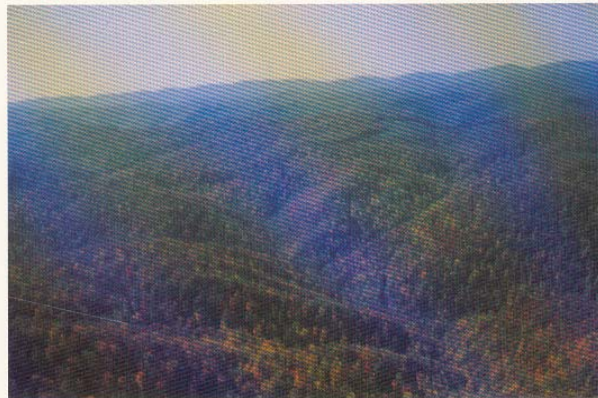


National Center of Forest Health Management

TECHNOLOGY
TRANSFER

*Non-target Impacts
Diflubenzuron*

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



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USDA

Forest Service

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Acknowledgments

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Use of most insecticides is regulated by State and Federal law. Applicable regulations must be obtained from appropriate regulatory agencies. **CAUTION:** Insecticides can be injurious to humans, domestic animals, plants, fish, or other wildlife if not handled or applied properly. Use all insecticides selectively and carefully. Follow recommended practices given on the label for use and disposal of insecticides and insecticide containers.

Table of Contents

Acknowledgments ... v

Contributors ... v

Part A. Project Description ... 1

Chapter 1. Introduction ... 2

Introduction ... 3
Diflubenzuron ... 3
The Nontarget Project ... 4
 Objectives ... 4
 Study Location ... 4
 Study Design ... 5
References ... 5

Chapter 2. Site Characteristics ... 6

Climate ... 7
Study Watersheds ... 7
References ... 9

Part B. Aquatic Organisms ... 13

Chapter 3. Aquatic Salamanders ... 14

Introduction ... 15
Laboratory Study Methods and Costs ... 15
Field Study Methods and Costs ... 16
 Advantages and Disadvantages ... 16
Results ... 17
 Laboratory Study ... 17
 Field Study ... 17
Discussion ... 17
Recommendations for Future Studies ... 18
References ... 18

Chapter 4. Macroinvertebrates ... 23

Introduction ... 24
Laboratory Study Methods ... 24
Field Study Methods ... 25
Results ... 26
 Laboratory Study ... 26
 Field Study ... 26
Discussion of the Laboratory Study ... 27
Recommendations for Future Studies ... 27
References ... 28

Chapter 5. Aquatic Fungi ... 31

Introduction ... 3

Field Study Methods ...	32
Results ...	33
Pretreatment 1991 ...	33
Treatment 1992 ...	33
Discussion ...	34
Recommendations For Future Studies ...	35
References ...	36

Part C. Terrestrial Organisms ... 41

Chapter 6. Terrestrial Salamanders ...	42
Introduction ...	43
Laboratory Study Methods ...	43
Field Study Methods and Costs ...	43
Advantages and Disadvantages ...	44
Results ...	44
Laboratory Study ...	44
Field Study ...	45
Discussion ...	45
Recommendations for Future Studies ...	46
References ...	46
Chapter 7. Canopy Arthropods ...	53
Introduction ...	54
Laboratory Study Methods ...	56
Field Study Methods and Costs ...	56
Advantages and Disadvantages ...	57
Results ...	58
Discussion ...	59
Recommendations for Future Studies ...	59
References ...	60
Chapter 8. Pollinating Insects – Native Species ...	66
Introduction ...	67
Field Study Methods ...	67
Results ...	69
Discussion ...	70
Recommendations for Future Studies ...	71
References ...	71
Chapter 9. Pollinating Insects – Honey Bees ...	77
Introduction ...	78
Field Study Methods ...	78
Methods for Diflubenzuron Extraction and Analysis ...	79
Results ...	80
References ...	80
Chapter 10. Invertebrates in Leaf Litter and Soil ...	81
Introduction ...	82
Field Study Methods ...	82

Results ...	85
Core Samples ...	85
Bag Samples ...	85
Discussion and Recommendations ...	86
References ...	86

Chapter 11. Soil Bacteria and Fungi ... 93

Introduction ...	94
Laboratory Study Methods ...	94
Field Study Methods ...	94
Results ...	94
Laboratory Study ...	95
Field Study ...	95
Discussion ...	96
References ...	96

Chapter 12. Decomposer and Ectomycorrhizal Fungi ... 106

Introduction ...	107
Field Study Methods ...	107
Results and Discussion ...	108
Conclusions ...	110
References ...	111

Chapter 13. Terrestrial Fungi In Leaf Litter ... 121

Introduction ...	122
Laboratory Study Methods ...	122
Results ...	124
Discussion ...	125
References ...	126

Chapter 14. Microorganisms In Soil ... 130

Introduction ...	131
Laboratory Study Methods ...	131
Results ...	132
Discussion ...	133
References ...	133

Part D. Residue Levels and Persistence ... 140

Chapter 15. Aquatic Environment ... 141

Introduction ...	142
Laboratory and Field Study Methods ...	142
Results ...	143
Discussion ...	144
References ...	145

Chapter 16. Terrestrial Environment ... 146

Introduction ...	147
Methods ...	148
Reporting of Diflubenzuron Analysis Data ...	151

Results ... 151
References ... 155

Chapter 17. Breakdown Products of Diflubenzuron ... 161

Introduction ... 162
Methods ... 162
Results and Discussion ... 163
Conclusions ... 164
References ... 164

Part E. Summary ... 168

Introduction ... 169
Aquatic Organisms ... 169
Terrestrial Organisms ... 170
Residue Levels and Persistence ... 172
Conclusion ... 174

Part A. Project Description

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 DEB 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 aurally 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 Femow 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 9° C, with lows occasionally falling to -29° C and highs rarely exceeding 32° 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 O1 and O2 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 m³/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 m³/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 m³/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 120° 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, NH₃, NO₃, SO₄, 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				Precipitation (mm)
	WS 1	WS 4	WS 7	WS 13 ²	
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				Precipitation
	WS 1	WS 4	WS 7	WS 13 ^a	
pH	6.22	5.93	6.21	6.18	4.16
Elect.Cond. ^b	44.6	22.4	26.6	26.7	36
Cl ^c	0.52	0.6	0.53	0.5	0.13
NO ₃	8.32	3.54	5.31	2.39	1.9
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
NH ₃	0.1	0.06	0.09	0.08	0.3

^a Data from 1984 to 1991

^b Electrical conductivity in $\mu\text{S}/\text{cm}$

^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

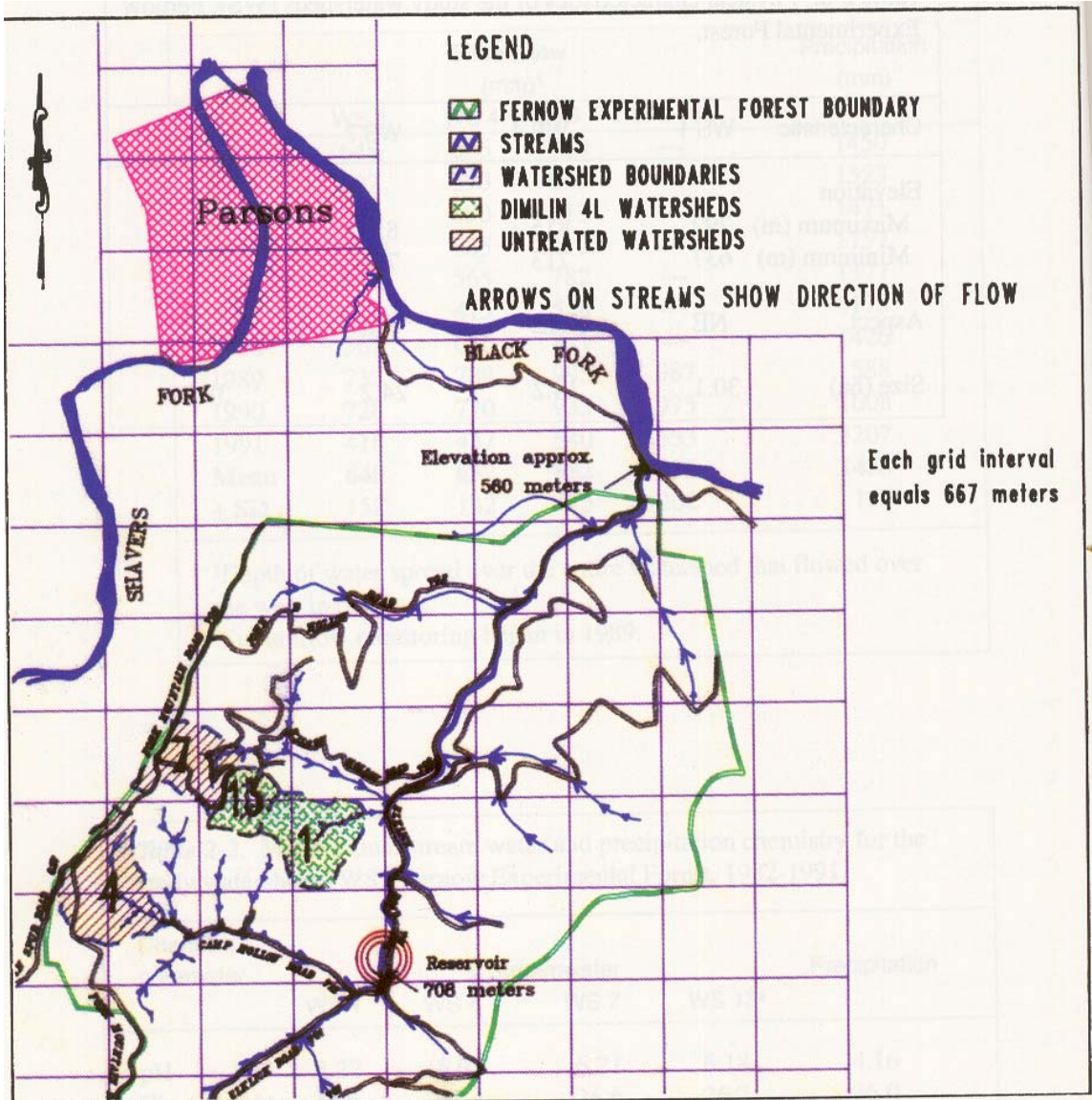


Figure 2-1. Study watersheds of the Fernow Experimental Forest near Parsons, West Virginia.

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. Ham 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 sphenoccephala* 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-in 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) (Fitzpatrick 1973, Kerr and others 1982, Rezmck 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 cm²) and 10 rocks (approximately 500 cm²) stacked on top of larger rocks (approximately 900 cm²). 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 cm²) 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).

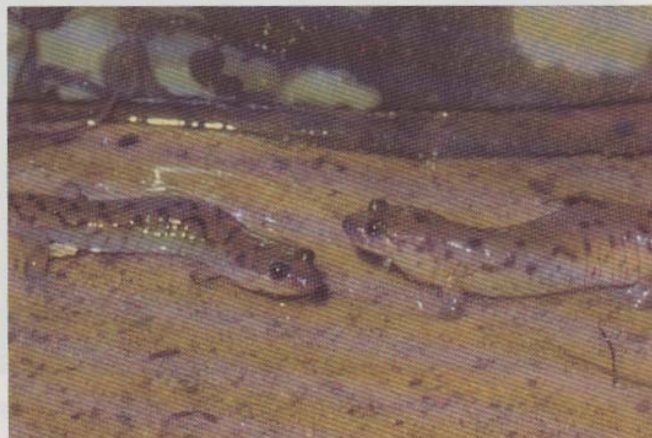


Figure 3-1. The seal salamander, *Desmognathus monticola*, a dominant aquatic species in the Fernow Experimental Forest.

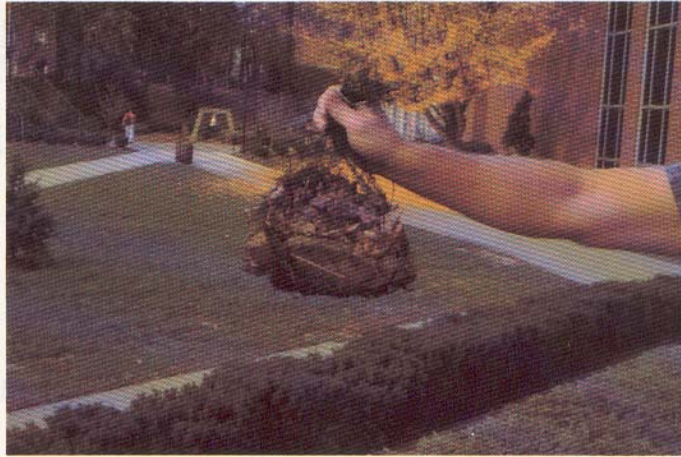


Figure 3-2. Refugium bags used to collect juvenile aquatic salamanders.

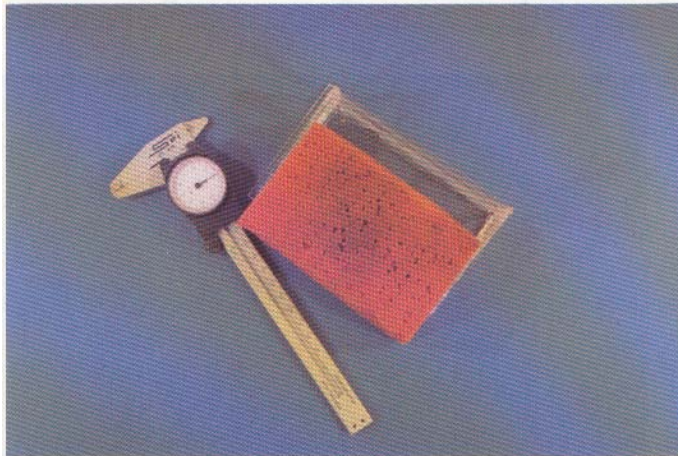


Figure 3-3. A device used to measure snout-vent-length of salamanders.

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 (*Cynogmula 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 100 C. Insects were maintained in glass test chambers, because preliminary testing showed that DEB 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 tines 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 ($P < 0.05$) 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-Qaten 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 DEB-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 ($P < 0.05$) 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 $P < 0.05$. 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 DEB 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.



Tara Dalbey

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 Kiug 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- μ 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 mm²) 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 NO₃ and NH₄ 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 DEB -- 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
<i>Number of taxa</i>							
1	Sugar Maple		6	8	15	3	8.0
	Red Oak		17	8	6	14	11.3
		19					
13	Sugar Maple		23	12	6	8	12.3
	Red Oak		19	19	12	17	16.8
		14					
4	Sugar Maple		15	10	14	16	13.8
	Red Oak		19	5	10	12	11.5
		13					
7	Sugar Maple		16	13	15	9	13.3
	Red Oak		14	16	21	12	15.8
		14					
^{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
<i>Number of taxa</i>							
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 i **975b**). 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-in 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 m² 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 m² (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 DEB (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 DEB. 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 DEB, 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
<i>Desmognathus ochrophaeus</i>				
% 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)
<i>Plethodon cinereus</i>				
% 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 ≤ 0.05).

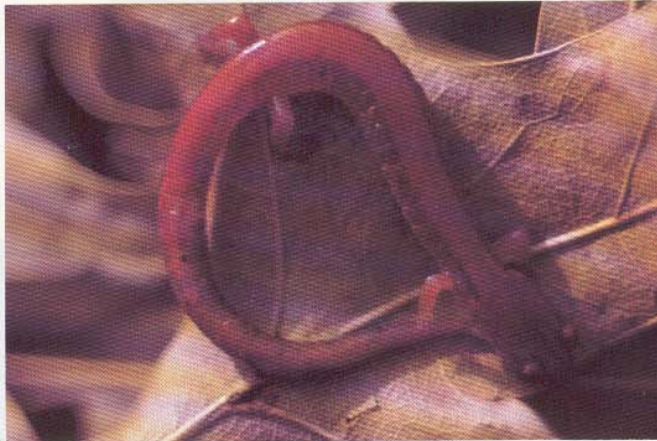
Table 6-4. Numbers of *Desmognathus ochrophaeus* and *Plethodon cinereus* observed in the treated (WS 1 and WS 13) and untreated (WS 4 and WS 7) watersheds (1989-1994 pooled data).

	Watershed	Numbers
<i>Desmognathus ochrophaeus</i>		
	1	531
	4	421
	7	173
	13	560
	Total	1685
<i>Plethodon cinereus</i>		
	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
<i>Plethodon cinereus</i>	613 (71.7 percent)	242 (28.3 percent)	855
<i>Desmognathus ochrophaeus</i>	1226 (68.6 percent)	561 (31.4 percent)	1787
Total	1839 (69.6 percent)	803 (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.

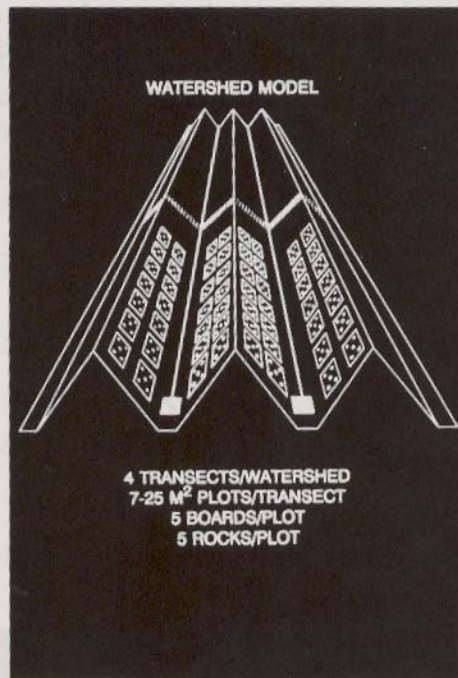


Figure 6-2. Transects of pine boards used to estimate surface abundance of salamanders.



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 DEB 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-m² 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 24° C for pupation and eclosion. For those species requiring a cold period, jars with pupae were held at 4° 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 (4° 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 DEB 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

equally high or higher richness and abundance of nontarget caterpillars (Butler and Kondo gypsy moth studies, other hardwood trees, such as maples, black cherry, and birches, yield 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 ($P < 0.05$) 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 ($P < 0.05$) 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

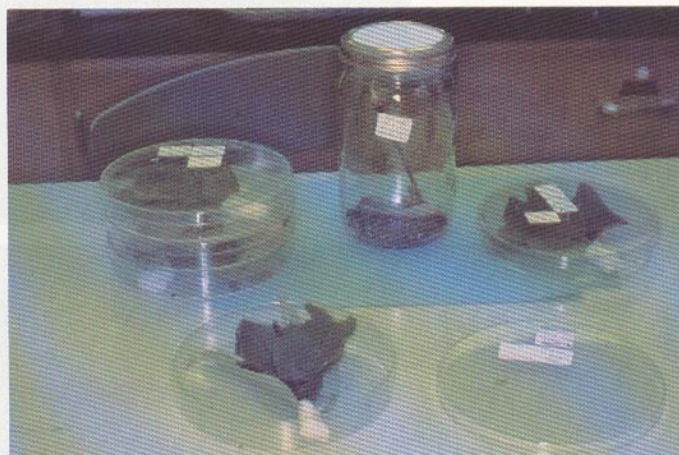


Figure 7-1. Rearing containers for field collected larvae, Fernow Experimental Forest.



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, bombyllid 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 0.61 alcohol per jar was used in April and September, and 0.81 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 90248-3 602). 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 Whiri-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 DEB 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 DEB 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_0 : 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_0 : 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 DEB-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 pompilid 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
<i>Dolichovespula adulterina</i> (Buyson)	0
<i>D. arenaria</i> (F.)	360
<i>D. maculata</i> (L.)	541
<i>D. norvegicoides</i> (L.)	1
<i>Vespula acadica</i> (Sladen)	1
<i>V. consobrina</i> (Saussure)	165
<i>V. flavopilosa</i> Jacobson	2,664
<i>V. maculifrons</i> (Bupson)	743
<i>V. vidua</i> (Saussure)	1
<i>V. vulgaris</i> (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.

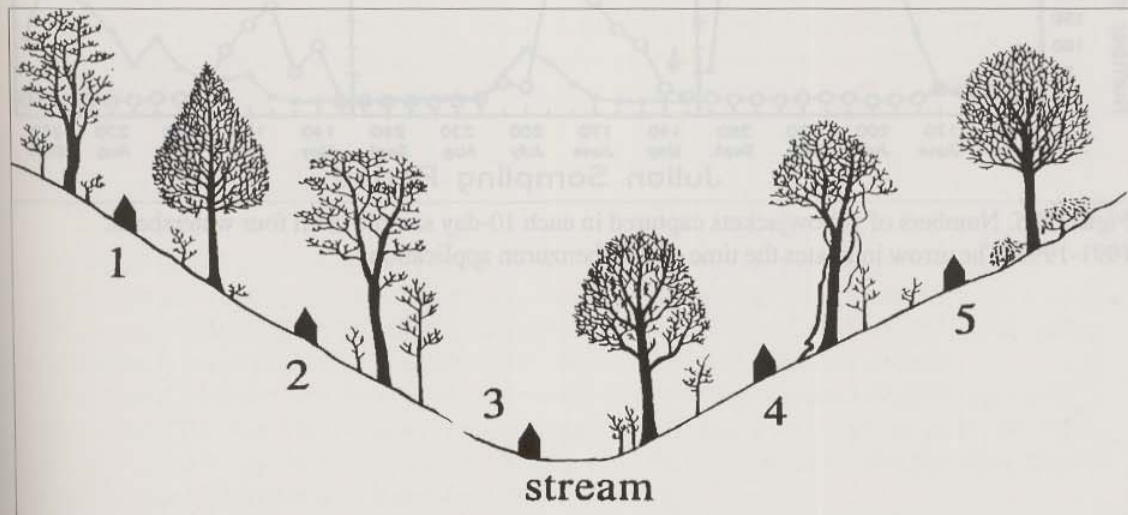


Figure 8-3. Positions of traps in a study watershed (diagram not to scale).

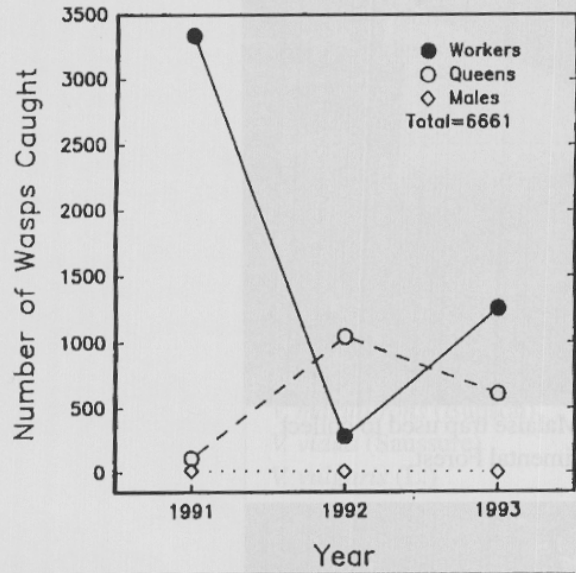


Figure 8-4. Numbers of yellowjackets captured in all four study watersheds, 1991-1993.

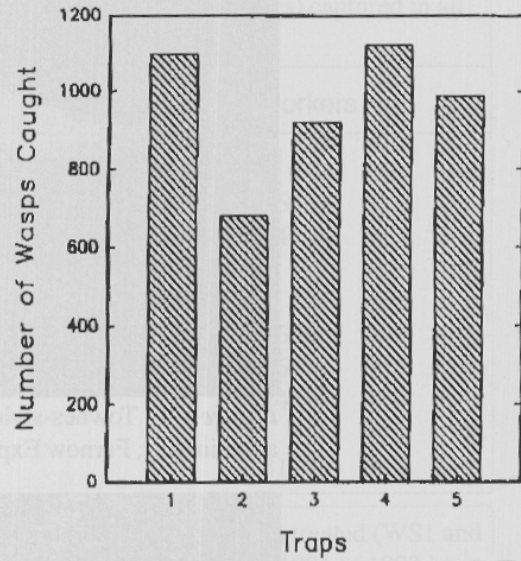


Figure 8-6. Numbers of yellowjackets captured by trap positions shown in figure 8-3, in all four watersheds, 1991-1993.

