UTILIZATION OF BLACK FLY LARVAE (DIPTERA: SIMULIIDAE) IN BIOMONITORING AND AQUATIC TOXICITY TESTING: ASSESSING THE EFFECTS OF LAWN-CARE CHEMICALS IN SUBURBAN STREAMS

by

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(Under the direction of Raymond Noblet)

ABSTRACT

Urban and suburban watersheds have the potential to be highly impacted by chemicals, especially insecticides used to control insect pests on lawns, ornamental plants, and home gardens. Three of the most common lawn-care insecticides detected in urbanized watersheds, carbaryl, chlorpyrifos, and malathion, have been evaluated in the laboratory to determine their acute toxicity, joint-toxicity as mixtures, bioavailability in the presence of organic matter, and effects on survival, growth and development to black fly larvae, Simulium vittatum cytospecies IS-7. An acute orbital shaker toxicity test was used to determine the 48 hr LC50 value of the three insecticides for S. vittatum IS-7 larvae. Results of the 48 hr LC50 tests showed chlorpyrifos to be the most toxic (LC50 = 0.28 µg/L) followed by carbaryl (LC50 = 23.72 µg/L), and malathion (LC50 = 54.2 µg/L). These insecticides were also tested as mixtures using a toxic unit (TU) approach. Toxicity was greater than additive for the ternary mixture of chlorpyrifos-carbaryl-malathion (LC50 = 0.56 TU), and the binary mixtures of chlorpyrifos-malathion (LC50 = 0.72 TU), and carbaryl-malathion (LC50 = 0.78 TU). The binary combination of chlorpyrifos and carbaryl was additive (LC50 = 0.98 TU). Bioavailability of the three insecticides to S. vittatum IS-7 was significantly altered in the presence of a laboratory diet. Chlorpyrifos bioavailability decreased whereas carbaryl and malathion bioavailability increased when diet concentrations were ≥ 150 mg/L. Natural seston had little effect on the bioavailability of these insecticides except in carbaryl dosed larvae where mortality was significantly increased when seston concentrations reached 150 mg/L in the flasks.

Multiple pulse-exposures of chlorpyrifos, carbaryl and malathion individually and as mixtures in a trough flow-through dosing system had little effect on survival, growth and development of S. vittatum IS-7. Survival in black flies exposed to chlorpyrifos was significantly lower than survival in malathion and carbaryl exposed insects. However, this effect was not significant compared to controls. No significant differences in growth or development were detected between insecticide-exposed and control larvae.
INDEX WORDS: Toxicity, Toxic Units, Insecticide Mixtures, Chlorpyrifos, Carbaryl, Malathion, Black Fly, Simulium vittatum, Suburban Watersheds, Bioavailability, Laboratory Diet, Seston, Life History, Survival, Growth, Development, Pulse-Exposure, Flow-Through
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DEDICATION

I would like to dedicate this dissertation to my family. To my wife Amanda, for the support and sacrifices she endured throughout my graduate career. To my daughter Gretta, for taking my mind off of work and greeting me with a big smile when I come home. To my parents, especially my father who suddenly passed away this March, for giving me the opportunity to achieve my goals. I love you all.
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CHAPTER 1

INTRODUCTION

Urban and suburban watersheds may be highly impacted by chemicals, especially insecticides used to control insect pests on lawns, ornamental plants, and in home gardens. An estimated 17 million pounds of insecticidal active ingredients are applied annually in domestic settings for control of insects on lawns and gardens (Aspelin and Grube 1999). Multiple lawn-care insecticides enter urban and suburban waterways primarily through runoff from rain events and are commonly detected in these aquatic systems (Struger et al. 1995, Hippe et al. 1994, Kimbrough and Litke 1994). In addition, lawn-care insecticides have been shown to cause detrimental effects in aquatic organisms (Kuivila and Foe 1995).

The organophosphate insecticides chlorpyrifos (O,O-Diethyl O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate), and malathion (Diethyl mercaptosuccinate, O,O-dimethyl-dithiophosphate), and the carbamate insecticide carbaryl (1-Naphthyl-N-methylcarbamate) are registered for use on lawns and gardens for control of insect pests in the U.S.A. Although chlorpyrifos is no longer registered for domestic use, it is registered for use on golf courses, which are often built in residential communities, to control turf pests. As a result of their widespread use, chlorpyrifos, carbaryl and malathion are three of the most commonly detected insecticides in urban and suburban streams such as those within the Flint River watershed of Georgia (Hippe et al. 1994). Thus, it is important to determine the impacts these chemicals may have on aquatic...
organisms to ensure the integrity of stream ecosystems and provide information required for watershed management.

One approach to assessing the effects of contaminants, such as lawn-care insecticides, on aquatic ecosystems is through monitoring the biological community inhabiting the waterway (biomonitoring). Macroinvertebrates are excellent biomonitors of contamination in streams and rivers because they are ubiquitous in diverse aquatic habitats, can be sensitive to environmental stress, and can aid in determining the effects of spatial and temporal impacts (Rosenberg and Resh 1996). Within the field of biomonitoring are several assessment scales ranging from the sub-organismal level (e.g., biochemical and physiological) to the ecosystem level along with several approaches including field experiments and laboratory toxicity tests (Rosenberg and Resh 1996).

Widely occurring within the macroinvertebrate community of streams and rivers are larval black flies (Diptera: Simuliidae). Simuliids are one of the most well defined groups of aquatic insects from a taxonomic and genetic perspective (Adler and McCreadie 1997). These attributes, in addition to their moderate susceptibility to contaminants, ubiquitous nature, and ability to be reared in large numbers in the laboratory, make black fly larvae excellent biomonitoring organisms. The University of Georgia maintains a colony of *Simulium vittatum* Zetterstedt cytospecies IS-7 that produces approximately 40,000 larvae/week for use in bioassays and toxicity tests.

Two toxicity tests have been developed in the laboratory which utilize colony-raised larvae in assessing the effects of contaminants, the orbital shaker toxicity test and the trough flow-through dosing system. Both testing systems are unique in that they provide a water current simulating flowing water, which is necessary for black fly larva
survival. The orbital shaker toxicity test is utilized for assessing the effects of contaminants on larval survival while the trough flow-through dosing system is used to assess the effects of contaminants on life-history endpoints such as growth, development and survival.

**Purpose of the Study**

This study was designed to investigate the toxic effects of carbaryl, chlorpyrifos and malathion, three lawn-care insecticides commonly detected in urban and suburban watersheds, when exposed to *S. vittatum* IS-7 colony larvae singly and as mixtures. The toxicity of the individual insecticides will be determined through analysis of the lethal concentration that will kill 50% of the test population (LC50) using the orbital shaker toxicity test. Results of the LC50 analyses will be used to determine the concentrations needed to assess impacts of the insecticides as mixtures using the modified toxic unit approach of Marking (1985) as described by Pape-Lindstrom and Lydy (1997) in the orbital shaker toxicity test.

Because food will be added in the trough flow-through dosing system, a series of experiments investigating the influence of laboratory diet and natural seston on the toxicity of the three insecticides will be conducted. These experiments will aid in determining what concentration of food (i.e. laboratory diet) can be added to the trough system without influencing the toxicity of the insecticides. With the trough flow-through dosing system, the effects of pulsed insecticide exposures on *S. vittatum* IS-7 survival, growth and development will be assessed. *Simulium vittatum* IS-7 larvae will be exposed to a series of 2 hr pulse insecticide exposures over the course of their larval life-stage.
Individual exposures of carbaryl, chlorpyrifos and malathion, as well as mixtures, will be assessed in this system.

