 2.1 | 1.5 |
| Carya glabra✓ | 3.3 | - | - | - |
| Liriodendron tulipifera | 5.0 | 5.7 | 6.9 | 15.7 |
| Ostrya virginiana✓ | 1.3 | - | - | - |
| Betula lenta✓ | 1.8 | - | 22.9 | 5.2 |
| Tsuga canadensis✓ | 1.0 | - | - | - |
| Acer pensylvanicum | - | 9.9 | 4.1 | - |
| Acer rubrum | - | 2.5 | - | 1.0 |
| Cornus florida | - | 0.9 | - | - |
| Hamamelis virginiana | 1.1 | 1.0 | - | - |
| Prunus serotina | - | - | 26.4 | 6.6 |
| Nyssa sylvatica | - | - | 5.1 | - |
| Aralia spinosa | 1.8 | - | - | - |
| Sassafras albidum | - | - | 3.3 | - |
| Amelanchier arborea | - | - | - | 1.0 |
| Quercus alba✓ | - | - | 0.6 | - |
| Quercus velutina✓ | - | - | 0.4 | - |
| Magnolia fraseri | - | - | - | 1.4 |
| Carpinus caroliniana✓ | - | - | 1.6 | - |
| Prunus pensylvanica | - | - | 0.4 | - |
| **Total** | **99.9** | **100.0** | **101.1** | **99.9** |

(✓ = Ectomycorrhizal tree species. Other species form endomycorrhizae.)

*Table 12-2.* Numbers of fruiting bodies representing 20 fungal families on two treated watersheds (WS 1 and WS 13) 3 years before and 2 years after Dimilin 4L application on the Fernow Experimental Forest.

**Watershed 1**

| Family | Year 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|---|
| Amanitaceae | 0 | 0 | 0 | 0 | 0 | 2 |
| Bolbitiaceae | 0 | 3 | 0 | 0 | 0 | 0 |
| Boletaceae | 6 | 9 | 2 | 5 | 4 | 2 |
| Calostomataceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Cantharellaceae | 0 | 0 | 34 | 8 | 0 | 0 |
| Clavariaceae | 0 | 6 | 0 | 1 | 0 | 0 |
| Coprinaceae | 0 | 1 | 4 | 0 | 0 | 0 |
| Cortinariaceae | 7 | 2 | 1 | 0 | 0 | 0 |
| Entolomataceae | 1 | 0 | 0 | 8 | 0 | 0 |
| Hygrophoraceae | 14 | 2 | 0 | 9 | 4 | 1 |
| Leotiaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Lepiotaceae | 1 | 0 | 0 | 0 | 0 | 0 |
| Lycoperdaceae | 1 | 11 | 3 | 53 | 4 | 1 |
| Nidulariaceae | 0 | 2 | 0 | 0 | 0 | 0 |
| Paxillaceae | 0 | 0 | 0 | 1 | 0 | 0 |
| Polyporaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Pyrenomycetaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Russulaceae | 4 | 15 | 6 | 15 | 3 | 6 |
| Sclerodermataceae | 1 | 0 | 0 | 4 | 0 | 0 |
| Strophariaceae | 0 | 0 | 0 | 1 | 0 | 0 |
| Tremellaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Tricholomataceae | 88 | 138 | 56 | 72 | 42 | 30 |
| **Total** | **123** | **190** | **105** | **177** | **55** | **42** |

*Table 12-2.* Continued

**Watershed 13**

| Family | Year 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|---|
| Amanitaceae | 0 | 3 | 0 | 1 | 0 | 0 |
| Bolbitiaceae | 0 | 2 | 0 | 0 | 0 | 0 |
| Boletaceae | 32 | 8 | 18 | 1 | 0 | 2 |
| Calostomataceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Cantharellaceae | 1 | 12 | 5 | 6 | 0 | 1 |
| Clavariaceae | 5 | 21 | 0 | 1 | 3 | 2 |
| Coprinaceae | 23 | 2 | 0 | 3 | 0 | 0 |
| Cortinariaceae | 11 | 0 | 0 | 5 | 0 | 0 |
| Entolomataceae | 1 | 7 | 0 | 0 | 1 | 0 |
| Hygrophoraceae | 4 | 0 | 10 | 14 | 0 | 3 |
| Leotiaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Lepiotaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Lycoperdaceae | 4 | 3 | 9 | 7 | 0 | 0 |
| Nidulariaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Paxillaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Polyporaceae | 0 | 0 | 0 | 0 | 1 | 1 |
| Pyrenomycetaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Russulaceae | 14 | 39 | 1 | 21 | 1 | 5 |
| Sclerodermataceae | 2 | 10 | 0 | 11 | 0 | 0 |
| Strophariaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Tremellaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Tricholomataceae | 119 | 79 | 89 | 81 | 31 | 33 |
| **Total** | **216** | **189** | **132** | **151** | **37** | **47** |

*Table 12-3.* Numbers of fruiting bodies representing 20 fungal families on two untreated watersheds (WS 7 and WS 4) 3 years before and 2 years after Dimilin 4L application on the Fernow Experimental Forest.

**Watershed 4**

| Family | Year 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|---|
| Amanitaceae | 14 | 2 | 0 | 5 | 0 | 4 |
| Bolbitiaceae | 0 | 2 | 0 | 0 | 0 | 0 |
| Boletaceae | 30 | 4 | 19 | 9 | 3 | 3 |
| Calostomataceae | 0 | 2 | 0 | 3 | 0 | 0 |
| Cantharellaceae | 0 | 5 | 0 | 16 | 0 | 2 |
| Clavariaceae | 5 | 5 | 21 | 1 | 2 | 1 |
| Coprinaceae | 1 | 4 | 0 | 5 | 0 | 0 |
| Cortinariaceae | 13 | 9 | 1 | 1 | 1 | 0 |
| Entolomataceae | 3 | 3 | 2 | 0 | 3 | 0 |
| Hygrophoraceae | 9 | 26 | 0 | 22 | 4 | 2 |
| Leotiaceae | 4 | 0 | 9 | 0 | 0 | 0 |
| Lepiotaceae | 2 | 0 | 0 | 0 | 0 | 0 |
| Lycoperdaceae | 27 | 1 | 0 | 15 | 0 | 0 |
| Nidulariaceae | 0 | 0 | 7 | 0 | 0 | 0 |
| Paxillaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Polyporaceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Pyrenomycetaceae | 0 | 5 | 0 | 0 | 0 | 0 |
| Russulaceae | 54 | 42 | 27 | 49 | 14 | 11 |
| Sclerodermataceae | 6 | 30 | 2 | 52 | 2 | 1 |
| Strophariaceae | 0 | 3 | 0 | 0 | 0 | 0 |
| Tremellaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Tricholomataceae | 152 | 85 | 18 | 53 | 20 | 16 |
| **Total** | **320** | **230** | **106** | **231** | **49** | **40** |

*Table 12-3.* Continued.

**Watershed 7**

| Family | Year 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|---|
| Amanitaceae | 3 | 0 | 0 | 1 | 1 | 1 |
| Bolbitiaceae | 0 | 6 | 0 | 0 | 0 | 0 |
| Boletaceae | 5 | 3 | 2 | 4 | 1 | 3 |
| Calostomataceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Cantharellaceae | 0 | 3 | 1 | 6 | 0 | 1 |
| Clavariaceae | 0 | 4 | 0 | 1 | 1 | 1 |
| Coprinaceae | 6 | 2 | 1 | 8 | 0 | 0 |
| Cortinariaceae | 7 | 6 | 4 | 5 | 1 | 2 |
| Entolomataceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Hygrophoraceae | 21 | 5 | 1 | 27 | 5 | 4 |
| Leotiaceae | 0 | 10 | 0 | 0 | 0 | 0 |
| Lepiotaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Lycoperdaceae | 1 | 0 | 5 | 21 | 0 | 0 |
| Nidulariaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Paxillaceae | 0 | 0 | 0 | 1 | 0 | 0 |
| Polyporaceae | 0 | 0 | 0 | 1 | 1 | 0 |
| Pyrenomycetaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Russulaceae | 40 | 23 | 5 | 11 | 4 | 7 |
| Sclerodermataceae | 1 | 2 | 0 | 2 | 0 | 1 |
| Strophariaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Tremellaceae | 0 | 2 | 0 | 0 | 0 | 0 |
| Tricholomataceae | 64 | 37 | 34 | 96 | 11 | 18 |
| **Total** | **148** | **104** | **53** | **184** | **25** | **38** |

*Table 12-3.* Continued

**Watershed 7**

| Family | Year 1989 | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|---|
| Amanitaceae | 3 | 0 | 0 | 1 | 1 | 1 |
| Bolbitiaceae | 0 | 6 | 0 | 0 | 0 | 0 |
| Boletaceae | 5 | 3 | 2 | 4 | 1 | 3 |
| Calostomataceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Cantharellaceae | 0 | 3 | 1 | 6 | 0 | 1 |
| Clavariaceae | 0 | 4 | 0 | 1 | 1 | 1 |
| Coprinaceae | 6 | 2 | 1 | 8 | 0 | 0 |
| Cortinariaceae | 7 | 6 | 4 | 5 | 1 | 2 |
| Entolomataceae | 0 | 1 | 0 | 0 | 0 | 0 |
| Hygrophoraceae | 21 | 5 | 1 | 27 | 5 | 4 |
| Leotiaceae | 0 | 10 | 0 | 0 | 0 | 0 |
| Lepiotaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Lycoperdaceae | 1 | 0 | 5 | 21 | 0 | 0 |
| Nidulariaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Paxillaceae | 0 | 0 | 0 | 1 | 0 | 0 |
| Polyporaceae | 0 | 0 | 0 | 1 | 1 | 0 |
| Pyrenomycetaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Russulaceae | 40 | 23 | 5 | 11 | 4 | 7 |
| Sclerodermataceae | 1 | 2 | 0 | 2 | 0 | 1 |
| Strophariaceae | 0 | 0 | 0 | 0 | 0 | 0 |
| Tremellaceae | 0 | 2 | 0 | 0 | 0 | 0 |
| Tricholomataceae | 64 | 37 | 34 | 96 | 11 | 18 |
| **Total** | **148** | **104** | **53** | **184** | **25** | **38** |

***Table 12-4.*** Relative abundance (percent) of tree species in the four watersheds on the Fernow Experimental Forest by type of mycorrhizal association. Data are derived from importance value indices calculated for all tree species in each watershed.

| | Watershed | | | |
|---|---|---|---|---|
| Type of association | 1 | 4 | 7 | 13 |
| Ectomycorrhizae[a] | 54 | 59 | 35 | 39 |
| Endomycorrhizae | 46 | 41 | 65 | 61 |

[a] Includes all members of Betulaceae, Fagaceae, Juglandaceae, Pinaceae, and Tiliaceae along with one member of the Oleaceae (*Fraxinus americana*) thought to form mycorrhizae with *Boletinellus merulioides*.

***Table 12-5.*** Major species of ectomycorrhizal fungi collected from four watersheds, Fernow Experimental Forest.

| Family of Basidiomycetes | Species |
|---|---|
| Amanitaceae | *Amanita citrina* |
| | *Amanita fulva* |
| | *Amanita vaginata* |
| Boletaceae | *Boletinellus merulioides* |
| Cortinariaceae | *Cortinarius* spp. |
| Russulaceae | *Lactarius camphoratus* |
| | *Russula krombholzii* |
| | *Russula silvicola* |
| | *Russula viresens* |
| Sclerodermataceae | *Scleroderma citrinum* |

***Table 12-6.*** Major species of litter decomposing fungi collected from four watersheds, Fernow Experimental Forest.

| Family of Basidiomycetes | Species |
|---|---|
| Tricholomataceae | *Clitocybe gibba* |
| | *Clitocybe* sp. |
| | *Collybia confluens* |
| | *Collybia dryophila* |
| | *Marasmius capillaris* |
| | *Marasmius siccus* |
| | *Mycena luteopallens* |
| | *Strobilurus conigenoides* |

*Table 12-7.* Coefficient of community indices calculated for pairwise combinations of individual watersheds, Fernow Experimental Forest.

| | Before Treatment | | | After Treatment | | |
|---|---|---|---|---|---|---|
| **Watershed 1 (treated)** | | | | | | |
| | **1989** | **1990** | **1991** | **1992** | **1993** | **1994** |
| **1989** | ------ | 0.600 | 0.625 | 0.700 | 0.714 | 0.666 |
| **1990** | ------ | ------- | 0.667 | 0.545 | 0.625 | 0.589 |
| **1991** | ------ | ------- | ------- | 0.556 | 0.667 | 0.615 |
| **1992** | ------ | ------- | ------- | ------- | 0.625 | 0.588 |
| **1993** | ------ | ------- | ------- | ------- | ------- | 0.909 |
| **1994** | ------ | ------- | ------- | ------- | ------- | ------- |
| **Watershed 13 (treated)** | | | | | | |
| | **1989** | **1990** | **1991** | **1992** | **1993** | **1994** |
| **1989** | ------ | 0.720 | 0.706 | 0.909 | 0.500 | 0.667 |
| **1990** | ------ | ------- | 0.500 | 0.880 | 0.421 | 0.476 |
| **1991** | ------ | ------- | ------- | 0.706 | 0.364 | 0.769 |
| **1992** | ------ | ------- | ------- | ------- | 0.375 | 0.667 |
| **1993** | ------ | ------- | ------- | ------- | ------- | 0.667 |
| **1994** | ------ | ------- | ------- | ------- | ------- | ------- |
| **Watershed 4 (untreated)** | | | | | | |
| | **1989** | **1990** | **1991** | **1992** | **1993** | **1994** |
| **1989** | ------ | 0.774 | 0.727 | 0.800 | 0.762 | 0.667 |
| **1990** | ------ | ------- | 0.593 | 0.800 | 0.615 | 0.615 |
| **1991** | ------ | ------- | ------- | 0.571 | 0.824 | 0.588 |
| **1992** | ------ | ------- | ------- | ------- | 0.700 | 0.800 |
| **1993** | ------ | ------- | ------- | ------- | ------- | 0.750 |
| **1994** | ------ | ------- | ------- | ------- | ------- | ------- |
| **Watershed 7 (untreated)** | | | | | | |
| | **1989** | **1990** | **1991** | **1992** | **1993** | **1994** |
| **1989** | ------ | 0.636 | 0.824 | 0.818 | 0.706 | 0.778 |
| **1990** | ------ | ------- | 0.667 | 0.692 | 0.571 | 0.727 |
| **1991** | ------ | ------- | ------- | 0.762 | 0.625 | 0.706 |
| **1992** | ------ | ------- | ------- | ------- | 0.762 | 0.818 |
| **1993** | ------ | ------- | ------- | ------- | ------- | 0.824 |
| **1994** | ------ | ------- | ------- | ------- | ------- | ------- |

# *Chapter 13. Terrestrial Fungi In Leaf Litter*

**Patricia Gundrum**
**Alan Iskra**
**Mary J. Wimmer**

## Introduction

Because chitin is found in the cell walls of many fungi (Moore-Landecker 1990), there is a concern about adverse effects diflubenzuron (DFB) may have on these organisms. When Dimilin is aerially applied to leaf surfaces, it adheres to the waxy layer (Wimmer and others 1993). When the leaves fall to the ground in autumn, leaf decomposers, including fungi, utilize these leaves as a nutrient source (Baker and Snyder 1970).

A study on cotton (Bull and Ivie 1978) showed DFB to be persistent through 9 months in soil. DFB also persisted as a surface deposit on foliage and was subject to washoff in heavy rain. Mansager and others (1979) found only trace amounts of $^{14}C$ labeled on leaves, stems, bolls, or squares of newly emerging cotton tissue 48 days after treatment with $^{14}C$ DFB.

Feofilova and others (1981) explored the effect of DFB on carotene production in *Blakeslea trispora* (Thax.) and found that in this fungus DFB did not affect chitin synthetase, but it inhibited carotene synthesis. Mushrooms grown in compost treated with DFB to control fungus gnats contained no detectable level of DFB (Argauer and Cantelo 1984, White 1986). Exposure of $^{14}C$ DFB to a culture of *Pseudomonas putida* (Trevisan) produced no evidence of degradation of the molecule upon extraction using thin-layer chromatography (TLC) and radioautography after a 6-h exposure at 30° C (Metcalf and others 1975). Dimilin altered normal development of the inner and outer spore wall, as well as the spore hairs at 400 ppm in *Streptomyces bambergiensis* Muller (Smucker and Simon 1986).

Gas chromatographic analysis was used to show the breakdown products of DFB by four soil microorganisms. *Rhodotorula* sp., *Cephalosporium* sp., and *Penicillium* sp., were able to break the urea bridge, but could not utilize DFB as a sole carbon source. Seuferer and others (1979) documented that *Fusarium* sp. was able to utilize DFB as a sole carbon source, and that rapid breakdown would occur by adding an acetone solution to induce the production to enzymes necessary for DFB metabolism. An environmental fate study by Booth and others (1987) showed no significant differences between the growth of six fungal species on nutrient medium with various concentrations of Dimilin 25-W and controls.

The objective of this study was to determine whether Dimilin 4L in the agar media of leaf inhabiting fungi would inhibit growth.

## Laboratory Study Methods

### *Collection Of Leaf Samples*

Leaf samples were collected from six locations at each of the four study watersheds (WS 1, WS 4, WS 7 and WS 13) from March 1991 through April 1992. The top 5.0 cm of leaf litter were collected every 2 weeks from March through September 1991 and March and April 1992 and placed in 5-gallon plastic bags. Collections were also made once per month from October 1991 through February 1992.

In the laboratory, 40 leaves per watershed were randomly selected and rinsed under running water to remove extraneous debris. Twenty leaves were from red oak (*Quercus rubra* L.); 10, American beech (*Fagus grandifolia* L.); and 10, species such as red maple (*Acer rubra* L.), sugar maple (*A. saccharum* Marsh.), and yellow-poplar (*Liriodendron tulipifera* L.). Three 1.0-$cm^2$ sections from each leaf (leaf at the midvein, on the leaf margin, and between the midvein and leaf margin) were surface sterilized with 70 percent ethyl alcohol for 1 minute and 10 percent chlorox solution for 1 minute to remove surface contaminants. The three leaf discs were plated on either malt agar (MA) medium or potato-dextrose agar (PDA) medium.