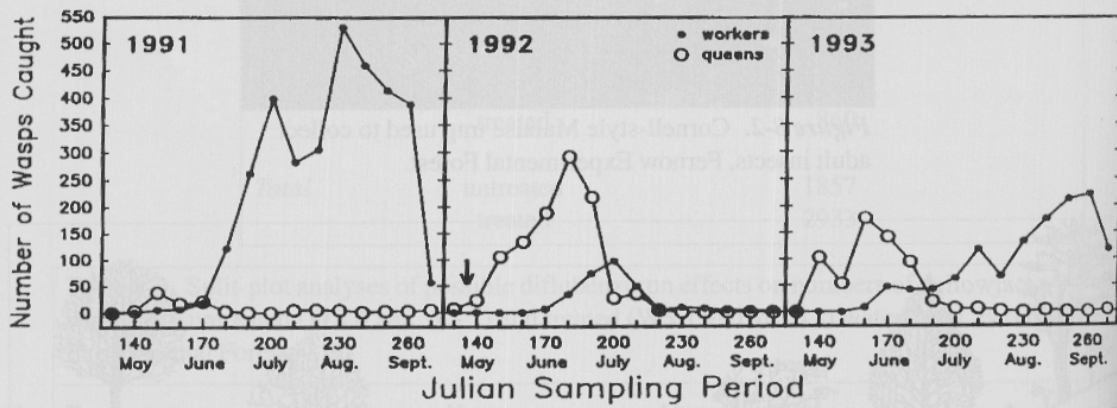


Figure 8-5. Numbers of yellowjackets captured in each 10-day sample in all four watersheds, 1991-1993. The arrow indicates the time of diflubenzuron application.

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 DEB 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 μ l of 500 ppm deuterated DEB 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 μ l acetonitrile. For partial purification to improve the signal-to-noise ratio during the GC and mass spectrometric analyses, 300 μ l (2 x 150 μ l) 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 μ l of acetonitrile, and stored at 4° C prior to GC 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 Femow 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 45° 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-Qaten 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-Qaten 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_0 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 purpureus*). 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 DEB 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
<i>Percent Composition</i>		
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
	<i>Ceratoppia</i> sp. A	31	0.4731
	<i>Ceratoppia</i> sp. C	16	0.1030
	<i>Oribatella</i> sp. A	20	0.3536
	<i>Diarthrohallus</i> sp. A	24	0.0833
	<i>Rhodacarus roseus</i>	30	0.1979
	<i>Heminothrus</i> sp. A	20	0.4667
	<i>Maerkelotritia gibbera</i>	30	0.4165
	<i>Cheiroseius</i> sp. A	9	0.0451*
	<i>Rhodacurus</i> sp. A	30	0.1979
	<i>Gamasellus concinnus</i>	29	0.0353*
<i>Tenuilala nuda</i>	17	0.3324	
Araneae	All	21	0.0452*
Collembola	All	34	0.0766
	<i>Onychiurus encarpatus</i>	30	0.2469
	<i>Isotomurus palustroides</i>	8	0.0838
	<i>Isotomurus japonica</i>	24	0.1920
	<i>Triacanthella copelandi</i>	14	0.1239
	<i>Hypoquastura brevispina</i>	18	0.1003
	<i>Isotomurus tricolor</i>	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
	<i>Ceratoppia</i> sp. C	0.3848	0.9983
	<i>Ceratoppia</i> sp. A	0.0869	0.1917
	<i>Diarthrohallus</i> sp. A	0.7285	0.9774
	<i>Oribatella</i> sp. A	0.0246*	0.4098
	<i>Cheiroseius</i> sp. A	0.2741	0.9917
	<i>Heminothrus</i> sp. A	0.5998	0.8407
	<i>Maerkeletritia gibbera</i>	0.0912	0.9958
	<i>Gamasellus concinnus</i>	0.1741	0.9352
Araneae	All	0.8845	0.7613
Collembola	All	0.0730	0.9958
	<i>Onychiurus encarpatus</i>	0.2193	0.9988
	<i>Triacanthella copelandi</i>	0.5605	0.4710
	<i>Sminthurus purpureus</i>	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-in 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 (1965 a).

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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Life cycle of *Dictyostelium discoideum*:

- a. individual amoeboid cells from germinated spores
- b. aggregation of individual cells
- c. migration of massed cells as a single unit
- d. fruiting body
- e. spores

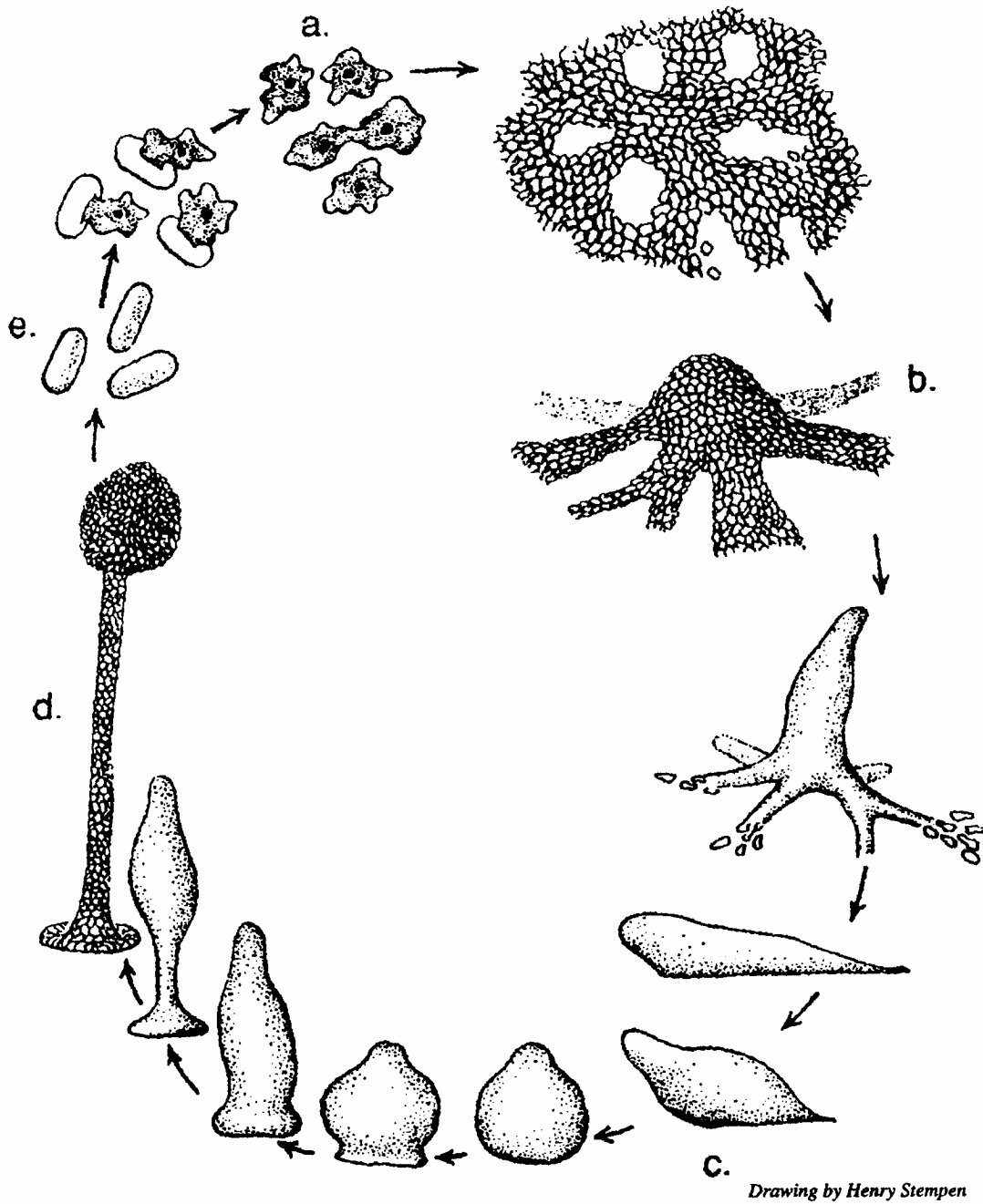
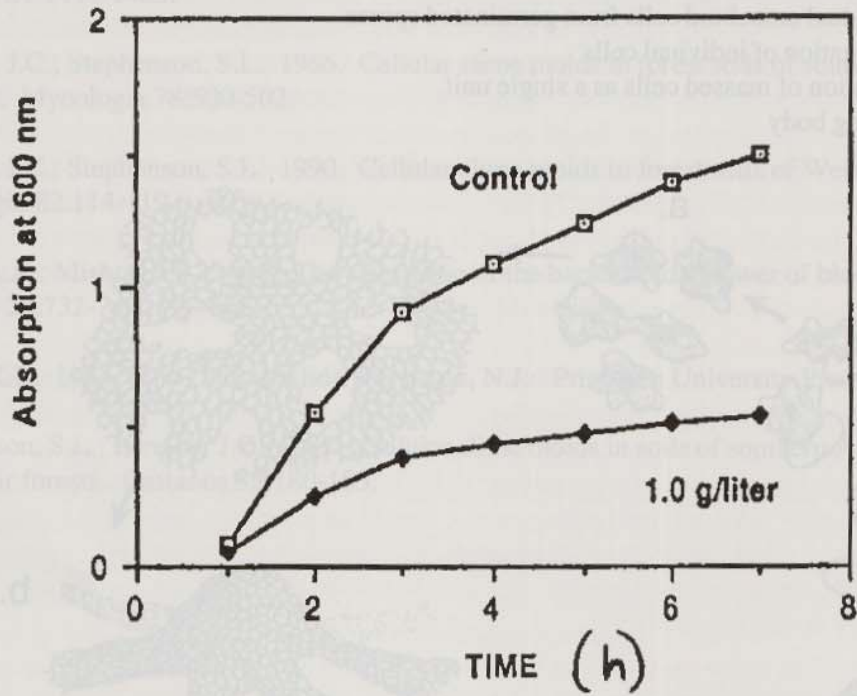
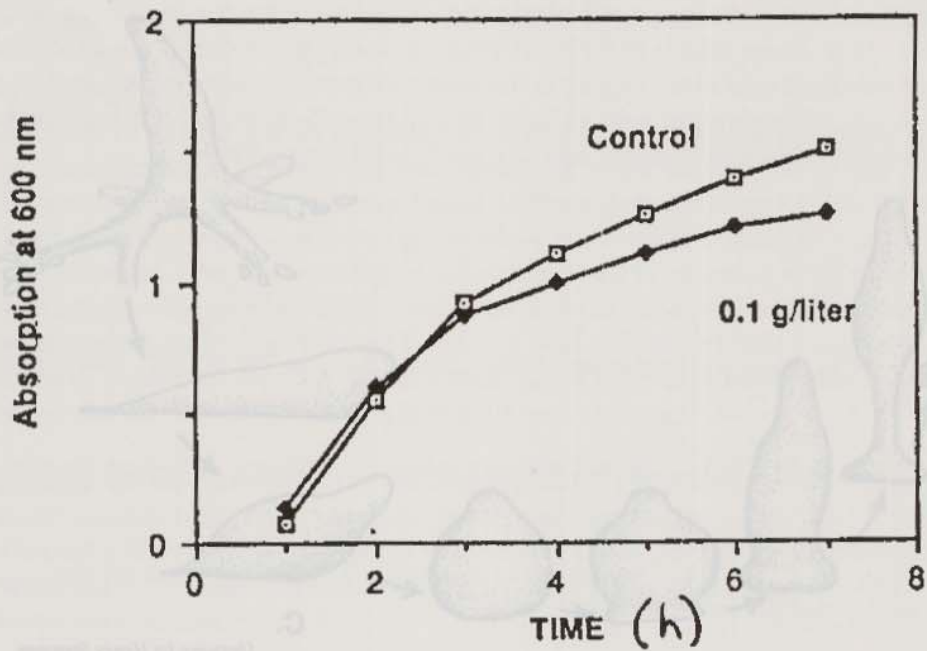


Figure 11-1. Life cycle of the cellular slime mold *Dictyostelium discoideum*, found in soil.

11-2a. BACILLUS CEREUS

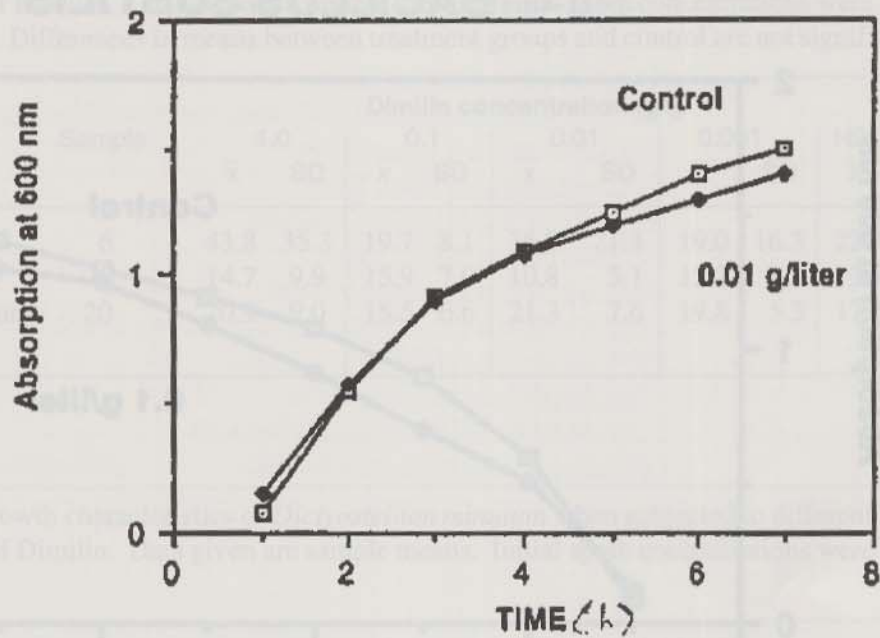


11-2b. BACILLUS CEREUS

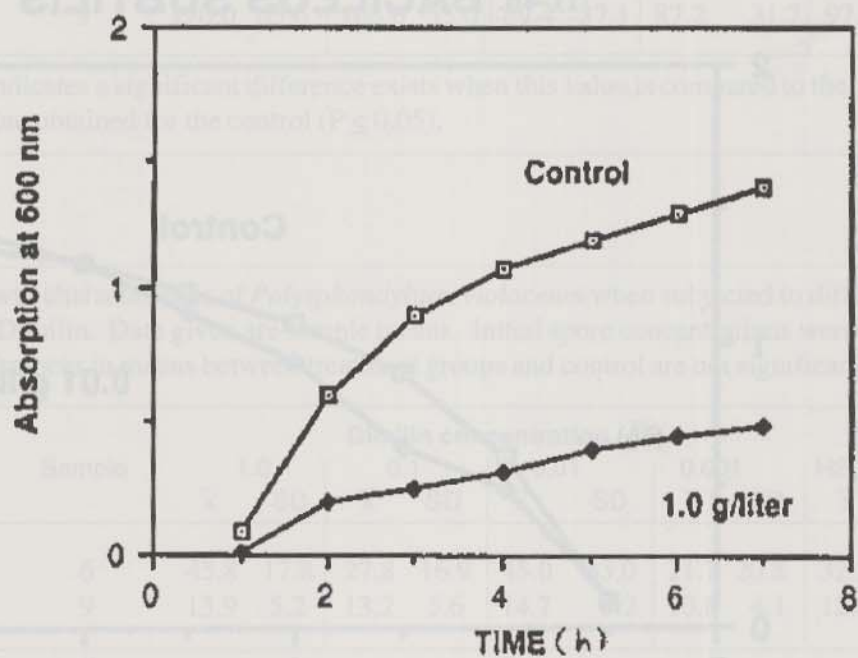


Figures 11-2a, b. Growth rate (each numbered time interval represents 8 hours) of *Bacillus cereus* in a nutrient broth with Dimilin present (a) at a concentration of 1.0 g per liter and (b) at a concentration of 0.1 g per liter. Growth rate of the same bacterium in the control flask is given for comparison.

11-3a. BACILLUS CEREUS

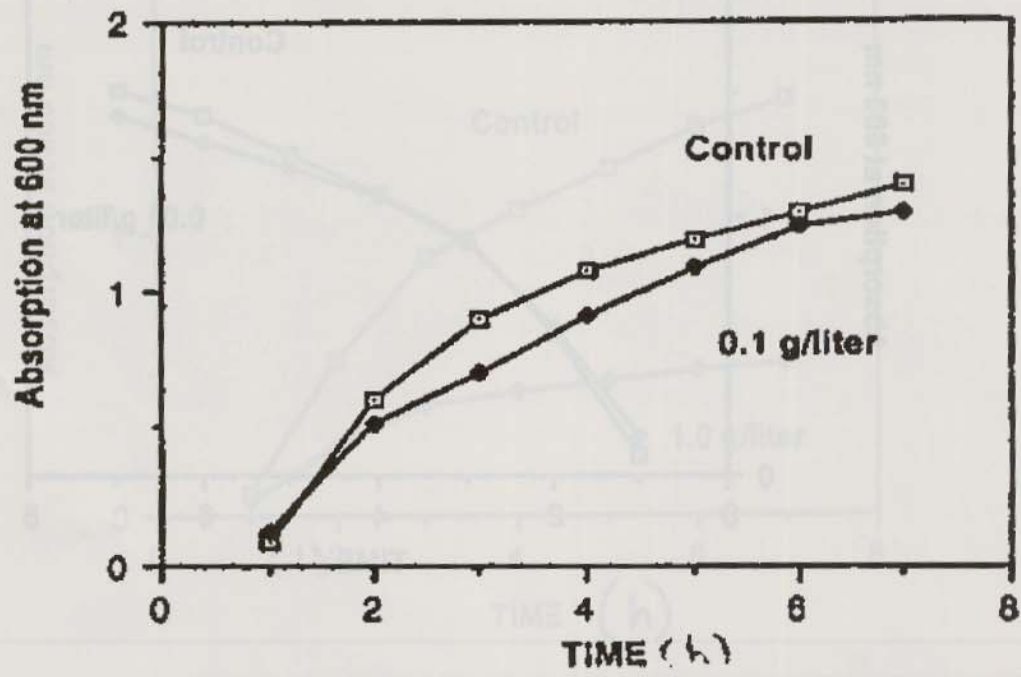


11-3b. BACILLUS SUBTILIS

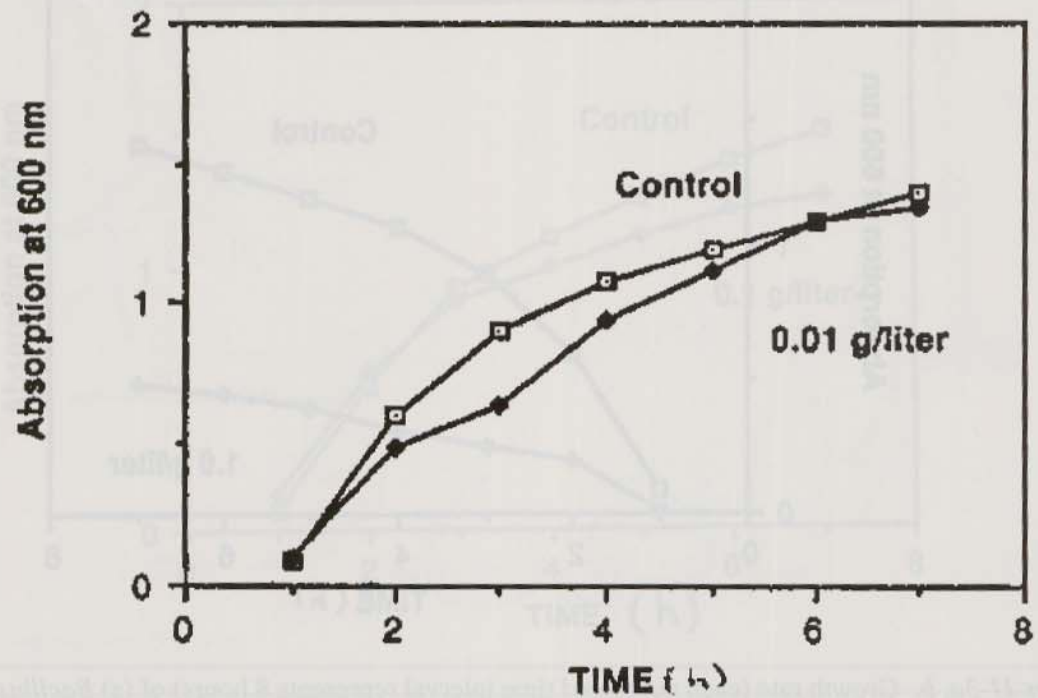


Figures 11-3a, b. Growth rate (each numbered time interval represents 8 hours) of (a) *Bacillus cereus* in a nutrient broth with Dimilin present at a concentration of 0.01 g per liter and (b) *Bacillus subtilis* in a nutrient broth with Dimilin present at a concentration of 1.0 g per liter. Growth rate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

11-4a. BACILLUS SUBTILIS



11-4b. BACILLUS SUBTILIS



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/cm ²	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/cm ²	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/cm ²	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.

Year	Watershed			
	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

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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 m² 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 ± 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 *Scieroderma* 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 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.

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

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.

Type of association	Watershed			
	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	<i>Amanita citrina</i>
	<i>Amanita fulva</i>
	<i>Amanita vaginata</i>
Boletaceae	<i>Boletinellus merulioides</i>
Cortinariaceae	<i>Cortinarius</i> spp.
Russulaceae	<i>Lactarius camphoratus</i>
	<i>Russula krombholzii</i>
	<i>Russula silvicola</i>
	<i>Russula viresens</i>
Sclerodermataceae	<i>Scleroderma citrinum</i>

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

Family of Basidiomycetes	Species
Tricholomataceae	<i>Clitocybe gibba</i>
	<i>Clitocybe</i> sp.
	<i>Collybia confluens</i>
	<i>Collybia dryophila</i>
	<i>Marasmius capillaris</i>
	<i>Marasmius siccus</i>
	<i>Mycena luteopallens</i>
	<i>Strobilurus conigenoides</i>

Chapter 13. Terrestrial Fungi In Leaf Litter

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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 DEB 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-cm² 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 DEB; in other words, an easily removable sample did not necessarily have less DEB 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	ppm
<i>Alternaria sp.</i>	3	0.024	UD ^a	UD
	3	0.033	UD	UD
	2	0.051	UD	UD
<i>Helminthosporium sp.</i>	3	0.184	UD	UD
	3	0.158	UD	UD
	3	0.159	UD	UD
<i>Trichoderma sp.</i>	4	0.186	2.68	14.4
	3	0.155	2.49	16.1
	3	0.196	0.69	7.2
<i>Pestalotia sp.</i>	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

^aUD 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

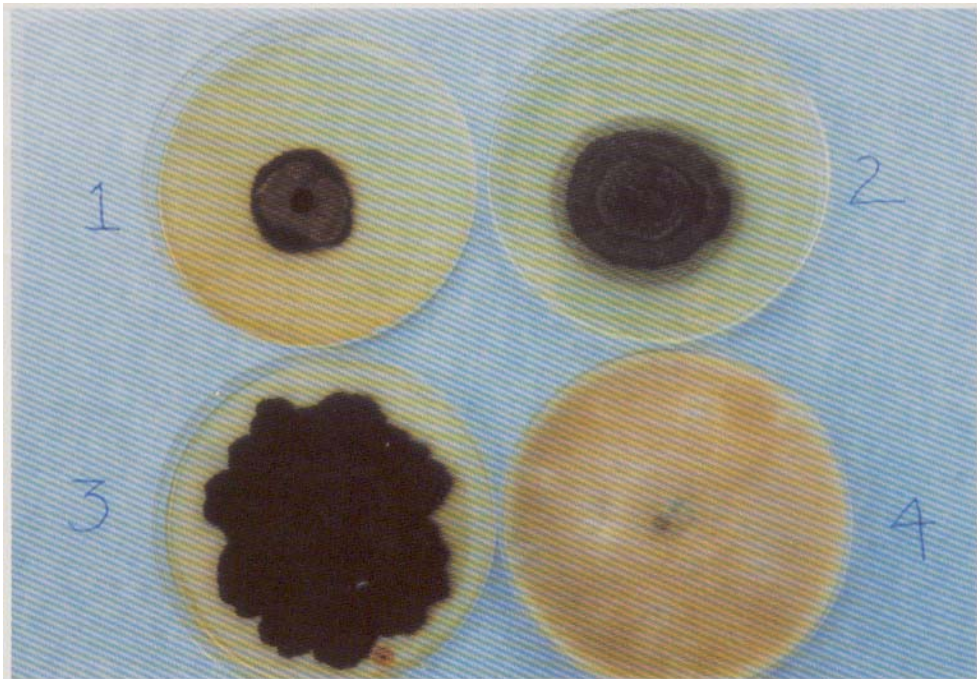


Figure 13-1. Categories of radial growth achieved by terrestrial fungi from leaf litter growing on agar from pure culture media amended with various concentrations of diflubenzuron, Fernow Experimental Forest.

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 CO₂ 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 cm²), 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-m² 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 K₂SO₄ 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 CO₂-C production per gram dry weight of soil per day. The denitrification rate of each sample was determined at the rate of N₂O-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 DEB 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.