**Expected Outcomes of Research**

Through the experiments designed in this study, a better understanding of the toxic effects lawn-care insecticides have on aquatic organisms will be achieved. Results will aid in determining whether these chemicals are less than additive, additive, or greater than additive in toxicity when organisms are exposed to them as mixtures. Results will also aid in determining the effects these insecticides may have on the fitness of black fly larvae and aquatic organisms in general.
CHAPTER 2
LITERATURE REVIEW

Biomonitoring

Biological monitoring or biomonitoring, is defined as the systematic use of living organisms or their responses to determine the quality of the environment (Matthews et al. 1982). Several organisms have been used in biomonitoring studies of aquatic systems, such as bacteria, protozoa, algae, macrophytes, fish, and benthic macroinvertebrates (Hellawell 1986). However, benthic macroinvertebrates, which are organisms that inhabit the bottom substrates in freshwater systems for at least part of their lifecycle, are most frequently used (Rosenberg and Resh 1993, 1996).

The popularity of benthic macroinvertebrates stems from the many advantages they offer as components of biomonitoring studies. First, they are ubiquitous; therefore, they provide measurable experimental units in many different aquatic habitats and systems impacted by pollutants (Lenat et al. 1980, Hellawell 1986, Rosenberg and Resh 1993). Second, the large number of species within this group exhibit a range of responses to contaminants (Hellawell 1986, Rosenberg and Resh 1996). Third, their sedentary nature allows for spatial analysis of contaminant effects (Hawkes 1979, Hellawell 1986, Rosenberg and Resh 1993). Finally, their relatively long life cycles enable investigators to do temporal analyses of contaminant effects (Lenat 1980, Hellawell 1986, Rosenberg and Resh 1993). Although there are many advantages in using macroinvertebrates as biomonitor, there are some disadvantages such as failure to indicate stress with certain
contaminants (Hawkes 1979), problems with study design (Hawkes 1979, Hellawell 1986, Roesenberg and Resh 1993), and analysis (Hellawell 1986, Roesenberg and Resh 1993). However, these problems are associated with other organisms used in biomonitoring as well (Hellawell 1986).

Within the field of biomonitoring, there are several hierarchical levels or scales ranging from the suborganismal to the ecosystem level (Rosenberg and Resh 1996). At the biochemical level, the effects of stressors on subcellular processes such as enzyme activities (Siegfried and Young 1993) and ion regulation (Havas and Hutchinson 1983) are studied (Rosenberg and Resh 1996). Disruption of one or more subcellular processes may lead to effects at the individual level where life-history endpoints may be affected (Johnson et al. 1993) or behavioral changes may take place (Petersen and Petersen 1984, Heinis et al. 1990, Vuori 1994, Rosenberg and Resh 1996). At the population level, groups of organisms are used to determine the degree of impairment within an aquatic ecosystem by determining the susceptibility of the organisms to a given stressor (Rosenberg and Resh 1996). Community-level assessments attempt to summarize the magnitude, ecological consequences, or significance of a particular stress on the structure of the community being examined (Johnson et al. 1993). At the top of biomonitoring hierarchy is the ecosystem level. Ecosystem-level assessments aid in determining if specific processes within the system are affected that may compromise the function of the system as a whole (Rosenberg and Resh 1996). This level is often the goal of many water quality assessments. However, quite often it is not achieved and data from lower levels of biomonitoring are often extrapolated or assumptions are made as to how the entire ecosystem might be affected.
In addition to the levels of biomonitoring previously mentioned, there are also other biomonitoring approaches that provide information for water quality assessments such as the analysis of head capsules from sediment samples (Paleolimnology), toxicity testing and field experimentation (Rosenberg and Resh 1996). Paleolimnology involves the study of benthic macroinvertebrate remains recovered from sediment core-samples, typically the head capsules of dipteran families such as Chironomidae and Chaoboridae, to determine the condition of the system prior to a stressor(s) or recovery time from the stressor(s) based on the species present (Kansanen 1985, 1986, Rosenberg and Resh 1996). Toxicity testing provides information for determining safe levels of toxicants, comparing relative toxicities of individual or mixtures of chemicals and predicting possible effects of toxicants on environmental systems (Rosenberg and Resh 1996). Field experimentation can assist in interpreting observed responses of aquatic insects to environmental change, calibrating biomonitoring programs, identifying sensitive species, distinguishing between direct and indirect effects of environmental stressors on organisms and direct and indirect effects of environmental stressors on an ecosystem (Cooper and Barmuta 1993, Rosenberg and Resh 1996).

Black Fly Larvae as Biomonitor

Black fly larvae have not been recognized as potential biomonitors in environmental assessments of aquatic ecosystems. Black flies are typically ignored or grouped as “Dipterans” or “Simuliids”. Because certain species of adult female black flies are voracious biters and are vectors of disease, research has primarily focused on control of these insects rather than their potential use as aquatic invertebrate biomonitors.
However, black fly larvae have many attributes that would potentially make them excellent research organisms in environmental assessments. First, they are the best known group of aquatic macroinvertebrates at the species level with 97% of the species described as larvae and 90% as pupae (Adler and McCreadie 1997). Second, black fly larvae are nearly ubiquitous in running waters (Adler and McCreadie 1997). Third, larvae possess giant salivary gland chromosomes that can be utilized in genetic assessments (Sanderson et al. 1974, Rothfels 1987). Fourth, larvae have high levels of detoxification enzymes that can be utilized in biomarker studies (Siegfried and Young 1993). Fifth, black fly larvae can be efficiently reared in the laboratory (Gray and Noblet 2000) enabling laboratory bioassays to be conducted in conjunction with field assessments. Finally, larvae are moderately to highly susceptible to environmental contaminants compared to organisms typically used in toxicity tests and environmental assessments (Chapter 3). These attributes allow black flies to be used in several scales of biomonitoring, which could possibly lead to a better understanding of contaminant impacts on aquatic biota.

**Black fly Biology**

**Life-History**

Black flies are members of the family Simuliidae in the dipteran order. They are holometabolous insects with the egg, larvae, and pupae developing in lotic systems (Peterson 1984). Depending on the water temperature and species type, it takes approximately 4 to 30 days for the eggs to hatch (Peterson 1981). After hatching, first instar larvae search for suitable attachment sites and adequate food supplies by drifting downstream on a silken strand extruded from their cephalotory silk glands (Peterson
Once a suitable substrate is located, the larvae begin feeding and develop through a series of instars. The number of larval instars is species specific and varies between six and eleven (Ross 1979, Colbo 1989). The rate of development is highly influenced by the quantity and quality of food and water temperature (Peterson 1981). Warm water species can complete larval development in four days while black flies that hatch in late Fall and overwinter in the larval stage, may take several months to pupate (Crosskey 1990).

The black fly pupal stage requires a minimum of two days and a maximum of two to three weeks to complete with duration dependent upon water temperature (Crosskey 1990). Larvae form a cocoon around themselves with respiratory gills extending from the anterolateral corners of the thorax (Peterson 1981). The final molt from pupa to adult occurs after the pharate adult splits the pupal skin (Hinton 1958).

Adults emerge through a T-shaped slit in the pupal skin on the back of the head extending along the median longitudinal line in the thorax (Peterson 1981). Adults rise to the water surface in an air bubble, fly to nearby rocks or vegetation and wait for the cuticle to harden before proceeding with adult activities (Peterson 1981). Females, especially anautogenous females, will seek a blood meal to aid in the development and maturation of eggs (Peterson 1981). Females can produce 200 to 500 eggs per gonotrophic cycle (Peterson 1981). Males may seek a sugar meal (e.g. nectar or honeydew) to replenish energy reserves before searching for a mate. Mating is species specific and can take place in flight or while stationary on the ground (Peterson 1981). Ovipositional method, strategy and site are also variable among species (Peterson 1981).
Larval Ecology

Black fly larvae are one of the most ubiquitous groups of macroinvertebrates in flowing waters (Adler and McCreadie 1997). They can inhabit a variety of habitats ranging from trickling, pristine headwater streams to large, eutrophic rivers. Habitat selection is species specific and often sibling species specific on a microdistribution scale (Colbo and Wotton 1981, Adler and McCreadie 1997). However, the one similar characteristic among all black fly habitats is that the water must be flowing. Flowing water is necessary for larvae to fulfill oxygen demands and perform their feeding behavior.