Subcultures were made from emerging fungal colonies within 3 to 10 days. The subcultured fungi were allowed to colonize the growing medium. Identification of each morphologically distinct isolate was attempted under the phase-contrast microscope. Names and numbers were delegated to the identified isolates, and numbers were assigned to the isolates that were not identified. Isolates were again subcultured onto MA and PDA media with 0, 10, 25, 100, and 1,000 ppm liquid formulated Dimilin 4L (suspended in the agar). The different concentrations were achieved by adding distilled water to the premixed Dimilin 4L formulation and mixing the desired concentration into nutrient medium after autoclaving and cooling to 43°C. Dimilin is added after autoclaving because DFB is heat labile and has been shown to be at least partially lost under normal autoclaving conditions (Wimmer, pers. comm.). Dimilin 4L was added before the nutrient medium solidified, and fungal isolations were made. Growth on untreated control and treated plates was measured radially within 21 days and based on a scale from 1 to 4, with 1 being within 1 cm of the inoculum plug; 2, fungal growth covering half of the Petri dish; 3, growth covering three-quarters of the Petri dish; and 4, mycelial growth covering the entire Petri dish (Fig. 13-1).

***Analysis Of Laboratory-Cultural Fungi For DFB Uptake***

To measure the uptake of DFB into that part of the fungus protruding above the agar plate, the following procedure was followed for four fungal isolates.

1. Mycelia were transferred to a mortar and pestle using micro-scissors, a spatula, forceps or a scalpel, depending on the height of the culture above the agar. Care was taken not to touch the agar, because it contained 100 ppm DFB. The concentration of DFB in the agar was confirmed by converting solid weight of agar to initial liquid volume, and then extracting and measuring the DFB from a known weight of solidified agar. Plates of each agar type were analyzed in random groups of two-four plates combined per sample.

2. Mycelia were ground for 2 minutes. Then 10 ml of acetone was added, along with 50 microliters of 500 ppm deuterated DFB as an internal standard.

3. Samples were ground for 1 minute more and poured into a 50-ml glass centrifuge tube.

4. Mortar and pestle were rinsed with 10 ml of acetone and this wash was added to the centrifuge tube.

5. The sample was centrifuged at 1700 rpm for 2 minutes, and the supernate was decanted into a 250-ml round bottom flask.

6. Steps 3, 4, and 5 were repeated two more times (for a total of three washes). Each time 10 ml of acetone was added to the pellet for transferring back into the mortar and pestle for grinding.

7. After the final centrifuging, the weight of a clean watch glass was recorded and the mycelia pellet was spread out on it to dry overnight. The dry weight of the pellet on the watch glass was recorded the next day.

8. The 250-ml round bottom flask was placed on a rotary evaporator to remove the acetone. The residue was transferred in methylene chloride through a sodium sulfate funnel into a 50-ml pear bottom flask.

9. After removing the methylene chloride by rotary evaporation, the sample was taken up in 75 microliters of acetonitrile by vortexing and was placed into a glass vial for storage at 4° C prior to GC and mass spectrometric analysis for DFB as described by Wimmer and others (1991).

Growth (by categories) on untreated control and treated plates were compared using the SAS ANOVA procedure and Duncan's multiple-range test.

## *Results*

Ninety-two morphologically distinct fungi were isolated from senescing leaves in the study watersheds. Seventy-two predominately-occurring fungi were tested with the different concentrations of Dimilin 4L. Some of the less frequently occurring isolates were also tested. The positively identified isolates were all tested with all four concentrations of Dimilin 4L (10, 25, 100 and 1000 ppm) (Table 13-1). Since only about 33 g AI of Dimilin is applied aerially per hectare in a gypsy moth suppression project, 100 ppm on nutrient agar in a Petri dish is a high dose.

Fourteen of the 35 isolates tested showed some significant difference ($P \leq 0.05$) in growth between the five concentrations of Dimilin 4L (Table 13-1). Seven of the fourteen showed a decrease in growth at the higher concentrations (100 and 1000 ppm). The remaining 7 isolates that showed significant differences also showed a spurious effect that was not consistent with the trend of decreasing radial growth with increasing concentration of Dimilin 4L. This effect could be attributed to causes other than Dimilin (e.g. natural variation in fungal growth under laboratory conditions). Significant differences between the higher concentrations (100 and 1000 ppm) and the lower concentrations (0, 10 and 25) could well be realized by the megadoses applied to the isolate in culture. The megadoses are unrealistic and the concentrations would never be applied during an operational project for managing gypsy moth.

The fungi samples varied in the ease with which they could be removed from the agar without agar contact. This ease did not correlate with finding or not finding DFB; in other words, an easily removable sample did not necessarily have less DFB than a less easily removable one. More importantly, there was consistency within each fungal type. The findings are shown on the next page.

| Sample | Number agar plates | Dry weight (g) | DFB µg | DFB ppm |
|---|---|---|---|---|
| *Alternaria sp.* | 3 | 0.024 | UD[a] | UD |
| | 3 | 0.033 | UD | UD |
| | 2 | 0.051 | UD | UD |
| *Helminthosporium sp.* | 3 | 0.184 | UD | UD |
| | 3 | 0.158 | UD | UD |
| | 3 | 0.159 | UD | UD |
| *Trichoderma sp.* | 4 | 0.186 | 2.68 | 14.4 |
| | 3 | 0.155 | 2.49 | 16.1 |
| | 3 | 0.196 | 0.69 | 7.2 |
| *Pestalotia sp.* | 3 | 0.062 | 1.78 | 28.7 |
| | 3 | 0.061 | 1.58 | 25.9 |
| | 3 | 0.028 | 0.49 | 17.5 |
| | 3 | 0.056 | 1.70 | 30.4 |

[a]UD indicates below detectability limit.

Some of the fungal types did not appear to take up DFB at detectable levels, whereas some do take up significant amounts. In all cases, no inhibition of growth was seen despite the fact that the fungi must produce chitin during their growth and development. Two possible explanations that could explain the observations of uninhibited growth in those species that take up DFB: 1) the fungal chitin synthetase system unlike the arthropod enzyme system, is not inhibited by DFB, and (2) DFB sensitive fungal enzymes are localized in an area of the fungal cell inaccessible to the DFB. The exact mechanism of uptake and location of the insecticide within the fungal system is unknown.

## Discussion

Although this is, by no means, an extensive study of all leaf inhabiting and degrading fungi, the consistent isolations made from March 1991 through September 1992 produced the morphologically distinct isolates listed in this study. This is a good cross-section representing the leaf litter decomposers in the four watersheds. Because DFB is known to inhibit chitin synthesis in arthropod cuticles, the high and even the moderate doses of Dimilin 4L applied to the growing medium would be expected to inhibit chitin synthetase and, hence, cell wall formation in fungi as well. There is a general lack of understanding of the different mechanisms involved in the synthesis of chitin and the difference between chitin synthesis in fungal cell walls and chitin synthesis in the arthropod exoskeleton. Some speculation has been made regarding the possible inhibition of enzymes by DFB through indirect activity of particular enzymes other than chitin synthetase (Flemming and others 1982). Regardless of the reasons or the biochemistry involved, the leaf inhabiting fungi do not seem to be adversely affected when growing on nutrient medium amended with varying low concentrations (10, 25 ppm) of Dimilin 4L.

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***Table 13-1.*** The maximum radial growth achieved by a representative species for various genera of terrestrial fungi from leaf litter on the Fernow Experimental Forest growing on agar from pure culture media amended with various concentrations of diflubenzuron.

| | Maximum radial growth *Various Concentrations (ppm) of DFB* | | | | |
|---|---|---|---|---|---|
| | 0 | 10 | 25 | 100 | 1000 |
| **Class - Basidiomycetes** | | | | | |
| •Scleroderma | 4[a] | 3 | 3 | 4 | 3 |
| Omphalina | 2 | 2 | 2 | 3 | 2 |
| Marasmius | 3 | 2 | 3 | 3 | 3 |
| Schizophyllum | 3 | 3 | 3 | 3 | 3 |
| •Amanita | 2 | 2 | 2 | 1 | 1 |
| •Cenococcum | 2 | 2 | 2 | 1 | 1 |
| **Class - Blastomycetes** | | | | | |
| Candida | 3 | 3 | 3 | 3 | 3 |
| Streptomyces | 3 | 3 | 3 | 3 | 3 |
| Sporobolomyces | 3 | 3 | 3 | 3 | 3 |
| **Class - Hyphomycetes** | | | | | |
| Aureobasidium | 4 | 4 | 4 | 4 | 4 |
| Monilia | 3 | 3 | 3 | 3 | 3 |
| Botrytis | 4 | 4 | 4 | 4 | 4 |
| Cladosporium | 4 | 4 | 4 | 4 | 4 |
| Alternaria | 4 | 4 | 4 | 4 | 4 |
| Humicola | 3 | 3 | 3 | 3 | 3 |
| Menispora | 3 | 3 | 3 | 3 | 3 |
| Gliocladium | 4 | 4 | 4 | 4 | 4 |
| Trichoderma | 4 | 4 | 4 | 4 | 4 |
| Aspergillus | 4 | 4 | 4 | 4 | 4 |
| Cephalosporium | 4 | 4 | 4 | 4 | 4 |
| Penicillium | 4 | 4 | 4 | 4 | 4 |
| •Calcarisporium | 3 | 3 | 3 | 2 | 2 |
| Bispora | 4 | 4 | 4 | 4 | 4 |
| Stemphylium | 3 | 3 | 2 | 3 | 2 |
| •Acrospeira | 3 | 3 | 2 | 2 | 2 |
| Myrothecium | 3 | 2 | 3 | 2 | 2 |
| •Thozetellopsis | 3 | 3 | 2 | 2 | 2 |
| Volutella | 3 | 3 | 2 | 3 | 2 |
| Epicoccum | 4 | 4 | 3 | 4 | 4 |
| Stilbum | 3 | 3 | 3 | 3 | 3 |
| •Graphium | 2 | 2 | 2 | 1 | 1 |
| **Class - Coelomycetes** | | | | | |
| Phoma | 3 | 2 | 3 | 2 | 2 |
| Actinopelte | 2 | 2 | 2 | 2 | 2 |
| Septoria | 2 | 2 | 2 | 2 | 2 |
| Pestalotia | 4 | 4 | 3 | 4 | 3 |

[a] *radial growth of fungal culture represented from 0 to 4. Where 0 = no growth and 4 = covering all media in plate.*

• *significant difference ($P \leq 0.05$) in growth attributable to Dimilin 4L*

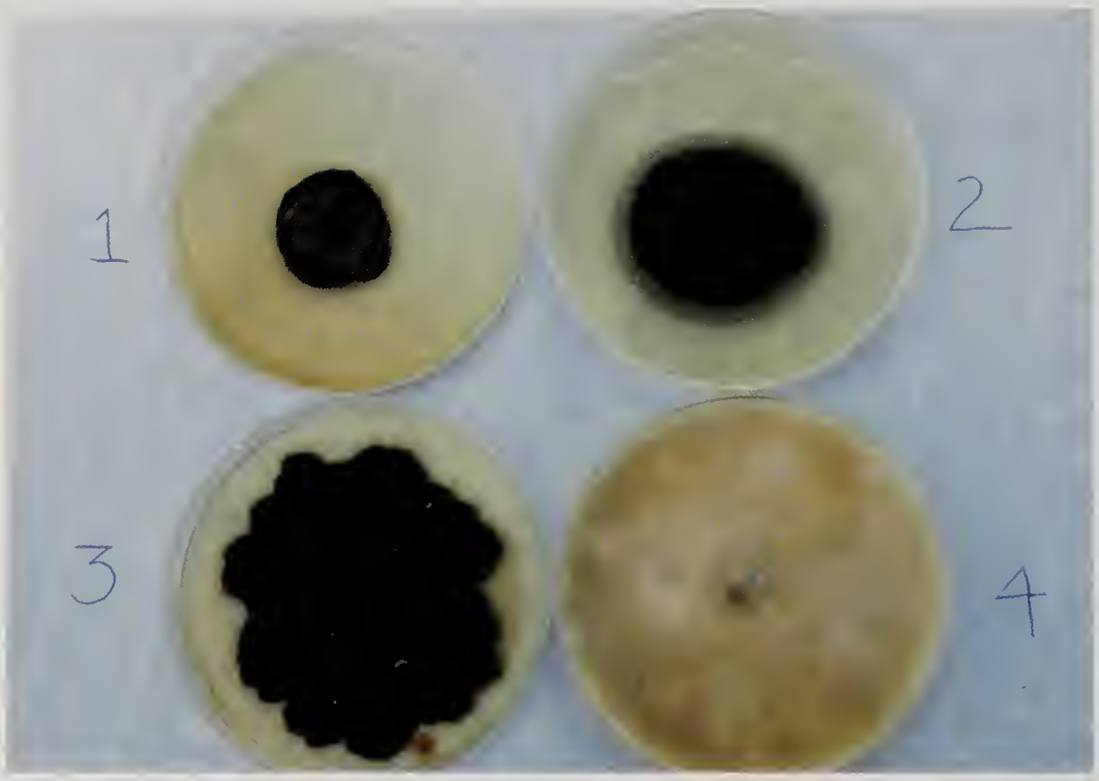


***Figure 13-1.*** Categories of radial growth achieved by terrestrial fungi from leaf litter growing on agar from pure culture media amended with various concentrations of diflubenzuron, Fernow Experimental Forest.

# *Chapter 14. Microorganisms In Soil*

**Alan Sexstone**

## Introduction

Measurement of microbial biomass carbon can be used to follow temporal changes in soil bacteria and fungi without the need for selective culture. Estimates of biomass carbon can reveal differences in total microbial populations between soils. Respiration, measured as $CO_2$ production, gives a good general picture of aerobic microbial activity. Denitrification is an anaerobic respiratory process carried out by many soil heterotrophic bacteria and can be measured as gaseous N production. Net nitrogen mineralization, measured as ammonia plus nitrate accumulation, can reveal negative impacts on microorganisms responsible for ammonification and nitrification. Comparison of all these processes in treated and untreated soils can reveal potential impacts of diflubenzuron (DFB) on a wide spectrum of the soil microbiota.

The purpose of this preliminary study was to quantify potential changes in microbial biomass carbon, respiration, denitrification, and nitrogen mineralization after DFB treatment of soil samples obtained from the Fernow Experimental Forest. These parameters were chosen as good general indicators of key microbial activities that are central to nutrient regeneration and cycling within forest soils. We also examined soils treated with gypsy moth frass to determine the potential impacts of defoliation on soil microbial processes, particularly rates of nitrogen transformation.

## Laboratory Study Methods

In July 1990, 239 undisturbed soil cores (7.5 cm by 7.5 cm) were obtained from WS 13 using a slide hammer and bulk density corer. Soil cores were transferred to pint-sized wide-mouth canning jars in a way that maintained the structural integrity of the samples. Structural integrity was important, because the rates of microbial activities are influenced by diffusional constraints imposed by soil structure. Cores were immediately returned to the laboratory where they were held at 5° C until initiation of experiments, which began within 48 h.

All cores were weighed to determine their moisture content, numbered, then randomly assigned to one of three treatment groups: untreated control, treated DFB, and frass. Untreated control cores were moistened to 65 percent water filled porosity (percent WFP) by surface addition of sterile distilled water. This percent WFP is considered to be optimum for microbial activity in soil. Dimilin treated cores first received 4.418 micrograms of DFB (formulated as a wettable powder) dispersed in 1 ml of water and added dropwise to the surface of the soil core (44.2 $cm^2$), followed by adjustment with water to 65 percent WFP. The third group of cores was treated by adding 1.6 g of freshly collected frass to the surface of each core. Frass was collected during a 48-h period from a 41-$m^2$ area during active gypsy moth defoliation in the West Virginia University Forest. Following frass additions, these cores were also adjusted to 65 percent WFP. Cores from all treatment groups were then covered with plastic wrap to minimize evaporative water loss and a pencil-sized hole was made in the cover to allow free gas exchange. Cores were incubated in the dark at 20° C and weighed every 2 to 3 days throughout the experiment. Water was added as necessary to maintain a constant moisture content.

Eight replicate jars from each treatment group were sacrificed for analysis periodically at 0, 1, 3, 5, 7, 8, 9, and 10 weeks. First, 30-g subsamples were removed from each replicate core and immediately refrigerated for later analysis of inorganic nitrogen (N) and microbial biomass.

Nitrate-N was determined using the Szechrome NAS colorimetric procedure, and ammonium-N was determined using a Wescan ammonia analyzer. Net nitrogen mineralization was determined as total inorganic N that accumulated with time per g soil dry weight. Microbial biomass was determined using chloroform fumigation to lyse microorganisms followed by extraction with 0.5 M $K_2SO_4$ and determination of soluble carbon by a dichromate digestion. Biomass carbon was estimated by subtracting soluble carbon in unfumigated soil from the fumigated sample, followed by multiplication with a conversion factor (2.64; Vance and others 1987). For this analysis the eight replicate samples were pooled, thoroughly mixed, and divided into four equal parts for duplicate determinations of soluble carbon on fumigated and unfumigated samples.

The jars containing the remaining soil were then sealed with gas-tight lids modified with a rubber septum to allow for amendment and sampling of the gaseous headspace with a syringe. Acetylene (10 percent v/v), an inhibitor of nitrous oxide reduction, was added to each sample in order to measure denitrification. Duplicate 5-ml gas samples were removed from each jar after 1 and 3-h then transferred to 3-ml evacuated glass tubes for storage. These samples were later analyzed for carbon dioxide and nitrous oxide production using electron capture gas chromatography. The microbial respiration rate was determined as the rate of $CO_2$-C production per gram dry weight of soil per day. The denitrification rate of each sample was determined at the rate of $N_2O$-N production per gram dry weight of soil per day.

## Results

### *Microbial biomass*

The effect of Dimilin and frass on microbial biomass in Fernow soil is shown in Figure 14-1. Biomass carbon was consistently higher in frass-amended soil during the first 50 days of the study. This carbon level likely reflects exogenous microorganisms growing in close association with the frass particles, since much higher microbial biomass was present at day 0 compared with that in Dimilin treated and untreated control soils. There is a trend of decreasing microbial biomass carbon in Dimilin treated soil during the first 35 days, followed by recovery to untreated control levels by the end of the experiment. In contrast, microbial biomass in untreated control soils exhibited an increasing trend throughout.