References

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Biomass Carbon (micrograms/grams soil)

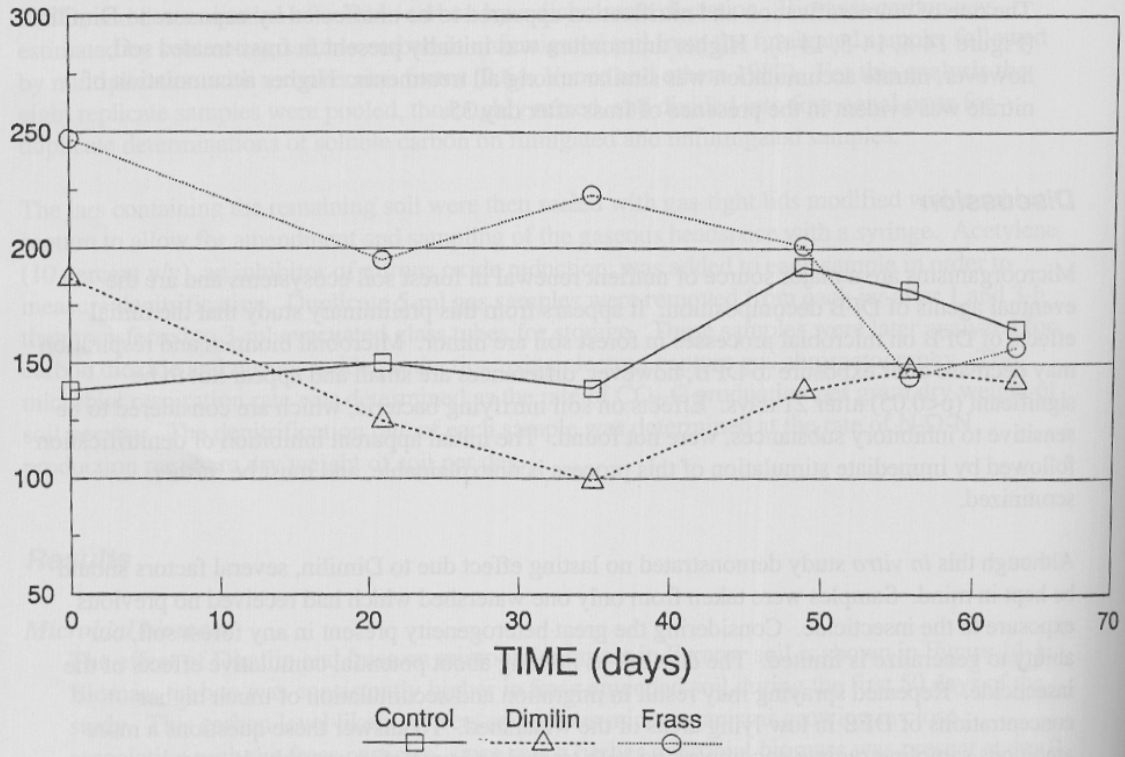


Figure 14-1. Microbial biomass in soil from the Fernow Experimental Forest. Data are the means of duplicate determinations. Eight 20-gram soil samples, one from each core, were pooled and then divided into two subsamples for the biomass determination using the chloroform-fumigation- and K₂SO₄-extraction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

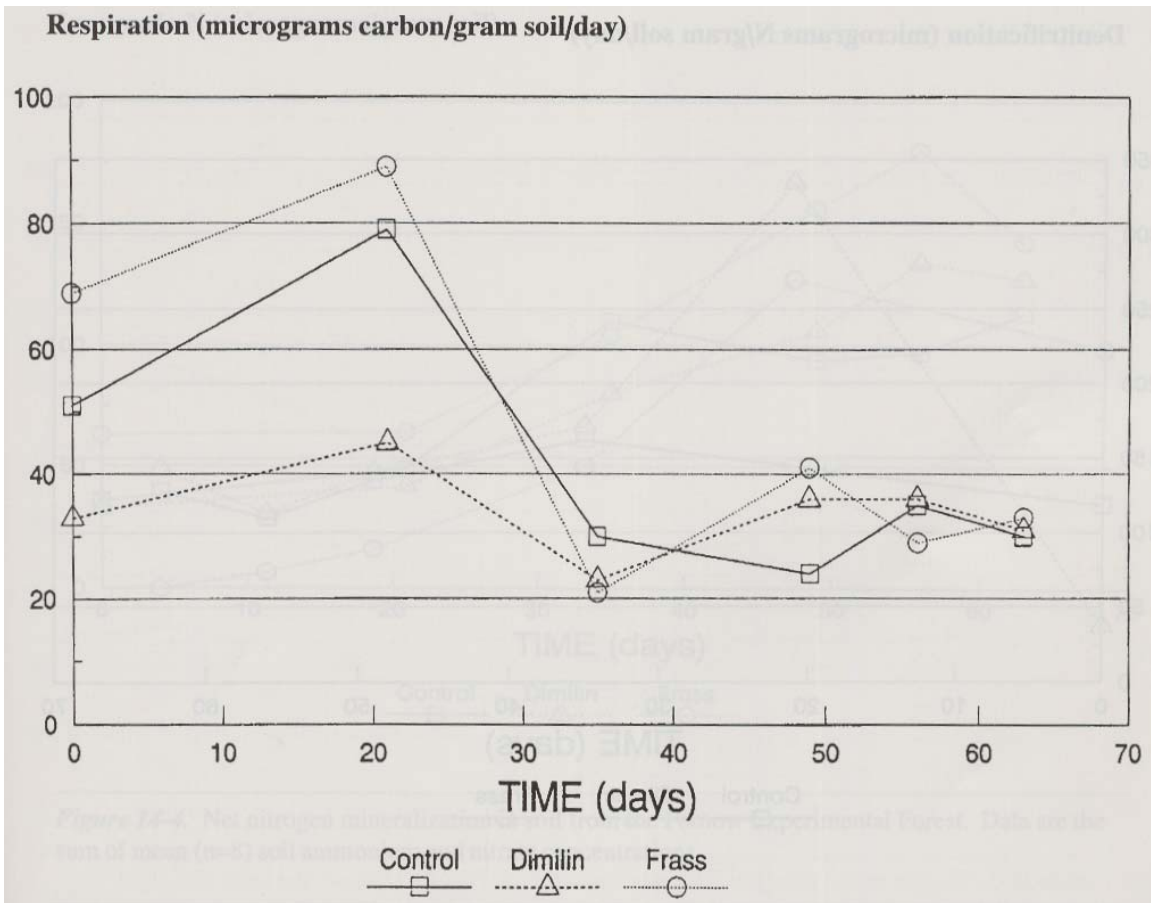


Figure 14-2. Microbial respiration in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Rates of carbon dioxide production were determined by gas chromatography.

Denitrification (micrograms N/gram soil/day)

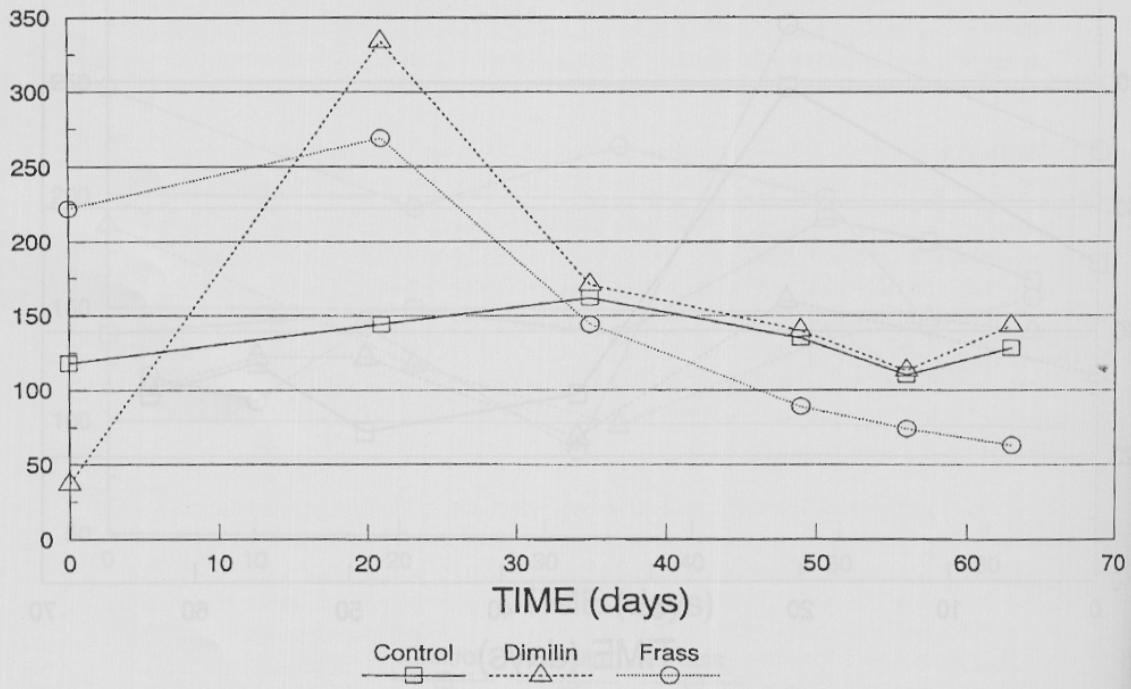


Figure 14-3. Denitrification in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Rates of nitrous oxide accumulation in the presence of 10 percent acetylene were determined by gas chromatography.

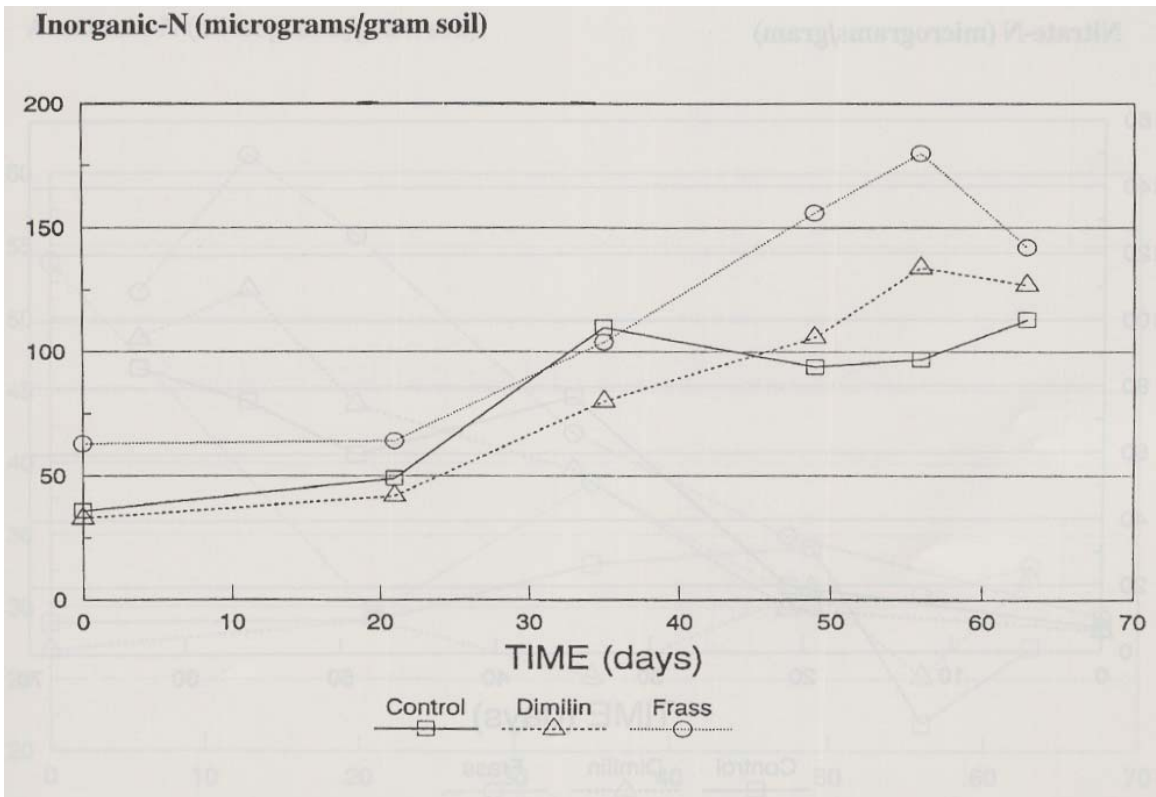


Figure 14-4. Net nitrogen mineralization in soil from the Fernow Experimental Forest. Data are the sum of mean (n=8) soil ammonium and nitrate concentrations

Nitrate-N (micrograms/gram)

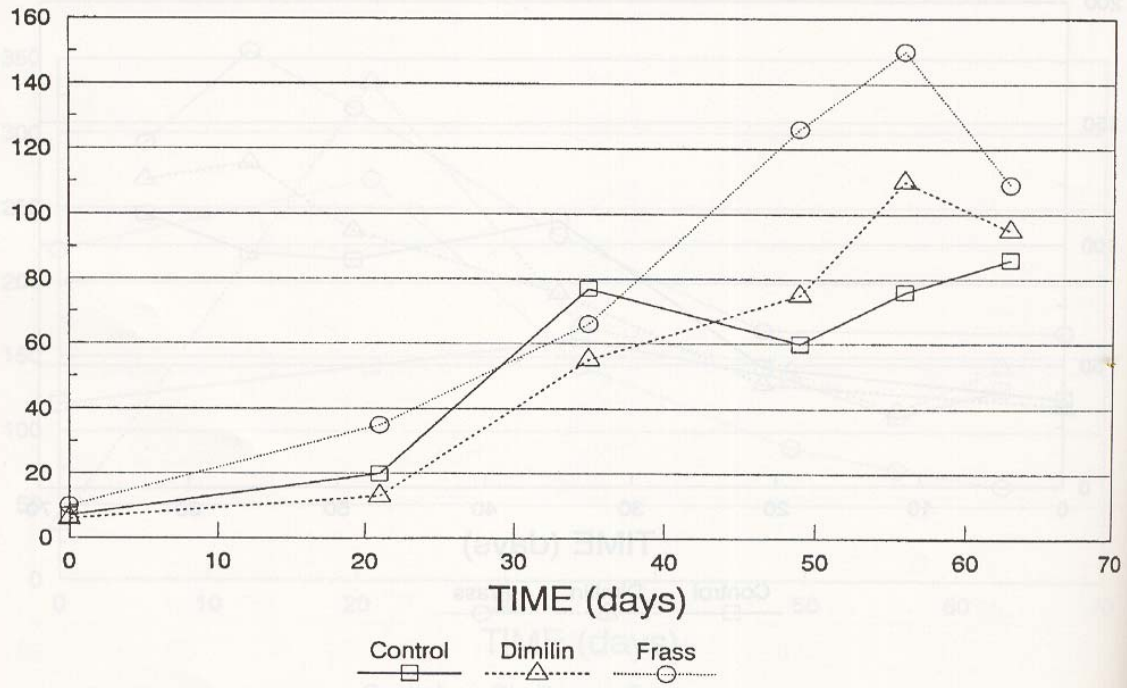


Figure 14-5. Nitrate in soil from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Nitrate was determined colorimetrically in 1.0 N KCl extracts using Szechrome NAS reagents.

Ammonia-N (micrograms/gram soil)

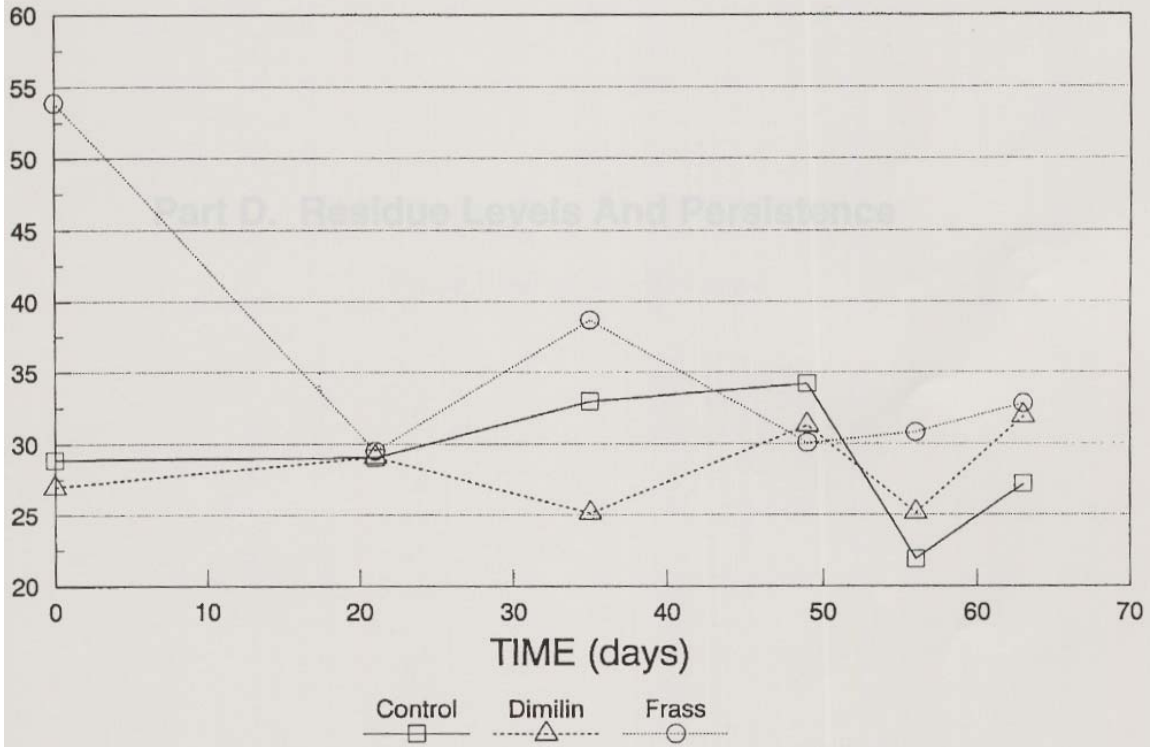


Figure 14-6. Ammonia in soils from the Fernow Experimental Forest. Data are the means of eight replicate determinations. Ammonium was determined in 1.0 N KCl extracts using a Wescan ammonia analyzer.

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 1 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 (MeGH) 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/mm 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

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-cm² pieces to make a composite sample for which the total area was usually 10,000-20,000 cm². 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-in intervals approximately 1-in 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-in 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 OC 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 J 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 cm² 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/cm²) 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 DEB 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 cm² of leaf on upper canopy leaves, and from 1.9 to 25 ng per cm² 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 cm² the upper canopy and from 9 to 270 ng per cm² 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/cm² 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 DEB coverage represented as ng/cm² of slide area. Values range from 25 to 167 ng/cm². The distribution along a vertical sector midway along the horizontal slides showed a similar pattern ranging from 5 to 116 ng/cm². (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/cm² for upper canopy leaves, and 74 and 271 ng/cm² 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/cm², respectively, considering the 0.1 m² of ground surface sampled per leaf litter composite. Thus, the DFB coverage found on slides 1 m off the ground (5 to 167 ng/cm²) 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

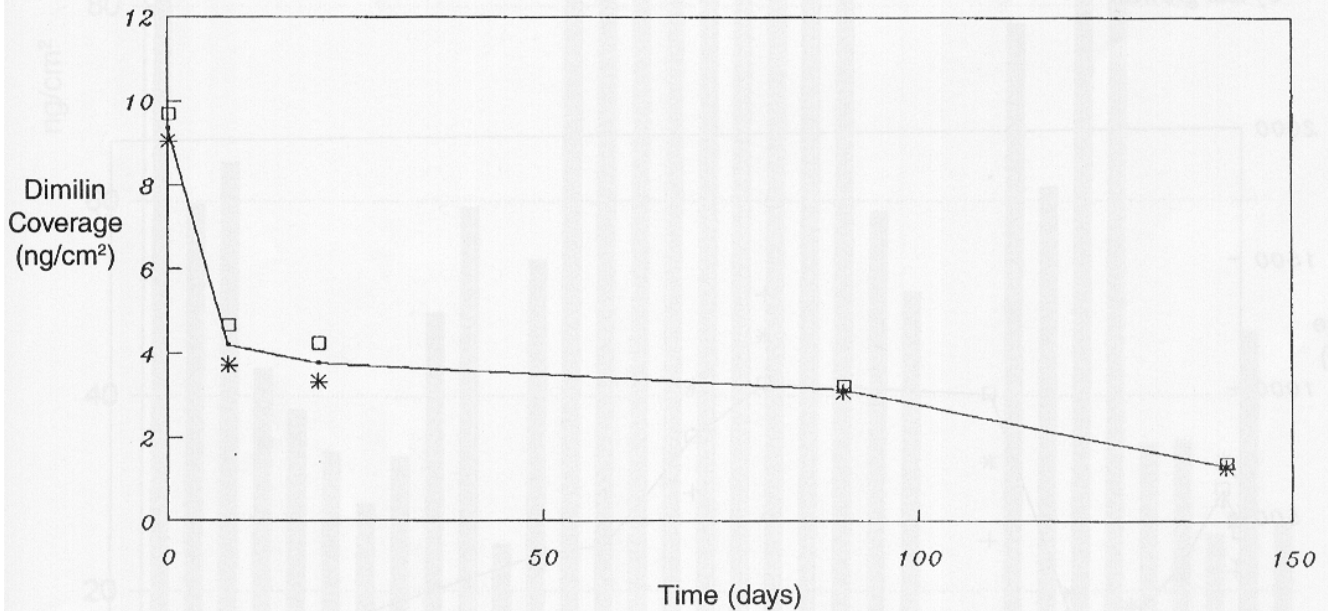


Figure 16-1. Persistence of DFB on upper canopy foliage of a WVU Experimental Forest yellow-poplar during the 1991 growing season. DFB concentrations on the leaf samples are reported as DFB coverage in ng/cm² of leaf surface area. The two coverage values shown at each time point are for the two subsamples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

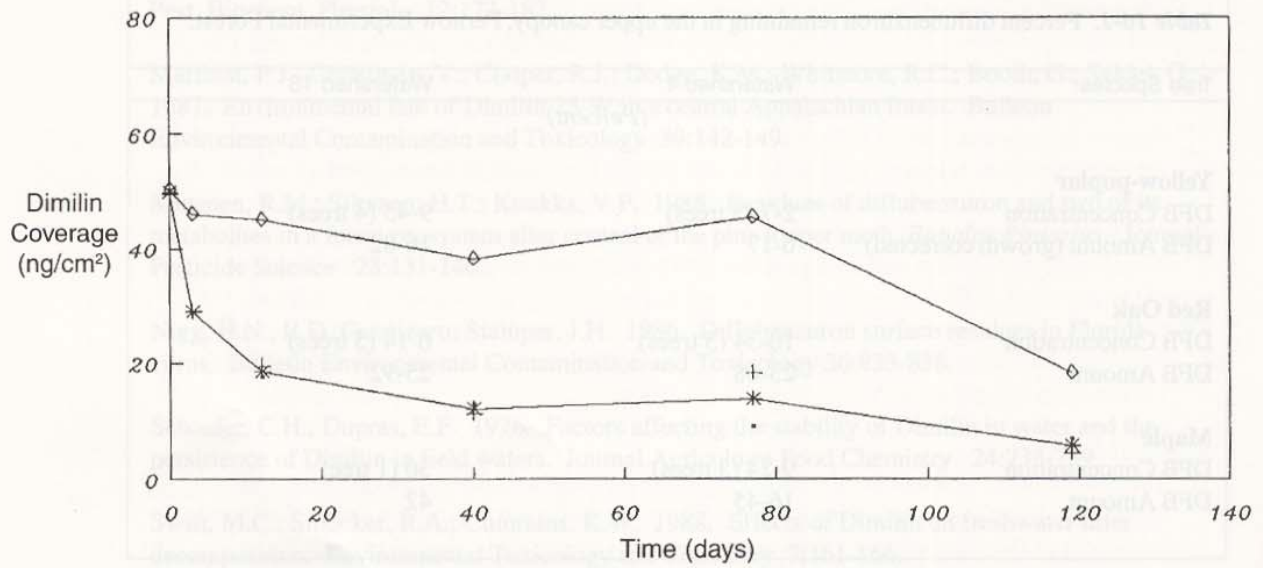


Figure 16-2. Persistence of DFB on the lower canopy of a Fernow Experimental Forest red oak: effect of leaf growth dilution on DFB coverage. The bottom line represents the concentration of DFB on leaves during the growing season as described in Figure 16-1. The top line shows the actual amount of DFB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

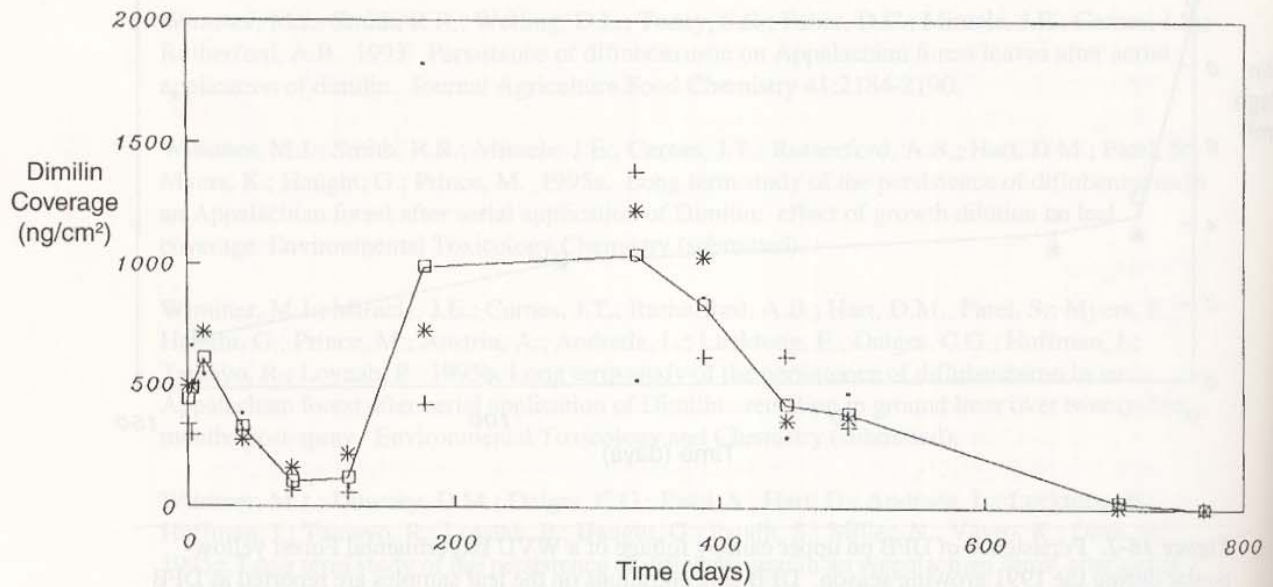


Figure 16-3. Persistence of DFB in Fernow Experimental Forest ground litter over 25 months post-spray. The average of two subsample values for each of three composite samples is shown for each time point. DFB concentration is reported as ppb DFB (ng DFB/g of litter dry weight).