Except for a few genera of black flies that do not possess or have reduced labral fans (i.e. Crozetia, Twinnia, Gymnopais), simuliiid larvae are primarily filter-feeding organisms (Currie and Craig 1987). Larvae can filter suspended particles ranging in size from 0.09 to 350 µm out of the water column with their cephalic labral fans (Wotton 1976, Chance 1977). The ability to filter extremely small particles allows black fly larvae to utilize bacteria and dissolved organic material (< 0.45 µm) as food sources (Edwards and Meyer 1987, Ciborowski et al. 1997). When substrate and food resources are optimal, black fly larvae can be dominant components of aquatic ecosystems especially at lake-outlets where some population densities have been estimated to be 1,200,000 larvae/m² (Wotton 1987).

Although simuliiids can be found at high densities and can filter a wide range of particles, they have very little influence on the quantity of organic matter exported in lotic systems (Merritt et al. 1984). This may be due, in part, to the low filtering efficiency of the larvae (Kurtak 1978). In contrast, they do play a significant role in enhancing the
quality of organic matter. Black fly larvae are able to convert seston particles from the ultra fine size class (< 50 µm) to the fine particulate organic matter category (50 µm – 1 mm) which can be utilized by other organisms in the system (Cummins 1987, Wotton et al. 1998). These larger particles also contain black fly gut microflora and enzymes, which may enable the consumer to better utilize the material (Cummins 1987). Larvae can also be considered a food source for predacious insects and insectivorous fish (Cummins 1987).

**Insecticides**

**Chlorpyrifos**

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate], is a broad-spectrum, chlorinated organophosphate insecticide that was first registered in 1965 to control foliage- and soil-borne insect pests on a variety of food and feed crops (USEPA 1999b). It is one of the most widely used organophosphate insecticides in the United States, and until recently was one of the major insecticides used in domestic settings for control of structural pests, turf and ornamental plant pests, and indoor pests (USEPA 1999b). Now it is a restricted-use pesticide and is no longer available for use in domestic settings (USEPA 1999b).

Although there is some discrepancy in the mode of action of organophosphate insecticides, such as chlorpyrifos, in insects, it is believed that they follow the same mode of action as in vertebrate animals, cholinesterase inhibition (Matsumura 1985). Chlorpyrifos, when activated from its phosphorothioate to its phosphate form through the action of cytochrome P-450 enzyme systems, mimics the neurotransmitter acetylcholine.
and actively binds acetylcholinesterase at the enzyme’s esteratic site (Matsumura 1985). This leaves the enzyme inhibited, causing acetylcholine to build-up in the synapse and subsequent overstimulation of the neuron (Matsumura 1985).

Although chlorpyrifos is applied to control pests in terrestrial systems, small amounts enter streams and rivers primarily through runoff from rain events (Racke 1993). Concentrations and persistence of chlorpyrifos in water varies depending on the type of formulation used on surrounding areas (Extoxnet 1996b). Concentrations in water rapidly decline as chlorpyrifos adheres to sediments and organic material (Extoxnet 1996a). The concentrations measured in surface water systems are usually less than 1 µg/L (Racke 1993). However, concentrations at or below 1 µg/L can significantly affect invertebrate survivorship (Muirhead-Thompson 1978, van der Hoeven and Gerritsen 1997, Moore et al. 1998, Naddy et al. 2000) and community structure (Ward et al. 1995). Due to its hydrophobic nature, chlorpyrifos is likely to bioconcentrate in aquatic organisms (Howard 1991).

Volatilization is the primary route of chlorpyrifos degradation in water with a reported half-life of 3.5 to 20 days in pond water (Racke 1993). Other degradation pathways for chlorpyrifos are photolysis (half-life of 21 to 28 days) and hydrolysis which is temperature and pH dependent (half-life 35 to 78 days; pH 7.0, 25°C) (Howard 1991). The major products of chlorpyrifos from hydrolysis are 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) and O-ethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate (desethyl chlorpyrifos) (Macalady and Wolfe 1985).
Malathion

Malathion (diethyl mercaptosuccinate, O,O-dimethyl-dithiophosphate) is a broad-spectrum organophosphate insecticide introduced in 1950 for control of sucking and chewing insects on fruits and vegetables, household insects and lice (Extoxnet 1996c). It has also been widely used in eradication efforts for Mediterranean fruit flies and boll weevils and in public health for the control of mosquitoes (USEPA 2000). However, in order to meet Food Quality Protection Act (FQPA) guidelines, it is no longer registered for use on pets, livestock, certain agricultural products and for indoor applications (USEPA 2000).

Like chlorpyrifos, malathion must be activated from its phosphorothioate form to its phosphate form in order to actively bind acetylcholinesterase and elicit toxicity (Matsumura 1985). However, unlike chlorpyrifos, malathion can be quickly detoxified in organisms that have high levels of carboxylesterases, such as mammals (Matsumura 1985). Carboxylesterases can hydrolyze one or both carboxyl esters associated with the structure of malathion before it is activated to its phosphate form, greatly reducing its toxicity (Pasarela et al. 1962). In general, insects have lower levels of carboxylesterases than mammals, which makes malathion an excellent insecticide (high insect toxicity; low mammalian toxicity) (Matsumura 1985).

Malathion can enter aquatic systems through spray drift from ground and aerial applications and from runoff due to rain events (Hippe et al. 1994). Malathion has a high water solubility (143 mg/L at 20°C, Bowman and Sans 1983) relative to other insecticides. Thus, it weakly partitions in sediments and is not likely to bioconcentrate in aquatic organisms (Howard 1991). Malathion degrades primarily through hydrolysis.
with half-lives ranging 1.4 days at pH 8.0 (Wolfe 1976) to 147 days at pH 6.0 (Howard 1991). Products of hydrolysis include malaoxon, malathion $\alpha$ and $\beta$ monoacids, diethyl fumarate, diethyl thiomalate, O,O-dimethylphosphorodithioic acid and O,O-dimethylphosphorothioic acid (Howard 1991). Malathion may also degrade in water through photolysis and biological processes (Howard 1991).

The impact of malathion on non-target aquatic invertebrates appears to be related to the level of assessment and type of organisms studied. Concentrations as low as 0.1 $\mu$g/L have been shown to affect the net-spinning behavior of the caddis fly Hydropsyche slossonae (Banks) (Tessier et al. 2000). However, typical applications of malathion for mosquito and arthropod pests had no effect on non-target invertebrate populations (Jensen et al. 1999) or the amphipod Gammarus pulex L. (Crane et al. 1995). Thus, although malathion is toxic to aquatic invertebrates (LC50 for Daphnia magna = 1 $\mu$g/L), the fate and influence of environmental factors on this chemical may make the amount available for uptake into non-target species too minimal for toxicity to be observed.

**Carbaryl**

Carbaryl (1-naphthyl-N-methylcarbamate) is a broad-spectrum carbamate insecticide. It was first registered for use in 1958 to control pests on agricultural crops, lawns, landscapes, livestock and pets (Back 1965, Extoxnet 1996a). Carbaryl, like the organophosphate insecticides chlorpyrifos and malathion, is a potent cholinesterase inhibitor (Matsumura 1985). However, unlike the organophosphates, carbaryl’s inhibition of cholinesterase is reversible and recovery of the enzyme is faster (Matsumura 1985).
Carbaryl can enter aquatic systems through spray drift from aerial applications (Coutant 1964, Courtemanch and Gibbs 1980) and from runoff due to rain events (Hippe et al 1994). It may be detected in the water and sediments in aquatic systems but is not likely to bioconcentrate (Howard 1991). Carbaryl has been shown to hydrolyze rapidly with the rate dependent on temperature and pH (Howard 1991). Karinen et al. (1967) reported that 93% of carbaryl was hydrolyzed after 8 days at 28°C compared to only 9% at 3.5°C. An increase in pH from 5 to 8 decreased the half-life of carbaryl from 1500 to 1.8 days (Howard 1991). Carbaryl may also undergo photolysis. The half-life recorded for carbaryl in direct sunlight was 6.6 days in distilled water (Wolfe et al. 1978). The major product formed from these degradation processes is 1-naphthol (Howard 1991).