### *Respiration*

During the first 35 days of the study, respiration was lower in Dimilin-treated samples when compared with that in the untreated control or frass-amended soil (Figure 14-2). After 35 days there was no difference among the treatments.

### *Denitrification*

Denitrification in frass-amended soil was initially higher compared with the other treatments (Figure 14-3). This result likely reflects stimulation of heterotrophic denitrifying bacteria due to increased available carbon and nitrogen available in the frass. Frass used in this study contained an average 2 percent total N and 13 percent protein. The stimulatory effect of frass on gaseous N loss, however, was short lived. After 35 days of incubation, the mean denitrification rate was slightly lower in the presence of frass compared with the other treatments. In contrast, there appeared to be a significant inhibition of denitrification in samples obtained immediately after Dimilin addition (day 0). By 21 days, however, the Dimilin treated soil had significantly higher denitrification compared with the untreated control. After 35 days of incubation, untreated control and Dimilin treated soils exhibited

similar denitrification activity. The reason for the initial inhibition and stimulation of denitrification following Dimilin addition is not readily apparent.

***Net nitrogen mineralization***

The rate of ammonification and nitrification appeared to be unaffected by exposure to Dimilin (Figure 14-4, 14-5, 14-6). Higher ammonium was initially present in frass-treated soil, however, nitrate accumulation was similar among all treatments. Higher accumulation of nitrate was evident in the presence of frass after day 35.

## Discussion

Microorganisms are a major source of nutrient renewal in forest soil ecosystems and are the eventual agents of DFB decomposition. It appears from this preliminary study that the initial effects of DFB on microbial processes in forest soil are minor. Microbial biomass and respiration may decrease after exposure to DFB, however, differences are small and appear not to be significant ($p \leq 0.05$) after 21 days. Effects on soil nitrifying bacteria, which are considered to be sensitive to inhibitory substances, were not found. The initial apparent inhibition of denitrification followed by immediate stimulation of this process is unexplainable, and must be further scrutinized.

Although this *in vitro* study demonstrated no lasting effect due to Dimilin, several factors should be kept in mind. Samples were taken from only one watershed which had received no previous exposure to the insecticide. Considering the great heterogeneity present in any forest soil, our ability to generalize is limited. The data tell us nothing about potential cumulative effects of the insecticide. Repeated spraying may result in migration and accumulation of much higher concentrations of DFB in low lying areas of the watershed. To answer these questions a more ambitious sampling regime encompassing both treated and untreated watersheds must be undertaken.

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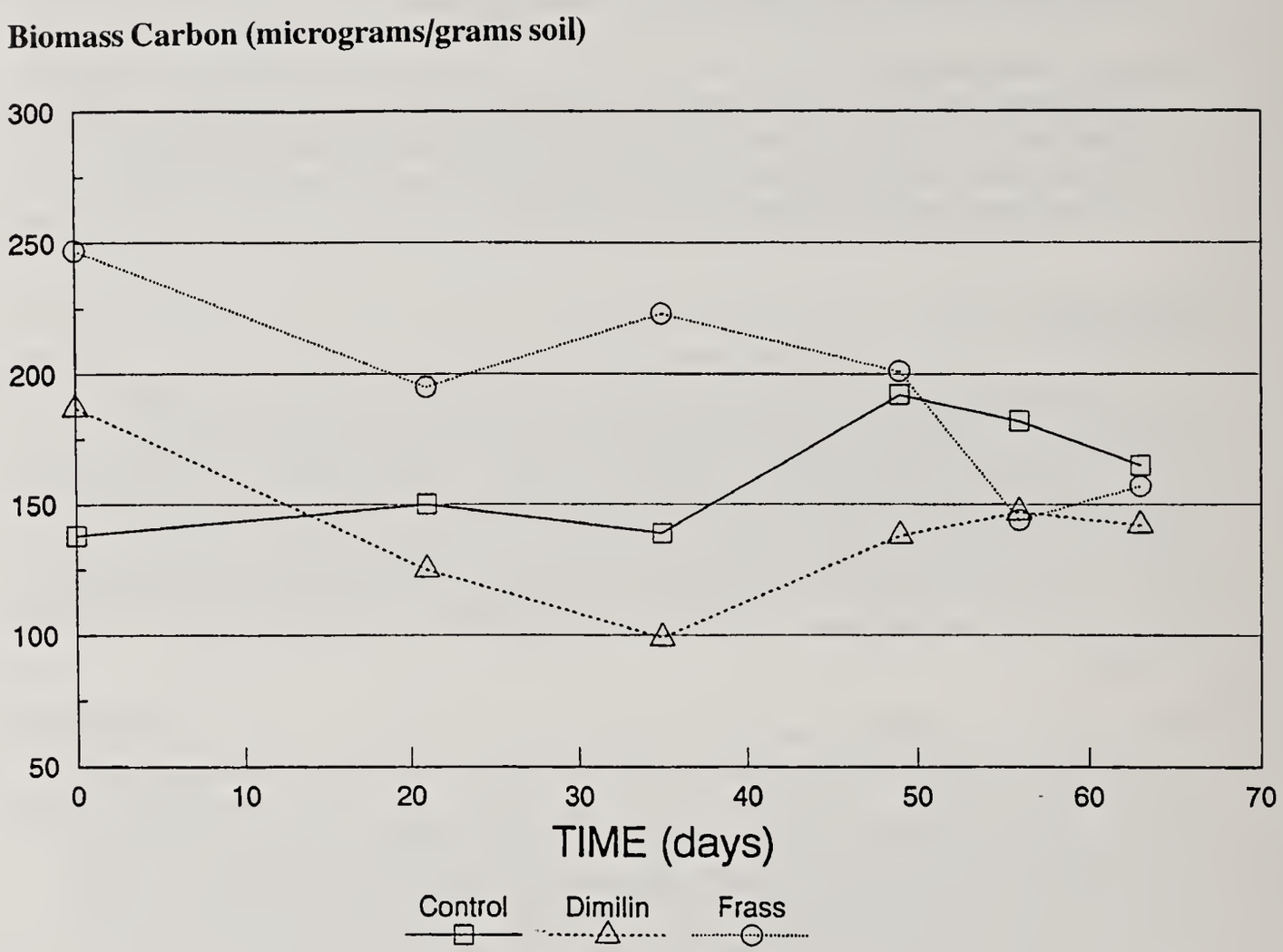


*Figure 14-1.* Microbial biomass in soil from the Fernow Experimental Forest. Data are the means of duplicate determinations. Eight 20-gram soil samples, one from each core, were pooled and then divided into two subsamples for the biomass determination using the chloroform-fumigation- and K2SO4 -extraction method of Vance and others (1987).

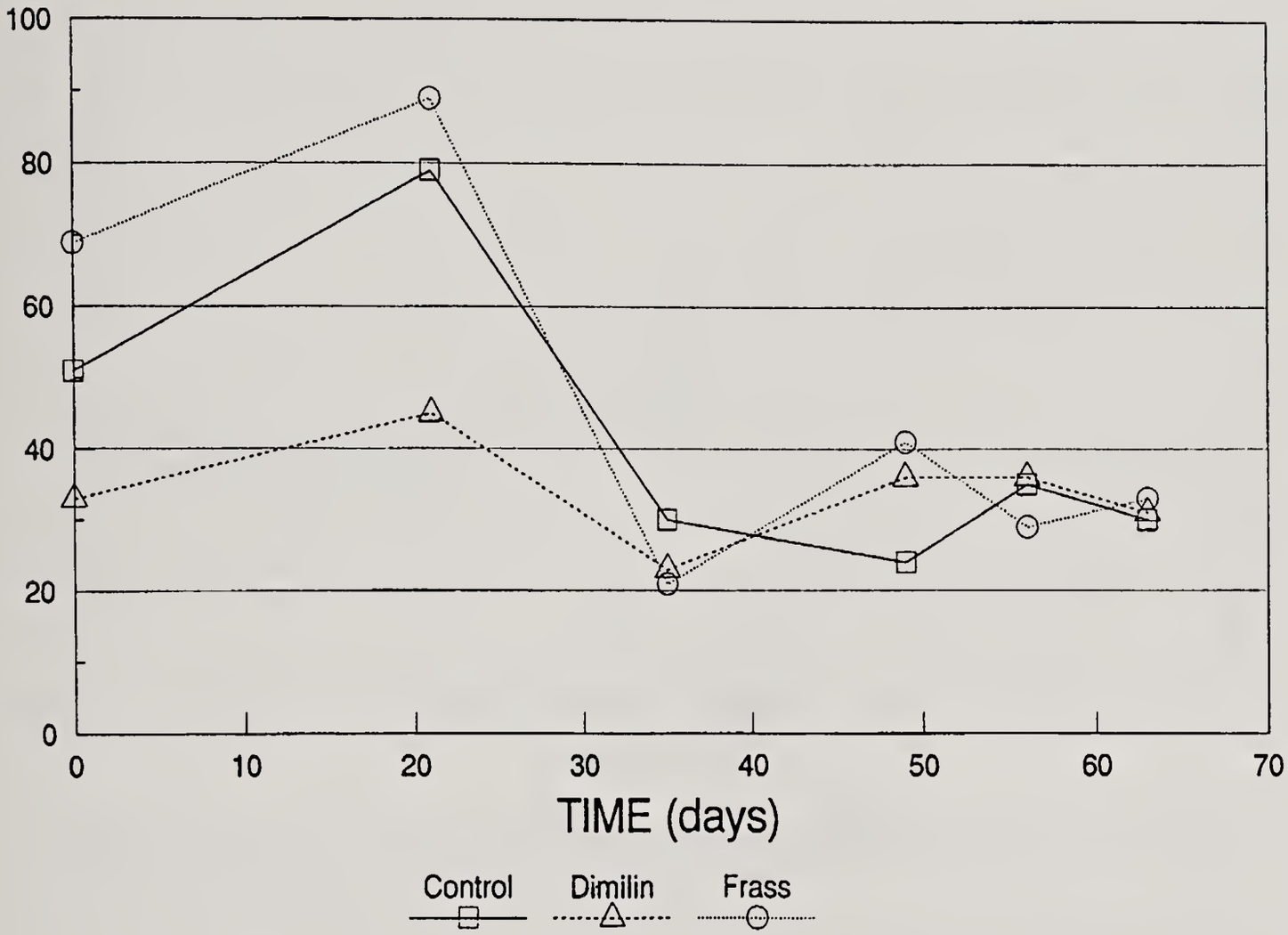


***Figure 14-2.*** Microbial respiration in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Rates of carbon dioxide production were determined by gas chromatography.

**Denitrification (micrograms N/gram soil/day)**

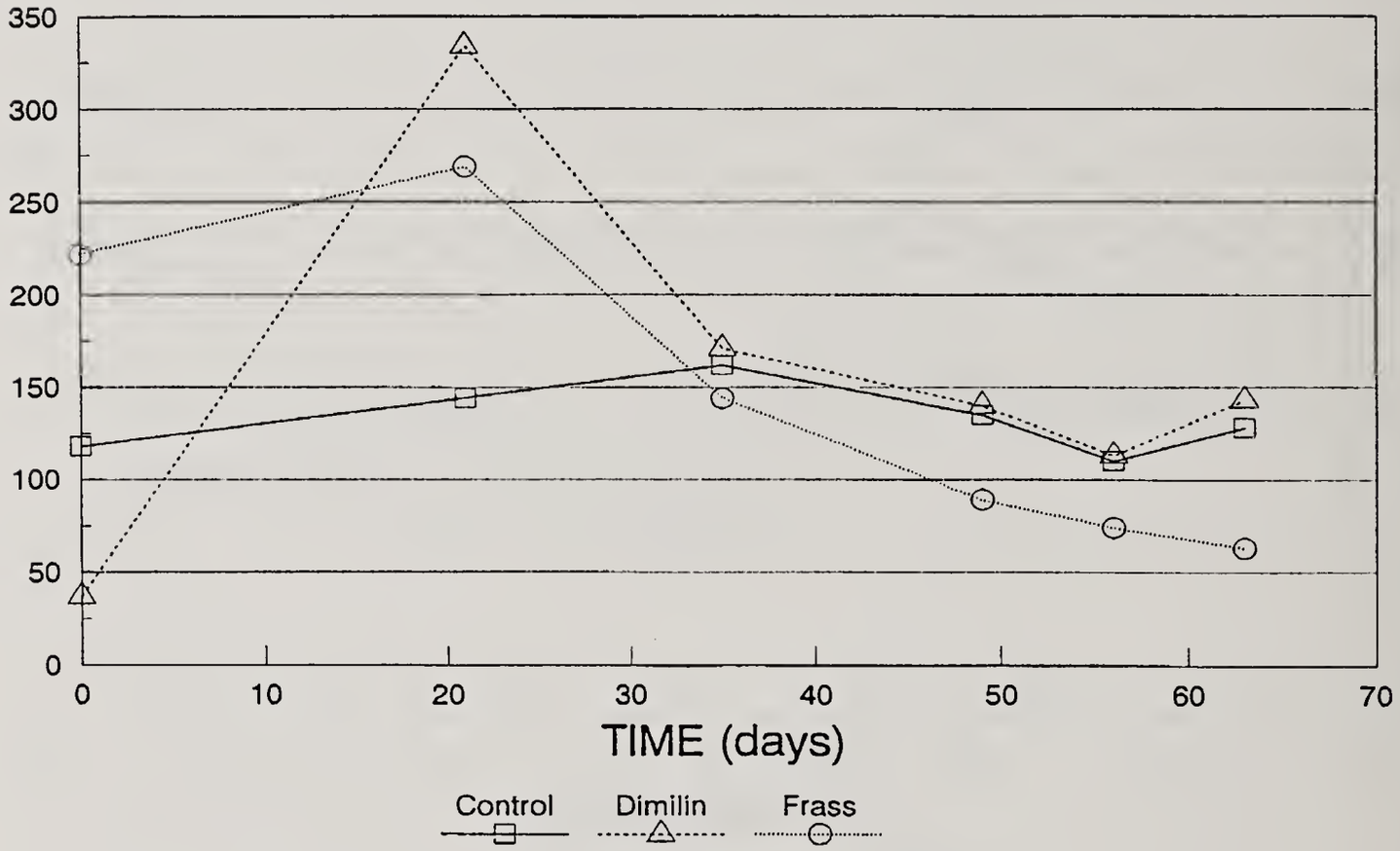


*Figure 14-3*. Denitrification in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Rates of nitrous oxide accumulation in the presence of 10 percent acetylene were determined by gas chromatography.

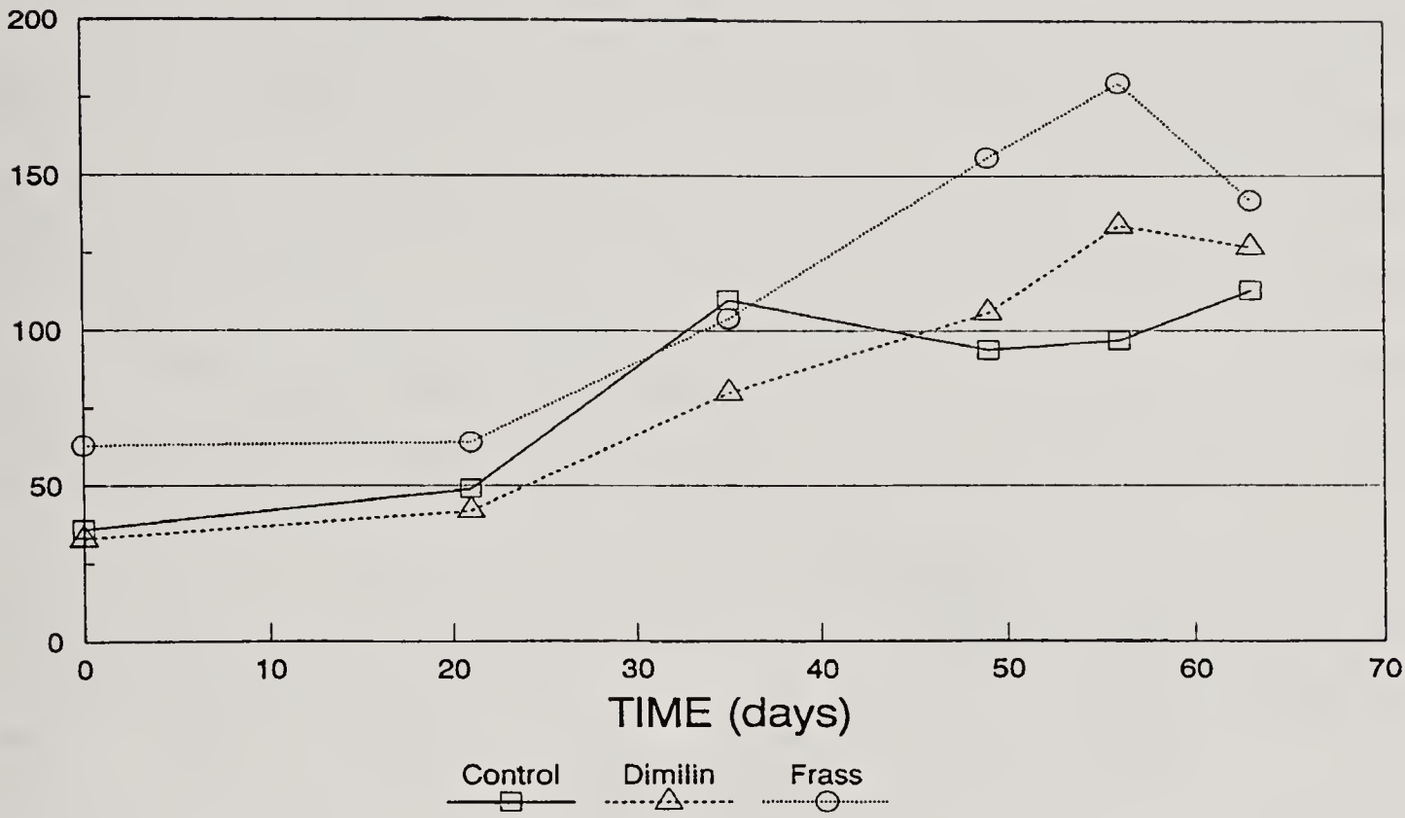


***Figure 14-4.*** Net nitrogen mineralization in soil from the Fernow Experimental Forest. Data are the sum of mean (n=8) soil ammonium and nitrate concentrations