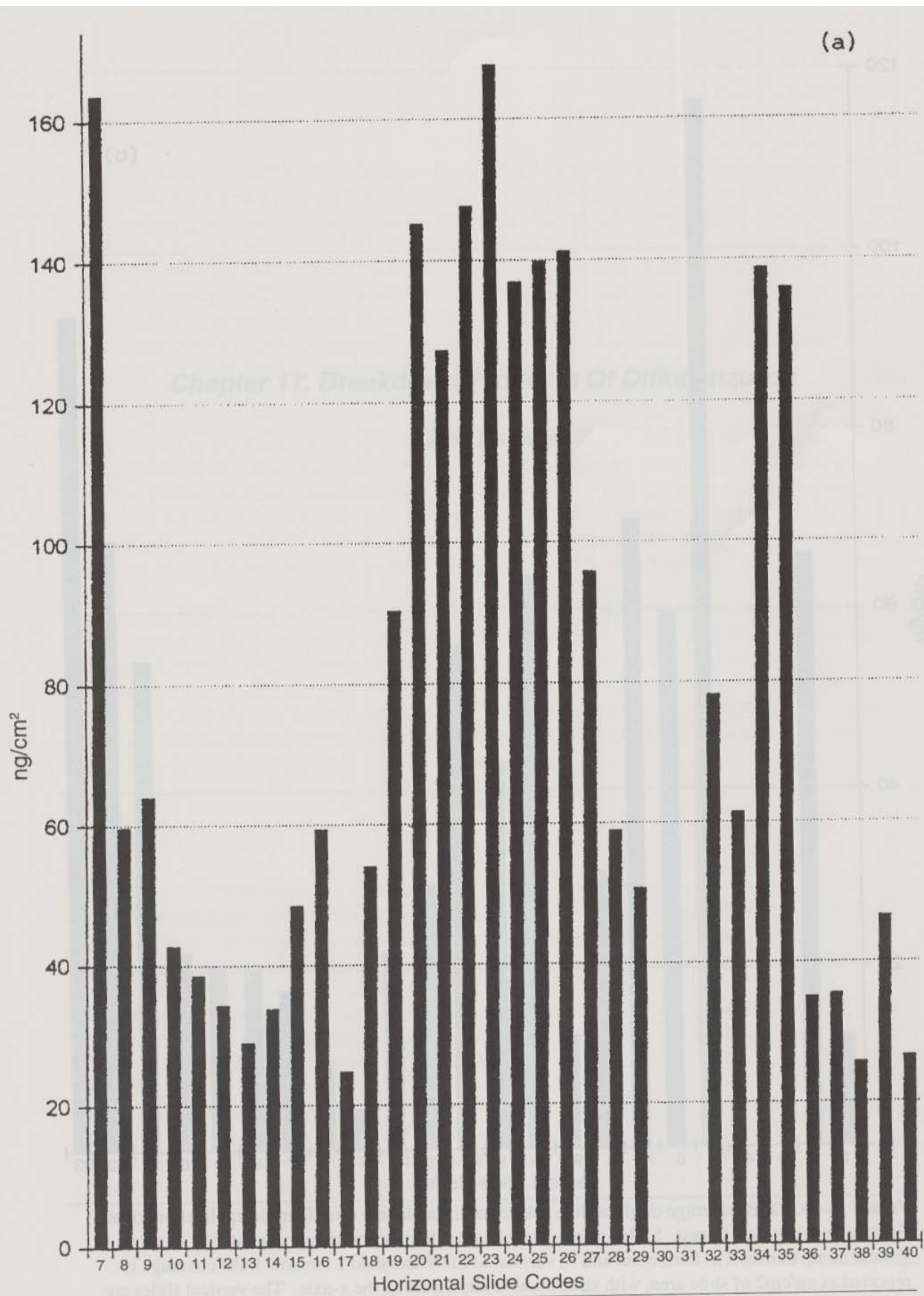


Figure 16-4a. See next page.

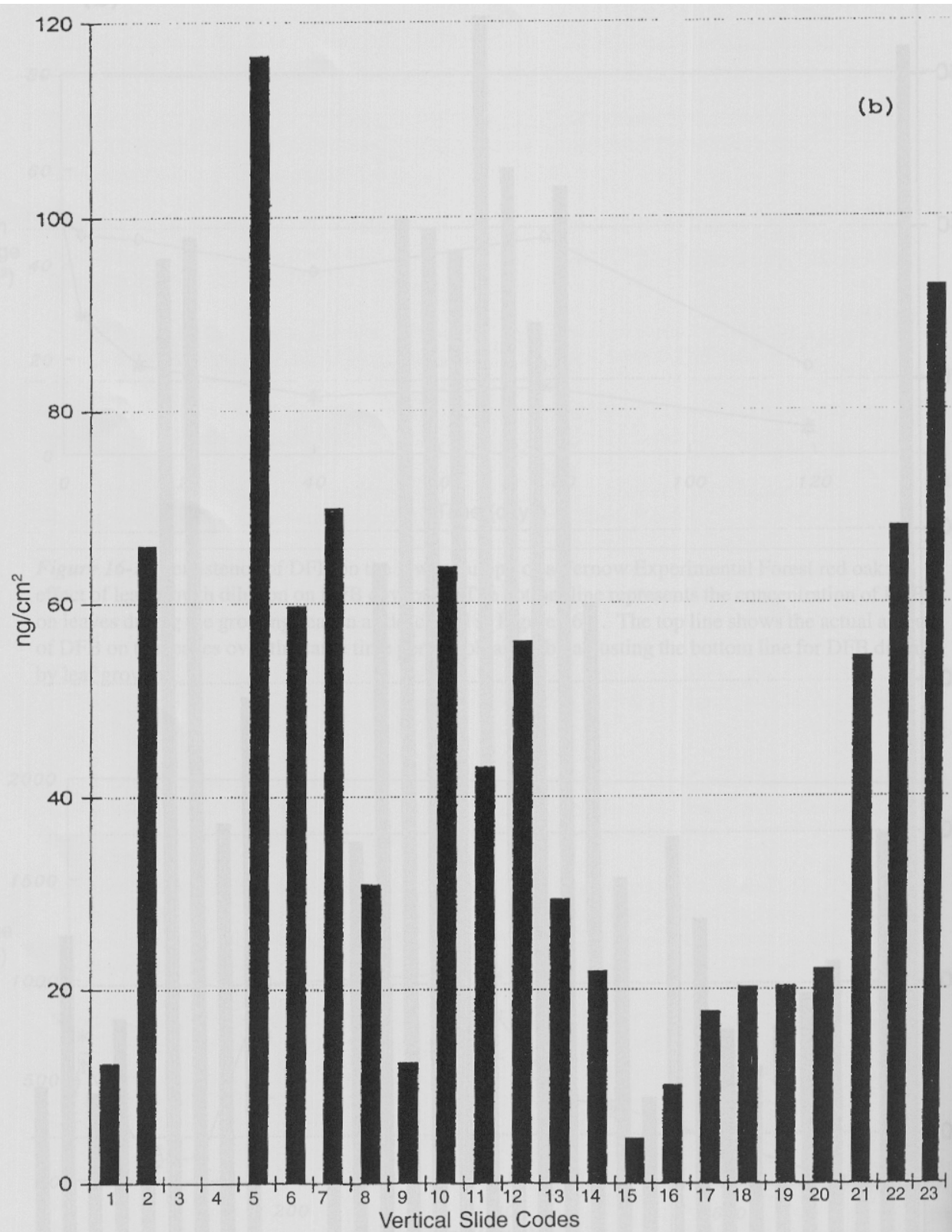


Figure 16-4b. DFB coverage of glass slide substrates immediately after Dimilin application to the Fernow Experimental Forest. Slides were placed flat at 1.6 m intervals approximately 1 m above ground along transects a) horizontal and b) vertical to the Watershed 1 stream. DFB coverage is reported as 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

Introduction

Diflubenzuron (DFB), which has been used to treat large areas of forests in the eastern United States, has been shown to degrade by hydrolytic cleavage to 2,6-difluorobenzoic acid (DEBA) and 4-chlorophenylurea (CPU) (Nimmo and others 1984). The CPU in turn degrades to 4-chloroaniline (parachloroaniline = PCA) by microbial action in soil and water (Ivie and others 1980, Nimmo and others 1986, Smucker 1987). Public concern has been expressed that PCA is a suspect carcinogen (Chhabra 1989). Relatively little is known, however, of its persistence in the environment as a consequence of the extensive spraying of Dimilin for control of gypsy moth. The acid-base chemistry of PCA causes it to bind tightly to soil material, such as humic acids, and persist in the environment (Hsu and Bartha 1974, Worobey and Webster 1982b,). This characteristic is apparently a result of both ion-pairing and absorption.

The binding of PCA to soils and organic matter, in general, is of sufficient tenacity to make its removal for analytical purposes difficult and time-consuming. Extraction methods, including acid or base hydrolysis, methylation, sonication, and pyrolysis have all been attempted with limited results (Mutanen and others 1988; Wegman and others 1984; Worobey and Webster 1982a, 1982b). Yet to obtain information on PCA persistence after spraying with DFB, the compound must be isolated from field collected samples in good, reproducible yields.

Adding to the difficulty of PCA determination in forest floor samples is the phenomenon that the parent material, DFB, is typically applied at the very low dosage of only 35 g AI/ha (0.25 ounces AI/acre). Even if DFB converts completely to PCA, and none of the PCA is removed by further mineralization from the system or other transport, the expected concentrations will be no greater than the parts per billion range when the insecticide is applied uniformly. The low concentrations coupled with the difficulty of recovery present a difficult analytical task for the evaluation of PCA persistence.

Methods

A series of field sampling plots was established within four watersheds on the Fernow Experimental Forest for the purpose of evaluating the environmental fate of DFB and its metabolite PCA. These plots and sample collection protocols are described in detail in Chapter 16. To evaluate PCA production and persistence, it was essential that the study watersheds were free of any PCA from other sources. Although PCA is widely used in the dye, chemical, and rubber industries, the Fernow Experimental Forest is in a relatively remote location well removed from industrial activity. Samples of leaf litter and soils analyzed prior to Dimilin 4L application confirmed the absence (below detection limit) of PCA in the treated watersheds.

An analytical method for the extraction of PCA from samples of soil and leaf litter collected in the treated watersheds was developed by modification of procedures described elsewhere. The goal of the extraction procedure was to recover as much PCA as possible while avoiding harsh treatments that could either increase its concentration artificially by catalyzing DFB degradation or decrease its concentration as a result of chemical decomposition. Samples with known quantities of DFB were used to assess recovery procedures.

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 HCl 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 3 μ C-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 μ l. 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 Winmer 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)

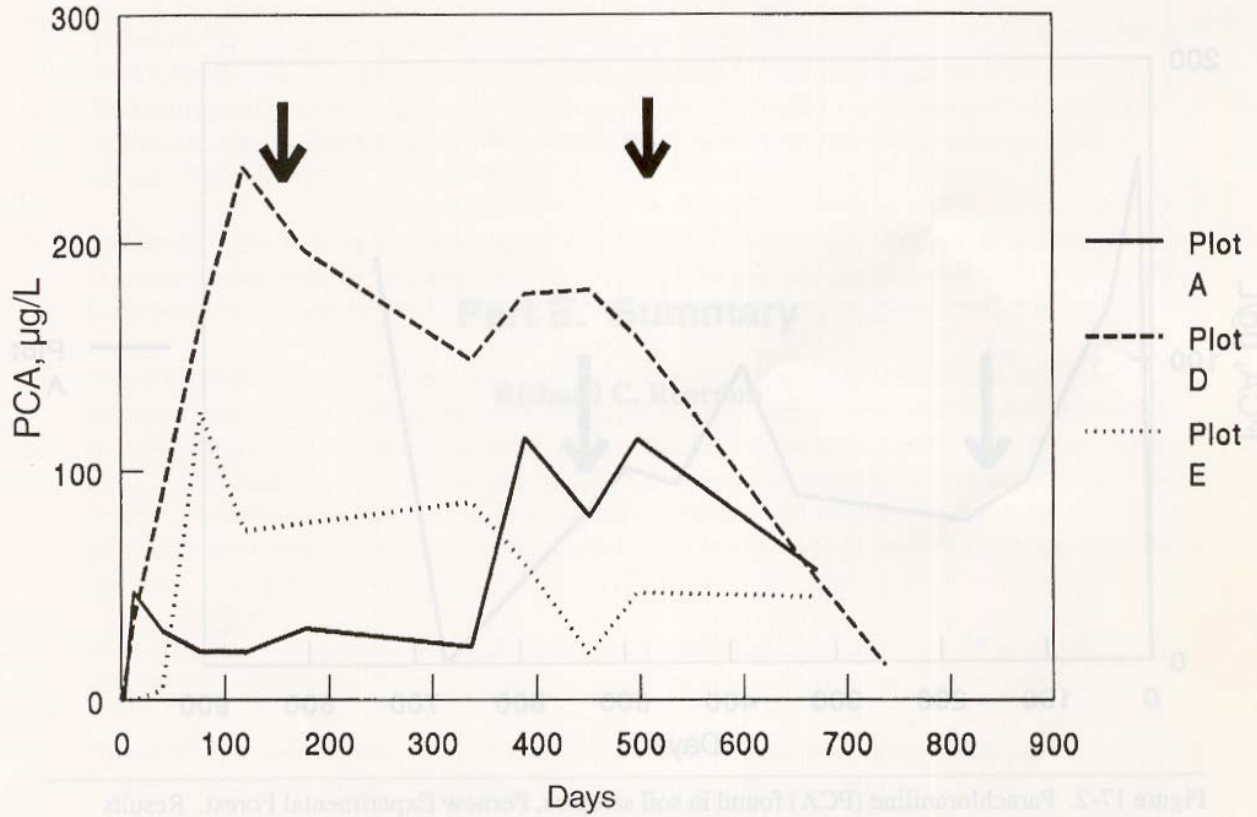


Figure 17-1. Parachloroaniline (PCA) found in ground litter samples, Fernow Experimental Forest. Results show average values for each time point taken in plots A, D, and E. Bold arrows denote dates of leaf fall.

Soil
Plot A (arrows denote leaf fall)

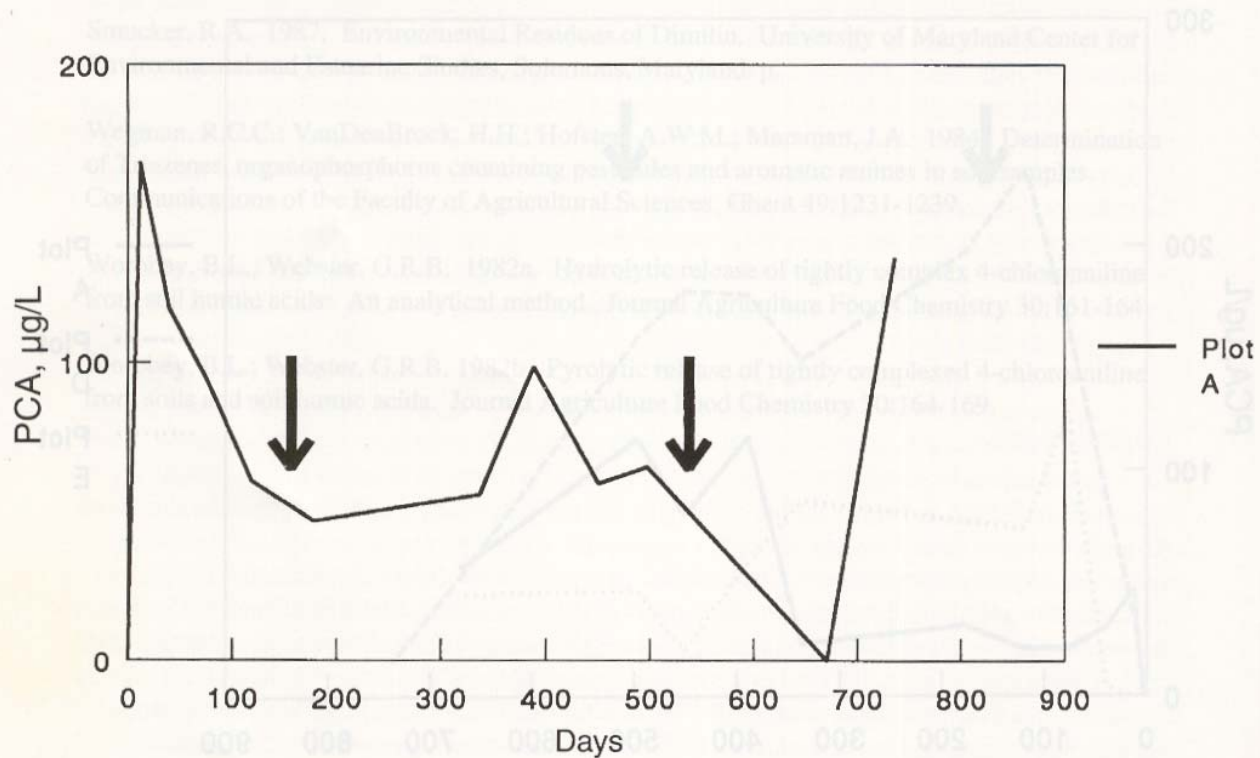


Figure 17-2. Parachloroaniline (PCA) found in soil samples, Fernow Experimental Forest. Results show average values for each time point taken in plot A. Bold arrows denote dates of leaf fall.

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 Allacre) 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-in 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 *Isoptera* sp., the mayfly *Paraleptophlebia* sp. and the crane fly *Hexatoma* sp. Mean shredder densities in treated streams declined significantly ($P < 0.05$) 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-in 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-in 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-m² 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- μ m 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 Femow 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.

Pesticide Precautionary Statement

This publication reports on the use of pesticides. It does not contain recommendations for pesticide use, nor does it imply that the uses discussed here have been registered. All uses of pesticides must be registered by appropriate State and Federal agencies before they can be recommended.

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Forest Health Technology Enterprise Team

TECHNOLOGY
TRANSFER

Mating Disruption

Using Mating Disruption to Manage Gypsy Moth: A Review



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Using Mating Disruption to Manage Gypsy Moth:

A Review

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Contents

Introduction.....	1
Mating Biology of the Gypsy Moth.....	2
Mating Disruption of the Gypsy Moth.....	2
Assessing Biological Effectiveness of Mating Disruption.....	3
Development of Mating Disruption—1971 through 1997.....	4
Before 1989 — Initial Development and Application.....	4
1989—A Transitional Year.....	5
Efficacy.....	6
Deposition.....	7
Residual Activity.....	8
Summary of 1989 Results.....	8
1990-1997 — Second Stage of Development and Application.....	8
1990 — A New Bead Formulation.....	8
Disrupt II.....	10
Efficacy.....	11
Residual Activity.....	11
Bead Formulation.....	12
Efficacy.....	12
Residual Activity.....	12
Deposition.....	14
Laboratory Evaluations.....	14
Summary of 1990 Results.....	14
1991 — Methods Development for the Bead Formulation.....	15
Airport Trials.....	15
Efficacy.....	15
Deposition.....	16
Residual Activity.....	16
Summary of 1991 Results.....	18
1992 — Dose-Response Evaluations.....	21
Airport and Laboratory Trials.....	21
Efficacy.....	21
Deposition.....	22
Residual Activity.....	23
Summary of 1992 Results.....	23

1993 -- Reduced Doses of Disparlure.....	23
Efficacy.....	23
Deposition and Residual Activity.....	24
Summary of 1993 Results.....	25
1994 -- Increased Doses of Disparlure.....	25
Efficacy.....	25
Deposition and Residual Activity.....	26
Summary of 1994 Results.....	26
1995 — Monitoring of Blocks Treated in 1994.....	26
Summary of 1995 Results.....	27
1996— Evaluation of Additional Tank Mixes.....	27
Laboratory Trials.....	27
Airport Trials.....	27
Field Trials.....	28
Summary of 1996 Results.....	28
1997 — Additional Trials of Flake Formulations.....	29
Efficacy.....	29
Alternative Tank Mix.....	29
Summary of 1997 Results.....	30
Current Disparlure Formulations and Use.....	30
Disrupt II.....	30
Decoy GM.....	32
Operational Use.....	32
Effects on Nontarget Organisms.....	33
Conclusions.....	33
Summary.....	34
Mating Biology of the Gypsy Moth.....	34
Mating Disruption of the Gypsy Moth.....	35
Development of Mating Disruption — 1971 through 1997.....	35
Current Disparlure Formulations and Use.....	36
References.....	37
Appendix A — Charts.....	39
Appendix B — Labels.....	49
Appendix C — Material Safety Data Sheets.....	55

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Preface

This review is published as part of a joint USDA program conducted by three of its agencies — Forest Service, Agricultural Research Service (ARS), and Animal and Plant Health Inspection Service (APHIS) — to develop specific methods for managing sparse-density expanding populations of the gypsy moth, *Lymantria dispar* (L.). The program is supported through the efforts of the Gypsy Moth Mating Disruption Working Group (hereafter, Working Group).

The Working Group was formed in 1990 and is composed of members from the USDA Forest Service, ARS and APHIS; U.S. Environmental Protection Agency (U.S. EPA); Virginia Department of Agriculture and Consumer Services (VDACS); Rockbridge County Virginia Gypsy Moth Program; Hercon Environmental Co.; Biosys, Inc.; and Nalco Chemical Co. In 1997, Thermo Trilogy Company (Columbia, MD) acquired biosys, Inc., and Loveland Industries (Greeley, CO) replaced Nalco Chemical Co. as representing private industry. Through cooperative efforts, the Working Group conducts field and laboratory studies to solve problems associated with the use of mating disruption to manage sparse-density gypsy moth populations. It also provides technical assistance to improve the quality of operational programs involving the aerial application of pheromones for managing gypsy moth.

This version (January 1998) of *Using Mating Disruption to Manage Gypsy Moth: A Review* is an update of handbook FHM-NC-08-95 printed in December 1995. It contains all of the information included in the December 1995 version as well as the results of studies conducted in 1996 and 1997.

Introduction

Semiochemicals, also called behavior-modifying chemicals, are volatile chemicals emitted by organisms to transmit information to other individuals. When the organisms are the same species, the semiochemical is a pheromone. Pheromones that act as attractants cause an organism to move towards the chemical source.

Insect pheromones that act as sex attractants show promise for suppressing pest populations through mating disruption. The idea behind mating disruption is to create interference with the sex pheromone emitted by the female to a level at which the male has difficulty locating her.

Mating disruption is accomplished by adding artificial pheromone sources to the environment. The effect is creating an atmosphere concentrated with the attractive material or creating many odor sources. Consequently, the males become confused and are prevented from finding female moths.

Scientists first learned how to synthesize many pheromones in sufficient quantities for field evaluations in the early 1970's. In Europe experiments using pheromones to control major insect pests in fruit orchards were started in 1975. In the United States the first pheromone registered for use as a mating disruptant was for control of the pink bollworm, *Pectinophora gossypiella* (Saunders), in 1978. Ridgeway et al. (1990) did an in-depth review of a wide range of uses for pheromones and other behavior-modifying chemicals.