The toxicity of carbaryl, like the chemical’s hydrolysis rate, has been shown to be temperature and pH dependent, with toxicity, in general, greater in water with low pH (≤ 6) and higher temperature (≥ 20°C) (Lohner and Fisher 1990). At pH 8, however, this relationship does not hold true. *Chironomus riparius* mortality was shown to be greater at 10°C than at 20°C and 30°C, while at 20°C, toxicity was less than at 30°C (Lohner and Fisher 1990). An explanation for this pattern is not certain but may be related to retention of carbaryl hydrolysis metabolites by *C. riparius* (Lohner and Fisher 1990).

Carbaryl has been shown to affect *Daphnia* spp. phenotypes; however, the effects reported are not consistent among studies. Hanazato (1991, 1992, 1993) showed that exposure to carbaryl induced the formation of neckteeth or high helmets and long tailspines in several species of *Daphnia*. In contrast, Barry (1999) reported that exposure to carbaryl caused a decrease in crest growth in *Daphnia longicephala* (Hebert). Possible
explanations for the discrepancy may be related to the species used in the studies as well as the concentration of carbaryl exposed to the organisms.

Aerial applications of carbaryl for control of forest pests have been shown to affect aquatic invertebrate populations in ponds and streams. Gibbs et al. (1984) reported a near disappearance of the amphipod community and reductions in the numbers of immature Ephemeroptera, Trichoptera, and Odonata inhabiting a woodland pond sprayed with carbaryl. Adverse effects were also observed in streams sprayed with carbaryl in which invertebrate drift significantly increased (Coutant 1964) as well as observed mortality in mayfly and stonefly taxa (Courtemanch and Gibbs 1980).

Chemical Mixtures

Methods of Assessing Chemical Mixtures

The original concept for determining the toxicity of toxicant mixtures, the isobole concept, was devised by Loewe and Muischnek (1926). They proposed that the toxicity of a mixture could be determined through analysis of lethal concentrations of the compounds administered to the organism at different ratios. In additive toxicity, proportions of the LC50 for the individual toxicants equaling 100% (i.e. 50% of A + 50% of B or 25% of A + 75 % of B) would produce 50% mortality in the test organisms across all ratios. Deviations from 50% mortality were considered to be greater than additive if the observed mortality was greater than 50% and less than additive if mortality was lower than 50%. However, these were often exceptions because additivity was considered the norm (Loewe and Muischnek 1926).
Bliss (1939) extended the work in analyzing mixture toxicity by modeling three types of joint action between or among toxicants, independent joint action, similar joint action, and synergistic action. In independent joint action, the toxicants act independently and have different modes of toxic action (Bliss 1939). The toxicity of the mixture can be determined from the dose-response curve for each component applied alone and the correlation in susceptibility to the two toxicants (Bliss 1939). In similar joint action, the toxicants produce similar but independent effects, so that one component can be substituted at a constant proportion for the other (Bliss 1939). The toxicity of the mixture is predictable directly from the proportions of the toxicants applied (Bliss 1939). In synergistic action, the effectiveness of the mixture cannot be assessed from the individual components alone (Bliss 1939). The degree of toxicity depends upon knowledge of the toxicants combined toxicity when applied in different proportions (Bliss 1939). The concepts of Bliss were further defined by Finney (1942) in which a means for testing the hypothesis that mixtures were additive could be achieved through analysis of harmonic means of the LC50s. However, Finney’s concepts were extremely complex and in some opinions, too complex to be widely applied (Sun and Johnson 1960, Marking 1985).

In the 1960’s, the basis for the toxic unit (TU) concept was developed and implemented in studies focusing on the toxicity of metal mixtures and industrial pollutants (Lloyd 1961, Herbert and Shurben 1964, Brown 1968, Brown and Dalton 1970). In this concept, the toxic strength of a mixture of compounds may be determined by summing the strengths of individual compounds (Marking 1985). Typically, the LC50 for a given compound is assigned a value of 1 TU. In a complex mixture, the concentration of a specific compound in solution is divided by the respective LC50 value.
to obtain the proportion of that compound contributing to the toxicity of the mixture (Brown 1968). If the sum of the proportions of all compounds present in the mixture is less than 1.0, then it is assumed that the mixture will not kill 50% of the test population in 48 hrs (Brown 1968). Likewise, if the sum of all proportions is greater than 1.0, more than 50% of the test population will be expected to die in 48 hrs (Brown 1968).

Keplinger and Deichmann (1967) developed a method for assessing the toxicity of pesticide mixtures similar to the toxic unit concept. In the assessment of two pesticides, 1/2 of the LD50 of the individual chemical would be administered to the test organism. Likewise, in the assessment of three pesticides, 1/3 of the LD50 of the individual chemical would be administered. Thus, the sum of the concentrations subjected to the organism would equal the average of the individual LD50s. Assessment of the mixtures was accomplished by dividing the observed mortality by the expected mortality (50%). Mixtures with ratios greater than 1 were more than additive while those less than 1 were less than additive.

Schubert et al. (1978) also developed a method of analyzing contaminant mixtures. In their approach, for a pair of contaminants, one contaminant was maintained at a constant concentration, such as the LD1 for that contaminant, while the concentration of the other was varied to obtain a dose-response curve (Schubert et al. 1978). From the dose response curve, an LD1, LD50 and LD99 were obtained. Then, the contaminants were reversed such that the one that was at a fixed concentration was varied and the one that was varied remained fixed. For mixtures of three contaminants, the LD1 for the mixture of the first two contaminants was held constant while the concentration of the third was varied. The results obtained with the combinations could be tested for
synergism, additivity, or antagonism by using the dose of the increased contaminant needed to attain the LD50 (LD50 –LD1) and the corresponding value for that agent in the presence of one of more of the other agents (Schubert et al. 1978). The ratio of these values provided an index of the degree and nature of the combined effect: synergism > 1, additivity = 1, antagonism < 1 (Schubert et al. 1978).

In addition to the many concepts developed for analyzing chemical mixtures, several indices have also been devised. A mixture co-toxicity coefficient index was developed by Sun and Johnson (1960) that utilized the LC50 values of the chemicals individually and as mixtures (actual toxicity index) and the percentage of the chemical in the mixture (theoretical toxicity index) as a ratio to determine similar, independent and synergistic action. Marking and Dawson (1975) developed a quantitative index for the toxicity of mixtures in water based on the isobole theory and involving the use of the TU concept to sum the action of various components of a mixture. Konemann (1981) devised the mixture toxicity index (MTI) based on the TU approach. In his opinion, a scale for the toxicity of a mixture should give constant values for the reference points of no addition and concentration addition and should have a logarithmic form because of the log-normal distribution of LC50 values. De March (1987) believed that his index could be applied to several models of joint action as opposed to the MTI.

From the literature obtained on the toxicity of chlorpyrifos, carbaryl and malathion as mixtures, many of the previously mentioned methods have been utilized. The methods chosen by researchers for use in their studies seems to be related to the type of study being conducted and the method they are most comfortable with. However, variations of the TU concept appear to be the most popular and accepted.
Chlorpyrifos, Malathion and Carbaryl in Mixture Studies

The toxic effects of chlorpyrifos in pesticide mixtures have been shown to vary. Chlorpyrifos has been shown to interact independently with dieldrin (Steevens and Benson 2001) and additively with diazinon (Bailey et al. 1997). Studies with environmentally realistic concentrations of pesticide mixtures containing chlorpyrifos from food residues (Dolara et al. 1994, Lodovici et al. 1997) and lawn care chemicals (Yeary 1984) had no apparent effect on human lymphocytes, rat liver DNA or beagle hemolytic values respectively. In contrast, Pape-Lindstrom and Lydy (1997) showed that the herbicide atrazine increased the toxicity of several organophosphate insecticides, including chlorpyrifos, in toxicity tests with the midge, *Chironomus tentans*. The increase in toxicity of the insecticides is thought to be due to an increase in biotransformation rates of the organophosphates, resulting in more O-analog within the organism (Belden and Lydy 2000). The timing of chlorpyrifos exposure with other pesticides has been shown to influence toxicity. Rats administered chlorpyrifos followed by treatment with parathion four hours later produced significantly greater mortality compared to animals given parathion first followed by chlorpyrifos (Karanth et al. 2001). The results suggest that organophosphate-selective differences in detoxification through nontarget esterases may be important in the sequence-dependent differential toxicity (Karanth et al. 2001).