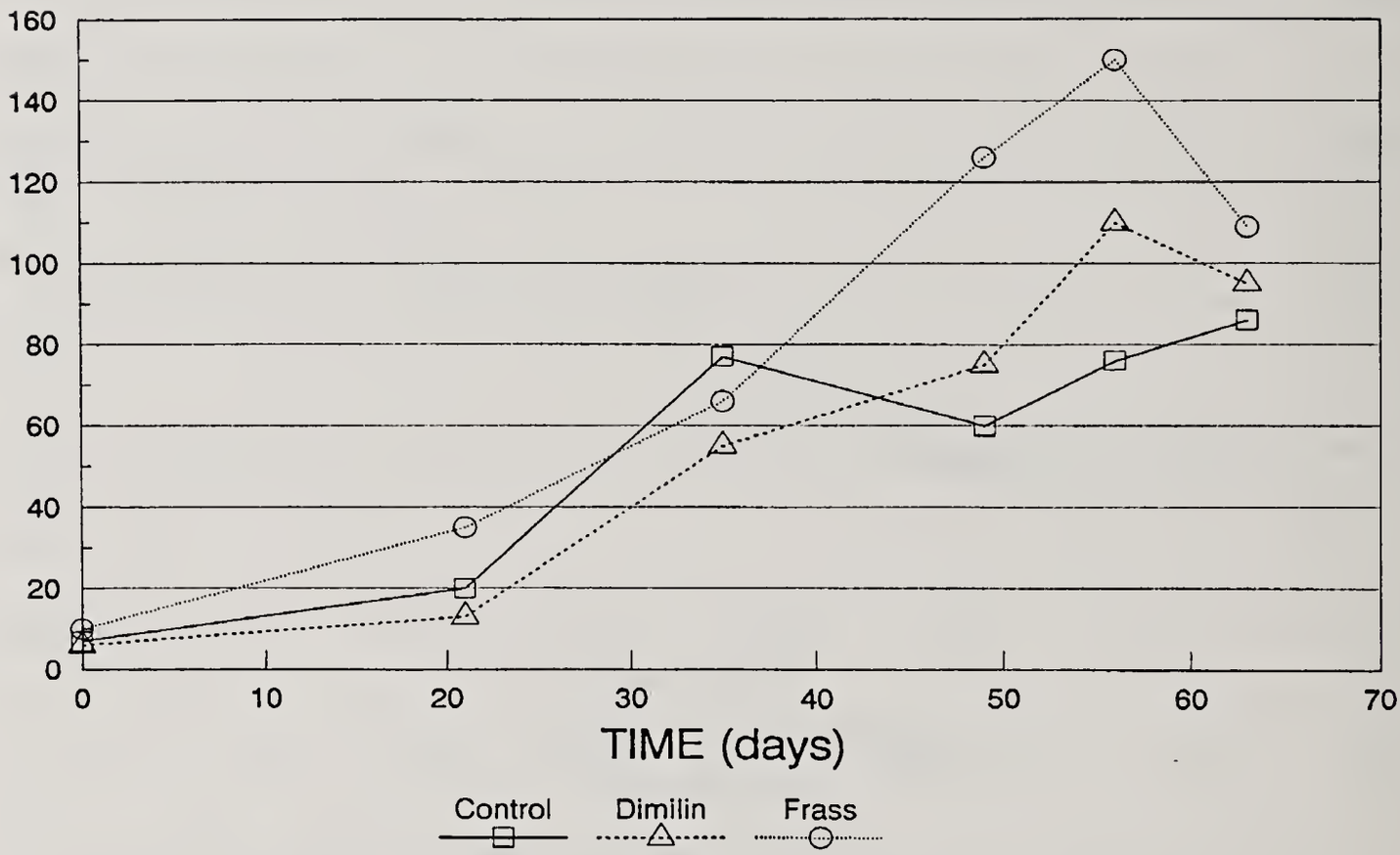


*Figure 14-5.* Nitrate in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Nitrate was determined colorimetrically in 1.0 N KCl extracts using Szechrome NAS reagents.

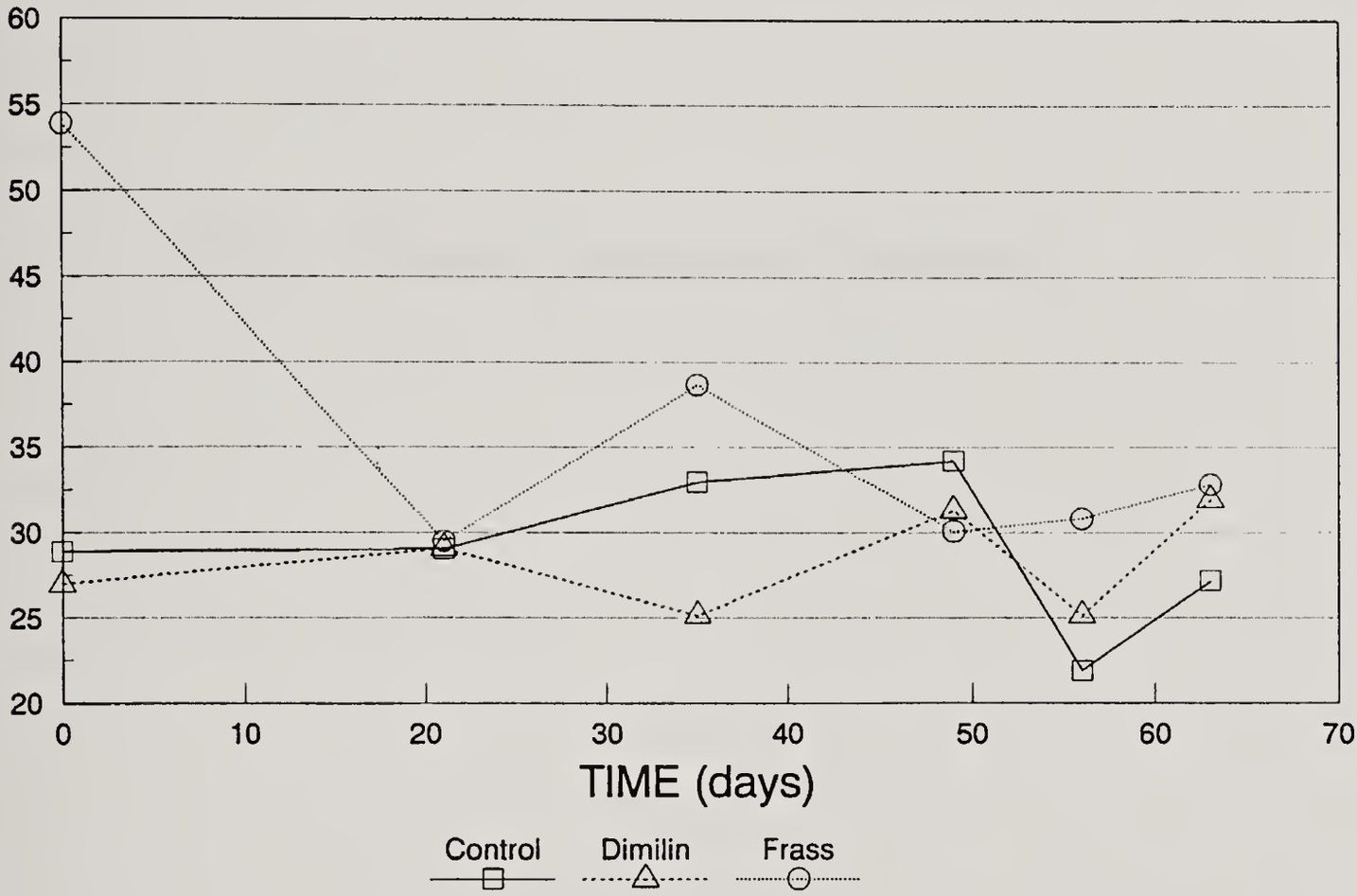


*Figure 14-6.* Ammonia in soils from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Ammonium was determined in 1.0 N KCl extracts using a Wescan ammonia analyzer.

# Part D. Residue Levels And Persistence

# *Chapter 15. Aquatic Environment*

**Felton Hastings and He Zhong**

## Introduction

The utilization of diflubenzuron (DFB) in or near water has prompted studies regarding its persistence in water. DFB residues rapidly dissipated from California ponds and lake water at a pH range of 7.7-8.6 and a temperature range of 20-25° C (Apperson and others 1978). Only 12 percent of initial levels remained in three ponds after 14 days but it required 35 days to reach this same level in the lake. Schaefer and Dupras (1976) concluded that DFB's lack of persistence in water was due to hydrolysis and absorption to organic matter. They recovered only 1 to 3 percent of the applied dose from three formulations (technical, wettable powder and flowable) in farm ponds after 192 h. Jones and Kochenderfer (1987) measured the persistence of DFB in a small eastern watershed following aerial application of 70 g AI/ha (0.06 lb AI per acre) of Dimilin 25-W. The generally low pH and temperature of these streams favor chemical stability of DFB (Schaefer and Dupras 1976, Ivie and others 1980). Although they measured ppt levels in water following the spray and ppb spikes following storm events, they concluded that the resident time of DFB in these high gradient headwater streams is very short.

The objectives of this study were to determine the levels of DFB in first- and second-order streams in treated watersheds and to determine whether any DFB entered the reservoir below the treated watersheds.

## Laboratory and Field Study Methods

Four watersheds were selected for the experiment: WS 1 and WS 13 were treated with Dimilin 4L at 0.08 l AI/ha (0.03 lb AI per acre), and WS 4 and WS 7 were untreated. An ISCO and grab sample were collected just before and during DFB application. Sampling continued at 15-minute intervals for the next 2 h, every 30 minutes for the next 2 h and hourly for the next 4 h. Grab samples were taken at the same time intervals from the upper, middle and lower portions of the reservoir. In WS 13, samples were processed within the hour of collection. This was to ascertain whether DFB was being lost either by degradation or adhesion to the glass walls of the collection vessels. All other samples collected on the first day (grab and ISCO) from the other watersheds and reservoir were processed within 14 h of collection. ISCO samples were also collected during the major storm events which occurred during the summer. These were also analyzed for DFB.

The HPLC analysis was similar to that used by Jones and Kochenderfer (1987) except we concentrated an additional 40-fold by freeze-drying, used a YMC C-18 column and eluted samples isocratically for 20 minutes with 60 percent acetonitrile at a rate of 1 ml/min. Water samples were vacuum-filtered through a 0.45 micron filter then loaded onto a pre-conditioned Sep Pak Octadecyl (C-18) cartridge at a flow rate of 10 ml/minute. The Sep Pak was prewashed with 3 ml of HPLC grade methanol (MeOH) followed by 5 ml of distilled water. After the sample was loaded onto the C-18 cartridge, it was placed back into the foil envelope and labeled. Sample volumes were determined by weighing each ISCO bottle before and after sample loading. All samples were partially cleaned by passing 30 ml of 30 percent acetonitrile (in HPLC grade water) through Sep Paks. Finally, the DFB was removed by passing 2 ml of 60 percent acetonitrile in water through the Sep Paks. This resulted in concentration of approximately 200- to 300-fold (400 ml or 600 ml reduced to 2 ml). The detection limit of DFB was 25 parts per trillion.

## *Results*

No level of DFB was found in any of the samples on the day of application (May 16, 1992); therefore, it is assumed that somewhere in the analysis, DFB was being lost. Because samples were loaded onto Sep Paks within the hour of collection from WS 13, loss by adherence to glass or hydrolytic breakdown was ruled out. However, concentrated amounts of Dimilin in acetonitrile left overnight in the refrigerator required shaking before HPLC analysis.

Four possibilities were tested to determine DFB loss due to our methods: freezing, washing with 30 percent and 60 percent acetonitrile, and adhesion to glass.

(1) Freezing. Known quantities of DFB were added to water from WS 4 (untreated) then loaded onto preconditioned Sep Paks and left in the freezer for 2 days. DFB was quantified with no apparent loss.

(2) Washing with 30 percent acetonitrile. The 30 percent washes were concentrated by freeze drying and resuspending the residue in 0.05 ml of acetonitrile. This gave an additional concentration of 60-fold (30 ml in 0.05 ml). No DFB was found.

(3) Washing with 60 percent acetonitrile. Although the method used by Jones and Kochenderfer (1987) worked well with our spiked samples, the sensitivity was increased to ascertain whether the 60 percent wash was in fact getting the DFB off the C-18 cartridge. This was accomplished by freeze drying these rinses (2 ml wash was picked up in 50 microliters or 40-fold concentration). Again, no DFB was found.

(4) Adhesion to glass. To assure that DFB was not adhering to the glass vessels, the ISCO jars were rinsed with methanol, evaporated to dryness and picked up the residue in 60 percent acetonitrile. No DFB was found.

Because no DFB was found in any of the samples, including those from the reservoir, Mary Wimmer of the Department of Biochemistry (West Virginia University) prepared standard samples of Dimilin as unknowns. These samples were analyzed by HPLC finding 2.6 and 2.2 micrograms per ml. This was in close agreement with Wimmer's values which were determined using deuterated DFB by gas chromatography (GC). As an additional check, Wimmer prepared six unknown water samples, half of which were sent to personnel at the USDA Forest Service Laboratory at Parsons, West Virginia. These samples were added to known volumes of water from an untreated watershed, passed through 0.25-micron filters and loaded onto preconditioned C-18 cartridges as per Jones and Kochenderfer (1987). The other half of each sample was treated with deuterated DFB, extracted with methylene chloride and analyzed by gas chromatography and mass spectrometry (Department of Biochemistry, West Virginia University). The findings are shown below.

| Sample | Calculated Concentration (ppb)[a] | Our Value (ppb) | Wimmer's Value (ppb) |
|---|---|---|---|
| 1 | 100 | 18.5 | 134 |
| 2 | 100 | 21.7 | 127 |
| 3 | 20 | 6.0 | 15 |
| 4 | 20 | 7.0 | 9 |
| 5 | 0.1 | 0.95 | 0.16 |
| 6 | 0.1 | 0.86 | 0.18 |

[a]An estimation based upon commercially available Dimilin 4L.

Our higher concentration values were 1/5 -1/6 of actual; our mid-range values were close to Wimmer's; and our lower values were higher by a factor of 5. Wimmer interpreted these data to suggest that some DFB passed through the C-18 cartridges. To test this, water samples recovered from the pass through C-18 cartridges were analyzed for DFB by GC/MS method. The results showed that DFB retention on the filters decreased as DFB concentration increased. We cannot unequivocally say that this is or is not the case for the actual field samples, although Sep Paks worked well when the sample was added to the cartridge at a rate of 10 ml/min in a previous study (Jones and Kochenderfer 1987). Our spiked samples gave us the confidence that DFB was being reliably quantified by HPLC.

Samples from storm events after the Dimilin application were selectively analyzed. Minor storm events occurred on May 18 and 24 (less than 1.3 cm), and June 14 and 18, with the first large storm on July 27. The June 14 storm affected only Watersheds 1 (3.4 cm) and 4 (2.2 cm). No DFB was found in the water following any storm event, including the large storm (greater than 7.6 cm) of July 29.

Most chromatograms from treated watersheds following storm events were similar to chromatograms from untreated watersheds but some had a peak with a retention time later than that of DFB. The common metabolites (2,6 - difluorobenzamide, 2,6-difluorobenzoic acid, 4-chlorophenyl urea and 4-chloroaniline) all had retention times earlier than DFB; therefore, the unknown peak was neither DFB nor any of the common metabolites.

## *Discussion*

Our inability to find residues of DFB in the treated watersheds was frustrating, because Jones and Kochenderfer (1987) found transient levels of DFB in a first-order stream after aerial application of 70 g AI/ha (0.06 lb AI per acre) of Dimilin 25-W. They found parts per trillion (ppt) levels during the first 5 h after treatment and 34 ppt after a small storm event (1.1 cm) after two days. These levels should have been retained by our filters. In their study, however, twice as much DFB was applied, leaf expansion was estimated at 40-50 percent, and a severe rain storm occurred approximately 1 h after application.

We corresponded with both Alice Jones (microbiologist, USDA Forest Service, retired) and Daniel Downey (Department of Chemistry, James Madison University) regarding these results. Both had conducted studies concerning the aerial application of DFB and residue levels in stream water; they were not surprised that we did not find DFB because a storm event occurred just before Dimilin was applied. They concurred that under such conditions the rate of flow in first- and second-order streams could have exceeded our limit of quantification.

We conclude that DFB was not detected due to the rapid rate of flow in the first- and second-order streams (dilution effect) and because of DFB's general insolubility in water (0.02 ppm). It also seems reasonable that this insolubility caused a "layering effect" on the water surface. Both the grab and ISCO sampling were taken below the water surface, thus reducing the chances of detecting DFB.

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# *Chapter 16. Terrestrial Environment*

**Mary J. Wimmer**

## *Introduction*

Studies of diflubenzuron (DFB) persistence on foliage, reviewed by Fischer and Hall (1992), showed that DFB applied to cotton leaves is resistant to photodegradation and not taken up or metabolized by the leaves (Bull and Ivie 1978, Mansager and others 1979, Schaefer and Dupras 1976); minimal losses were observed over a 14-day period due to volatilization or weathering; and 23 percent of the original DFB was retained after exposure to 7 to 8 cm of rain the following week. When applied to citrus leaves, DFB persisted with no loss over 1 month if conditions were cool and dry, or with a 50 percent loss over 1 month if conditions were hot and wet (Nigg and others 1986).

A short-term (21-day) study of DFB persistence on three Appalachian oak trees in West Virginia reported an initial loss of one-third of the DFB without rain (Martinat and others 1987), decreasing to 20 percent of the application level within 10 days after more than 3 cm of rain. From 10 to 21 days, although more rain had occurred, no further loss of DFB was detected.

In a research summary, 25 percent of the original DFB residue was reported to persist on southern Appalachian forest leaves after 63 days (Van den Berg 1986). Diflubenzuron persistence on foliage has also been suggested by the finding of an increase in DFB concentration in leaf litter the year after application in a coniferous forest, attributed to residual insecticide on needles that fell the previous autumn (Mutanen and others 1988). To date, no comprehensive DFB persistence study in a forest throughout the growing season, as well as after leaf fall, has been reported.