Preliminary demonstrations of the effectiveness of mating disruption were shown for several forest pests—the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough); western pineshoot borer, *Eucosma sonomana* Kearfott; ponderosa pine tip moth, *Rhyacionia zozana* (Kearfott); several bark beetles; and the gypsy moth, *Lymantria dispar* (L.).

In the late 1980's the trend towards using more environmentally friendly control agents and developing prevention strategies for managing the gypsy moth resulted in greater emphasis on developing mating disruption. Concurrently, the 5-year congressionally mandated Appalachian Integrated Pest Management (AIPM) Gypsy Moth Project was initiated in a 38-county area in West Virginia and Virginia. A major emphasis of this project was developing technology, including mating disruption, for managing sparse gypsy moth populations (less than 10 egg masses per acre).

This review is a compilation of historical and current information on the use of mating disruption to manage sparse-density populations of the European strain of the gypsy moth. Included is information on development, registration, and formulations of the synthetic pheromone disparlure, as well as effects on nontarget organisms. Previously unpublished studies of efficacy, deposition, and residual activity of disparlure are reported.

Mating Biology of the Gypsy Moth

In the European strain of the gypsy moth, the adult male is guided to the flightless adult female primarily by pheromone, identified by Bierl et al. (1970) as Z-7,8-epoxy-2-methyloctadecane, also called disparlure. Although zig-zag flight along the plume of disparlure is the primary mechanism for initial orientation, other behavioral cues are important for successful location and recognition of a mate. Visual cues, principally those presented by tree trunks, are important in inducing landing and the walking search for a female. Recognition at close range and mating are evoked by contact cues such as wing tip touching. Thus, although location of the female over long distances is mediated by pheromone, additional visual and contact stimuli are critical to successful reproduction (Charlton and Carde 1990).

The gypsy moth is univoltine, that is, has one generation per year, and the mating season occurs over a 3-6 week period in late summer. Most mating normally occurs daily between 1000-1700 hours. Some males mate more than once, and some of the males do not mate at all (Mastro 1995). A small portion (about 20%) of the females in dense populations, and an unknown but probably small percentage in sparse populations, mate more than once. Females are capable of high pheromone release rates for 3 days but then their ability to release pheromone and mate decreases. Once mated, females stop releasing pheromone and begin ovipositing. Unmated females near the end of their life will lay unfertilized eggs.

Mating Disruption of the Gypsy Moth

The synthetic version of the gypsy moth sex pheromone used in mating disruption is structurally similar to the pheromone produced by the female gypsy moth. A 50:50 mixture of the (+) and (—) enantiomers (molecular structures with a mirror-image relationship) of disparlure has been applied using ground and aerial application methods. In both methods dispensers impregnated with this racemic mixture of pheromone release it slowly into the environment.

The ideal formulation of disparlure would release the pheromone at a constant rate and discharge all of the active ingredient in a specified period of time. The physical characteristics of the formulation and tank mix adjuvants have an effect on the discharge of disparlure. Variable meteorological conditions such as temperature and relative humidity, however, can exert unpredictable influences on disparlure release from a formulation.

For mating disruption to be effective, the synthetic pheromone must be present in sufficient quantities for the entire mating period. Since disparlure is volatile, denser than air, and is dispersed by air currents, complete initial coverage of an area is probably not essential. Due to the vertical distribution of females on tree trunks, however, there is a need for vertical distribution of the pheromone.

Failure of males to locate mates in air permeated with disparlure, probably results from desensitization of the chemoreceptors in the males' antennae, as well as from disorientation by following false pheromone trails or leaving the pheromone treated area (Carde and Minks 1995).

The mating disruption technique is more effective as gypsy moth populations decrease in density, because males locate females primarily by the pheromone at low population densities (Beroza and Knipling 1972, Knipling 1979). At high population densities males can more easily locate females using visual cues, as well as by chance encounters.

The gypsy moth is not an ideal candidate for mating disruption due to the high fecundity of females producing 300-1200 eggs per mass, the polygamous nature of males, and the clumped or aggregated distribution pattern of adult females, which is probably due to their sedentary nature and clustering at suitable larval resting and pupation sites. Nevertheless, some characteristics of the European strain of the gypsy moth are suited to the use of mating disruption, including the flightlessness of females, limited dispersal of the majority of males beyond a few hundred meters, low mating success at sparse densities, and the characteristic of having one generation per year. Unlike the European strain, some recently introduced gypsy moth females of the Asian strain are capable of strong flight, and how this characteristic would influence the usefulness of the mating disruption technique is unknown.

Assessing Biological Effectiveness of Mating Disruption

The effectiveness of mating disruption of the gypsy moth is assessed biologically on the basis of using several techniques in combination: counting life stages under bands, monitoring flight of males, monitoring females for mating success, examining eggs for embryonation, and conducting egg mass surveys. These techniques were used to determine efficacy in the methods development trials reported for 1990-1995.

Counting life stages under bands—Numbers of larvae, pupae and egg masses under bands (such as burlap) on tree boles at breast height are counted. A decrease in abundance or the absence of larvae and pupae as compared with levels in the previous year indicates effectiveness of mating disruption in the previous year. A decrease in abundance or the absence of viable egg masses in the treated area as compared with an untreated area indicates the effectiveness of mating disruption in the year of treatment.

Monitoring flight of males—Traps are baited with 500 µg of (+) - disparlure to attract males during the flight period and deployed at the rate of one trap per 15 acres. If mating disruption is effective, males will not be caught because they will not be able to locate the pheromone in the trap just as they will not be able to locate females. In the year disparlure is applied, the number of males captured does not provide an estimate of population density but rather a measure of the effectiveness of communication disruption.

Monitoring females for mating success—Virgin 1-day-old females are placed untethered in shelters—such as modified delta traps (triangular cardboard traps usually used to capture adult male gypsy moths). The inability of males to locate the females in the shelters (monitor females) as well as resident wild females from under bands indicates effectiveness of mating disruption.

Examining eggs for embryonation—Egg masses are collected from monitor females and resident wild females under bands, held an additional 30 days in an outdoor insectary under

ambient conditions, and then examined for the presence or absence of embryos. The absence of embryos indicates effectiveness of mating disruption.

Conducting egg mass surveys—A visual search for egg masses is conducted and egg masses are counted. Absence of egg masses or their presence in lower numbers indicates effectiveness of mating disruption.

Traps baited with disparlure have some competitive advantage over females in that traps emit pheromone continuously over the entire mating season. Traps do not, however, present all of the orientation and recognition cues that females do. Therefore a trap catches only 20-30% of the males that visit the area within 3 m of the trap (Carde 1996). Reduction in the number of males trapped after application of various disparlure formulations has not been equated with reduction in mating success. Monitor females provide a more direct measure of mating success. Such monitoring has not been directly related to changes in density of native egg masses, however, mainly because no accurate technique has been developed for quantifying egg mass densities in sparse populations.

Development of Mating Disruption—1971 through 1997

Before 1989 – Initial Development and Application

Federal and State agencies and private companies have attempted to use mating disruption to manage populations of the gypsy moth since 1971 (e.g., Granett and Doane 1975, Schwalbe et al. 1974, Stevens and Beroza 1972, Webb et al. 1988). Before 1989 various entities conducted mating disruption activities independently, with mixed results. Many research and operational trials of mating disruption were complicated by numerous problems, such as inappropriate and inconsistent formulations of disparlure, relatively insensitive evaluation techniques, and lack of data on seasonal release rates of disparlure. Nevertheless, the following generalizations were derived from these early efforts at mating disruption (Kolodny-Hirsch and Schwalbe 1990): (1) a direct dose-response relationship exists both for disruption of mating communication and for disruption of mating (Webb et al. 1988), (2) the degree of mating reduction is inversely related to male population density (Webb et al. 1988), and (3) a peak in mating occurs during peak male flight. In addition, in 11 of 15 mating disruption trials with the gypsy moth published between 1972 and 1988, evidence for mating disruption was based entirely on the reduction of male moths caught in traps and on the mating success of laboratory-reared or field-collected females placed in the test plots. In only one report were changes in native population trends statistically tested and shown to be significant (Beroza et al. 1974).

Numerous formulations containing the active ingredient (AI) disparlure were evaluated during these early years, for example, hollow plastic fibers (Conrel Inc.), gelatin microcapsules (National Cash Register Capsular Products, Stauffer Co., Penwalt Co.), and plastic laminated flakes (Hercon Environmental Inc.). In general, these formulations provided a relatively uniform distribution of pheromone under laboratory conditions. In field tests, however, the disparlure was inefficiently released, and major problems were encountered in the aerial application of these formulations due to the spray systems available for aircraft at the time.

In earlier trials, low doses of disparlure, e.g., 50 mg AI per ha (20 mg AI per acre), in various controlled release formulations reduced male trap catch for the entire season and in a few cases suppressed mating. In general, however, results were inconsistent and discouraging. In a dense population of gypsy moth, ground application of disparlure at doses of 5, 50 and 500 g AI per ha (2, 20 and 200 g AI per acre) dispensed from plastic laminate tape stapled on tree boles decreased mating success by 7%, 34% and 84% respectively, as measured against controls in small-plot tests (Schwalbe and Mastro 1988). Even in the plots treated with 500 g AI per ha some monitor females were mated, probably due to the high density of gypsy moth males, short range communication (i.e., visual cues) and chance encounters. In later trials, Webb et al. (1988) demonstrated more consistent efficacy by the use of 75 g AI per ha (30.4 g AI per acre) disparlure applied aerially to disrupt mating.

From 1983 to 1989 only one commercial product containing racemic disparlure, the plastic laminated flakes Disrupt II (Hercon Environmental Inc., Emigsville, PA), was registered by the U.S. EPA for use in mating disruption. Therefore, operational use of mating disruption was limited to use of these layered plastic flakes.

1989—A Transitional Year

In July 1989, an eradication program was conducted in Giles County, Virginia, with the aerial application of the bacterial insecticide *Bacillus thuringiensis* (*B.t.*) and the growth regulator diflubenzuron as the primary treatments, and on approximately 2,500 acres disparlure was applied in the form of Disrupt II at a dose of 30.4 g AI per acre (Leonard et al. 1992). The mating disruption technique was selected for that portion of the project area where the National Science Foundation had an ongoing study on behavior of dark-eyed junco (*Junco hyemalis hyemalis*). Since part of the diet of juncos is lepidopteran larvae, *B.t.* or diflubenzuron could not be used. The project area included uninhabited forest land ranging in elevation from 1,000 to 1,300 m, with oak as the primary overstory vegetation and gypsy moth populations below 10 egg masses per acre. An adjacent untreated area was used for comparison.

The mating disruption portion of this eradication effort used previously developed technology for dispensing and evaluating disparlure but also acquired new data on vertical deposition and release rates of Disrupt II. The 1989 trials are described here in detail because the methods were also used in 1990-1995.

Specialized equipment developed in the 1970's by Schweitzer Aircraft for applying flakes was mounted, one pod on each side, under the wing of a Cessna 206 (Fig. 1). The dispensing rate of the flakes is controlled by an

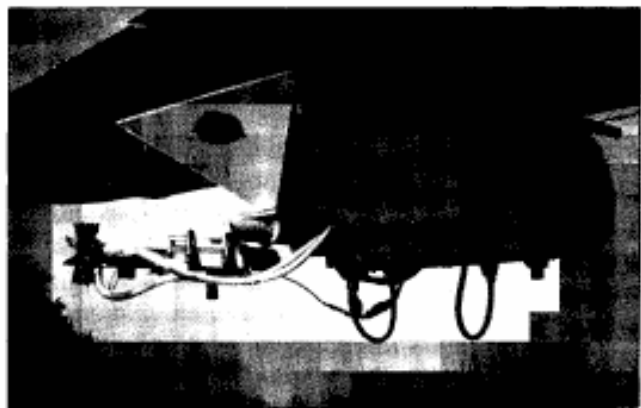


Figure 1. Specialized application equipment mounted under the wing of a Cessna 206 for dispensing the flake formulation of racemic disparlure (Disrupt II).

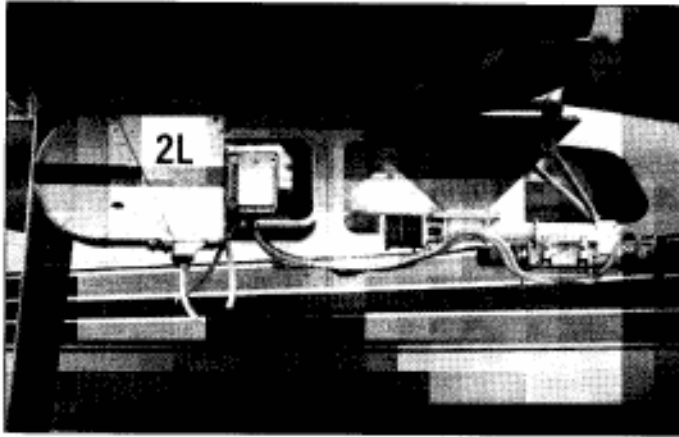


Figure 2. Flakes and sticker are mixed in a chamber then dispensed through a spinner.

auger, and the sticker is controlled by a pump and tubing system. The flakes and sticker are mixed in a chamber then dispensed through a spinner (Fig. 2). During gypsy moth suppression projects using an aqueous formulation, this aircraft typically is assigned a 75-foot swath width, but due to the inability of the motor controlling the auger on each pod to deliver sufficient flakes, a 45-foot swath was assigned to realize the desired deposit rate of approximately 41 flakes per square meter (3.7 flakes per square foot).

During characterization trials, the flake deposition pattern within the swath was uneven, with peaks occurring directly under the pods and valleys under the fuselage and wing tips. Additionally, the pod motors for augering the flakes and pumping the sticker malfunctioned periodically and bridging (binding) of the flakes in the hopper was a constant problem. During applications, a total of 165 g of flakes (dose of 30.4 g Al), talc powder to prevent bridging of the flakes, and 4 oz of the sticker/extender Gelva -1990 (Monsanto, St. Louis, MO) to adhere the flakes to the foliage were mixed and applied per acre. The flake treatment was initiated in July just before anticipated adult male flight. The size of the flake hoppers limited treatment to 125 acres per load at the 30.4 g Al dose.

Efficacy

No egg masses were found before treatment (spring 1989) in the pheromone treated and untreated areas, while both areas had similar male moth catches (summer 1988). In 1989 after treatment, no males were captured in the treated area, and 26% of the traps captured male moths in the untreated area. Pheromone traps placed on a 250 m grid for 2 years after treatment (1990 and 1991) captured one moth on the edge of the pheromone treated area in 1991 and an average of four moths per trap in the untreated area. Both of these areas were trapped from 1992 through 1995 on a maximum spacing of a 1-km grid. In 1995 the pheromone treated area was still relatively free of male moths, while populations in the untreated area had received insecticide treatment in 1993.

Laboratory-strain 10K irradiated sterile gypsy moth females (male parent was irradiated with 10 Krads as a pupa) were used as monitor females. Sterile females were used to satisfy regulatory L concerns because the project was located outside the quarantine regulated area. When mated with a normal male, these sterile females produce an egg mass that embryonates, but most eggs do not hatch. If these sterile females do not mate, an egg mass can be produced but it will not embryonate. Monitor females were deployed at 100 stations twice weekly for 3 weeks coinciding with male moth flight. They were left overnight and retrieved the next day. None of the monitor

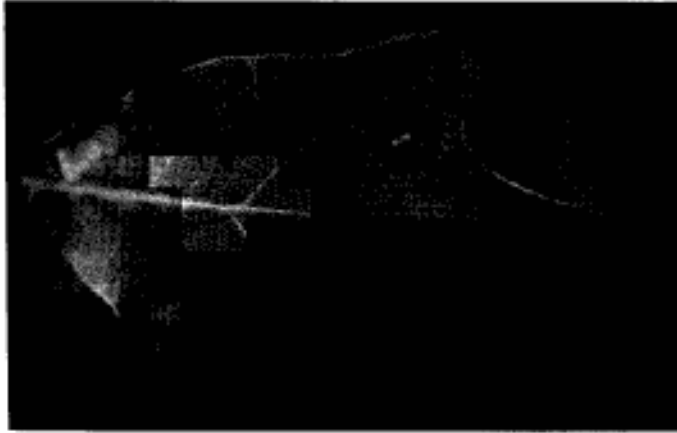


Figure 3. *Deposition of green plastic laminated flakes on foliage.*

females were mated in the treated or untreated areas. This led to speculation that sterile females were not appropriate for use as monitors and plans to test an escape resistant mating station in which fertile females could be deployed when working outside of the quarantine regulated area were formulated.

Deposition

In addition to the efficacy results, a bucket truck and ground tarps were used to evaluate deposition of 10 times the normal application of flakes (1 x dose flakes containing dispartlure and 9 x dose of blank flakes) applied to a 4 ha (10 acre) site within the dispartlure-treated area. This high application rate was used to ensure detection of a sufficiently high number of flakes. Twenty overstory and twenty understory trees were sampled. Rakes were deposited throughout all layers of the canopy, including the understory foliage (Fig 3). flakes were inventoried at 160 sampling points in the canopy and in 40 ground deposit nets. At application, only 10% of the flakes penetrated all levels of foliage and were deposited on the ground beneath the forest. Over 6 weeks, an additional 6% of the flakes fell to ground level indicating excellent performance of the sticker (Fig. 4).

Figure 4. *Flake deposition after application of 10 times normal Disrupt II in Giles County, Virginia, 1989. The ground level was sampled on all dates. The canopy was sampled only on July 25, August 1, and September 12.*

Residual Activity

During aerial application, sections of black roofing paper were placed on the ground in open areas to collect flakes for release rate analysis. These flakes were weathered on the roofing paper placed beneath the forest canopy and collected weekly for 8 weeks. Initially the flakes contained 17% disparlure by weight. Results of gas chromatographic analysis of the flakes indicated that they lost approximately 65% of their disparlure content over the first 10 days, 10% over the next 20 days, and 12% in the last 26 days of the test. Moth flight started approximately 15 days after application. This rapid initial release of lure from the flakes was unexpected and prompted additional sampling to confirm suspicions that elevated temperatures on the dark surface of the roofing paper from which the flake samples were collected had increased the release of disparlure. On day 100 after treatment, 20 flake samples were collected from foliage in the sun, foliage in the shade, and roofing paper. On average the flakes collected from foliage contained twice as much lure as the flakes aged on and collected from the roofing paper.

Summary of 1989 Results

The Giles County project was considered a success as only one male moth was recovered from the pheromone treated area for 2 years after treatment (1990 and 1991). These efficacy results were better than anticipated based on the inconsistent operability of the pods, the nonuniform distribution of flakes, and the small proportion of disparlure released during moth flight and mating activity.

1990-1997 Second Stage of Development and Application

The ALPM Gypsy Moth Project of the USDA Forest Service reviewed all available information gathered through 1989 and determined which information was adequate and what questions remained unanswered. This review showed the need for methods development and operational evaluation of mating disruption. This need led in fall 1990 to the formation of the Gypsy Moth Mating Disruption Working Group, which was composed of members from Federal, State, and county governmental agencies; and private companies.

The newly formed Working Group proposed a series of methods improvement projects for implementation over the next 7 years. The broad objectives of these efforts were to (1) refine techniques for evaluating efficacy and equipment for applying Disrupt II, (2) quantify the lowest efficacious dose and number of applications of Disrupt II, and (3) develop an additional formulation of disparlure that could be applied through conventional boom and nozzle spray systems. Therefore, unlike the separate activities conducted from 1971 to 1989, activities from 1990 through 1997 represent a concerted effort. Methods improvement trials are discussed by year in the following sections. Operational uses of Disrupt II are summarized in Table 1.

1990 — A New Bead Formulation

The studies initiated in 1990 had two specific objectives: (1) to evaluate the impact of a single yearly application of disparlure and of an initial double application of disparlure on gypsy moth

Table 1. Acres of operational treatments using disparlure flakes, Disrupt II, applied at 30.4 g AI per acre to manage gypsy moth, 1989 through 1997.

Year	Location	Acres treated
1989	Giles Co., VA	2,500
1990	Sequatchie Co., TN	200
1991	Roanoke/Bedford Co., VA	2,900
	Allegheny/Botetourt Co., VA	400
	Mercer Co., WV	1,700
<i>Subtotal 1989-1 991</i>		
1992	Bedford/Botetourt Co., VA	4,829
	Floyd/Carroll Co., VA	1,000
	Mercer/Monroe/Pocahontas Co., WV	4,230
<i>Subtotal 1992</i>		<i>10,059</i>
1993	Giles Co., VA	2,600
	Mercer Co., WV	350
<i>Subtotal 1993</i>		<i>2,950</i>
1994	Craig Co., VA	775
	Monroe Co., WV	3,385
	Raleigh Co., WV	270
<i>Subtotal 1994</i>		<i>4,430</i>
1995	Pulaski/Giles Co., VA	2,450
	Mercer/Summers Co., WV	259
	Franklin Co., OH	235
<i>Subtotal 1995</i>		<i>2,944</i>
1996	Halifax/Dare Co., NC	1,900
	Greensville/Southampton Co., VA	2,926
	Giles/Craig/Botetourt/Roanoke Co., VA	3,578
	Carroll Co., VA	425
	Monroe Co., WV	3,887
	Mercer Co., WV	355
	Unicoi/Carter Co., TN	252
	Sheboygan Co., WI	2,200
<i>Subtotal 1996</i>		<i>15,523</i>
1997	Southampton Co., VA	560
	Floyd Co., VA	567
	Roanoke Co., VA	5,151
	Allegheny Co., VA	1,227
	Greenbrier Co., WV	100
	Monroe Co., WV	1,156
	Summers Co., WV	2,052
<i>Subtotal 1997</i>		
Total - All Years		51,719

mating success and population trends over 5 years, and (2) to field test an additional commercial disparlure formulation that could be applied through conventional boom and nozzle systems. Rockbridge and Augusta Counties in Virginia were selected as the project area for these method improvement evaluations for these reasons: (1) this area was within the AIPM project 2-km grid of pheromone traps, and male moth data were available for 1989 (average 27 moths per trap, range from 11 to 200 per trap); (2) county technical support personnel were already in place; and (3) isolated woodlots were abundant.

Disrupt II

Three woodlots (each 35-160 acres) were treated with two applications of flakes (Disrupt II) in 1990 only, each at a dose of 30.4 g AI per acre per application. Treatments were applied just before anticipated initial male flight (June 17) and before anticipated peak flight (July 20). Three additional woodlots were treated just before male flight in 1990 and again every year from 1991 through 1993 with a single application of flakes (Disrupt II) at a dose of 30.4 g AI per acre. An additional three woodlots were not treated. All applications of flakes were applied at 165 g per acre with 4 oz of Gelva-1990 as sticker and talc to prevent bridging.

Density of gypsy moth populations were estimated yearly using three techniques: surveying pre- and post-treatment egg mass densities (1/40 acre subplots on uniform 50 m grids); surveying larval, pupal and egg mass densities under burlap bands (checked when most gypsy moth caterpillars reached late instars and pupal stages, and again after the male flight period); and trapping male moths (one trap per 15 acres, with a minimum of three traps in woodlots smaller than 45 acres). To monitor mating success, laboratory-strain females were placed in modified delta trap mating stations in each woodlot at the rate of 30 females three times a week for 3 weeks during the peak moth flight period. The modified delta trap (not coated on the inside with adhesive, ends open, and female placed on a piece of burlap inside) was found to be the most escape resistant as well as maintained the attractiveness of the female to males (Fig 5). In addition to the laboratory-strain females placed in mating stations for monitoring, resident wild females under bands were monitored, and their egg masses collected and assessed for fertility within treated and untreated woodlots. A total of 1,165 1/40-acre subplots (2.5-4.6% of total woodlot area) was established for egg mass surveys (Leonhardt et al. 1996).

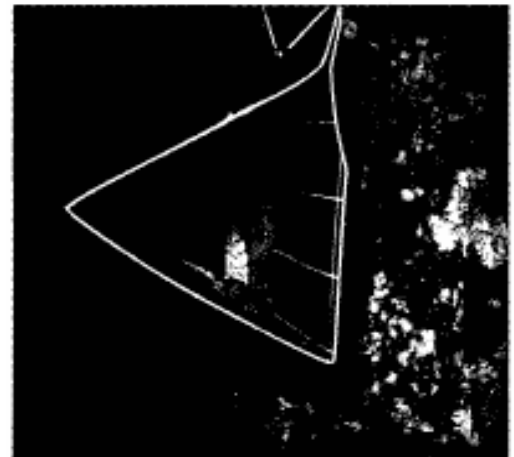


Figure 5. Modified delta trap used as a mating station for monitor adult females.