The majority of pesticide mixture studies with malathion have shown an increase in toxic effects. Synergistic mortality was observed in Japanese quail, *Coturnix coturix*, and pheasant, *Phasianus colchicus*, chicks, after feeding on a diet containing a mixture of malathion and EPN (Kreitzer and Spann 1973). The insecticides malathion and dioxathion have been shown to be synergistic in rainbow trout, *Salmo gairdnen*
Likewise, specific combinations of the insecticides chlordane, malathion, and furadan, have shown synergistic toxicity in test fish, *Notopterus notopterus* (Mor) (Gupta et al. 1994). Macek (1975) showed that parathion, fenthion, carbaryl, EPN, and perthane, when added in combination with malathion, produced greater than additive toxicity in bluegill sunfish, *Lepomis macrochirus*. Synergistic decreases in cholinesterase activity have also been observed in rat plasma combined with a mixture of malathion and diazinon (Iyaniwura 1990) and in the marine copepod *Tigriopus brevicornis* exposed to a mixture of malathion and dichlorvos (Forget et al. 1999).

Exposure to carbaryl has been shown in some studies to influence the toxicity of other pesticides while in other studies, its toxicity has been altered. Statham and Lech (1975) reported that rainbow trout, *Salmo gairdnen*, co-exposed to a sublethal dose of carbaryl combined with 2,4-D, dieldrin, rotenone, and pentachlorophenol separately, had significantly greater mortality than controls and carbaryl alone while Johnston et al. (1994) reported that pretreatment of hybrid red-legged partridges, *Alectoris rufa* cross, with malathion potentiated the toxicity of carbaryl. Carbaryl, in combination with methyl parathion, has been shown to significantly reduce the rates of feeding, food absorption and conversion, and oxygen consumption in the fish, *Heteropeustes fossilis* (Bloch) (James and Sampath 1994). Carbaryl, in combination with malathion (Abdel-Rahman et al. 1985) and phenthoate (Sambasiva Rao and Ramana Rao 1989) significantly increased blood glutathione and decreased hepatic glutathione in rats and synergistically inhibited acetylcholinesterase activity in the fish *Channa punctatus* (Bloch) respectively. In contrast, Kaushik and Kumar (1993) reported that the combination of aldrin,
monocrotophos and carbaryl were antagonistic to the freshwater crab *Paratelphusa masoniana* (Henserson) in 96 hr LC50 experiments.

**Insecticide Bioavailability**

The presence of particulate and dissolved organic matter in aquatic systems can have a profound influence on the bioavailability and subsequent toxicity of many insecticides. Although a few studies have shown that the addition of organic matter has increased the bioavailability of insecticides (Hermsen et al. 1994, Ekelund et al. 1987), the majority of authors have reported decreases (Muir et al. 1985, Hall et al. 1986, Ramakrishna Rao and Sarma 1986, Day 1991, Kadlec and Benson 1995, Kusk 1996, Gama Flores et al. 1999, Sarma et al. 2001, Schultz and Liess 2001b). Loss of free chemical in the aqueous phase due to binding of the insecticide to the organic material is believed to be the primary reason for reduced bioavailability (Carter and Suffet 1982, Hall et al. 1986, Day 1991). The manner and extent to which insecticides bind to organic material is related to the chemical characteristics of the insecticide as well as the size and composition of the organic material (Carter and Suffet 1982, Saint-Fort and Visser 1988, Kadlec and Benson 1995). Possible binding mechanisms for the adsorption of insecticides to organic matter include H-bonding, ion exchange, Van der Waals forces, protonation, charge transfer, ligand exchange, hydrophobic bonding, and coordination via a metal ion (Saint-Fort and Visser 1988).

The addition of particulate organic material for food in laboratory toxicity tests has been shown to influence the toxicity of insecticides. Rotifers exposed to DDT or methyl parathion in the presence of *Chlorella* showed reduced mortality levels with
increasing concentrations of the green algae (Ramakrishna Rao and Sarma 1986, Gama Flores et al. 1999, Sarma et al. 2001). In contrast, the bivalve, *Mytilus edulis*, exposed to lindane in the presence of suspended solids, which are utilized as food, had significantly reduced feeding rates compared to controls (Hermsen et al. 1994). A similar study with the bivalve, *Abra nitida* (Muller), exposed to hexachlorobenzene (HCB) in the presence of suspended solids showed that more of the insecticide was accumulated as opposed to bivalves exposed to HCB in clean water (Ekelund et al. 1987). However, no toxic effects were reported. Others have also reported that suspended solids can produce detrimental effects in aquatic organisms (Schultz and Leiss 2001a); however, the majority of studies have shown that suspended solids protect organisms from insecticide exposure by reducing their bioavailability (Muir et al. 1985, Hall et al. 1986, Hill 1989, Coats et al. 1989, Schultz and Liess 2001b).

The presence of dissolved organic material can also affect the bioavailability and subsequent toxicity of insecticides. Day (1991) reported that decreases in bioaccumulation and toxicity of fenvalerate, deltamethrin, and cyhalothrin were observed in *Daphnia magna* with increasing dissolved organic carbon concentrations. Reductions in the toxicity of azinphos methyl, fenvalerate and methyl parathion were observed in waters containing varying concentrations of natural organic matter in a bacterial bioluminescence assay (Kadlec and Benson 1995). Kusk (1996) attributed the reduction in pirimicarb toxicity to *Daphnia magna* to the release of dissolved organic material from sediments added to the test vessels.
Effects of Sublethal Insecticide Exposure on Organismal Life History

Organisms in aquatic systems are often continuously exposed to low concentrations of insecticides and/or intermittently exposed to high concentrations for short periods of time. These exposures may not cause the organism to perish. However, many life history parameters such as growth (McKenney 1986, Fairchild et al. 1992, Barry et al. 1995, Blockwell et al. 1996, Ferrando et al. 1996, McKenney et al. 1998, Nebeker et al. 1998, Stuijfzand et al. 1999, Hamm and Hinton 2000, Sanchez et al. 2000), generation time (Ferrando et al. 1996) and reproduction (McKenney 1986, Holdway and Dixon 1986, Fairchild et al. 1992, Wong 1996, Sanchez et al. 2000) become compromised due to sublethal insecticide exposure. Thus, the overall fitness of the organism may become reduced leading to possible population decreases and subsequent alteration of ecosystem function. Reduced organismal fitness may result from allocating assimilated energy towards detoxification and homeostatic mechanisms required for survival and away from activities that support growth and reproductive processes (Blockwell et al. 1996).

The insecticides chlorpyrifos, malathion and carbaryl have been shown in numerous studies to affect life history parameters. Reduced fecundity and population growth was observed in the benthic copepod, *Amphiascus tenuiremis* cf. Mielke, chronically exposed to chlorpyrifos in sediments (Green and Chandler 1996). These authors also demonstrated that early life-stages of the copepod exposed to chlorpyrifos have more of an effect on population dynamics than adults exposed to similar concentrations (Chandler and Green 2001). Sediment associated chlorpyrifos has also been shown to reduce the weight of emerging female chironomids, *Chironomus riparius*.
Meigen, leading to possible reductions in fecundity (Callaghan et al. 2001). Aqueous concentrations of chlorpyrifos have been shown to reduce growth and fecundity in fish, *Orechromis mossambicus* (Thankamoni Amma and Konar 1996).

The greatest effect of sublethal malathion exposures on organismal life history has been associated with growth. Reduced growth rates have been observed in the fish species *Jordanella floridae* (Hermanutz et al. 1985), *Macrapodus cupanus* (Muniandy 1987), *Puntis stigma* (Khillare and Wagh 1988), *Orechromis mossambicus* (Ramakrishnan et al. 1997), and *Clarias gariepinus* Burchell (Nguyen and Janssen 2002). Fordham et al. (2001) also showed that growth, as well as development, were significantly altered in bullfrog tadpoles, *Rana catesbeiana*, exposed to malathion in water during 28 d static renewal tests.