The persistence of DFB on foliage once Dimilin-coated leaves enter a stream has not been thoroughly studied (Fischer and Hall 1992). DFB persisted in artificial leaf packs in a second order stream for 4 months beginning in November (Swift and others 1988). That study, however, used yellow-poplar (*Liriodendron tulipifera* L.) leaves that had been manually sprayed with Dimilin wettable powder, and more than 60 percent of the DFB was lost initially from the leaves when they were placed in the water. The residual level of DFB was determined to be toxic to shredders in a laboratory bioassay, although both shredders and collectors were well represented in the leaf packs throughout the stream study.

Critical to a thorough environmental fate study is the availability of a rapid, sensitive and precise method for analyzing DFB in complex environmental samples, such as extracts of foliage, leaf litter or soil. Methods for analyzing DFB have been limited in the past due to various factors; however, a new procedure takes advantage of the heat-induced fragmentation of DFB during gas chromatography (Wimmer and others 1991). In mass spectrometric detection of the resulting fragments using selected ion monitoring along with deuterated DFB as an internal standard, the interference from other compounds in leaf extracts is removed without purification of the DFB. Thus, rapid and sensitive analyses can be done, making a comprehensive environmental fate study feasible.

This chapter describes 1991 and 1992 studies of the persistence of DFB in Appalachian forest environments over entire growing seasons. The natural substrates studied were leaves of broadleaf trees, leaf litter, soil, beehive components, and reservoir water. The fate of DFB when leaves containing the insecticide enter a stream environment was also studied. The sampling methods are detailed, and the new GC and mass spectrometric analytical method for DFB was used.

## Methods

### *New Diflubenzuron Analysis Method*

A new method for analyzing DFB, the active ingredient in Dimilin, enables rapid and precise measurement of the insecticide on complex environmental samples such as leaves and leaf litter (Wimmer and others 1991). The method uses gas chromatography and mass spectrometry with deuterated Dimilin as an internal standard. No purification of DFB in extracts from leaves or from simple samples (water, artificial substrates) is required. A quick HPLC partial purification is used for extracts from leaf litter, bees, pollen, and other more complex samples.

### *Dimilin Applications*

In a separate 1991-92 study (Wimmer and others 1993), Dimilin was applied to 448 ha in the West Virginia University Experimental Forest outside Morgantown, WV by the WV Department of Agriculture as part of their 1991 Dimilin spray program for control of the gypsy moth. Application of an aqueous suspension of the wettable powder (Dimilin 25-W) was done at a dose of 35 g AI/ha (0.03 lb AI acre) in 9.47 l/ha (1 gal/acre) acre using a Twin Beech flying just above the canopy. In the center of the block, 20 trees of 7 different species, 15-30 m apart along an approximately linear transect, were labeled for sampling throughout the season.

In the Fernow Experimental Forest Study in 1992, the liquid formulation of Dimilin (4L) was applied at a dose of 0.08 l AI/ha (0.03 lb AI acre) and in 9.47 l/ha (1 gal/acre) to Watersheds 1 and 13 with a helicopter flying just above canopy level under ideal application conditions. Ten trees of three different species distributed throughout each watershed were monitored for DFB coverage throughout the season.

### *Sampling Leaves In The Upper And Lower Canopy*

In determining the environmental fate of DFB aerially applied as Dimilin to the leaves of Appalachian hardwood trees, a sampling protocol that accurately reflects the amount of insecticide on these 60- to 70-year old trees during the growing season was necessary. The decision was made to determine the DFB levels on the upper and lower canopy of each tree by collecting a representative leaf sample from each part of the canopy and averaging the total DFB over these leaves (Wimmer and others 1993). After making a homogeneous composite from the leaves, subsamples were analyzed for DFB, and the amount extrapolated back to the whole. Therefore, approximately 200 leaves each from upper and lower canopy were removed from branches that were manually cut by tree climbers from the four cardinal directions. Leaves were removed at six different times after Dimilin application.

A factor that affects measurement of insecticide persistence is the growth of new leaves that were not present at the time of application. These new leaves have zero DFB coverage unless they receive runoff from adjacent leaves. Lack of systemic transfer of insecticide to the new leaves is indicated by the finding that DFB is not taken up by leaves or roots (Bull and Ivie 1978, Mansager and others 1979). In sampling a tree, any new leaves contributing to the total will result in a false low value for insecticide persistence that cannot be corrected by growth dilution. Therefore, care was taken to not remove new growth.

A key part of the analysis was the development of a procedure for making a homogeneous composite sample from the 200 sample leaves, and then subsampling the composite for

replicate analyses. The leaves were counted and the total leaf area measured using an automatic leaf area meter (Li-Cor Co., Lincoln, Nebraska). The leaves were then cut with scissors into approximately 1-$cm^2$ pieces to make a composite sample for which the total area was usually 10,000-20,000 $cm^2$. The pieces were well mixed by shaking in an air-filled storage bag for two minutes. The total composite weight was recorded, and two subsamples of different weights, 8 to 12 g each, were taken out for extraction of DFB and analysis. Subsamples typically were 4 to 10 percent of the total composite weight.

All samples were stored frozen at -23° C in non-frost-free freezers until DFB was extracted from subsamples. DFB has been extracted from leaves stored frozen for over a year and no loss of insecticide was seen. No dry weight of leaves was taken because the normal drying process (heating) would likely decompose at least some of the heat-labile DFB as well as potentially affect its binding to, and extraction from the leaf surface.

***Leaf Litter And Soil Sampling***

The leaf litter sampling procedure used by the producers of Dimilin, Duphar/Uniroyal, for the U.S. EPA registration of Dimilin was used in both WVU Forest and Fernow studies. Plots of 15 by 15 m were measured off in areas below the sampled trees in the Fernow Experimental Forest. Within each plot, five 3.1- by 3.1-m subplots were flagged. At each sampling time, three leaf litter and three soil composites were made from each plot, each consisting of a sampling from the five subplots as follows. Using random number generated coordinates to locate points within each subplot, 10- by 20-cm sections were cut with stiff 10-cm putty knives, through the leaf litter and into the top inch of soil underneath. The litter and soil were transferred into separate zipper-lock plastic bags. This was done for each of the five subplots to generate a composite sample of litter and one of the corresponding soil. Two additional composites of each substrate were made by the same sampling method.

The leaf litter samples, which contained organic material (e.g., leaves, sticks, acorns, roots) at various stages of decomposition, were cut manually into 1-cm square pieces. The leaf litter and soil composites were mixed to homogeneity by shaking, as done with the green leaves. Two subsamples were weighed after the total wet composite weight was recorded, and all samples were frozen until subsample extraction. The dry weight of each leaf litter and soil subsample, used in all calculations, was measured after the acetone extraction step (below) which removes water essentially the same as heat drying. Soil samples from all time points were analyzed for the two plots, A and D, containing the highest litter DFB levels.

***Extraction And Analysis Of Diflubenzuron From Leaf, Ground Litter, And Soil Subsamples***

The details of extracting DFB from leaves and its subsequent measurement are described in Wimmer and others (1991, 1993, 1995a), and from leaf litter and soil in Wimmer and others (1995b, c). Briefly, the leaf, leaf litter, or soil subsample was shaken with acetone after addition of the deuterated DFB internal standard. The acetone was removed, leaving the DFB in a water suspension. The insecticide was extracted into methylene chloride and after solvent removal, was transferred into 0.8-1.2 ml of acetonitrile for refrigerated storage before GC and mass spectrometric analysis. Leaf litter and soil extracts were first subjected to HPLC partial cleanup. After the acetone washes were combined, the ratio of the protonated-to-deuterated DFB species was fixed. Therefore, the yield in subsequent steps was not critical. This is one of the major advantages of isotope ratio methods for quantifying insecticides. The protonated DFB originates solely from the environmental sample. From the known amount of deuterated

DFB added and the H/D isotope ratio of the resulting DFB fragments measured by mass spectrometry, the amount of protonated form can be calculated (Wimmer and others 1991).

***In-Stream Leaf Persistence Study***

Subsamples of leaves from the above composites, with their DFB coverage known, were placed in screened polyethylene tubes in a headwater stream in the WVU Experimental Forest to determine how long the DFB would remain on these leaves in the water (Harrahy and others 1994). At each time point, replicate samples were removed from the stream and analyzed for DFB by the method described above for fresh leaves. HPLC partial purification was necessary for late time points due to leaf colonization by microorganisms.

***Beehive components***

Pollen, bees, and honey were sampled from hives at various times throughout the season. All samples were first refrigerated, then frozen before sample work-up. Honey was diluted with distilled water and extracted with methylene chloride in a separatory funnel, after addition of deuterated DFB as an internal standard. Frozen bee and pollen samples were homogenized with a blender before extraction with acetone. The rest of the work-up was similar to that for leaf litter. All samples were partially cleaned up with HPLC before GC and mass spectrometric analysis. The results of this study are reported elsewhere in this publication (see chapter on Pollinators-- Honey Bees).

***Parsons Reservoir***

Water (400-1000 ml) was sampled from the Parsons Reservoir inlet, deuterated DFB was added, and the DFB was extracted into methylene chloride by shaking in a separatory funnel. After solvent removal, the sample was dissolved in 1.0 ml of acetonitrile for GC and mass spectrometric analysis (see chapter on Aquatic Environment).

***Artificial substrates***

Glass slides were placed horizontally on wire stands at 2-m intervals approximately 1-m above the ground in Watershed 1 of the Fernow Experimental Forest to monitor Dimilin deposition at ground level. Forty slides were placed parallel to the ridge line and 23 slides were placed along one vertical transect from stream to ridge line. Also, nylon spheres were hung at 2-m intervals along a string (approximately 2 m above the ground) to monitor deposition. The DFB was dissolved off the surface of these artificial substrates with methylene chloride. The solvent was removed, and the sample was taken up in acetonitrile for analysis. If GC and mass spectrometric analysis were used instead of HPLC, deuterated DFB was added to the sample.

***Fungi***

See chapter by Gundrum, Iskra and Wimmer in this publication (Terrestrial Fungi in Leaf Litter).

***Field weather data***

Weather data for the WVU Experimental Forest study were obtained with the cooperation of the Federal Aviation Administration/Department of Transportation and the National Climatic Data Center, NOAA Environmental Data Service, Asheville, NC. The data were recorded at the Morgantown Municipal Airport monitoring station located approximately 13 km southwest of the WVU Experimental Forest spray block and at a similar elevation.

Weather data for the Fernow study were obtained from the USDA Forest Service Timber and Watershed Laboratory, Parsons, WV. The data were recorded at a station located in the Fernow Experimental Forest within 0.8 km of the treatment watersheds.

## Reporting of Diflubenzuron Analysis Data

***Leaves.*** The amount of DFB can be expressed in ng per $cm^2$ of leaf area (coverage on the leaf surface), or µg per kg (ppb, concentration by non-dried leaf weight). To determine the actual amount of DFB remaining where leaf growth has occurred, a growth dilution correction is made; the DFB coverage ($ng/cm^2$) is multiplied by the relevant growth factor determined by dividing the area per leaf at the time of interest by the initial area per leaf at time zero, the first time immediately after DFB application (Wimmer and others 1995a).

***Leaf litter and soil.*** The amount of DFB is expressed as µg per kg (ppb) based upon the dried weight of the subsample measured after extraction with acetone. This weight agrees with the usual heat-dried weight, and avoids not only an extra step in sample work-up, but also the problem of the heat lability of DFB.

***Beehive components.*** The amount of DFB in honey is given as ng per ml (ppb) based on the volume of honey extracted, while that in bees and pollen is expressed as µg per kg (ppb) based on initial weight of the extracted sample.

## Results

A comprehensive study of the environmental fate of DFB aerially applied to an Appalachian forest ecosystem begins by tracing its persistence on tree foliage, a major initial receptor, throughout the growing season, followed by its persistence in the underlying leaf litter, soil, and stream debris. Stream debris is especially important after leaf fall if there is a significant amount of DFB from the initial spray left on leaves at abscission.

### *Diflubenzuron Persistence On Leaves*

In the study of 20 trees representing seven tree species in the West Virginia University Experimental Forest, the amount of aerially applied DFB on the leaves was monitored for 141 days after treatment (just prior to leaf fall) to determine its persistence on the foliage. The yellow-poplar had lost its leaves by the 141-day sampling. The rainfall and air temperature data for each sampling period indicate that the 1991 growing season at the site received low amounts of precipitation during an unusually long, warm growing season.

A representative DFB persistence curve (Fig. 16-1) illustrates the utility of the new GC and mass spectrometric quantification method. For each time, the two coverage values shown are for the two subsamples taken from each of the 200-leaf composite samples. The excellent agreement seen between the two values supports the homogeneous nature of the composite when subsampled in this manner. Therefore, the amount of DFB can be precisely measured in a foliage sample using the GC and mass spectrometric method, with DFB level variability resting not on the sample work-up and analysis, but on the sampling of the tree itself during the season and on sample handling prior to freezing.

The initial DFB coverage on the 20 WVU Forest sample trees was found to vary from 2.9 to 75 ng per $cm^2$ of leaf on upper canopy leaves, and from 1.9 to 25 ng per $cm^2$ on lower canopy leaves which, as expected due to nearly full leaf expansion at the time of spray, typically received less DFB than the leaves above them. The effect of a rain event immediately after treatment was to wash nearly half the DFB off the upper leaf canopies of the two trees sampled. The re-application of insecticide for the season-long persistence study did not raise the net coverage to an unusual level when compared with the Fernow coverage (below).

A significant loss of DFB from the foliage, ranging from 20 to 80 percent, was observed within the first 3 weeks after application. Only a non-measurable trace of rain fell during the first time period (8 days post-spray), implying that the initial loss of DFB seen was due to wind or other physical removal of the dried powder formulation. The remaining DFB was generally found to persist for the rest of the growing season until leaf fall, at which 13 out of the 20 trees retained more than 20 percent of the original insecticide applied. Seven trees, consisting of all five yellow-poplars and the two black oaks, showed from 5 to 20 percent DFB retention, primarily due to a greater initial drop. A publication of this study is in print (Wimmer and others 1993).

The Fernow Experimental Forest study differed from the WVU study in several ways. Dimilin application was done under more ideal weather conditions using a smaller and rotary-winged aircraft, and no rain occurred for over 24 h after spray. The liquid formulation of Dimilin (4L) was applied, not the wettable powder (W-25); although one might expect that less evaporative loss would occur than with the powder, no sampling was possible prior to the initial rain event to investigate this. More care was taken in sampling to avoid removal of leaves emerging posttreatment, and samples were prepared more efficiently for freezing to minimize decomposition. The most significant difference, however, was that in the 1992 study, leaf expansion was more typical for gypsy moth spraying, giving the opportunity to study significant growth dilution of the DFB on the leaves throughout the growing season. Furthermore, this study was coupled with several studies of nontarget organisms to determine the impact of DFB on these sensitive populations (see elsewhere in this publication).

The concentration of DFB on the Fernow leaf samples declined in a similar fashion as in the WVU study, with a significant initial drop followed by a much more gradual decrease (Wimmer and others 1995a). Initial coverages were generally higher than in the WVU study, ranging from 7 to 300 ng per $cm^2$ on the upper canopy and from 9 to 270 ng per $cm^2$ on the lower canopy. The more equal coverage between upper and lower canopy leaves was likely due to greater penetration of spray because of reduced leaf expansion than in the WVU Forest study. Table 16-1 shows the percent of the original DFB concentration on the upper canopy leaves just prior to leaf fall.

### *Growth Dilution*

In an environmental fate study of an insecticide, two questions can be asked: (1) How long does the total insecticide originally introduced into the environment remain?; and (2) How does the efficacy of the insecticide, expressed by its concentration in the environmental medium of interest, change during its lifetime? The first question was answered by determining the total amount of insecticide in the environmental medium as a function of time -- in this case on leaf surfaces during the growing season. The µg of DFB in each leaf composite subsample was

measured, converted to a normalized unit of concentration (either ng/cm$^2$ or µg/kg), and then compared with the initial value.

A problem arises when a significant size change occurs in the environmental medium over time, such as the increases in leaf area and weight during the growing season. This change alters the normalization factor, which enables comparison with original pesticide levels, and results in false, lower-than-actual numbers for the amount of insecticide remaining.

In 1991 at the West Virginia University Forest, the early onset of warm weather caused most leaf expansion to have occurred by the time of Dimilin application (May 14-15). Growth was accurately monitored by measuring the total area of each leaf sample and dividing that by the actual number of leaves; thus, the area per leaf was averaged over the entire approximately 200-leaf sample. The only trees to show slight growth of the older leaves during the course of this DFB persistence study were the yellow-poplars, and then only the tail end of their growth curve was seen. The highest growth dilution factor, 1.2-1.3, does not significantly change the 10 to 20 percent range of DFB persistence discussed above. The maples and oaks did not show any consistent growth pattern indicating that their older leaf growth was essentially complete at the time of spray.

In the 1992 Fernow study, significant growth of the leaves out at the time of spray occurred after the Dimilin 4L application. The actual amount of the original DFB retained by the leaves was determined by multiplying by the growth factor. Growth was accurately monitored by measuring the total area of each leaf sample and dividing that by the actual number of leaves; thus the area per leaf is averaged over the entire approximately 200-leaf sample. Of 19 trees, 14 retained 14-45 percent of the original upper canopy DFB; four trees exceeded 65 percent, and one retained 6 percent. A summary of the two types of measurement for the percent of DFB remaining in the upper canopy is shown in Table 16-1 and Figure 16-2 illustrates DFB persistence for one tree during the season.

Lower canopy results were similar. The overall average DFB concentrations at leaf fall were 15 percent and 18 percent of original for the upper and lower canopies, respectively. The overall average DFB amounts left at leaf fall were 38 percent and 46 percent for the two canopies. The residual DFB was delivered along with the leaves to the streams and leaf litter underlying the canopy. A manuscript of this study has been submitted for publication (Wimmer and others 1995a).

***Diflubenzuron Persistence In Leaf Litter***

In the West Virginia University Forest, leaf litter was monitored for DFB from immediately after aerial application to the forest (mid-May 1991) until just before leaf fall the following year (late September 1992). After a rise in DFB level during the first 2 weeks after spray consistent with the loss from overlying foliage, the insecticide declined to low levels until leaf abscission when a significant rise in DFB was seen. This rise was presumably due to the residual DFB known to persist on the leaves during the growing season, as discussed above.