To assess relative competitiveness, laboratory-strain and resident wild females were deployed in a moderately dense population of gypsy moth located to the north of the study woodlots in Rockbridge County.

Efficacy

The *efficacy* results over all years (1990 through 1994) are presented in Chart A (pages 41-43). Population densities were significantly reduced by either the double application in 1990 or single application every year from 1991 through 1993. Compared with untreated woodlots, the abundance of immature life stages and fertilized egg masses under burlap bands, the percentage of mating success in monitored females, and the number of trapped adult males all remained low in woodlots treated yearly with one application of 30.4 g AI per acre (Leonhardt et al. 1996). The low degree of mating success in monitor and wild females indicated there was significant mating disruption in those treated woodlots in all four years of treatment. Although monitor females were not deployed in these woodlots in 1994, the year after the last pheromone treatment, all of the other measures of assessment showed that the gypsy moth population remained suppressed. In the woodlots receiving a double application, all measures of population density were low in the year of treatment (1990) and i year after treatment (1991). In 1992, however, the population began an upward trend although at a reduced rate of increase as compared with the untreated woodlots (Leonhardt et al. 1996). The evaluation of resident wild and laboratory-strain females in 1990 demonstrated similarity in their attractiveness, behavior and mating success. Therefore only laboratory-strain females were used in all future work (1991 through 1997).

Residual Activity

To evaluate consistency of Disrupt II over years, the release rates for the 1989 (used in Giles County) and 1990 (used in Rockbridge County) Disrupt II products were determined after multiple passes over 23 by 30 cm (9 by 12 inch) white canvas coated art paper cards (Strathmore Paper Co., Westfield, MA) placed on the ground in an open area at the airport. The cards were allowed to dry and were hung vertically on string beneath the forest canopy for aging. The amount of disparlure per flake was determined as a function of duration of exposure. Using this data, the calculated release rates for both products were slow and uniform over time and did not differ significantly (Fig. 6). Both the 1989 and 1990 Disrupt II products released only approximately 50% of their racemic disparlure content after 42 days, which is the approximate duration of the male moth flight period.

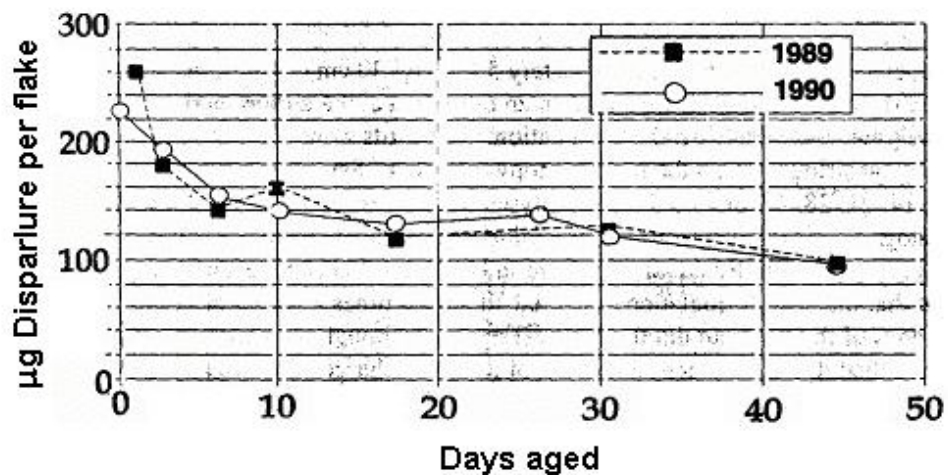


Figure 6. Release rates of disparlure from Disrupt II flakes produced in 1989 and 1990.

Bead Formulation

AgriSense (Fresno, CA) developed a new polymeric flowable bead formulation for mating disruption of pink bollworm in cotton. To determine the release characteristics of this bead formulation containing dispartlure, laboratory evaluations were conducted by Leonhardt (ARS, Beltsville, MD) in 1990. The bead formulation released dispartlure at a faster rate than did the flakes but field release and efficacy data were needed to support laboratory results.

Efficacy

A field test was proposed using this flowable bead formulation containing dispartlure (AGRIS1029). The bead formulation was to be applied to a 10 acre woodlot, to evaluate efficacy, and deposition and release rate profiles on foliage. A similar 10 acre woodlot was treated with flakes (1990 Disrupt II). The beads contained 40 % AI (by weight), and ranged from 50 to 800 microns with a volume median diameter (VMD)—the droplet size that divides the spray volume in half—of 275 microns. The tank mix consisted of 75 g of beads, 124 oz water, 2% (by volume) Gelva- 1990, applied at 1 gal per acre to yield a dose of dispartlure of 30.4 g AI per acre.

The application was attempted with a small fixed wing aircraft (Ag Cat) equipped with standard spray booms and flat fan nozzles. This aircraft spray configuration had been used effectively to apply the flowable bead formulation for pink bollworm, although the desired dose rate of 30.4 g AI per acre of dispartlure (AGRIS-1029) required the application of between 21 to 53 times the amount of beads per gallon of tank mix as had been used effectively for pink bollworm.

Numerous problems were encountered during airport characterization trials: (1) beads collected on the flat fan diaphragms, and the nozzles would not shut off, (2) beads clumped together clogging the nozzles, boom and pump motor, and (3) beads would not stay suspended in the tank mix. Eventually the proposed 10 acre treatment had to be abandoned and a few trees near the airport were sprayed.

Residual Activity

To characterize release rates, approximately 50 23 by 30 cm (9 by 12 inch) canvas coated paper cards were placed on the ground in an open area at the airport, and each group sprayed repeatedly with the bead or flake formulation. The cards were allowed to dry and were hung vertically on string beneath the forest canopy for aging. Samples (3-5 cards) were taken after 0, 3, 7, 14, 21, 28, 42, and 56 days. Analyses were conducted on a Model 6C-9A gas chromatograph (Shimadzu Instruments, Columbia, MD) with results calculated as micrograms per flake or per bead. Residual dispartlure in beads and flakes recovered from the spray cards showed that the bead formulation delivered about 3-4 times as much dispartlure to the air over the 42 days of the test than did the flakes (Fig. 7). Although the beads released considerably more lure than the flakes, more than 70% of it was discharged prior to the start of male flight (day 10). The accumulated dispartlure delivered by both formulations showed the beads released 22% of their content and the flakes 14% of their content during peak moth flight.

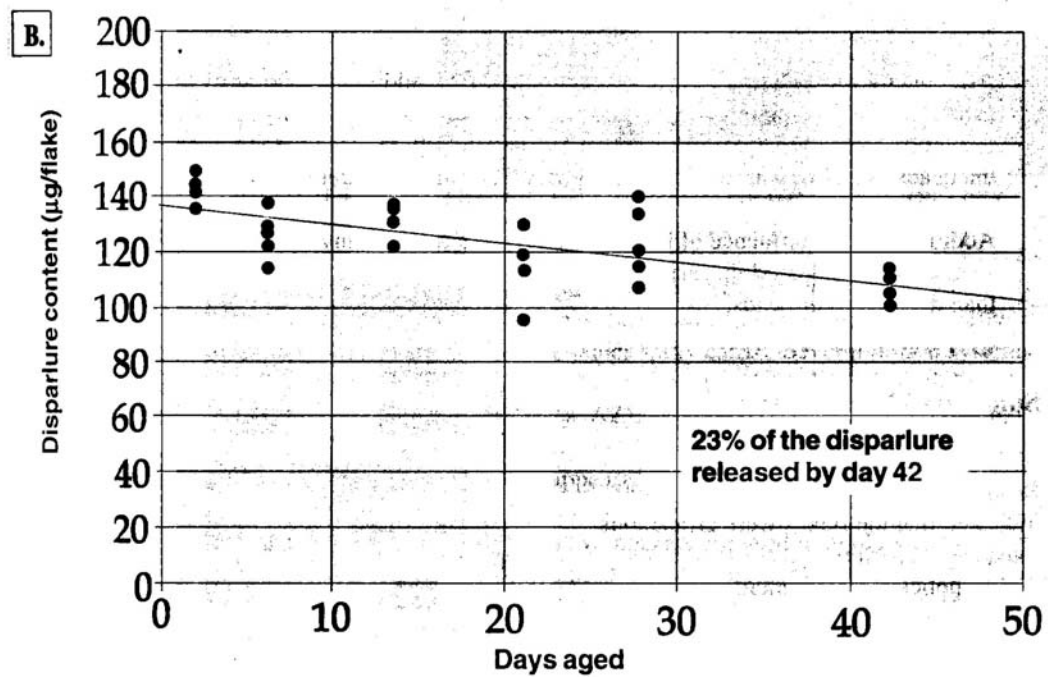
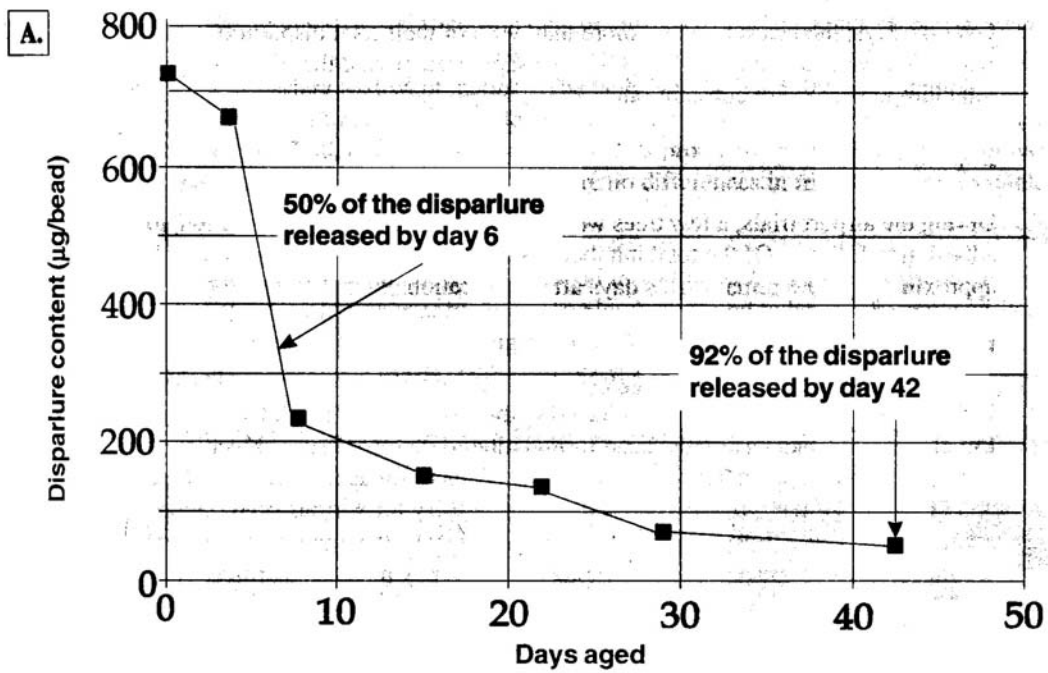


Figure 7. Disparlure content in (A) AgriSense beads and (B) Disrupt II flakes picked from spray cards aged in the field, Rockbridge County, Virginia, 1990.

Over 42 days, the beads released more than 90% of their total disparlure content, but the release was not at a constant rate. Over the same time period the flakes released 23% of their total lure at a slow but constant rate.

Deposition

During the airport trials, a few trees were sprayed with the bead formulation, to evaluate its adhesion to foliage. Of the total number of beads that originally adhered to foliage only approximately 20% remained 18 days after application.

Laboratory Evaluations

In fall 1990, an evaluation was conducted in the laboratory (APHIS Otis Methods Development Center) using the bead formulation to identify a suitable viscosity modifier to suspend the beads and a sticker to adhere the beads to foliage. Various tank mixes were applied to potted red oak seedlings and allowed to dry for various times before the application of rain. Before and between the rain events, the number of beads present within marked areas on replicate leaves were counted visually under a microscope.

The viscosity modifiers Nalquatic and StaPut (Nalco Chemical Co., Naperville, IL) provided favorable suspension of the beads. Nalquatic and StaPut acted as thickening agents and when each was combined with 2% Bond sticker (Loveland Industries, Greeley, CO) made the most promising tank mixes.

After the most promising tank mixes were identified, additional laboratory tests were conducted to determine if any of the tank mix additives significantly affected release of disparlure from the beads. All of the additives slightly reduced the initial release rate from the beads but there was no significant differences in release rates between the additives.

Additionally, the influence of bead size on release rate was investigated. As expected, the smaller beads released disparlure faster than did the larger beads.

Summary of 1990 Results

Results of trials conducted during 1990 indicated that the flakes were efficacious, releasing disparlure at a constant but slow rate. Flake application equipment problems persisted (e.g., bridging of flakes, uneven deposition of flakes beneath the aircraft) (Fig 8). The flowable bead formulation released disparlure erratically and too fast, probably because the majority of the beads were too small (106-205 microns). The rapid

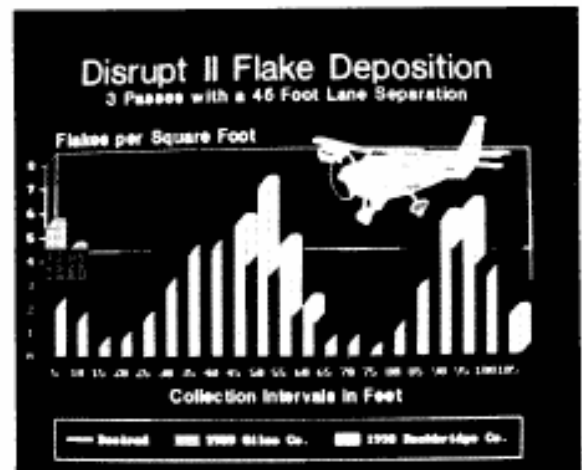


Figure 8. Uneven deposition of flakes beneath application aircraft.

release of lure documented in 1990 led to the conclusion that double applications of beads, 10 to 14 days apart, would be required in all subsequent applications in order to cover the entire flight period with adequate disparlure to disrupt mating. The tank mix (Gelva- 1990) used with the beads plugged standard aircraft boom and nozzle systems. Subsequent laboratory tests identified 2% Bond sticker combined with either Nalquatic or StaPut as promising tank mixes. All additives slightly reduced the initial release of lure, but there were no differences in release among the tank additives.

The modified delta trap mating station design developed in 1990 was used for all subsequent methods improvement projects. Laboratory-strain females were found to be equally competitive with wild females in attracting males and mating success. Therefore only laboratory-strain females were used in all subsequent tests.

1991 -- Methods Development for the Bead Formulation

Airport Trials

In January 1991, a series of field trials were conducted at the APHIS Aircraft Operations facility in Mission, Texas. The objectives of the trials were to evaluate (1) tank mixes that provided favorable deposition, rainfastness and suspendability of beads in laboratory evaluations in 1990, and (2) various types of dispensers for application of the beads.



Figure 9. CP nozzle with check valve used to apply bead formulation of racemic disparlure.

The viscosity modifiers Nalquatic (0.25%) and StaPut (20%) with Bond sticker (2%) provided favorable suspendability and deposition of beads when tank mixes were aerially applied to 23 by 30 cm (4 by 5 inch) Kromekote paper spray cards. Several nozzle systems (e.g., hollow cone, open pipes extending from the boom) were evaluated for sprayability of beads with CP nozzles (C and E Enterprises, Mesa, AZ), performing with minimal clogging (Fig. 9). The degree of clustering of beads (thought to be an important factor for release rate of lure and adhesion to foliage) on spray cards appeared to be similar for the StaPut and Nalquatic tank mixes when applied using CP nozzles with check valves and set on the largest orifice with 90° deflection plate. These inexpensive nozzles are used to apply various pesticides to agricultural crops and easily attach to standard spray booms.

Efficacy

In June and July 1991, six blocks, each approximately 50 acres, were treated in Rockbridge County, Virginia: three blocks with a double application (12 days apart) of beads containing 40% racemic disparlure at a dose of 30.4 g AI per acre per application (75 g beads per acre

per application) and three blocks with a double application of Disrupt II at 30.4 g AI per acre per application (**165** g flakes per acre per application). The beads were applied using a Cessna 188 (AgTruck) equipped with six CP nozzles, 75 ft swath, and tank mix consisting of 20% StaPut, 3% Bond, and 77% water. A Cessna 206 equipped with 2 pods was used to apply the flakes using a 45 ft swath and tank mix consisting of 4 oz of Gelva- 1990 per acre. Three additional blocks were not treated.

Prior to 1991, the diatomaceous earth that Hercon adds to the flakes to reduce static electricity during the manufacturing process was sifted out prior to final packaging of the Disrupt formulation. Starting in 1991, some of the diatomaceous earth (12 - 14% by weight) was left in the flakes in order to prevent bridging of the flakes in the hoppers. This eliminated the need to add talc to the hoppers.

AgriSense adjusted the bead size so that there were fewer beads in the 106-250 micron range and more beads in the 300-425 micron range (Fig. 10). The objective was to reduce the release rate of disparlure from the beads (Fig. 11) without creating so large a bead as to cause clogging in the spray system as well as problems with large beads adhering to foliage.

The 1991 posttreatment efficacy results for the bead treatment indicated that it was effective, as no fertilized egg masses were found under burlap bands or in 1/40-acre subplots and none of the monitor females were mated (Chart B, page 44). The bead treatment effect evident in 1991 was not evident in 1992, i year after treatment.

The 1991 posttreatment efficacy results for the flake treatment were complicated by gypsy moth populations expanding in the general area surrounding the northernmost block (fertile egg masses were recovered in and surrounding the treated block) while no fertile egg masses were recovered in the other two flake-treated blocks (Chart B). Also, none of the monitor females in the flake-treated blocks were mated. In 1992, no treatment effect was evident.

Deposition

As part of the 1991 evaluations, two forested sites received 10 times the normal application of flakes or beads to allow recovery for determination of sticker performance on foliage. The formulations were applied in the same tank mixes used on the efficacy blocks. On day 12, coinciding with the start of peak flight, 79% of the flakes and 68% of the beads remained attached to foliage. On day 56 after application, only 59% of the flakes and only 28% of the beads remained attached to the foliage, due to failure of the sticker.

Residual Activity

Emission rates of disparlure from beads or flakes were essentially the same whether the sample was collected from foliage or canvas coated cards. On day 42 after application, the flakes still contained about 60-70% of their original disparlure content (Fig. 1 2A) and on day 88 in late September the flakes still contained 40% of their original lure content. Additional analysis showed that 2.5 ***g per flake of disparlure or about 2% of the original dose remained after 12 months of exposure in the field. In contrast, on day 42 after application, the beads

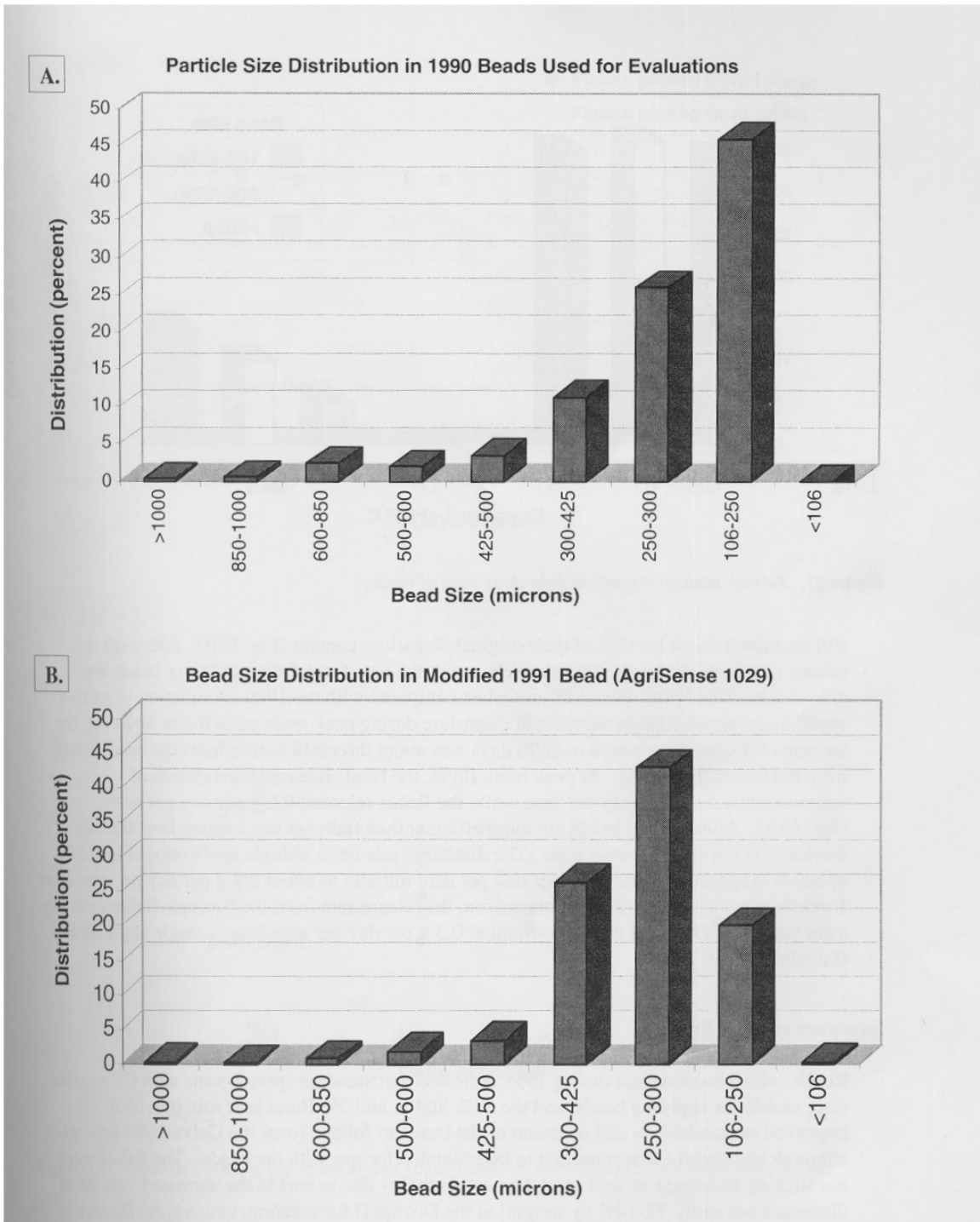


Figure 10. Size distribution of beads produced (A) in 1990 and (B) in 1991.

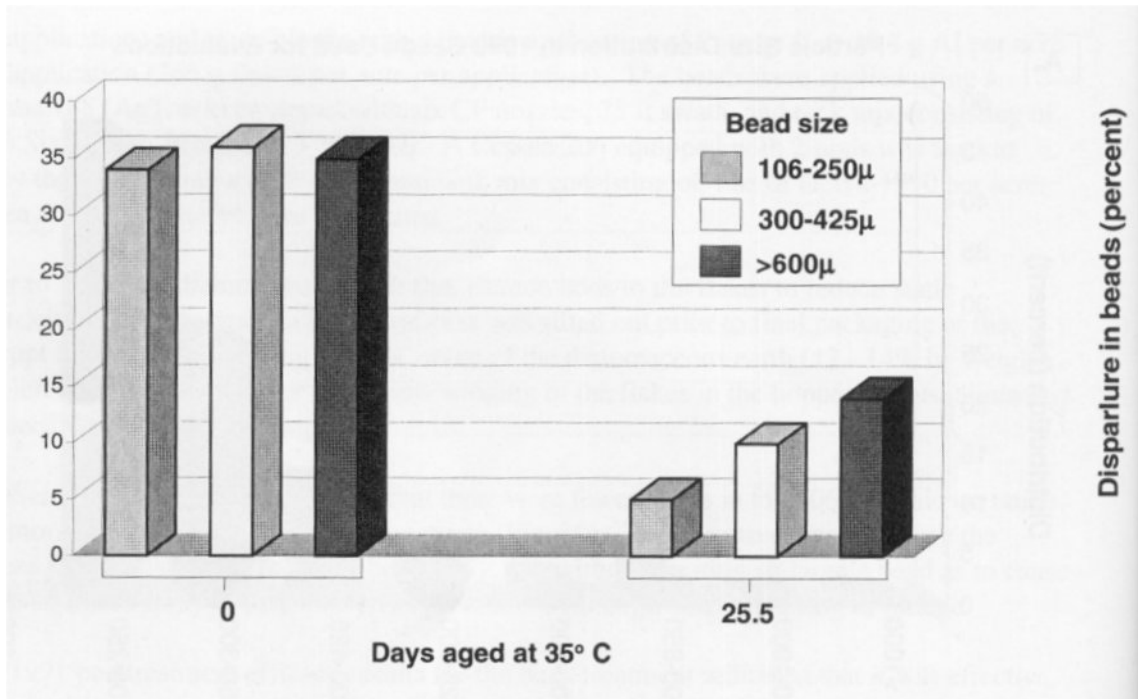


Figure 11. Release rates of disparlure from three sizes of beads.

still contained about 10-15% of their original disparlure content (Fig. 12B). Although the release rate from the beads was relatively rapid, the new formulation of larger beads was effective in slowing the release of lure when compared with the 1990 formulation. The two applications provided high amounts of disparlure during peak male moth flight, however, the amount of disparlure released over 90 days was about threefold higher from the beads than from the flakes (Fig. 13A). At peak moth flight, the beads released a maximum of approximately 1.9 g per day per acre while the flakes released 0.6 g per day per acre (Fig. 13A). Although the beads are more efficient than flakes at discharging lure, the rate of discharge is not constant over time. The discharge rate from a single application (Fig. 13B) of beads drops from about 1.4 g per day per acre initially to about 0.8 g per day per acre after 1 month (June 17 - July 18). In comparison, the release rate from the laminate flakes over the same period in 1991 was nearly constant at 0.3 g per day per acre from a single application (Leonhardt et al. 1992).