Carbaryl has been shown to affect life history endpoints as well. Marian et al. (1983) reported that growth and production efficiency were significantly affected in bullfrog tadpoles, *Rana tigrina* Daud, chronically exposed to carbaryl. Freshwater snails, *Lymnaea stagnalis*, have been shown to have reduced fecundity and growth after 5 weeks of carbaryl exposure (Seuge and Bluzat 1983). James and Sampath (1994) also reported reduced growth in relation to feeding inhibition in the fish, *Heteropneustes fossilis* (Bloch) exposed to sublethal levels of carbaryl.
CHAPTER 3

SUSCEPTIBILITY OF BLACK FLY LARVAE (DIPTERA: SIMULIIDAE) TO LAWN-CARE INSECTICIDES INDIVIDUALLY AND AS MIXTURES

Introduction

Urban and suburban watersheds have the potential to be highly impacted by chemicals, especially insecticides used to control insect pests on lawns, ornamental plants, and in home gardens. An estimated 17 million pounds of insecticidal active ingredients are applied annually in domestic settings for control of insects on lawns and gardens (Aspelin and Grube 1999). Multiple lawn-care insecticides enter urban and suburban waterways primarily through runoff from rain events and are commonly detected in these aquatic systems (Struger et al. 1995, Hippe et al. 1994, Kimbrough and Litke 1994). Consequently, organisms inhabiting streams and rivers receiving insecticide runoff from lawns and gardens are exposed to these chemicals as mixtures, which may lead to possible increases or decreases in the toxic effect of the individual insecticide (Kaushik and Kumar 1993, Macek 1975).

It has been perceived that chemicals with the same quantitative structure-activity relationship (QSAR) or mode of action produce additive toxicity when applied jointly (Konemann 1981, Lloyd 1985, Broderius et al. 1995, McCarty et al. 1992). However,

studies with organophosphate insecticides applied jointly have shown that these chemicals elicit greater than additive effects when organisms are exposed to them as mixtures (Macek 1975, Durairaj and Selvarajan 1995). Thus, it is erroneous to assume that all structurally and mechanistically similar chemicals behave additively as mixtures. Experimental data are needed on specific chemical mixtures, particularly those commonly occurring in urban and suburban watersheds to determine their joint activity.

The organophosphate insecticides chlorpyrifos (O,O-Diethyl O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate), and malathion (Diethyl mercaptosuccinate, O,O-dimethyl-dithiophosphate), and the carbamate insecticide carbaryl (1-Naphthyl-N-methylcarbamate) are registered for use on lawns and gardens for control of insect pests in the U.S.A. (Fig. 3.1). Although chlorpyrifos is no longer registered for domestic use, it is registered for use on golf courses, which are often built in residential communities, to control turf pests. As a result of their widespread use, chlorpyrifos, carbaryl and malathion are three of the most commonly detected insecticides in urban and suburban watersheds such as the Flint River watershed of Georgia (Hippe et al. 1994). Thus, it is imperative to determine the cumulative toxicity of these insecticides in order to predict potential impacts on organisms inhabiting these streams.

Figure 3.1. Chemical structures of carbaryl, chlorpyrifos, and malathion.
The purpose of this study was to evaluate the toxicities of chlorpyrifos, malathion, and carbaryl individually and as mixtures using black fly larvae, *Simulium vittatum* Zetterstedt cytospecies IS-7 as the test organism in an acute orbital shaker toxicity test. Acute toxicity will be determined for each insecticide through analyzing the lethal concentration required to kill 50% of the test organisms (LC50), followed by assessing the joint toxicity as mixtures using the modified toxic unit approach of Marking (1985) as described by Pape-Lindstom and Lydy (1997). Data from this study should provide a better understanding of the toxicities resulting from the use of multiple lawn-care insecticides.

**Materials and Methods**

**Organisms**

Black fly larvae (Diptera: Simuliidae) are often dominant species in stream ecosystems. They are holometabolous, filter-feeding insects commonly found on rocks, trailing vegetation, snags, and other substrates in streams and rivers. Their primary role in aquatic ecosystems is converting ultra fine particulate organic matter into fine particulate organic matter that can be utilized by other organisms. Larval Simuliids also serve as a food source for fish and other invertebrates (Cummins 1987). Larvae used in experiments were reared in temperature controlled, simulated stream systems at the University of Georgia (UGA) where a colony of *Simulium vittatum* IS-7 is reared under the methods outlined by Gray and Noblet (1999).
Chemicals

Analytical-grade standards of chlorpyrifos, malathion and carbaryl were purchased from Chemservice (Westchester, PA). Standards were certified to be ≥ 98% pure. Stock solutions of all standards were prepared by dissolving a weighed amount of insecticide into 100 ml of pesticide-grade acetone and stored at 4°C in amber bottles with Teflon®-lined caps.

LC50 determination

Moderately-hard reconstituted test water was prepared using the method of Weber (1993). Temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness were measured before and after each test except alkalinity and hardness, which were measured pre-test only (Table 3.1). Temperature, dissolved oxygen, and conductivity were measured using a YSI 85 water quality meter and pH was measured with a Corning 440 pH meter. Alkalinity and hardness concentrations were determined through titration with 0.2 N H₂SO₄ and 0.1 M EDTA, respectively (APHA, AWWA, and WPCF 1995). Testing was conducted with ambient air temperatures in the laboratory measuring 20°C (± 0.5) and a 16:8 light:dark photoperiod.

For each toxicity test, sections of nylon screen substrate with several hundred larvae attached were removed from the rearing tank and placed into enamel pans with 1L of culture water. Fifteen 4th-5th instar larvae were transferred with larval forceps into 250 ml Pyrex® flat-bottom extraction flasks containing 145 ml of moderately-hard reconstituted water. Instar number was determined post-testing through measurement of the cephalic apotome width (Hudson 1994) using an ocular micrometer at 45X on a Meiji
Techno dissecting scope. Flasks were placed on a New Brunswick Scientific G-10 Gyratory® shaker and allowed to acclimate for 10 min at 100 rpm, 10 min at 125 rpm and 40 min at 150 rpm, the final speed for the duration of the experiment, for a total 1 hr acclimation period. The G-10 shaker produces a current in the flasks simulating flowing water. This acclimation period allows the larvae to choose a preferred attachment site and position themselves in the optimal flow pattern within the flasks.

**Table 3.1.** Mean values ± standard error for water quality parameters measured before and after acute orbital shaker toxicity tests. Line 1 represents pre-testing values and line 2 represents post-testing values.

<table>
<thead>
<tr>
<th>Dissolved Oxygen (mg/L)</th>
<th>pH</th>
<th>Conductivity (µS/cm)</th>
<th>Temperature (°C)</th>
<th>Alkalinity (mg/L as CaCO₃)</th>
<th>Hardness (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 8.94 ± 0.11</td>
<td>8.01 ± 0.01</td>
<td>291.70 ± 3.60</td>
<td>20.30 ± 0.10</td>
<td>63.00 ± 1.00</td>
<td>90.00 ±1.00</td>
</tr>
<tr>
<td>2 8.90 ± 0.09</td>
<td>7.94 ± 0.01</td>
<td>284.10 ± 8.90</td>
<td>21.00 ± 0.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prior to dosing the flasks, six insecticide concentrations were prepared in 100 ml volumetric flasks by spiking reconstituted water with insecticide stock solution. The maximum volumes of stock solution used for preparation of the dosing solutions were 95.05, 56.22 and 707.21 µl for chlorpyrifos, carbaryl and malathion, respectively. The contents were then emptied into 250 ml amber bottles until dosing. After the acclimation period, the flasks were treated with 5 ml of the appropriate insecticide concentration with a 5 ml glass pipet using a Drummond pipet-aid® to bring the final volume in each flask to 150 ml. Immediately after treatment, flasks were capped with aluminum foil to minimize evaporative water loss and prohibit external debris from entering the flasks. Blank flasks
containing 145 ml of reconstituted water were treated in a similar manner for each concentration and the contents were collected for analysis in 250 ml amber bottles with Teflon® lids and stored at -20°C. After 48 hrs, larvae were removed from the flasks and mortality assessed. Any larva that did not respond to a gentle probing was considered dead. Approximately 250 ml of test water for each insecticide concentration were collected at the end of the test and stored for chemical analysis.