Diflubenzuron levels in the leaf litter remained significant over the next 8 months, declining back to pre-leaf fall levels prior to the falling of unsprayed leaves from the 1992 season. No sampling has been done since then because the DFB remaining would presumably be diluted to barely detectable levels by the fresh organic matter.

In the Fernow Experimental Forest study conducted over a longer time period, a very similar pattern of DFB behavior was seen in the nine plots monitored. In the majority of plots, DFB is still detectable after the second leaf fall. A representative plot of DFB levels in ground litter over two growing seasons after spray is shown in Figure 16-3. A manuscript of the ground litter studies has been submitted for publication (Wimmer and others 1995b).

***Diflubenzuron Persistence In Forest Soil***

The soil within the first 2.5 cm underlying the leaf litter samples did not show measurable amounts of DFB regardless of the time of year taken. One can postulate that when the DFB dissipates from the leaf litter by the end of each summer (Fig. 16-3), the loss is due either to biodegradation as the leaf litter is biodegraded, or to movement of the DFB further down into the soil layer. Considering the tendency of hydrophobic DFB to bind to organic material, its mobility in these soils would be expected to be low. The results of the soil studies are being prepared for publication along with the 4-chloroaniline study below (Wimmer and others 1995c).

***Search For Diflubenzuron Metabolite 4-Chloroaniline***

To further the environmental fate study of DFB, collaboration was done with Dan Downey (Department of Chemistry, James Madison University) to measure the DFB metabolite 4-chloroaniline in leaf litter and soil samples from selected Fernow plots. For the methods and results, see the chapter on breakdown products of diflubenzuron by Downey.

***Diflubenzuron Persistence On Leaves Placed In Stream Water***

Leaves for the in-stream study at the WVU Experimental Forest came from trees aerially sprayed with Dimilin in the spring and left to weather during the growing season. Rain exposure minimizes loss of insecticide when the treated leaves are first immersed, and closely mimics the natural situation at leaf fall. After measuring DFB coverage, leaf samples were placed in a headwater stream and residual DFB monitored as a function of time. During July and August, the amount of DFB on white oak (*Quercus alba* L.) decreased significantly (by 36 percent and 23 percent, respectively) within the first 48 h of stream incubation, reaching less than 10-percent of the original concentration within 3 weeks. In December studies with yellow-poplar, red maple, and white oak leaves, the rate of loss of DFB was slow. After 54 days in the stream, yellow poplar and red maple leaves retained 45 percent and 40 percent, respectively, of the original DFB, and white oak showed less a loss. The most likely cause of the difference in summer and winter persistence was the much cooler average water temperature in the December study.

The results of our December stream study extend the research of Swift and others (1988). In their study (begun in November), in which a water suspension of DFB wettable powder was manually sprayed to dose the leaf material, immediate loss of over 60 percent of the DFB was seen upon introduction of the leaves into the stream. Our study was done under more natural conditions using three species of rain-weathered leaves that had been dosed with DFB by a normal aerial application of Dimilin wettable powder. The initial loss of DFB when such leaves were placed in a stream was lessened (0 to 30 percent), as predicted by Swift and others (1988). In both studies, the DFB left after the initial immersion was found to persist on the foliage for more than 2 months.

In view of the persistence of DFB on hardwood leaves observed through the growing season to leaf fall, at low stream temperatures, nontarget aquatic organisms that consume these fallen

leaves may be exposed to the insecticide for a significant period of time. A full-length publication of this study is in print (Harrahy and others 1994).

***Parsons Reservoir***

A water sample from the Parsons Reservoir taken immediately after Dimilin 4L application to Fernow Watersheds 1 and 13 showed no detectable level of DFB. With our analytical method, the detection limit is in the range of 50 to 100 ppt for the liter of water sampled.

***Beehive Components***

See chapter by Cochran and Poling in this publication (Pollinators -- Honey Bees).

***Fungi***

See chapter by Gundrum, Iskra, and Wimmer in this publication (Terrestrial Fungi in Leaf Litter).

***Artificial Substrates***

The pattern of DFB found on glass slides placed approximately 1 m above the ground in Watershed 1 during the aerial application of Dimilin shows a non-uniform distribution along the length of the sampled area. The coverage for the line of slides each 2 m apart placed horizontal to the stream approximately midway up the slope is shown in Figure 16-4a, with DFB coverage represented as $ng/cm^2$ of slide area. Values range from 25 to 167 $ng/cm^2$. The distribution along a vertical sector midway along the horizontal slides showed a similar pattern ranging from 5 to 116 $ng/cm^2$ (Figure 16-4b). A look at the levels of DFB coverage on the two trees samples in this study located closest to the slides (Trees 5 and 6) showed, respectively, 134 and 302 $ng/cm^2$ for upper canopy leaves, and 74 and 271 $ng/cm^2$ for the lower canopy leaves. The initial on-the-ground coverage based upon ground litter data after spray for the closest two plots (G and H) ranges from 9 to 65 $ng/cm^2$, respectively, considering the 0.1 $m^2$ of ground surface sampled per leaf litter composite. Thus, the DFB coverage found on slides 1 m off the ground (5 to 167 $ng/cm^2$) was consistent with that found on surrounding foliage surfaces, and was less than that on the upper canopy leaves as expected considering the DFB caught by overstory foliage.

The nylon spheres hung 0.6-1.2 m apart along a string approximately 3 m above ground level showed no detectable levels of DFB coverage.

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*Table 16-1.* Percent diflubenzuron remaining in the upper canopy, Fernow Experimental Forest.

| Tree Species | Watershed 1 | Watershed 13 |
|---|---|---|
| | *(Percent)* | |
| **Yellow-poplar** | | |
| DFB Concentration | 2-7 (3 trees) | 9-45 (4 trees) |
| DFB Amount (growth corrected) | 6-17 | 16-82 |
| **Red Oak** | | |
| DFB Concentration | 10-34 (3 trees) | 6-14 (5 trees) |
| DFB Amount | 23-68 | 25-92 |
| **Maple** | | |
| DFB Concentration | 9-24 (3 trees) | 30 (1 tree) |
| DFB Amount | 16-45 | 42 |

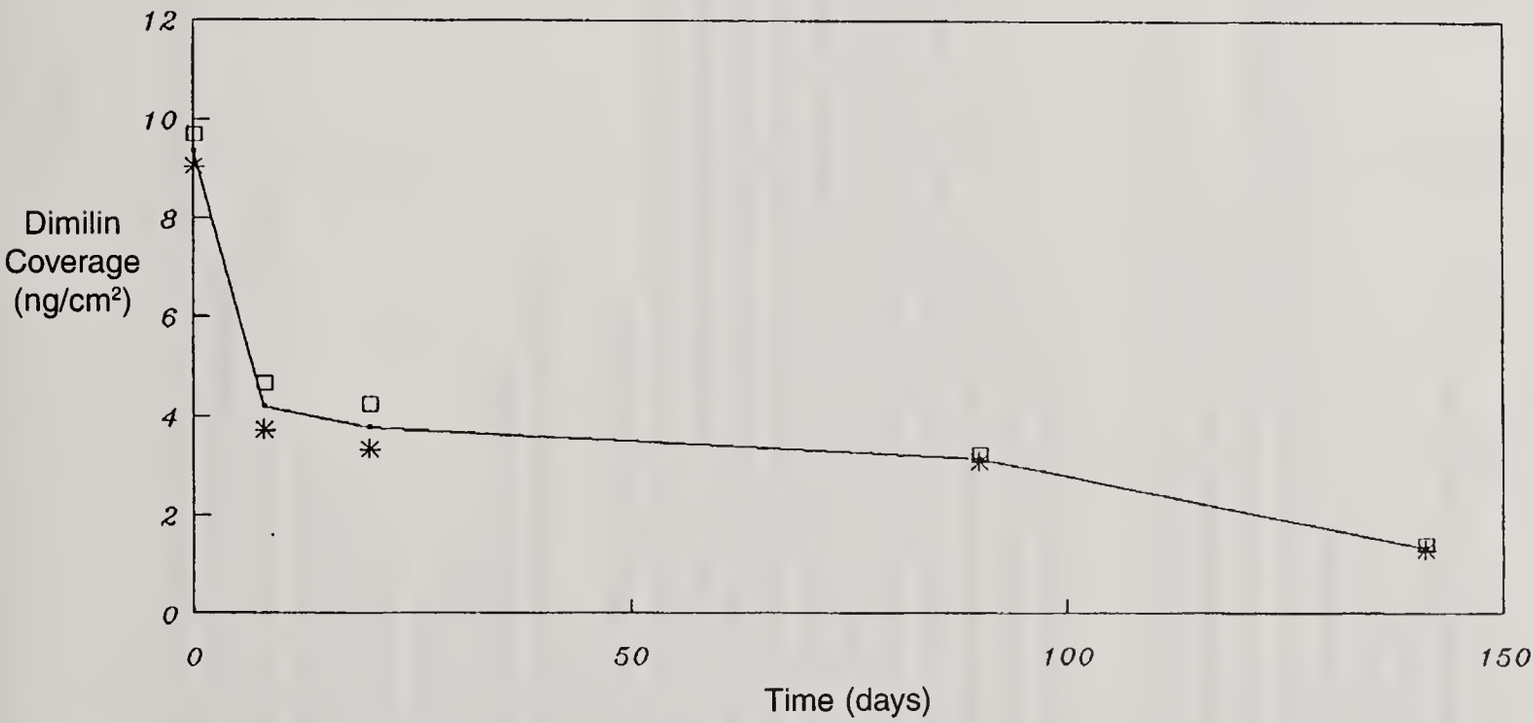


***Figure 16-1.*** Persistence of DFB on upper canopy foliage of a WVU Experimental Forest yellow-poplar during the 1991 growing season. DFB concentrations on the leaf samples are reported as DFB coverage in ng/cm2 of leaf surface area. The two coverage values shown at each time point are for the two subsamples analyzed from the 200-leaf composite sample.

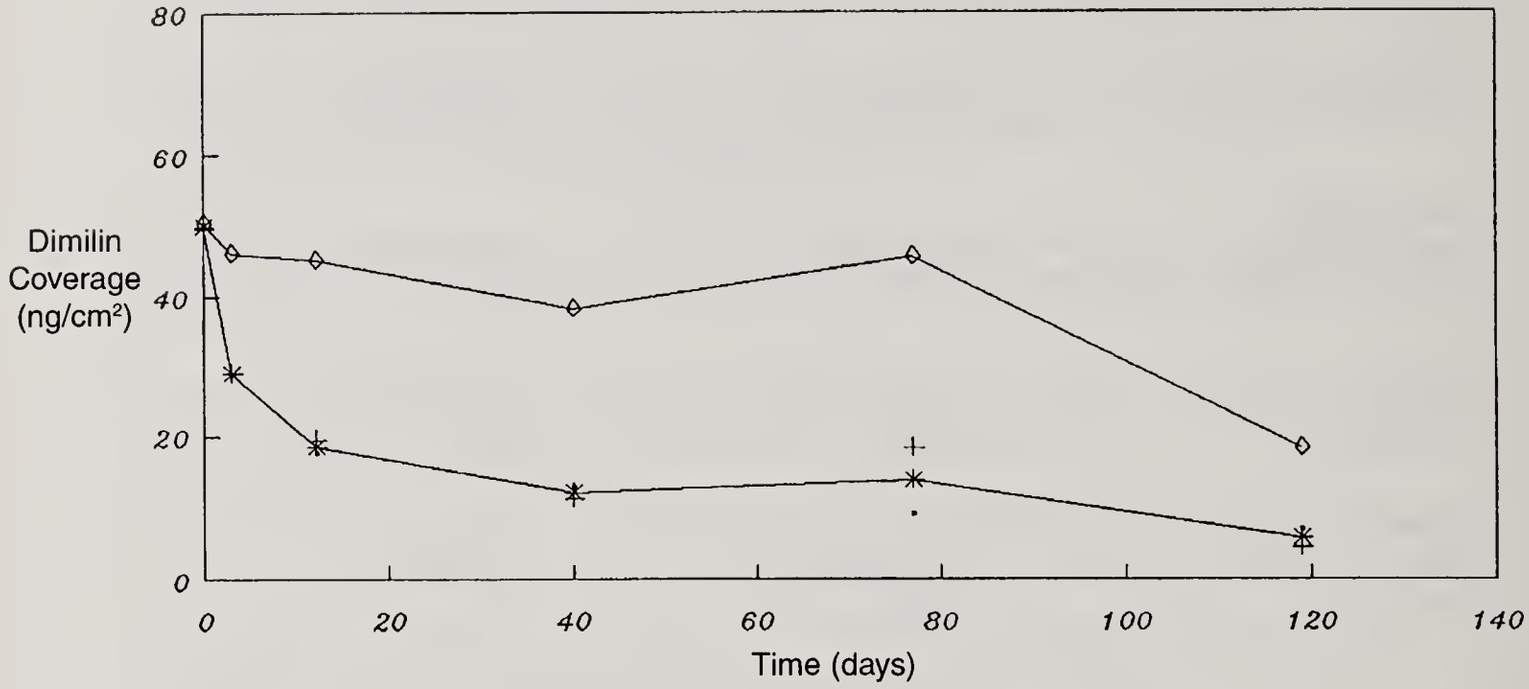


*Figure 16-2.* Persistence of DFB on the lower canopy of a Fernow Experimental Forest red oak: effect of leaf growth dilution on DFB coverage. The bottom line represents the concentration of DFB on leaves during the growing season as described in Figure 16-1. The top line shows the actual amount of DFB on the leaves over the same time period, obtained by adjusting the bottom line for DFB dilution by leaf growth.

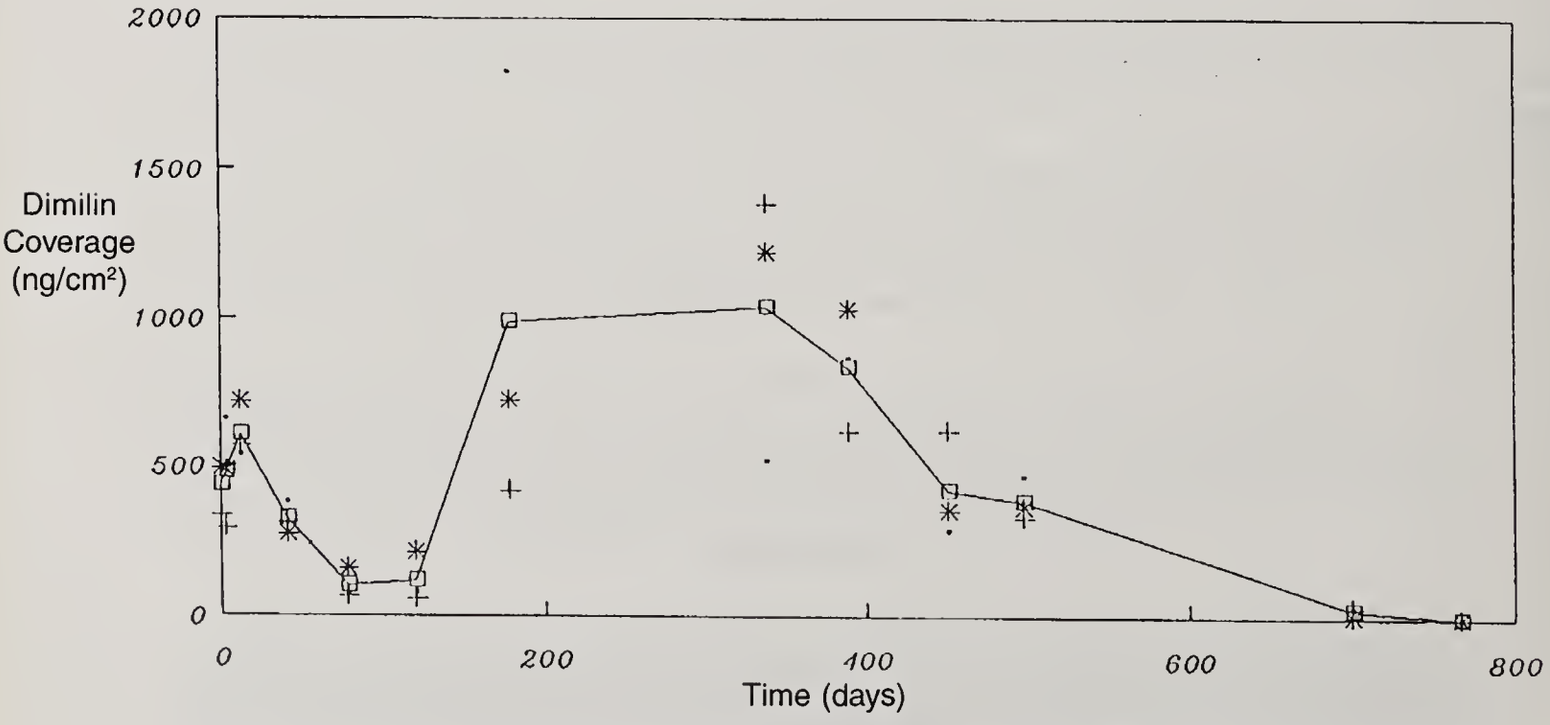


*Figure 16-3.* Persistence of DFB in Fernow Experimental Forest ground litter over 25 months post-spray. The average of two subsample values for each of three composite samples is shown for each time point. DFB concentration is reported as ppb DFB (ng DFB/g of litter dry weight).