Summary of 1991 Results

Results of trials conducted during 1991 indicated that standard spray booms with CP nozzles were usable for applying beads, and the 20% StaPut and 3% Bond tank mix provided improved suspendability and adhesion of the beads to foliage (over the Gelva- 1990 tank mix), although additional carriers needed to be evaluated for use with the beads. The flakes were not sticking to foliage as well as in the past, probably due in part to the increased volume of diatomaceous earth (12-14% by weight) in the Disrupt II formulation, which was effective in preventing bridging. Also, the pod application equipment continued to perform erratically, in

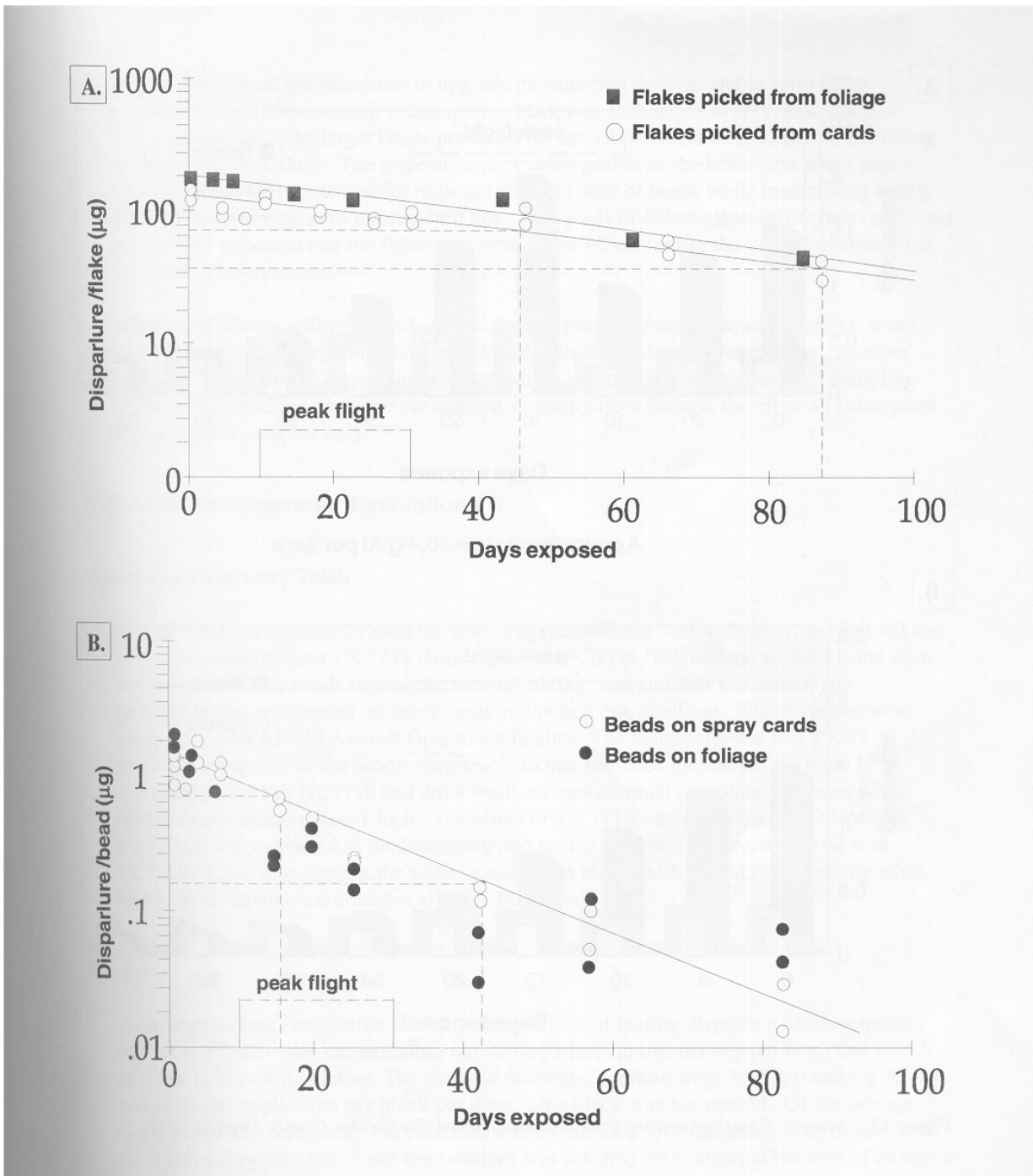


Figure 12. Disparlure content (A) of flakes and (B) of beads recovered from spray cards and foliage.

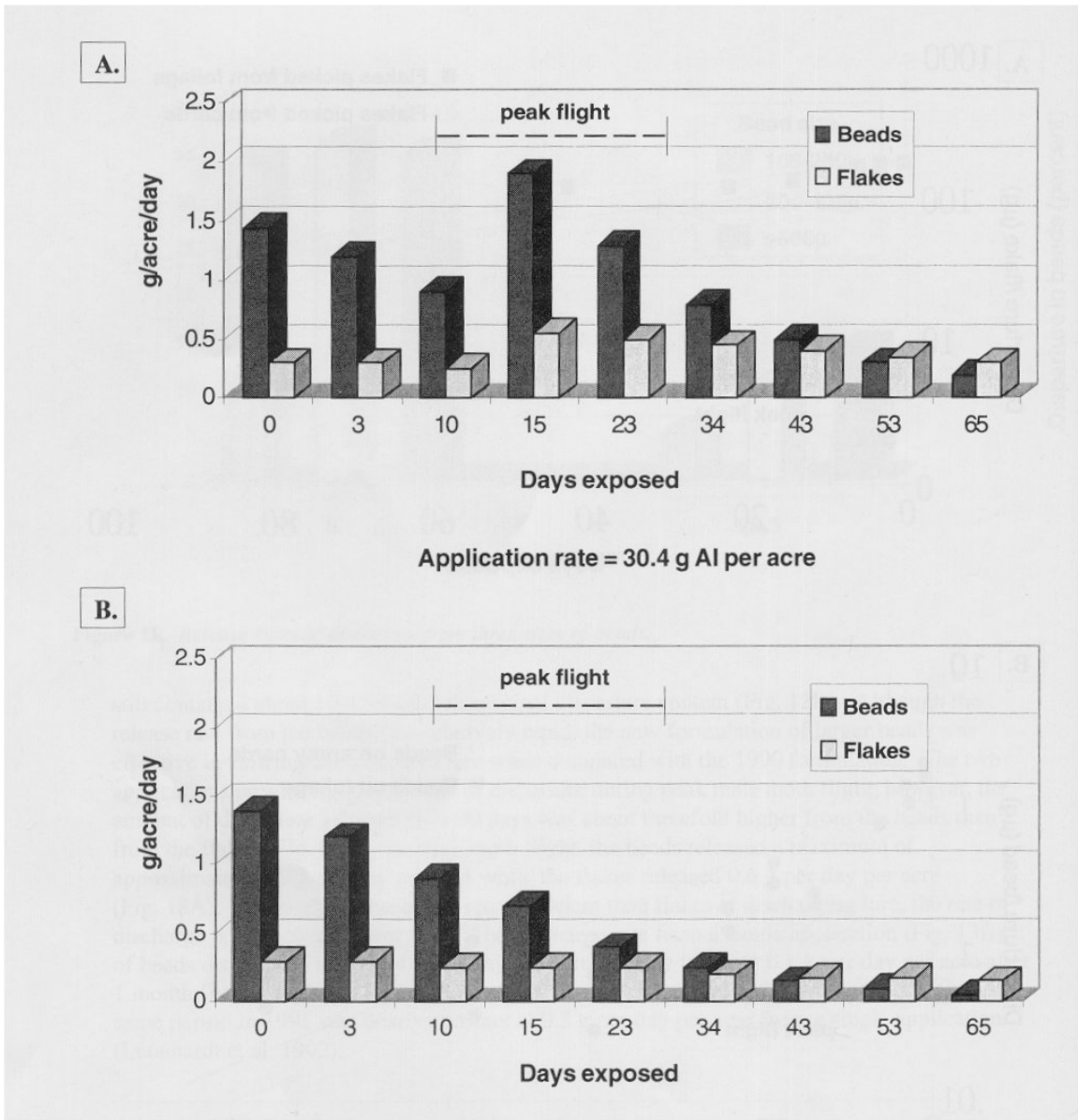


Figure 13. Average disparlure emitted from beads and flakes, Rockbridge County, Virginia, 1991.

spite of mechanical modifications to upgrade the augering systems and in spite of the application of a Teflon coating to the spinner blades on each auger to prevent buildup of flakes and sticker. The larger beads produced for the 1991 season were effective in slowing the release of disparlure. The more efficient release profile of the beads compared with flakes means there is potential for reducing the total dose of beads while maintaining a daily emission rate equivalent to the standard lure (30.4 g AI) of flakes. Results of trials conducted during 1991 indicated that the flakes and beads were efficacious in the year of treatment but not 1 year after treatment.

Although the flakes still contained 40% of their disparlure after 88 days in the field, at the start of the next year they contained only 2% of their original disparlure content and were unlikely to emit enough disparlure to affect mating in the year after application. Sampling from cards yielded the same release rates on sampling from foliage, therefore all subsequent tests used card samples only.

1992 -- Dose-Response Evaluations

Airport and Laboratory Trials

In April 1992, the previously used thickening agent StaPut at 20% volume with 3% Bond and another thickening agent TX 7719 (Nalco Chemical Co.) at 10% volume without Bond were each mixed with the beads to evaluate ease of mixing, suspendability of beads, and sprayability and weathering on spray cards and potted oak seedlings. These studies were conducted at the APHIS Aircraft Operations facility. The results showed that TX 7719 provided suspension of the beads comparable to that provided by StaPut, but more importantly once the TX 7719 had dried wash-off was minimal (less than 5%) even with rainfall events of more than 1 inch. The effect of TX 7719 on the discharge of disparlure from beads was evaluated in the laboratory and results indicated that beads mixed with TX 7719 release disparlure at the same rate as when mixed with StaPut or water only when held in an environmental chamber at 35°C for 30 days.

Efficacy

A pre-season (June) evaluation of dose versus degree of mating disruption (dose-response) was set up to determine the minimum rate of disparlure (grams per day per acre) that is effective in disrupting mating. The doses of racemic disparlure were 30, 15, 6 and 3 g AI per acre, with one application per block per dose. One block was not treated. Of the average block size of 200 acres, only the center 20 acres were used for evaluation. The bead formulation (instead of the flake formulation) was selected for evaluation because of its rapid release characteristics and because it would provide more "point sources" of pheromone than would the flake formulation at a given dose. An AgTruck equipped with 6 CP nozzles was used to apply the beads in a 60 ft swath at 1 gal per acre. Over the course of the 8-week evaluation a simulated population of 10 gypsy moth mating pairs per acre (10 mating pairs per acre at each of 4 times over a 2-week interval) was created by deployment of newly emerged

adults from laboratory-strain pupae supplied by APHIS and held outdoors at the evaluation site until eclosion. Males were released on tree boles and females were placed in mating stations (Leonard 1994).

All doses appeared to suppress mating to a similar extent. Only 0.05 % of the egg masses recovered from the treated blocks were fertile while 24% of those recovered from the untreated block were fertile. These results were unanticipated and confusing based on the wide range of doses and previous efficacy results of other investigators, although the combination an evenly distributed simulated low population versus a clumped or aggregate distribution in nature and cold rainy weather probably limited adult activity and confounded the sensitivity of the evaluation. Of the 720 males released into the untreated block only 67 (9.3%) were recaptured. No males were captured in any of the treated blocks.

Under more typical summer weather conditions, the test was repeated with essentially the same results.

A post-season (August) dose-response evaluation was conducted using doses of 0, 3, 6, 15 and 30 g AI per acre. The percentage of fertile egg masses recovered was .69% in the treated blocks and 43% in the untreated block. Of the 720 males released into the untreated block only 139 (19.3%) were recaptured. No males were captured in any of the treated blocks.

An additional efficacy evaluation was conducted during the gypsy moth mating season (July) to determine whether two applications of beads at 6 g AI per acre would disrupt mating. This dose was selected based on 1991 release rates: an application of beads at 6 g AI per acre initially released disparlure at the same rate as an application of flakes at 30.4 g AI per acre and was more cost effective. A second application of beads, 10-14 days after the first application, was needed to augment the dose of racemic disparlure since the beads release their pheromone content quickly (Chart C, page 45). Also, the 6 g AI per acre dose for each of two applications was considered economically competitive (\$8-\$13 for AI and formulation for 12 g dose) with other operational control options (\$15 per application per acre for the gypsy moth nucleopolyhedrosis virus product Gypchek and \$5 per application per acre for *B.t.*). Six blocks (three treated and three untreated) were established in Rockbridge County, Virginia.

In 1992, fertile egg masses per acre were 0 in all three treated blocks compared with an average of 3.6 fertile egg masses in all three untreated blocks (Chart D, page 46). The percentage of monitor females mated was substantially reduced by the treatment, but mating was not entirely disrupted as fertile egg masses were recovered from under burlap bands in one of the bead plots in the year of treatment. In 1993, two of the treated blocks had 0 fertile egg masses per acre and the other 12.6, whereas the untreated averaged 28 egg masses per acre.

Deposition

Sticker performance of two bead tank mixes (10% TX 7719, and 20% StaPut and 3% Bond) and one flake tank mix (Gelva- 1990 at an increased rate of 6 oz per acre) was evaluated on

blocks, treated at 30.4 g AI per acre. Each tank mix was applied multiple times to ensure good coverage. Eight days after treatment and coinciding with the start of moth flight, 80% of the beads and TX 7719; 89% of the beads, StaPut and Bond; and 93% of the flakes remained adhered to foliage. At the conclusion of the test (44 days after treatment), 63% of the beads and TX 7719; 60% of the beads, StaPut and Bond; and 71% of the flakes remained adhered to foliage.

Residual Activity

Sets of 35 canvas coated cards were sprayed with beads mixed with 10% TX 7719, or with 20% StaPut and 3% Bond and hung in the woods for periodic collection, to determine disparlure release rates. The beads in both tank mixes released lure at approximately the same rate and at a slightly slower rate than in 1991, probably due to the fact that the bead size distribution again shifted slightly upwards. Both tank mixes released approximately 50% of the lure by day 20 and 70% of the lure by day 42.

Summary of 1992 Results

In and out of season tests using simulated populations of 10 mating pairs per acre indicated that a 3 g AI per acre dose of beads was equally effective as a 30 g AI per acre dose in disrupting mating. The beads applied at 6 g AI per acre for each of 2 applications during the normal gypsy moth mating season provided mating disruption. Results of trials conducted during 1992 demonstrated that the sticker/extender TX 7719 provided adequate suspension of beads and provided equal adhesion of beads to foliage and release rates as did the 20% StaPut and 3% Bond mixture. For all subsequent tests with beads TX 7719 was used as the sticker/extender because less volume is required which results in less cost. The bead size shifted upward again in 1992 bringing to attention the fact that different bead lots are not exactly repeatable in terms of bead size distribution or release rates.

The pods used to apply the flakes continued to malfunction. Therefore, a contract was developed with K&K Aircraft (Bridgewater, VA) to modify the Hercon pod system or to develop another dispensing system for flakes that could be used on small (e.g., AgCat) and large (e.g., Twin Beach) aircraft.

Flakes applied in 1992 were mixed with 6 oz Gelva-1990 per acre and contained 5% (by weight) diatomaceous earth. This reduction in diatomaceous earth and increase in sticker volume improved the adhesion of flakes to foliage when compared with flakes applied in 1991.

1993 – Reduced Doses of Disparlure

Efficacy

To determine the efficacy of reduced doses of disparlure, a trial was conducted in Rockbridge County, Virginia, using a total of 12 blocks: 4 blocks treated with beads applied at 6 g AI per

acre per application in two applications; 4 blocks treated with flakes at 20 g AI per acre in one application; and 4 blocks untreated. The 6 g AI per acre per application dose of beads applied in two applications was used because results were favorable in 1992. These blocks used for the 1993 evaluations had been used as untreated blocks for previous pheromone evaluations (Thorpe et al. 1998). The treatments were blocked on the basis of population density (numbers of immature life stages under burlap just prior to treatment). Two of the four replicates in each treatment had more dense populations than would normally be selected for mating disruption.

Mating was disrupted in all treated blocks (greatest in the two replicates with lowest pretreatment densities) when compared with untreated blocks, although fertile egg masses were recovered in two flake-treated blocks and three bead-treated blocks (Chart E, page 47). An average of 1 male moth per trap was captured in the flake-treated blocks, 3 male moths per trap in the bead-treated blocks, and 200 male moths per trap in the untreated blocks. In general, there were no differences in efficacy between the bead or flake treatments. After treatment egg mass surveys conducted in 1993 yielded an average of 28, 6.7 and 4.4 egg masses per acre respectively in the untreated, flake and bead treated blocks (Thorpe et al. 1998). In 1994 the gypsy moth populations in the untreated blocks continued to increase above 1993 levels (average 402 males per trap and 63 egg masses per acre) and increases also occurred in the treated blocks (an average 38 males per trap and 13.9 egg masses per acre in flake-treated blocks, and an average 107 males per trap and 11 egg masses per acre in bead-treated blocks). In 1995 populations in all treated and untreated blocks decreased sharply, probably as a result of the increased incidence of the fungus *Entomophaga maimaiga* and nucleopolyhedrosis virus in the general area (Thorpe et al. 1998).

Deposition and Residual Activity

Release rates were evaluated by aerially applying flakes with 6 oz of several sticker! extenders (Gelva-1990, Gelva-2333) and a Nalco product (RA-8554) to 23 by 30 cm (9 by 12 inch) canvas cards for analysis of residual disparlure content and to foliage for sticker performance. Monsanto decided not to manufacture sticker Gelva- 1990 beyond this year; therefore, evaluation of other products was needed. The cards were hung in a nearby wooded area for aging. Periodically, 3 to 5 replicate cards for each treatment were removed for determination of residual disparlure content in the flakes and beads. Beads applied at 1 gal per acre with 10% TX 7719 were sprayed on 23 by 30 cm (9 by 12 inch) spray cards for analysis of residual disparlure content as well as on foliage to evaluate adhesion.

Gelva-2333 was selected to replace Gelva-1990 based on good performance on a sticker and no noticeable changes in release characteristics. At 13 and 47 days after treatment, 90% and 84% of the flakes and Gelva-2333 were still adhered to foliage. This compared favorably with the flakes and Gelva-1990 mix where 88% and 72% were still adhered to foliage on days 13 and 47. The Nalco 8554 did not perform well with only 51% and 28% of the flakes remaining at days 13 and 47. The beads and TX 7719 mix did not perform as well in the past when only 57% and 31% of the beads still adhered 13 and 47 days after treatment.

Shin-Etzu Chemical Co. (Tokyo, Japan) provided a batch of an experimental slow release powder formulation and a commercially produced carrier for mixing with the powder in the tank. The powder contained racemic disparlure (9.5% AI) and was evaluated only for release rates on canvas cards, as the powder did not stay suspended in the carrier.

K & K Aircraft developed a system for dispensing the flakes, which consisted of one augering unit mounted beneath each side of the front wing and one unit beneath the fuselage. The system was developed for use on a Twin Beech with the potential of carrying sufficient flakes to treat approximately 3,000 acres per aircraft load. The system was evaluated at the airport during characterization trials but has never been used operationally. The swath width was 125 feet with peaks and valleys of deposition beneath the aircraft. This system needed (1) additional development to prevent clogging of the auger with flakes and sticker, and (2) larger holding tanks in the fuselage.

Summary of 1993 Results

Results of trials conducted during 1993 demonstrated that lower doses of disparlure (beads applied at 6 g AI per acre in each of two applications and flakes applied at 20 g AI per acre in one application) suppressed mating when compared with untreated blocks but did not prevent the production of some fertile egg masses in the blocks with higher population densities. Therefore, there was a need to reevaluate these doses of disparlure against low density populations. Gelva-2333 was selected to replace the soon to be discontinued Gelva-1990 as a sticker for the flakes. Suspension of the Shin-Etzu powder when mixed with the carrier and water in the tank was unsatisfactory. Also, the pheromone release rate from the powder was more rapid than the release rate from beads. These unfavorable characteristics resulted in a request to Shin-Etzu to modify their formulation and carrier before the initiation of future trials. The prototype system for dispensing flakes from large aircraft developed by K & K aircraft needed additional modification but was not pursued due to anticipated limited use of mating disruption over large areas in the next 5 years.

1994 - Increased Doses of Disparlure

Efficacy

Twelve blocks, four per treatment, were established farther south in Rockbridge County, Virginia, where there were less dense gypsy moth populations as determined by the capture of fewer male moths. These blocks were not isolated woodlots as were used in the past, but part of the general forest. Each block was approximately 100 acres with only the center 20 acres used for evaluation. The treatments were (1) beads at 15.2 g AI per acre with 10% TX 7719 (now designated Biogrip or 93SD1 55) in 1 gal per acre for each of two applications; (2) flakes at 30.4 g AI per acre (175 g of flakes and diatomaceous earth per acre) with 6 oz Gelva-2333 per acre for one application; and (3) untreated. Increased doses for both flakes and beads were used in 1994 because in 1993, even though lower doses of disparlure in both flakes and beads suppressed mating, fertile egg masses were produced in blocks with higher population densities. The beads manufactured for use in 1994 again increased in size. This emphasized the continuing problem of variability in bead size distribution between manufacturing lots.

The treatment blocks were established on the basis of the number of life stages found under burlap bands just prior to treatment.

The 1994 posttreatment results indicated that gypsy moth populations were effectively suppressed by both formulations; however, neither treatment was 100% effective in all blocks (Chart F, page 48; Thorpe et al. 1998).

Deposition and Residual Activity

The release rate and stickability of the larger beads were compared with the 1993 beads for release rate of dispartlure on canvas cards and for deposition on foliage. The beads were applied at the 30.4 g AI per acre dose in i gallon per acre with 10% Biogrip.

No differences were detected in the percentage of dispartlure released over 30 days for “old” beads (77%) and “new” beads (74%). Also, there was no detectable difference in adhesion to foliage.

In 1994, some nozzle clogging was encountered using the larger beads. After each load the CP nozzles were cleaned, and usually at least one of the outer nozzles was completely plugged.

Summary of 1994 Results

Results of methods improvement trials conducted during 1994 demonstrated that the bead and flake treatments suppressed population in the treatment year (1994) as determined by the number of fertile egg masses and egg masses per acre recovered in the treated versus untreated blocks. There were no detectable differences between the 1993 beads and the 1994 beads based on release rates and adhesion to foliage. Unfortunately, the 1994 beads applied at 15.2 g AI with 10% Biogrip clogged CP nozzles. Preliminary thoughts concerning the cause of this problem were larger sized beads, unpublished change in the Biogrip additive, pH of the water in the tank mix, or a combination of these factors. The original pods manufactured for Hercon by Schweitzer aircraft in the 1970's were no longer reliable for use. The applications were plagued with breakdowns, primarily of the motors and motor controls for the flake augers. This performance of the original pods during the methods improvement trials was in contrast to more efficient performance of a much modified set of pods (i.e., with new motors and motor controls, a different design of augers, and larger flake hoppers) developed and used by Harold's Flying Service (Leland, IL) to apply the flakes to operational blocks.

1995 – Monitoring of Blocks Treated in 1994

No new efficacy and residue trials were initiated in 1995, but monitoring continued in blocks treated in 1994. The bead and flake treatments provided comparable population suppression (as compared with untreated plots), but results were complicated by an increased incidence of the fungus *Entomophaga maimaiga* and nucleopolyhedrosis virus, which also suppressed the gypsy moth populations in the treated and untreated blocks (Chart F, page 48).

In response to plugging of CP nozzles by beads in 1994, a contract was developed with Schiffer Flying Service (Ovid, MI) to apply several tank mixes containing the bead formulation from the ground and air. Several nozzle/atomizers (e.g., flat fan, hollow cone, Micronair) were used to apply the bead formulation. Micronair AU-5000 atomizers (without screens) (Fig. 14) performed well and did not plug during ground and aerial trials. The other nozzle systems (including the CP nozzles) plugged during application using ground equipment.