Six insecticide concentrations and two controls, a moderately-hard water and a carrier (acetone) control, were tested on one shaker with five flasks per concentration and control, bringing the final totals to 40 flasks and 600 larvae. The carrier control for each insecticide had an acetone concentration equivalent to the maximum volume of stock insecticide used in preparation of the treatment solutions for the chemical being tested. Each of the three insecticides was tested on a separate shaker. Three orbital shaker toxicity tests were conducted for each insecticide.

All data were adjusted for control mortality before statistical analysis using Abbott’s formula (Abbott 1925). Data were analyzed using the maximum likelihood method fitting normal (probit) and logistic models to the data (Newman 1995). The model with the best fit across all repetitions was used for determining the LC50 for the specific insecticide. Toxicity tests were considered valid if control mortality was <10% and the p-value for the Chi-square goodness of fit test was >0.05.

Mixture Assessment

LC50 values generated for each insecticide in the first set of experiments previously described, were used as the basis for the analysis of these chemicals as
mixtures in the modified toxic unit (TU) approach to investigating joint toxicity (Marking 1985, Pape-Lindstrom and Lydy 1997). In this model, a value of 1 TU is assigned to the LC50 for each insecticide. Concentrations of individual insecticides contributing to the mixture were entered into the following equation to determine the expected toxicity of the mixture:

\[
\sum TU = \frac{CW_1}{LC50_1} + \frac{CW_2}{LC50_2} + \ldots + \frac{CW_i}{LC50_i}
\]

where \( CW_i \) is the concentration of an insecticide in a mixture and \( LC50_i \) is the LC50 for the respective component insecticide of the mixture from 1 to \( i \) (McCarty et al. 1992). Binary and ternary mixtures with expected toxicities ranging from \( \Sigma 2.00 \) TU to \( \Sigma 0.25 \) TU (2.00, 1.50, 1.00, 0.75, 0.50, 0.25 TU) or \( \Sigma 2.50 \) TU to \( \Sigma 0.50 \) TU (2.50, 2.00, 1.50, 1.00, 0.75, 0.50 TU), respectively, were tested in the acute orbital shaker toxicity test as previously described to determine the actual LC50 of the mixtures. Insecticide mixtures that produced an LC50 value significantly greater than 1 TU (i.e., the 95 % confidence interval did not encompass 1 TU) were determined to be less than additive in toxicity. Mixtures that produced an LC50 value significantly less than 1 TU were determined to be greater than additive.

Six TU values of the mixture and two controls, a moderately-hard water and a carrier (acetone) control were tested on one shaker with five flasks per concentration and control, bringing the final totals to 40 flasks and 600 larvae. The carrier control had an acetone concentration equivalent to the maximum volume of the stock insecticides used in preparation of the mixture. Each mixture combination (chlorpyrifos-carbaryl, chlorpyrifos-malathion, carbaryl-malathion, and chlorpyrifos-carbaryl-malathion) was
tested on a separate shaker. Three shaker tests were conducted for each mixture combination. Data were analyzed in a similar manner as in the LC50 determination tests; however, the TU values were used for statistical analysis instead of the actual insecticide concentrations.

**Chemical Analysis**

Concentrations of all three chemicals were analytically determined from water samples collected before and after the 48 hr toxicity test. For chlorpyrifos and malathion, a 50 ml volume of water was pulled under vacuum through a Bakerbond C-18 solid-phase extraction cartridge. Insecticides were eluted from the cartridge with two 0.5 ml aliquots of a 50:50 v/v ethylacetate-diethyl ether mixture. Eluates were dried over anhydrous sodium sulfate and the volume adjusted to 1 ml. Chlorpyrifos and malathion residues in eluates were quantified using a Hewlett Packard 6890 Gas Chromatograph interfaced with a HP 5973 mass selective detector using methane chemical ionization operated in the negative ion mode. The instrument was outfitted with a 30 m x 0.25 mm HP-5MS column with a 0.25 µm film thickness. The column was set initially at 60°C and ramped to 180°C at 15°C/min after an initial 0.5 min hold time. It was then ramped to 270°C at 6°C/min.

For carbaryl, 10 µl of concentrated HCL were added to a 2 ml aliquot of the water sample. The water sample was then analyzed directly by high performance liquid chromatography (HPLC) using a 1 ml injection volume. A Waters 2690 Alliance system with a 996 photodiode array detector was used for analysis. The HPLC was outfitted with an Alltech Associates 25 cm x 4.6 mm x 5 µm C-8 column. Residues of carbaryl
were eluted from the column with an acetonitrile-water gradient ramped from 10 – 95% acetonitrile over 20 min. Carbaryl was detected at 220 nm.

**Results**

**Chemical analysis**

Insecticide residues in water samples before the experiments were much lower than nominal, except for chlorpyrifos (Table 3.2). Because the same techniques were used to prepare all three stock solutions and to prepare the dosing solutions, we determined that the losses associated with carbaryl and malathion were related to their chemical properties rather than human error. Carbaryl and malathion are more susceptible to hydrolysis than chlorpyrifos especially at the pH of the test water (pH = 8.01). In addition, > 90% of the chemicals were recovered from extracts during spike and recovery tests and stock solutions in acetone were analytical determined to be accurate, further supporting chemical loss rather than error. Losses of all three insecticides over the 48 hr test period were likely due to volatilization. Henry’s law constants for chlorpyrifos, carbaryl and malathion are 0.74, 2.8 x 10^{-4} and 1.1x 10^{-3} Pa m^{3}/mol, respectively. Hydrolysis may also have been a factor in the loss of carbaryl and malathion with half-lives < 24 hr at pH 8. In the analysis of the water containing mixtures of the insecticides, the analytical method used for carbaryl was not sensitive enough to detect the nominal concentrations (nominal values < 10 µg/L). Consequently, the mixture data was not supported with analytical concentrations.
**LC50 determination**

Because concentrations of the insecticides detected in the water of the flasks after 48 hrs were much lower than the initial concentrations, the geometric mean of the initial and final concentrations was calculated for use in determination of the LC50 value for each insecticide. For comparison, Table 3.3 lists the median LC50 values produced using nominal concentrations and the geometric means of the analytical concentrations for each insecticide. The median LC50 of three repetitions was reported to account for variation among larvae in separate rearings. Variation among larvae in separate rearings is illustrated in Figure 3.2. The logit model provided the best fit to the data for determining the LC50 values for the individual insecticides (p >0.10). Chlorpyrifos proved to be the most toxic to *S. vittatum* IS-7 followed by carbaryl and malathion.