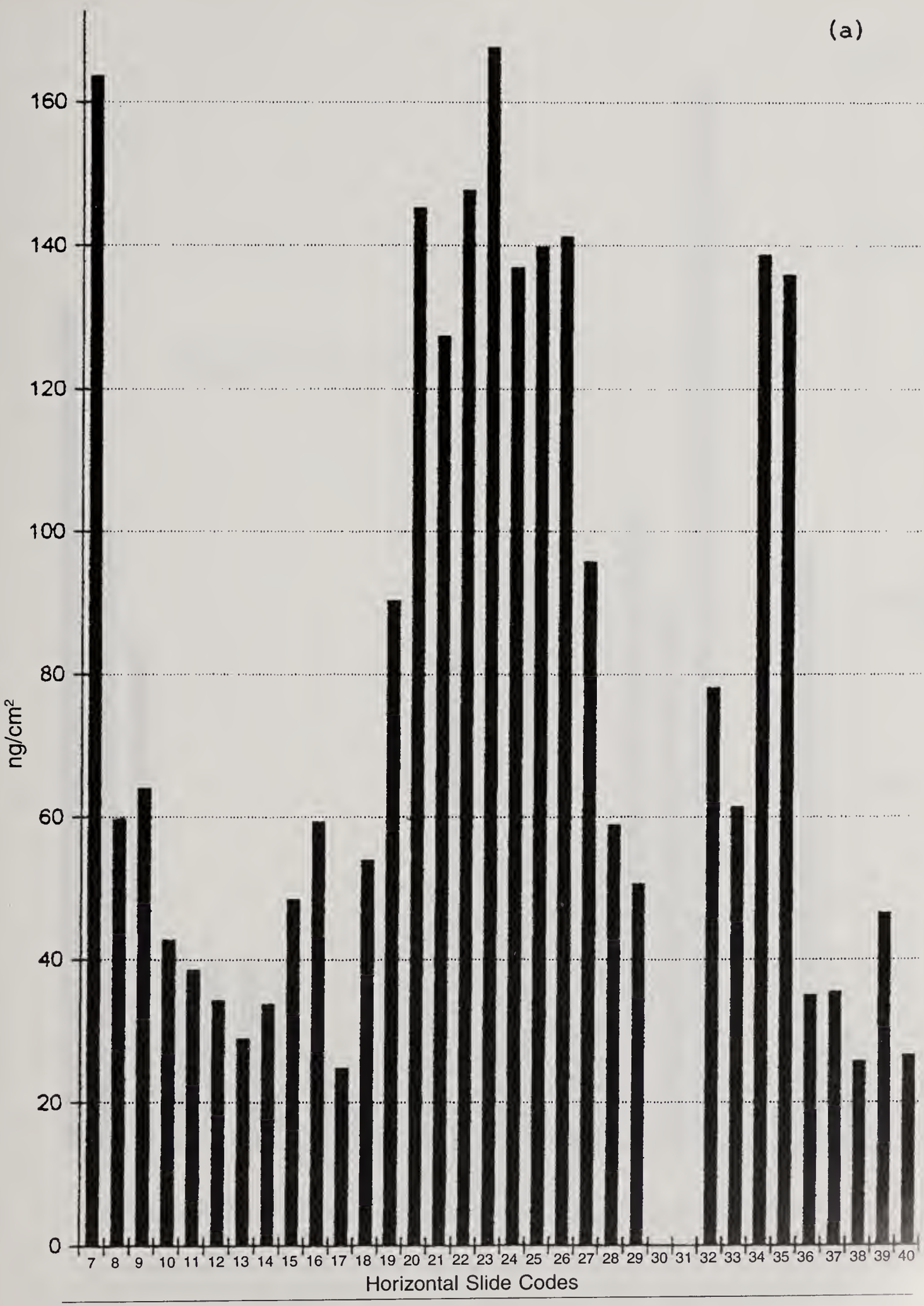


***Figure 16-4a.*** See next page.

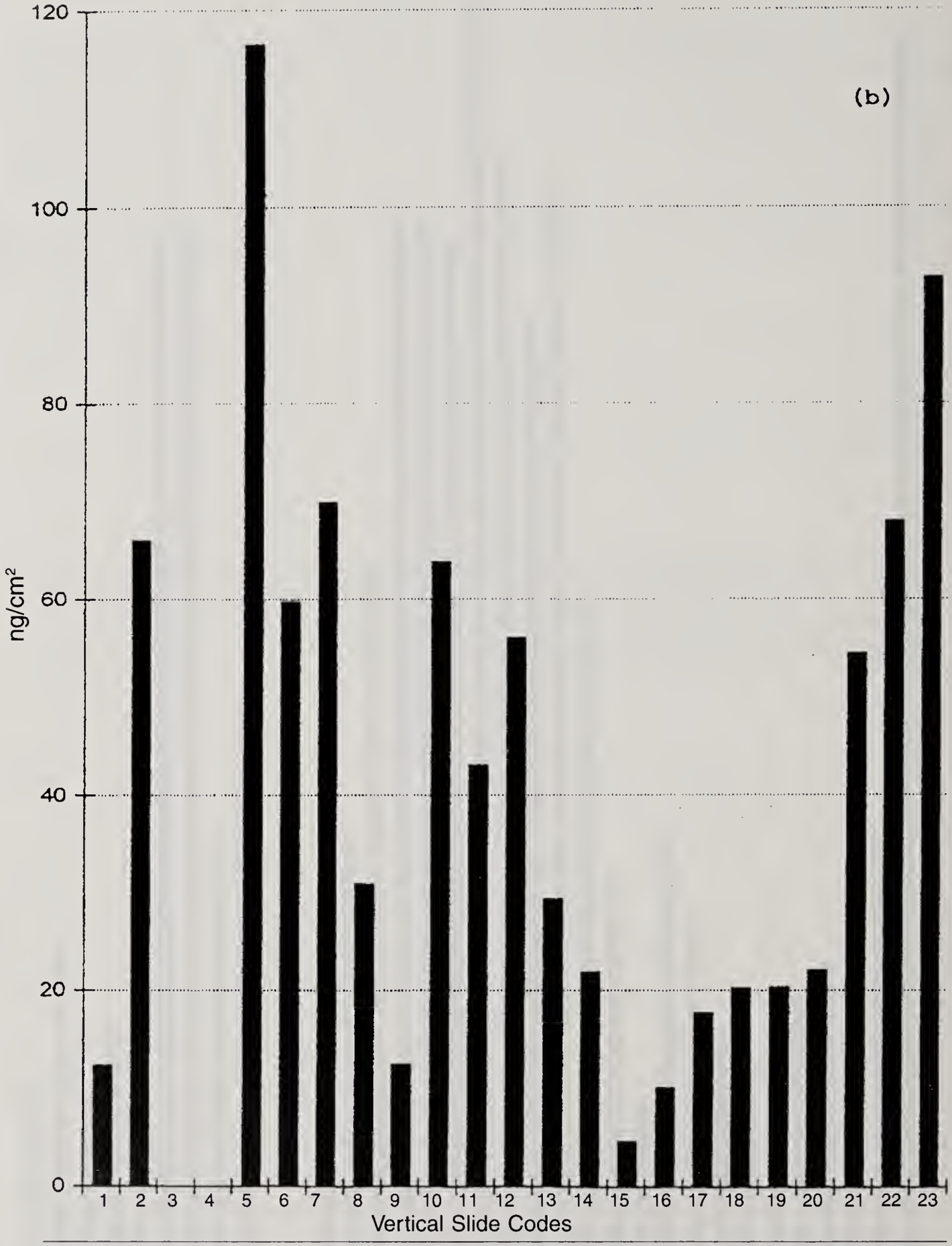


***Figure 16-4b.*** DFB coverage of glass slide substrates immediately after Dimilin application to the Fernow Experimental Forest. Slides were placed flat at 1.6 m intervals approximately 1 m above ground along transects a) horizontal and b) vertical to the Watershed 1 stream. DFB coverage is reported as ng/cm2 of slide area, with slide number shown along the x-axis. The vertical slides are numbered beginning from stream toward ridge. Missing slides show as zero coverage.

# *Chapter 17. Breakdown Products Of Diflubenzuron*

**Daniel Downey**

The final procedure selected for extraction begins with a weighed 5 g leaf litter or soil composite subsample (Chapter 16) being slurried with 50-ml of 6M HCl. This step ensures complete conversion of PCA to $HPCA^+$ with concomitant replacement of the $HPCA^+$ with $H^+$ on cation exchange sites. A 30-minute ultrasonication at 25° C was then done to accelerate removal of the analyte from the matrix. After filtration and washing with additional HCl, the recovered liquid was neutralized slowly with sodium carbonate. After neutralization, the pH was raised to pH 10.00 with 0.1 M NaOH. These steps were done to remove the PCA from the matrix and reconvert it to the uncharged base form. Three extractions with 50, 25, and 15 ml HPLC grade methylene chloride were made of the aqueous solution, which was then discarded. The three portions of methylene chloride were combined with 2 ml of 0.1 M HCI and 10 ml of 0.001 M heptansulfonic acid solution. The mixture was rotoevaporated slowly until only the aqueous mixture remained. Meanwhile, a C-18 Sep Pak cartridge was prepared by washing with 5 ml of HPLC acetonitrile, 5 ml HPLC water and 5 ml of 0.10 M heptanesulfonic acid solution. The residual contents of the rotoevaporatory flask, which contained the extracted PCA in the $HPCA^+$ form, were then passed through the conditioned cartridge as well. The $HPCA^+$ was desorbed along with the surfactant by passing 2 ml HPLC acetonitrile through the cartridge and collecting the eluant in a test tube. These steps were done to purify the PCA while concentrating the sample. An additional 0.2 ml of HPLC methanol was added to the recovered solution to prevent precipitation of the surfactant. All chemicals were analytical reagent grade.

The analytical finish for this analysis was ion-pair, reversed phase high pressure liquid chromatography (HPLC) with a diode array UV-VIS absorbance detector. The column was a 3μC-18 reversed phase, 10 cm narrow bore column (Hewlett-Packard). The mobile phase was 30 percent methanol/70 percent buffered surfactant solution. The latter solution contained 0.001 M heptanesulfonic acid and enough HPLC potassium monohydrogen phosphate to raise the pH to 7.5. The mobile phase was delivered at 3.0 ml per min. flow rate, and analysis times were less than 10 minutes. The detector was set at 243 nm and the injector volume was 4 μl. The identity of the PCA peak was confirmed by comparison of its UV spectrum to that of a standard. The analytical detection limit obtained by this method was 10 ppb PCA.

## *Results and Discussion*

In the study watersheds, PCA was below detectable levels in the soil or leaf litter before the application of Dimilin 4L to the foliage canopy. After application, as DFB was washed from the leaves and stems and as it degraded, measurable quantities of PCA were found. In some cases, concentrations on a dry weight basis of sample rose to values nearing one part per million. But typically the values were much lower, in the range of 50-400 ppb. A number of both the leaf litter and soil samples collected by the Wimmer group have been analyzed. The results for samples processed to date are provided for leaf litter (Figure 17-1) and soil (Figure 17-2). Replicates of the plotted average values for each time point deviated by no more than 20 percent.

Initially there was little PCA in the leaf litter samples. Very little of the sprayed parent DFB made its way through the forest canopy. As the season progressed, PCA concentrations increased as DFB directly washed off the canopy into the leaf litter and there degraded. After the first leaf fall, the PCA concentration did not change much, apparently because of low biological activity during the cold winter months. The PCA concentration increased in the leaf litter the following summer through September concomitant with the disappearance and degradation of DFB (see Terrestrial

Environment). After the second leaf fall, the PCA concentration was reduced as leaves that had not been treated with DFB entered the leaf litter.

The only soil analyzed to date was from plot A, giving some interesting results. An initial absence of PCA was followed by a significant increase in concentration in the soil. The concentration then decreased throughout the summer. We suspect the initial high PCA concentration was due to a storm washing residual PCA, which is a contaminant in the spray, from the canopy directly into the soil, where it was effectively bound as described above. After leaf fall, the concentration of PCA increased during the following season as biological activity increased. After the second leaf fall, the concentration again decreased through the winter months to near detection limits, then another dramatic increase in concentration occurred the following spring. This was the last sample processed to date, so it is not known what changes have occurred since that time. The most recent increase in PCA is probably the consequence of DFB breaking down in the leaf litter and being transported to the soil.

## Conclusions

The analysis of PCA in forest leaf litter and soils is a tedious, time consuming task. The results of the analysis based on dry weight are highly variable for a variety of reasons. The soil moisture varies considerably from season to season in the poor, thin soils of the study watersheds. The concentration of PCA is dynamic; it increases presumably as a result of DFB degradation, while almost simultaneously decreasing due to its own degradation or physical transport, or dilution with fresh organic matter, or both. The results to date indicate that PCA is present in low concentrations in areas sprayed with DFB. The concentration of PCA tends to increase with microbiological degradation of the parent DFB, and PCA does not appear to persist in the environment. Results obtained for both the leaf litter and soil should be confirmed by additional testing.

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**Ground Litter**
**Various Plots (arrows denote leaf fall)**

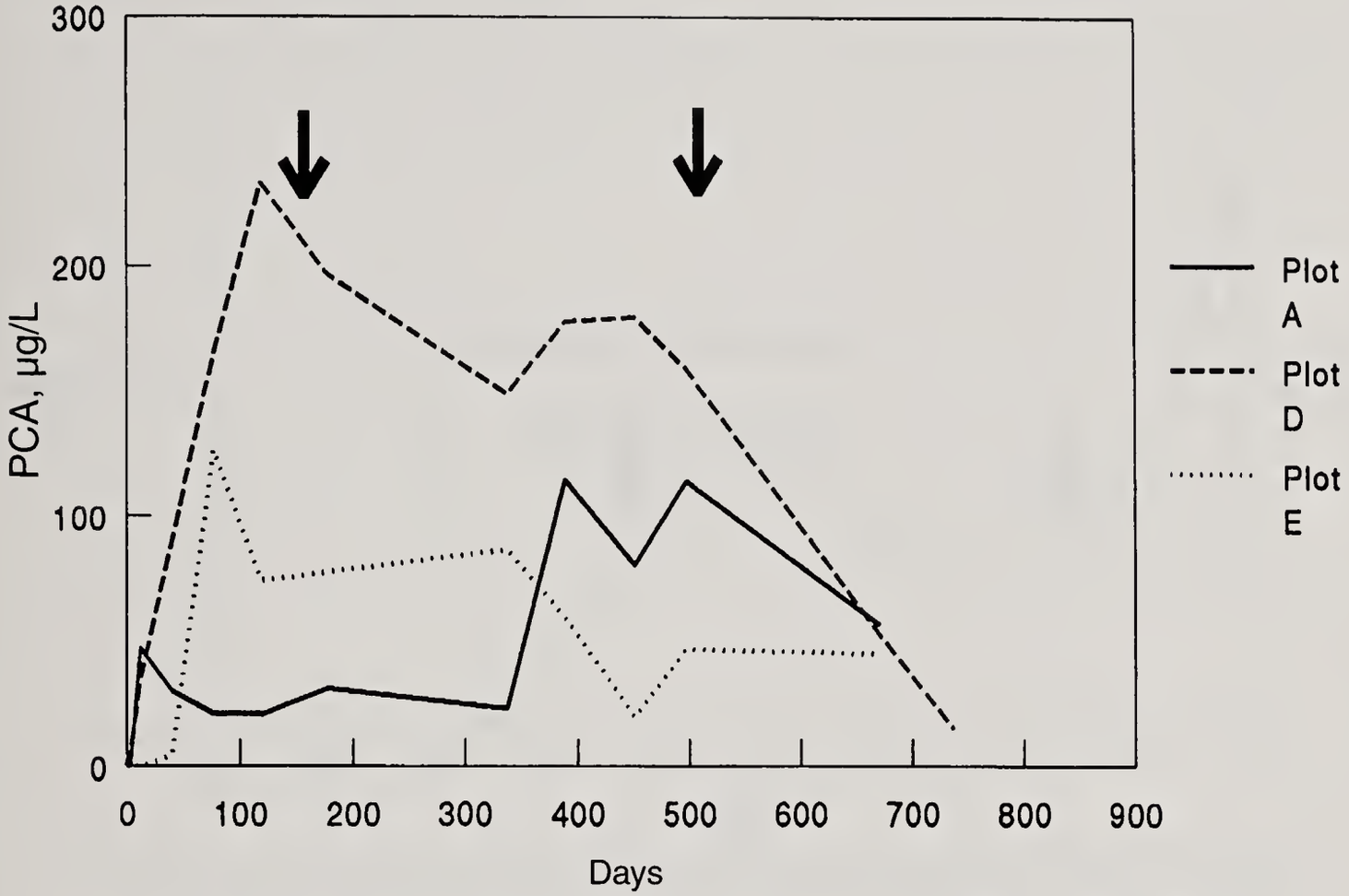


Figure 17-1. Parachloroaniline (PCA) found in ground litter samples, Fernow Experimental Forest. Results show average values for each time point taken in plots A, D, and E. Bold arrows denote dates of leaf fall.

**Soil**
**Plot A (arrows denote leaf fall)**

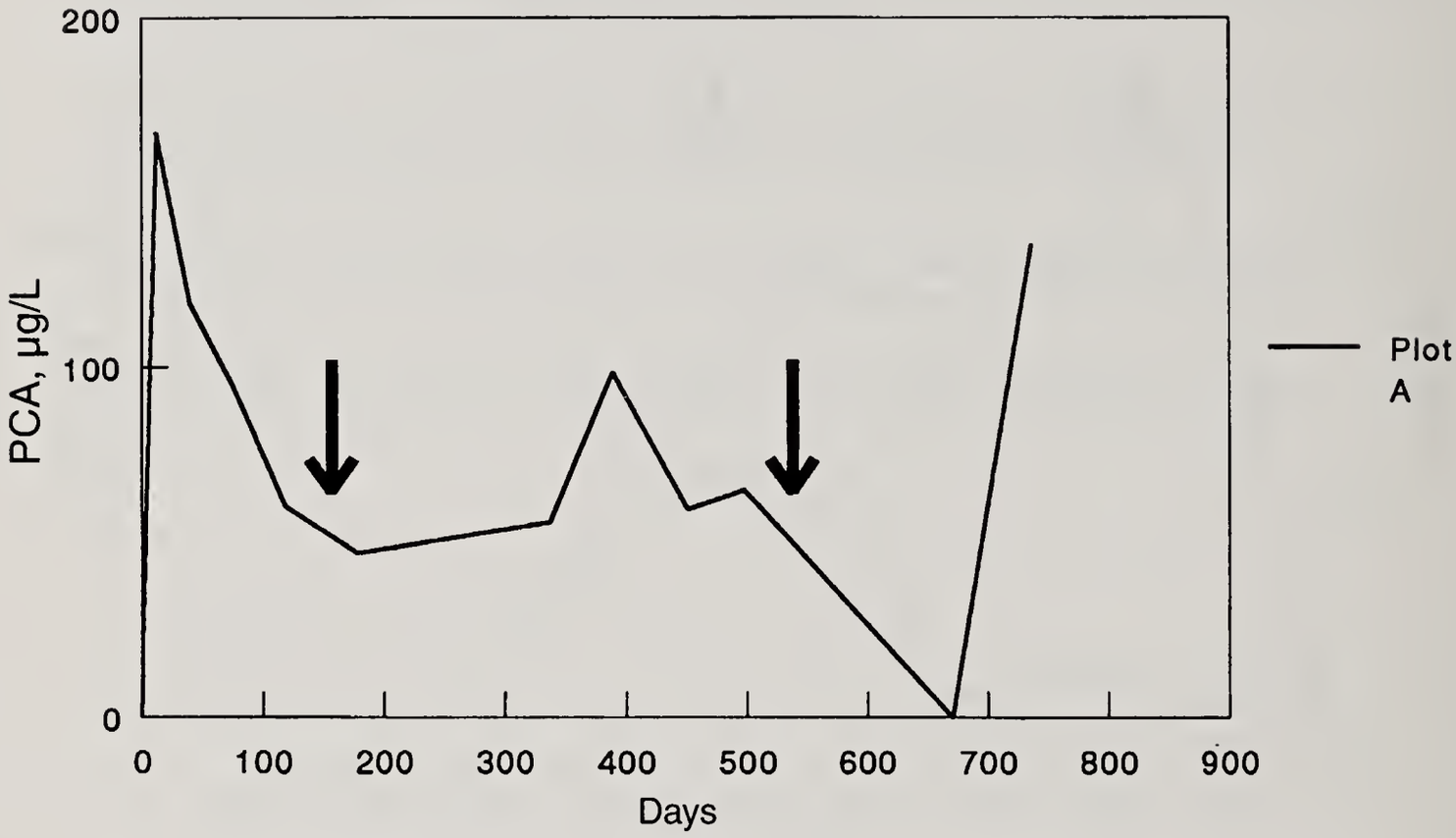


Figure 17-2. Parachloraniline (PCA) found in soil samples, Fernow Experimental Forest. Results show average values for each time point taken in plot A. Bold arrows denote dates of leaf fall.