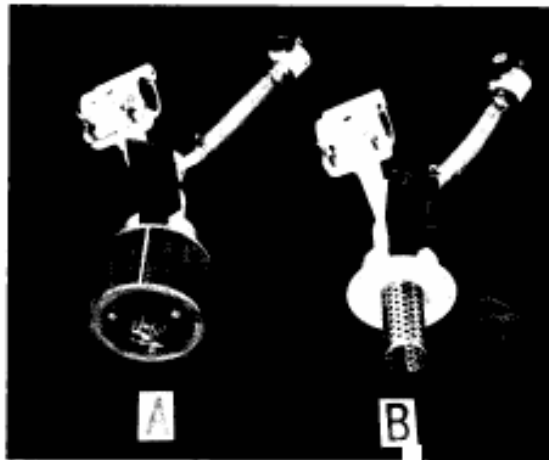


Figure 14. *Micronair AU-5000 atomizer (A) with cage screen used to apply insecticides and (B) without cage screen used to apply the bead formulation of racemic disparture.*

Summary of 1995 Results

The increasing incidences of gypsy moth fungus and virus throughout Rockbridge and Augusta Counties, Virginia, further complicated the 1994 efficacy trials. It was decided not to initiate additional trials in 1996 until another area could be located with suitable low density gypsy moth population, modifications were completed to equipment for applying the flakes, and nozzles and tank mixes for applying the beads were reevaluated.

1996 - Evaluation of Additional Tank Mixes

Laboratory Trials

In the laboratory (APHIS Otis Methods Development Center), the tank mixes were deposited on seasoned foliage of potted red oak seedlings, and the plants were exposed to accumulated rainfall totaling 5.0 inches. A surfactant, TX 8815 (now designated Nalco 8815), that had earlier shown promise in preventing clogging of nozzles and aiding in mixing did not appreciably contribute to suspendability of the tank mixes evaluated and substantially increased wash-off of the standard tank mix. Of the tank mixes tested, the following four were most resistant to wash-off: (1) Biogrip, Nalquatic, and Nalco 8815, (2) Biogrip and Nalquatic, (3) Bond and Nalquatic, and (4) Bond, Biogrip, Nalquatic, and Nalco 8815.

Airport Trials

At the airport (APHIS Aircraft Operations), the Bond and Nalquatic tank mix (with and without 0.5% Nalco 8815) were selected for evaluation in an aircraft based on performance during laboratory trials. Nalco 8815 did not aid in keeping beads in suspension once mixed but did aid in the initial mixing of beads into a slurry. The Micronair atomizers without cages did not malfunction using these tank mixes.

Field Trials

An AgTruck was used to aerially apply large volumes of several tank mixes to experimental blocks in Rockbridge County, Virginia, in July 1996. The Bond and Nalquatic mix was chosen as the basis for evaluation due to its higher resistance to wash-off as compared with the standard (Biogrip). Each tank mix was applied through CP nozzles and AU5000 Micronair atomizers. Mixes were evaluated with and without Nalco 8815.

Clogging did not occur in CP nozzles or Micronair atomizers with any of the tank mixes. Past efficacy trials in which clogging occurred used CP nozzles with stainless steel bodies. These trials used CP nozzles with plastic bodies. Flow dynamics of the bead mixes through plastic bodies is different than through steel bodies (discussion with C and E Enterprises, Mesa, AZ). Further evaluation of the types of nozzle bodies is needed.

Both types of nozzles were configured to deliver a range of droplet sizes. Over an 8-week period after spraying, beads on foliage were counted to determine wash-off. None of the tank mixes applied through Micronair atomizers performed well. The best performing mix was 0.75% Nalquatic, 3% Bond, and 0.5% Nalco 8815 in water. When applied through CP nozzles set at maximum deflection and at maximum orifice size, this mix resulted in greater retention of beads on foliage than when applied through Micronair atomizers set at VRU of 12 and 90° blade angle with cages removed.

Retention of beads on foliage:

Days after application	CP nozzle	Micronair atomizer
14	90%	71%
28	80%	55%
42	50%	31%
56	35%	1%

The addition of Nalco 8815 surfactant did not cause undue foaming and appeared not to have increased wash-off of beads from foliage.

Summary of 1996 Results

Increased incidence of the gypsy moth fungus dramatically reduced gypsy moth populations throughout Rockbridge County and continued to complicate evaluation of previous efficacy trials. Evaluations of various tank mixes showed that combinations of Nalquatic, Bond, and bead mixes in water, with and without Nalco 8815, could be applied through CP nozzles with plastic bodies and Micronair atomizers without cages with no clogging.

Wash-off data for the Nalquatic, Bond, and Nalco 8815 tank mix compared favorably with the original 1993 wash-off data for the Biogrip tank mix.

1997 - Additional Trials of Flake Formulations

Efficacy

Because of concerns over delayed U.S. EPA registration of the bead formulation, the Working Group's focus shifted back to evaluation of the flake formulation. Based on results of earlier tests with hand-applied pheromone dispensers (Kolodny-Hirsch and Schwalbe 1990), it was originally concluded that any successful pheromone dispenser must be distributed throughout the forest canopy to be effective. The effect of dispenser distribution on the efficacy of mating disruption, however, has never been determined experimentally for the Hercon flakes. To distribute the flakes throughout the canopy, a sticker is required to adhere the dispensers to foliage for the duration of male flight. The mixing and application of flakes and sticker requires special equipment and frequently results in application problems. If flakes could effectively disrupt mating when applied without sticker, application could be simplified and costs reduced.

Twelve blocks that supported very low gypsy moth populations were established in the northern half of Rockbridge County, Virginia. Four of these blocks were treated with the standard flake tank mix, which consists of Hercon Disrupt II at 75 g AI in 9.9 oz of Gelva2333 per ha and 3% diatomaceous earth. Four blocks were treated with flakes at the same dose and diatomaceous earth alone, and four controls were left untreated.

Biological efficacy was assessed using techniques described on pages 3 and 4. USDA milk carton traps to catch males and delta traps to deploy monitor females were placed both at ground level (2.5 m) and in the forest canopy (approximately 19 in). Burlap bands and pre-season and post-season 0.01 ha (1/40 acre) fixed-radius egg mass surveys were used to evaluate population levels and trends.

Communication between males and females was suppressed but not severed on treatment plots as measured by the number of males caught per trap per day. Average capture was 0.00013 males/trap/day on plots with sticker, 0.02 males/trap/day with no sticker, and 0.25 males/trap/day on control plots. Communication suppression was more successful in plots treated with sticker than in plots with no sticker. Mating success, as measured by the percent of fertile egg masses recovered from deployed females, was substantially higher in control plots (9.3%) than in either treatment (1.9% in plots with no sticker, and 1.4% in plots with sticker), and there appeared to be no difference between treatments. These combined data are ambiguous in that they do not show clear differences between the sticker and no-sticker treatments.

Alternative Tank Mix

Although the flakes have consistently been shown to adequately disrupt mating in efficacy trials in the past, the application equipment used to apply the flakes (dispenser pods developed in the 1970's by Schweitzer Aircraft and modified by Hercon Environmental, USDA APHIS, and Harold's flying Service) is expensive, specialized, and performs inconsistently. A slurry of flakes in an appropriate carrier that could be applied with conventional spray equipment would offer an advantage.

In 1997, field trials evaluated a tank mix slurry using a new carrier, LI108 (Loveland Industries, Greeley, CO). LI108 is both a thickening agent and adhesive; therefore, no other adjuvants were used in this mix. The slurry was pumped through (1) special pinch valves and 8050 Tee Jet flat fan tips fitted to adjustable stainless steel bodies and (2) large diaphragm check valves with 8085 Tee Jet flat fan tips and round orifices. Some clogging did occur in the stainless steel valve bodies, and boom pressures were lower than desired; however, the slurry passed through both nozzle configurations well and there was positive shutoff. Unfortunately, when the flakes remain in the slurry there is a release of pheromone from the flakes into the slurry and, subsequently, to the air. Tests are underway to determine these release rates. Additional field testing of various slurry and nozzle configurations, as well as weatherability studies of the LI108 flake slurry, are planned for 1998.

Summary of 1997 Results

Results of the sticker versus no-sticker efficacy tests were inconclusive, but provided some evidence that it may not be critical for pheromone dispensers (e.g., Hercon flakes) to be applied to a target area in such a way that they adhere to foliage. Under some circumstances it may be possible to simplify application procedures and reduce application costs by using a tank mix with no sticker. The Mating Disruption Working Group identified this area of inquiry as a high priority for 1998.

The Hercon flake pods worked well for applying the standard mix with sticker, and no clogging occurred. The no-sticker mix was also successfully applied, except that, despite initial attempts to calibrate the system, the pods appeared to be putting out the total amount of material before the plot was actually finished. Although no specific factor could be identified as causative, a combination of factors could have contributed to this phenomenon, including these: (a) slight changes in calibration, (b) inaccurate acreage estimates, (c) delayed initiation or termination of pod auger operation or both, (d) flake dribbling after shutoff, and (e) overlap of previous swaths (the aircraft was fitted with a global positioning guidance system, but it was not functioning properly at the time of application).

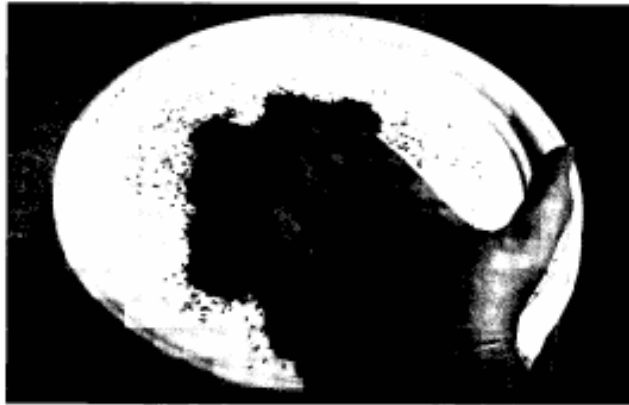
Preliminary tests of an alternative tank mix (LI108-flake slurry) applied through specially designed nozzles attached to conventional spray equipment are encouraging, although the rapid leaching of pheromone from the flakes into the slurry needs to be resolved.

Current Disparlure Formulations and Use

Disrupt II

The current formulation of disparlure flakes, Disrupt II (Hercon Environmental Co., Emigsville, PA), was granted full registration by the U.S. EPA (Reg. No. 8730-55) in 1992. It contains a different plasticizer than the earlier formulation, whose registration was granted in October 1983 (EPA Reg. No. 8730-6) but not renewed in 1989. The current label specifies application of 30-40 g AI per acre to forested and residential areas (more than one house occurring per 10 acres).

The Disrupt II controlled release formulation of disparlure consists of multilayered plastic flakes or confetti, each 1/32 by 3/32 inch (1 by 3 mm) (Fig. 15). The flakes contain 18.5% AI of racemic disparlure. The active ingredient is implanted and protectively sealed in a layer between outer polymeric layers. The inner layer serves as a reservoir of the active ingredient, which migrates continuously through the permeable barrier layer. The



flakes themselves contain 18.5% AI but they are packaged with diatomaceous earth (3% by weight), therefore the label for the final product is 17.9% AI. The flakes are mixed and applied with 4 oz per acre of the sticker/extender Gelva-2333. This mix is applied using an augering system modified from the system originally developed by Schweitzer Aircraft.

The flakes are applied at 30.4 g AI per acre (170 g flakes per acre) in one application at the estimated start of adult male emergence. On average the flakes release approximately 30-40% of their pheromone by day 42 after treatment. At the 30.4 g AI per acre dose, 0.3 g per acre per day is released over 2 weeks. The release rate is consistently low during the male moth flight period; therefore, much of the pheromone is released after the flight period and is wasted. The acrylic multipolymer resin emulsion Gelva-2333 (replaces Gelva-1990, which is no longer produced by Monsanto) is the sticker/extender mixed with the flake formulation. This sticker/extender has two major components: (1) an adhesive agent to adhere the flakes to foliage or other plant surfaces and (2) a surfactant. The specific components are found on the inert ingredient list provided by U.S. EPA and are considered exempt from the requirement of a tolerance when used as an inert ingredient in a pesticide formulation applied to growing crops. This sticker/extender, which was first used in 1994, has not been as intensively evaluated as Gelva-1990 but seems to perform equally well. In general, about 80% of the flakes deposited remain adhered after 42 days of exposure.

Disrupt II is delivered in 5 gallon buckets or plastic bags (approx. 18.7 lb or 8.5 kg) each containing sufficient flakes to cover 50 acres at the dose of 30.4 g AI per acre. The cost to incorporate the disparlure into and manufacture the plastic flakes is approximately \$8 to \$20 per acre, depending on the quantity ordered, which does not include the cost of the racemic disparlure (also approximately \$12 to \$20 per acre). The plastic components of the flakes can persist in the environment for 10-15 years, but usually are not noticed due to their small size, green color, and minimal deposition (average 4 per square foot). When applied using the original hoppers developed by Schweitzer aircraft, each load will treat a maximum of 125 acres. When applied using the system modified by Harold's flying Service, each load will treat approximately 400 acres.

Decoy GM

An application to register the Decoy GM bead formulation of disparlure was submitted by biosys (Columbia, MD) to the U.S. EPA in February 1995. In 1997, Thermo Trilogy Corporation (Columbia, MD) acquired biosys and is pursuing registration of the bead formulation.

The Decoy GM controlled release formulation of disparlure is a flowable bead formulation consisting of an open-pore polymer containing 34.04% AI of racemic disparlure. The beads range from 50 to 1000 microns with a volume median diameter (VMD)—the droplet size that divides the spray volumes in half—of approximately 275 microns. The particle size distribution of the beads varies for different production runs and this will affect the release characteristics. The beads appeared to release pheromone at a rapid rate although recent data indicates a release rate similar to flakes. The most widely used bead tank mix consists of 90% water, and 10% Biogrip (Nalco Chemical Co., Naperville, IL). It is applied at 1 gal per acre using AU-5000 atomizers without cage screens, or using CP nozzles with plastic bodies attached directly to the aircraft boom (without using a connector) and set on the largest orifice and 900 deflection plate.

The beads are applied at 15.2 g AI per acre in each of two applications, the first at the start of adult male emergence and the second to coincide with peak male flight. These beads rapidly release disparlure (75% over 8 weeks) although not at sufficient levels beyond the first 3 weeks. The beads release 50% of the AI by day 20 and 70% by the end of the season. Approximately 40% of the beads are lost from foliage over the season. Decoy GM is delivered in aluminum lined pouches containing 750 g of beads per pouch, which is sufficient to treat 17 acres at 15.2 g AI per acre. The bead formulation costs approximately \$3.00 per acre, which does not include the cost of the racemic disparlure (approximately \$12 to \$20 per acre).

The suspending agent Biogrip (93SD 155) is an aqueous acrylamide/acrylate polymer that has been used since 1992 with the bead formulation. Biogrip must be added to a water slurry of the beads to suspend them in the tank mix and to adhere them to the foliage. Biogrip's components also appear on the U.S. EPA ingredient list exempting it from the requirement of a tolerance. During one field trial, Biogrip caused milky stains on some car finishes, which required buffing or compounding for removal. Also, preliminary laboratory evaluations indicate that Nalquatic (0.75%) and Bond (3%) added to a water slurry of the beads provide better suspension in the tank mix and comparable adhesion to foliage. Therefore, recommendations for operational use of beads remains limited until tank mix and release rate problems are resolved.

Operational Use

Although the current Disrupt II flake product has been used operationally since 1990, many of the minor problems encountered with this product then still exist today. One of the problems is the slow release of the pheromone during the application year—specifically, during the male moth flight period. Then much of the pheromone is released after male moth activity and is consequently wasted. This problem requires the application of a high dose (30.4 g AI per acre) of disparlure with its associated high costs. The application equipment continues to perform erratically, although recent upgrades in the motors for the flake augering system and enlargement

of the flake hoppers have improved overall performance. The pods still mandate special aircraft requirements: at least a 24 volt electrical system, high wing for pilot to observe proper functioning and FAA approval. The persistence of the three-layer plastic laminate in the field continues to be an environmental concern about the application of flakes to residential areas. Hercon is evaluating, in the laboratory, a slow release flake formulation consisting of biodegradable plastics, as well as a paper matrix for use with racemic disparlure.

The Decoy GM flowable bead formulation, which is not registered by the U.S. EPA, has been aerially applied only during methods improvement projects and one pilot project. One of the major problems with the beads is the fast release rate of the active ingredient and, therefore, the need for a double application at additional cost. Maintaining optimum suspendability and stickability for the bead formulation is difficult; the recommended sticker/extender Biogrip needs additional evaluation relative to car finishes and may be discontinued.

Effects on Nontarget Organisms

The toxicity of insect pheromones to mammals is relatively low, and the U.S. EPA requires less rigorous testing of these products than it requires of insecticides. Therefore the toxicity data on disparlure is limited (Beroza et al. 1975, USDA 1995). Data regarding the toxicity of disparlure to animals or humans after subchronic or chronic exposures were not found in the available literature. Moreover, the acute toxicity of disparlure for endpoints other than mortality is poorly characterized (USDA 1995). Cameron (1995) reported an apparent persistence of disparlure in the human body based on attractancy to male gypsy moths for a minimum of 16 years.

In one laboratory exposure study, concentrations of racemic disparlure greater than 100 mg per liter of water resulted in some mortality of the test population of rainbow trout and bluegill (USD1 Fish and Wildlife Service 1972). This should not be interpreted to mean that racemic disparlure is toxic to fish when used for mating disruption of the gypsy moth, only that excessively large doses might be toxic.

Few studies appear in the published literature on the potential effects of disparlure on invertebrates.

Conclusions

The mating disruption technique should be used only to manage isolated or area-wide low density populations of the European strain of the gypsy moth. The exact biological parameters for its successful use have not been identified, although since 1990 there have been several successes in reducing populations (compared with untreated areas) within a range of low level populations. Criteria currently recommended for its use are these: (1) traps should capture no greater than 30 male moths per trap, and the average capture should be less than 15 per trap in the year before treatment, (2) populations should be well delimited (i.e., at least nine traps per square mile), (3) the treated area should be at least 5 miles from a source of large numbers of migrating male moths, and (4) the treated area should be large enough to offset anticipated male moth migration

(e.g., at least 2,500 ft on a side). In operational uses, monitoring for treatment effectiveness is the same as that used with traditional insecticides on eradication projects (e.g., delimiting grids of at least 9 traps per acre deployed for at least 2 years after treatment). However, with mating disruption, 0 captures in the year of treatment does not necessarily equate with successful mating disruption. Rather, the captures in the year after treatment are used to evaluate effectiveness.

Costs associated with the manufacture and application of racemic disparlure and Disrupt II have been high compared with costs associated with aerial application of traditional insecticides.

Additionally, the cost of monitoring in methods development areas (e.g., Rockbridge County) is quite high. The use of females obtained as pupae from natural populations or laboratory-strain to monitor mating success is labor intensive in holding the pupae until adult emergence, in placing and collecting 1-day-old females at monitoring locations, and in determining mating and embryonation of the collected females and egg masses. Laboratory experiments and field evaluations indicate that monitor females, both laboratory-strain and wild, are comparable in attractiveness to wild male populations. Although intensive surveys for native egg masses indicate efficacy, such surveys are time consuming and expensive, and due to difficulty of sampling, egg mass surveys alone do not demonstrate efficacy (Roelofs 1979).

In spite of numerous successes in using Disrupt II to reduce gypsy moth populations, basic questions remain concerning the behavior of adult male gypsy moths in locating and mating with females, the spatial and temporal distribution of racemic disparlure, and the dose-response relationship of disparlure and effective mating disruption. The mating disruption technique has great potential for managing low density populations of the gypsy moth without associated impacts on nontarget organisms.

Summary

The use of pheromones to manage pest species has proven an effective technique in agriculture and forestry. Insect pheromones that act as sex attractants are used to suppress pest populations through mating disruption. This publication is a compilation of historical and current information on the use of mating disruption to manage sparse-density populations of the European strain of the gypsy moth.

Mating Biology of the Gypsy Moth

The mating disruption technique is more effective as populations decrease in density since at higher population densities males can locate females visually, by chance encounter, as well as by following plumes of pheromone emitted by females. The gypsy moth has one generation per year, and the mating season occurs over a 3-6 week period in late summer. Adult flight activity and mating are temperature mediated but most mating normally occurs daily between 1000-1700 hours.

Mating Disruption of the Gypsy Moth

The identification and production of the synthetic gypsy moth sex pheromone (Bieri et al. 1970) or disparlure provided the opportunity to manage gypsy moth populations by mating disruption. Failure of males to locate females in air saturated with disparlure probably results from desensitization of the chemoreceptors in the males' antennae, as well as from disorientation by following false pheromone trails or leaving the pheromone-treated area (Carde 1996). The gypsy moth is not an ideal candidate for mating disruption due to its high fecundity. In addition, males are highly polygamous, and natural distribution patterns of adult females are not random but clumped or aggregated. Good characteristics of the European strain of the gypsy moth for mating disruption include flightless females, low mating success of females at sparse densities, limited dispersal of the majority of males beyond a few hundred meters, and one generation per year. Because some recently introduced females of the Asian strain of the gypsy moth are capable of flight, this strain may be less suited to the use of mating disruption.

Development of Mating Disruption – 1971 through 1997

Since 1971, many attempts have been made to use mating disruption to manage populations of the gypsy moth. Kolodny-Hirsch and Schwalbe (1990) reviewed the results of research and operational trials before 1989. In general, these results were inconsistent in terms of efficacy and formulation performance, and disparlure release profiles were not monitored during the treatment year.

In 1989, an eradication program was conducted on 2,500 acres in Giles County, Virginia, using the Disrupt II plastic flake slow release formulation (Hercon Environmental Inc.). The flakes were applied at a dose of 30.4 g AI per acre of racemic disparlure in one application using a small fixed-wing aircraft. The efficacy results were excellent as no adult male moths were captured within the treated block from 1989 through 1991 (male moths were recovered in an associated untreated block). In spite of the favorable efficacy results, the application equipment which intermittently malfunctioned and produced an uneven deposition pattern of flakes across the swath raised concerns for future use. Also, the flakes released only a small percentage of their disparlure content during male moth flight.

In 1990, a replicated study was conducted in Rockbridge County, Virginia, to evaluate three treatments: (1) two applications of flakes in 1990 only, each at a dose of 30.4 g AI per acre, (2) a single application of flakes in 1990 and again every year from 1991 through 1993 at a dose of 30.4 g AI per acre, and (3) untreated. The efficacy results over all years (1990 through 1994) showed that gypsy moth populations were significantly reduced by either type of flake treatment (Leonhardt et al. 1996). The flake application equipment continued to malfunction and, based on anticipated high costs to replace or redesign the equipment, an effort was made to locate another controlled-release formulation that could be applied through conventional booms and nozzles.

In 1990, AgriSense (formerly biosys and now Thermo Trilogy, Columbia, MD) developed a polymeric flowable bead formulation containing disparlure (Decoy GM). Release rate evaluations showed that the bead formulation released disparlure at a faster rate than the plastic laminate flake formulation.

In 1991 through 1994, various trials were conducted using the beads applied in two applications at doses from 6 to 30.4 g AI per acre per application in 1 gal of tank mix per acre per application. Although efficacy results were inconsistent over the various doses, in general results were favorable at doses as low as 6 g AI per acre per application for two applications. A bead formulation containing a greater portion of larger diameter beads was manufactured in an effort to slow the disparlure release rate.

The flakes were used operationally in several States. The operability of the application equipment for the flakes was improved by upgrading the motors for turning the augers and enlarging the holding capacity of the flake hoppers.

Current Disparlure Formulations and Use

The plastic flake formulation Disrupt II is registered by the U.S. EPA and used operationally to manage low density populations of the gypsy moth. The plastic flakes are applied at 30.4 g AI per acre of disparlure for one application using pods mounted on the underside of the aircraft wing. The polymeric bead formulation Decoy GM is not registered by the U.S. EPA, but an application for registration was submitted in February 1995. The beads are applied at 15.2 g AI per acre per application in two applications, using CP or Micronair AU-5000 atomizers with screen cages removed and attached to standard spray booms. Formulation costs are approximately \$8 per acre for the flakes and \$3 per acre for the beads. The active ingredient which is not included in the cost of formulation costs between \$400-\$700 per kg, which equates to \$12-\$20 per acre for a 30.4 g AI dose.

The exact biological parameters for the successful use of the mating disruption technique have not been identified although the technique should be used only to manage isolated or area-wide (to reduce the possibility of insect movement into the treated area) low density populations of the European strain. The technique is specific for the gypsy moth and has no known impacts on nontarget organisms.

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