# Part E. Summary

**Richard C. Reardon**

## Introduction

The synthetic arthropod growth regulator diflubenzuron (DFB), Dimilin (Uniroyal Chemical Co.), is aerially applied to control populations of the gypsy moth. Since its registration by the U.S. Environmental Protection Agency in 1976, approximately 4 million acres infested with the gypsy moth were treated with Dimilin 25-W as part of the Federal and State cooperative gypsy moth suppression program.

This study in the Fernow Experimental Forest (Tucker County, West Virginia) is the first to evaluate impacts on selected nontarget organisms and the environmental fate of DFB within entire broadleaf forested watersheds in the Appalachian Mountains using the Dimilin 4L formulation.

The Fernow Experimental Forest was selected as the study site due to the duration of the project (at least 6 years), application of an insecticide, the need for similar broadleaf forested watersheds in the Appalachian Integrated Pest Management (AIPM) Project area, and the availability of long-term data on stream water chemistry and flow rate, and a known history of stand succession. Four watersheds were selected, with two as untreated (WS 4 and WS 7) and two (WS 1 and WS 13) aerially treated with Dimilin 4L at 0.08 *l*/ha (0.03 lb AI/acre) in 9.4 *l* water/ha (1 gal/acre) on May 16, 1992.

During the study, gypsy moth populations on the Fernow Experimental Forest were negligible, so the potential additional effects on nontarget organisms due to defoliation were avoided.

Five nontarget groups were monitored: (1) aquatic and terrestrial salamanders; (2) canopy arthropods; (3) pollinating insects; (4) fungi, bacteria and invertebrates in leaf litter and soil; and (5) aquatic macroinvertebrates. Initial and degradation levels of DFB on tree surfaces, in leaf litter, in soil, and in water were also determined.

All nontarget groups were monitored on all four study watersheds before treatment (1989-1991) and after treatment (1992-1993). Additional posttreatment monitoring was conducted for salamanders, canopy arthropods, and residue levels in leaf litter and soil on all watersheds in 1994.

## Aquatic Organisms

***Salamanders.*** - The seal salamander (*Desmognathus monticola* Dunn) comprises nearly 90 percent of the aquatic salamander species in these four streams. In September and October of each year (1989-1994), 20 adult male and 20 adult female *D. monticola* were collected from all watersheds. Stomach contents, percentages of tail fat and carcass fat, and weight of total fat as well as number and volume of follicles were determined. Abundance and growth of adult and juvenile salamanders were studied in two 25-m long sections in each stream twice per month from May through September, 1989 to 1994. Major food items of *D. monticola* in the treated watersheds shifted from soft-bodied prey items (e.g. winged hymenopterans) before treatment to hard-bodied prey items (e.g. ants and coleopteran adults) after treatment but not in the untreated watersheds. *D. monticola* consumed significantly greater numbers of ants in the treated watersheds in 1992 than before and after treatment years as well as in the untreated watersheds for all years.

No significant differences were detected in snout-vent-length (SVL) growth, surface densities, percentages of tail fat and carcass fat, weight of total fat, or follicle number and volume as a result of exposure to Dimilin 4L.

***Macroinvertebrates.*** - Two types of laboratory toxicity tests, exposure in water and feeding, were conducted to determine the effects of DFB on nontarget aquatic insects. Four species of heptageniid mayflies and the stonefly *Peltoperla arcuata* Needham were exposed to Dimilin 25-W in water for 96 h and then transferred to insecticide-free water for 36 days. The mayflies were exposed to 0, 0.6, 5.6, 55.7, and 557.2 ppb and the stonefly to 0, 1.0, 10.2, 101.5, and 1,015 ppb Dimilin 25-W in water. In the feeding studies, the stoneflies *P. arcuata* and *Pteronarcys proteus* Newman were fed Dimilin 25-W treated and weathered yellow-poplar leaves and observed for 60 and 90 days, respectively. The heptageniid mayflies were sensitive to DFB in water at concentrations as low as 0.6 ppb. In the feeding studies, survival of treated *P. arcuata* was significantly different from the untreated at day 60 of the test; mean survival was 60 percent in the treated group versus 82 percent in the untreated watersheds. In the field, samples were collected monthly from October 1989 through April 1993 using a multi-level artificial substrate basket sampler. Samples were also collected during fall 1990 and 1992 using leaf pack colonization samplers. Species that showed significant differences ($P \leq 0.05$) in densities and decreased in treated streams included the stoneflies *Leuctra* sp. and *Isoperla* sp., the mayfly *Paraleptophlebia* sp. and the cranefly *Hexatoma* sp. Mean shredder densities in treated streams declined significantly ($P \leq 0.05$) after treatment whereas densities rose in the untreated watersheds.

***Aquatic Fungi.*** - Aquatic hyphomycetes play a major role in decaying leaves in flowing water. Their distributions in the four watersheds were studied by membrane filtration of stream water samples and by leaf bag incubation in both streams and weir ponds. A total of 33 taxa of aquatic hyphomycetes was recorded from leaf bags placed in streams. In general, the number of taxa present tended to decrease over time in all watersheds except watershed 4 (untreated). A total of 108 fungal taxa was recorded by membrane filtration of water samples from all four watersheds. Only 25 taxa were common to all four watersheds. No direct impacts due to application of Dimilin 4L were detected on aquatic hyphomycetes.

## *Terrestrial Organisms*

***Salamanders.*** - The redback salamander, *Plethodon cinereus* (Green), and the mountain dusky salamander, *Desmognathus ochrophaeus* Cope, were the dominant terrestrial salamander species on the four watersheds. In September and October of each year (1989-1994), 20 adult male and 20 adult female *P. cinereus* and *D. ochrophaeus* were collected from each watershed. Stomach contents, percentages of tail fat and carcass fat, weight of total fat as well as number and volume of follicles were determined for each species. Surface abundance and growth data were based on specimens collected under pine boards and natural cover objects along two 100-m horizontal transects located 20 and 40 m upslope from each stream in each watershed from 1989 to 1994. Both species showed a shift to hymenopterans (ants) and mites in the treated and untreated watersheds after Dimilin 4L application. No significant differences were detected in percentages of carcass fat, total fat, or follicle numbers or volume, growth, or surface abundance for the two salamander species between treated and untreated watersheds before or after application of Dimilin 4L.

***Canopy arthropods.*** - During each year of the study (1989-1994), samples were collected from early May through mid-August by means of blacklight traps, foliage pruning and burlap banding. One blacklight trap was operated in each watershed on the same night, one night each week. Foliage samples were collected once each week from black birch, black cherry, mixed maple and mixed oak trees on two sites of each watershed. A total of 40 trees was banded per watershed with equal numbers of black birch, black cherry, mixed maples and mixed oaks.

A total of 47,788 arthropods representing 188 families was identified under burlap bands. While overall abundance of arthropods was not reduced on treated sites in 1992, there was a significant ($P \leq 0.05$) reduction in arthropod richness in the treatment year. Some recovery in richness occurred in 1993. The most dramatic decline of arthropods under bands on treated watersheds was seen in the nontarget macrolepidopterous larvae during the treatment year. Total foliage arthropod richness (determined using pole pruners) was 225 families, and foliage arthropod abundance was 115,551. Significant ($P \leq 0.05$) decline occurred in taxa richness on treated watersheds in 1993, the year after treatment, and in arthropod abundance during 1992 and 1994. Among nontarget macrolepidopterous larvae on foliage, total richness for the study was 111 species and total abundance was 7,277. Caterpillar richness was significantly reduced ($P \leq 0.05$) on treated watersheds in the treatment year. Caterpillar abundance declined in 1992, the treatment year, and did not recover by the end of the study in the fall of 1994. Macrolepidoptera abundance was 71 percent lower on the treated watersheds in 1992 and remained significantly lower ($P \leq 0.01$) through the sampling period in 1994.

***Pollinating Insects -- Native Species.*** - Samples of adult pollinators were collected in 1991 to 1993 using Townes-style and Cornell-style Malaise traps. Five Townes-style Malaise traps were located in each watershed to collect insects for quantitative studies. In addition, Cornell-style Malaise traps were positioned in openings near forest edges. Significantly fewer ($P \leq 0.05$) yellowjacket workers were recovered from the treated watersheds from May through late September in the treatment and posttreatment years, respectively.

***Pollinating Insects -- Honey bees.*** - Ten colonies of honey bees were located approximately 0.6 km away from the treated watersheds. This site has supported a healthy apiary for many years. All honey bee colonies were sampled at bi-weekly intervals before treatment, during spray and after treatment (primarily May 1 through June 28, 1992). Samples consisted of 1 liter per week of adult honey bees, bee larvae, beeswax, honey, and pollen. The samples of bees, honey and pollen were analyzed for DFB residue levels using a technique described by Wimmer. No detectable levels of DFB were found in any of the samples indicating either a lack of DFB transport by the bees, or lack of bee foraging in Dimilin 4L treated watersheds, or both.

***Invertebrates in Leaf Litter and Soil.*** - Soil core samples and litter bag samples were taken from a 15- by 30-m plot established in each of the four watersheds. Over 39,800 organisms in 17 orders were identified during the study. Overall, samples were dominated by mites (49 percent) and springtails (28 percent). Although a total of 143 taxa was counted from soil core samples, only 19 were present consistently enough to be used for analysis of DFB effects. There was a significant ($P \leq 0.05$) negative difference (a decline in treated watersheds) for the order Araneida (spiders) and two species of mites between treated and untreated watersheds. In leaf litter bags, only 19 taxa were present consistently enough to be used for analysis of DFB effects. Significant ($P \leq 0.05$) negative differences were observed for only one species of springtail.

*Soil Organisms.* - In the laboratory, Dimilin 25-W was applied to broth cultures of two species of bacteria and three species of cellular slime molds. In the field, 10 samples of soil-leaf litter were collected for cellular slime mold analysis from each of the four study watersheds on eight occasions in May to June and five times in September to October from 1989-1992. The watersheds were sampled once in spring 1993. Grab samples of leaf litter from the watersheds were taken for bacterial counts in May and September to October 1991 and 1992. No DFB impacts were detected for the cellular slime molds in laboratory and field evaluations. In the laboratory, decreases in bacterial growth occurred when exposed to the high DFB concentration.

*Decomposer and Ectomycorrhizal Fungi.* - Five permanent 100-$m^2$ study plots were established in each of the four watersheds. These study plots were visited at 14-day intervals during July 1-October 8 from 1989 through 1992. Additional data on fruiting body occurrences was collected in 1993 and 1994 by searching throughout each watershed. A total of 2,659 fruiting bodies of macrofungi was collected from the four watersheds from 1989 through 1992. Species of *Amanita* and *Russula* spp. were the most important ectomycorrhizal fungi present in the four watersheds, whereas species of *Collybia* and *Marasmius* spp. were among the most important fungi associated with decomposing leaf litter. General trends at the fungal family level indicate that Dimilin 4L had no effect on mycorrhizal or litter decomposing fungi based on fruiting body counts.

*Terrestrial Fungi in Leaf Litter.* - Leaf samples were collected from six locations at each of four study watersheds from March 1991 through September 1992. Subcultured fungi were allowed to colonize on malt agar or potato-dextrose agar with 0, 10, 25, 100 and 1,000 ppm Dimilin 4L. Ninety-two morphologically distinct fungi were isolated from senescing leaves in the study watersheds. Seventy-two predominately occurring fungi were tested with the different concentrations of Dimilin 4L. The radial growth of fungal isolates did differ significantly ($P \leq 0.05$) for seven fungi but only at the 100 and 1000 ppm Dimilin 4L concentrations. These high concentrations of DFB would not be encountered during operational programs for gypsy moth.

*Microorganisms in Soil.* - In July 1990, 239 undisturbed soil cores (7.5 cm by 7.5 cm) were obtained from one of the study watersheds using a slide hammer and bulk density corer. Structured integrity of the soil cores was maintained. All cores were weighed to determine their moisture content and randomly assigned to one of three treatment groups: untreated, treated DFB, and frass. Microbial biomass and respiration decreased after exposure to DFB, however, differences were small and were not significant after 21 days.

## Residue Levels and Persistence

*Aquatic Environment.* - ISCO and grab water samples were vacuum-filtered through 0.45-µm filters and then loaded onto pre-conditioned Sep Pak cartridges. The processed samples were analyzed using HPLC. DFB was not detected in any of the water samples collected posttreatment probably because of its dilution in rapidly flowing first- and second-order streams, its general insolubility in water, and sample size. The levels of DFB expected in the field (ppb, ppt) should have been retained by the C-18 cartridges.

*Terrestrial Environment.* - DFB was extracted from all samples with acetone, and the extracts were prepared and analyzed using gas chromatography and mass spectrometry with deuterated

DFB as an internal standard. Samples, such as leaves, were analyzed without HPLC cleanup, while others, such as litter and soil, were analyzed after a partial HPLC cleanup.

***Leaves.*** - DFB concentration on leaves drops significantly during the first 2 to 3 weeks after application, followed by a much more gradual decline over the rest of the season. At season's end, the concentration was found to drop to an average of 15% (range 5-35% on 20 trees, four species) of the concentration after spray. Much of this drop resulted from growth dilution, not to actual loss of insecticide. Adjusting for growth dilution, a significant amount of DFB, an average of 40% (range 15-80%) of that applied, remained on leaf surfaces at the end of the season, when leaf fall delivers the residual pesticide into the ground litter. This leaf persistence pattern was consistent with the non-target canopy arthropod impacts that were observed throughout the growing season, even on species that appeared weeks after the spray.

***Ground litter.*** - A portion of the Dimilin 4L spray reaches the ground as evidenced by DFB appearing in the ground litter immediately after application. An initial rise in this DFB occurred 2 to 3 weeks after spray, paralleling loss from overlying leaves, followed by a gradual decline to low levels during the summer months. At leaf fall, DFB concentration rises significantly, presumably due to residual pesticide on leaves. These levels remained high over the winter months, declining during the second summer. Just prior to leaf fall the second year, most sample plots still contained easily-quantifiable DFB levels that subsequently became diluted to low or undetectable levels by the fall of untreated leaves. DFB, therefore, persists in ground litter for over 17 months post spray in this forest environment in the absence of Dimilin application in the second year.

***Soil.*** - All time points for the two sample plots with the highest litter concentration of DFB were worked up for analysis. In no soil sample, regardless of time point, was a detectable level of DFB found. Spiked soil samples showed that DFB was extracted from soil with the procedure used. The most likely explanation for this result is that DFB was biodegraded along with the ground litter during the warm summer months.

***Bark.*** - None of the bark samples analyzed from the trees with highest DFB coverage showed a detectable level of DFB, likely due to the small sample size.

***Artificial substrates.*** - Glass slides positioned approximately 1 m above the ground along a 70-m. horizontal transect and along a 30 m intersecting vertical transect near the center of Watershed 1 showed easily-detectable DFB in a variable pattern of coverage. No detectable DFB, however, was found on nylon spheres hung above ground in the same area of the same treated watershed.

***Breakdown Products of Diflubenzuron.*** - DFB degrades by hydrolytic cleavage to 2,6 - difluorobenzoic acid (DFBA) and 4-chlorophenylurea (CPU). The CPU in turn degrades to 4-chloroaniline (parachloroaniline or PCA) by microbial action in soil and water. Some people are concerned that PCA is a possible carcinogen. In the study watersheds, PCA was below detectable levels in soil or leaf litter before the application of Dimilin 4L to the foliage canopy. Later, as DFB moved into the ground litter, measurable quantities of PCA were found in litter and underlying soil. Typically, values were in the range of 50-400 ppb.

## *Conclusion*

This evaluation of the potential impacts of DFB on selected nontarget organisms in entire broadleaf forested watersheds was initiated in response to an information gap identified by the AIPM Project. In general, DFB effects on selected species of macrolepidoptera and aquatic macroinvertebrates were as previously documented for partial watersheds. The continued significant impact of DFB on selected canopy arthropods 27 months posttreatment is new data. No detectable DFB impacts on aquatic and terrestrial fungi, pollinating insects (except yellowjackets); invertebrates in leaf litter and soil (except two species of mites and on species of springtail); soil organisms (bacteria and cellular slime molds), and aquatic and terrestrial salamanders are new data. The development of a rapid, sensitive and precise method for analyzing DFB in complex environmental samples is a significant contribution from this study and for future studies of the environmental fate and persistence of DFB. These nontarget data recorded on the Fernow Experimental Forest should be considered representative for other entire broadleaf forested watersheds in the Appalachian Mountains. Our limitations for this evaluation included: (1) small watershed size, (2) minimal replication of treated and untreated watersheds, and (3) inability to use these watersheds exclusively beyond 6 years.