**Mixture assessment**

Binary and ternary mixtures of chlorpyrifos, carbaryl and malathion were tested using a range of TU values derived from median individual LC50 values generated from nominal concentrations (Table 3.3) for each insecticide. As in the LC50’s were used due to the lack of analytical data to support the concentrations of carbaryl in the mixtures and need for consistency in the concentrations of the numerator and denominator of the $\Sigma TU$ equation. As in the LC50 analysis for the individual chemicals, the logit model fit the data best and was used for determining the mixture LC50s (p > 0.26). Three tests were conducted for each insecticide mixture to account for variation among larvae in separate rearings. Variation among larvae in separate rearings is illustrated in Figure 3.2. The median value of the three tests was reported as the actual LC50.
Table 3.2. Concentrations of chlorpyrifos, carbaryl, and malathion detected from water samples in a 48 hr orbital shaker toxicity test. Initial concentrations were determined from spiked water samples at the time of dosing. Final concentrations were determined from water samples collected at the end of the experiments. Initial and final concentrations are reported as the average ± standard error of the three repetitions. Concentrations were analytically determined using gas chromatography for chlorpyrifos and malathion and high performance liquid chromatography for carbaryl.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Nominal Concentration (µg/L)</th>
<th>Initial Concentration (µg/L)</th>
<th>Final Concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>3.20</td>
<td>4.15 ± 0.35</td>
<td>1.02 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>1.55 ± 0.11</td>
<td>0.42 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.82 ± 0.04</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.57 ± 0.00</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.44 ± 0.02</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.19 ± 0.02</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>75.00</td>
<td>50.30 ± 0.65</td>
<td>32.37 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>65.00</td>
<td>44.67 ± 2.21</td>
<td>18.57 ± 5.17</td>
</tr>
<tr>
<td></td>
<td>55.00</td>
<td>37.83 ± 0.47</td>
<td>23.67 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>45.00</td>
<td>30.90 ± 0.49</td>
<td>16.47 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>35.00</td>
<td>24.13 ± 0.52</td>
<td>14.63 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>25.00</td>
<td>16.60 ± 0.97</td>
<td>9.50 ± 0.59</td>
</tr>
<tr>
<td>Malathion</td>
<td>1000.00</td>
<td>343.03 ± 32.15</td>
<td>178.20 ± 18.74</td>
</tr>
<tr>
<td></td>
<td>500.00</td>
<td>146.83 ± 2.27</td>
<td>80.30 ± 2.35</td>
</tr>
<tr>
<td></td>
<td>250.00</td>
<td>110.63 ± 12.12</td>
<td>34.00 ± 2.76</td>
</tr>
<tr>
<td></td>
<td>125.00</td>
<td>49.33 ± 5.58</td>
<td>9.47 ± 1.99</td>
</tr>
<tr>
<td></td>
<td>62.00</td>
<td>22.03 ± 2.80</td>
<td>6.63 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>31.00</td>
<td>11.07 ± 0.73</td>
<td>5.60 ± 0.29</td>
</tr>
</tbody>
</table>
Figure 3.2. LC50 values generated from three separate rearings of *S. vittatum* IS-7 larvae exposed to chlorpyrifos, carbaryl and malathion individually and as mixtures. Numbers on the x-axis of each graph refer to repetition. Error bars represent the 95% confidence interval associated with the LC50. LC50 values reported were generated from nominal concentrations.
Table 3.3. Median 48 hr LC50 values for *Simulium vittatum* IS-7 larvae exposed to three insecticides in an acute orbital shaker toxicity test using a logit model for analysis. LC50 values are accompanied by the fiducial limits (F.L.) and slope. P-values for the Chi-square goodness of fit test were >0.05. Analytical concentrations used in the analysis were the geometric mean of the initial and final concentrations detected in water samples. Analytical concentrations were determined using gas chromatography for chlorpyrifos and malathion and high performance liquid chromatography for carbaryl.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Concentration</th>
<th>LC50 (µg/L)</th>
<th>F.L.</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>Nominal</td>
<td>0.57</td>
<td>0.50 – 0.64</td>
<td>5.12 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>Analytical</td>
<td>0.28</td>
<td>0.25 – 0.32</td>
<td>5.36 ± 0.68</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>Nominal</td>
<td>44.34</td>
<td>42.37 – 46.31</td>
<td>15.02 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>Analytical</td>
<td>23.72</td>
<td>22.52 – 24.93</td>
<td>14.60 ± 2.04</td>
</tr>
<tr>
<td>Malathion</td>
<td>Nominal</td>
<td>283.00</td>
<td>237.69 – 340.79</td>
<td>3.09 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>Analytical</td>
<td>54.20</td>
<td>44.70 – 66.43</td>
<td>2.84 ± 0.26</td>
</tr>
</tbody>
</table>

The ternary mixture of chlorpyrifos-carbaryl-malathion was shown to be the most toxic mixture with an LC50 of 0.57 TU and a 95% confidence interval of 0.47 – 0.66 TU. Because the 95% confidence interval did not encompass 1 TU, we determined this mixture to be greater than additive. The binary mixtures of chlorpyrifos-malathion and carbaryl-malathion also had greater than additive toxicity while the binary mixture of chlorpyrifos-carbaryl was additive (Table 3.4).
Table 3.4. Median 48 hr LC50 values for S. vittatum IS-7 larvae exposed to mixtures of chlorpyrifos (Chp), carbaryl (Carb) and malathion (Mal) in an acute orbital shaker toxicity test using a logit model for analysis. LC50 values are accompanied by the 95% confidence interval and slope ± standard error. P-values for the Chi-square goodness of fit test were >0.05. Mixtures in which the 95% confidence interval encompasses 1 TU are considered additive in toxicity.

<table>
<thead>
<tr>
<th>Insecticide Mixture</th>
<th>LC50 (TU)</th>
<th>95% Confidence Interval</th>
<th>Slope ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chp-Carb-Mal</td>
<td>0.56</td>
<td>0.47 – 0.66 (G)</td>
<td>3.15 ± 0.40</td>
</tr>
<tr>
<td>Chp-Mal</td>
<td>0.72</td>
<td>0.60 – 0.82 (G)</td>
<td>4.02 ± 0.56</td>
</tr>
<tr>
<td>Carb-Mal</td>
<td>0.78</td>
<td>0.70 – 0.85 (G)</td>
<td>6.26 ± 0.70</td>
</tr>
<tr>
<td>Chp-Carb</td>
<td>0.98</td>
<td>0.90 – 1.05 (A)</td>
<td>9.63 ± 1.03</td>
</tr>
</tbody>
</table>

(G) = Greater than additive toxicity
(A) = Additive toxicity

Discussion

The intensive management required to produce green lawns, landscapes and gardens has driven the purchase and yearly application of millions of pounds of pesticides. Of the three main types of pesticides (herbicides, insecticides and fungicides) used on lawns and gardens, insecticides account for 23% of the total amount applied annually (Aspelin and Grube 1999). Although insecticides are not the most heavily used pesticide in domestic settings, they are the most acutely toxic to non-target animals, especially aquatic invertebrates exposed to runoff from residential areas. Insecticides have this effect on non-target species because they disrupt similar systems that lead to the death of target pests. Data reported in this study provide insight into the toxic effects these chemicals may elicit individually and as mixtures in aquatic ecosystems.
The insecticide common in all three mixtures that produced greater than additive toxicity was malathion. Greater than additive toxicity has been reported with several other insecticide mixtures containing malathion. Synergistic mortality was observed in Japanese quail, *Coturnix coturix*, and pheasant, *Phasianus colchicus*, chicks, after feeding on diet containing a mixture of malathion and EPN (Kreitzer and Spann 1973). Macek (1975) showed that parathion, fenthion, carbaryl, EPN, and perthane, when added in combination with malathion, produced greater than additive toxicity in bluegill sunfish, *Lepomis macrochirus*. The insecticides malathion and dioxathion have been shown to be synergistic in rainbow trout, *Salmo gairdnen* (Marking 1977). Likewise, specific combinations of the insecticides chlordane, malathion, and carbofuran, have shown synergistic toxicity in test fish, *Notopterus notopterus* (Mor) (Gupta et al. 1994). Johnston and others (1994) reported that carbaryl toxicity was potentiated in hybrid red-legged partridges, *Alectoris rufa* cross, pretreated with malathion.

Malathion, like other phosphorothioate insecticides, must be activated from its phosphorothioate form to a phosphate through the action of P-450 enzyme systems. The phosphate form of the chemical is then able to mimic acetylcholine and inhibit acetylcholinesterase activity by binding to the enzyme at the esteratic site (Matsumura 1985). However, unlike most organophosphates, malathion toxicity is dependent upon the levels of carboxylesterases present in the organism. Carboxylesterases can hydrolyze one or both carboxyl esters associated with the structure of malathion before it is activated to its phosphate form, greatly reducing its toxicity (Pasarela et al. 1962). In general, insects have lower levels of carboxylesterases than mammals, which makes malathion an excellent insecticide (high insect toxicity; low mammalian toxicity) (Matsumura 1985).
However, *S. vitattum* has been shown to possess high levels of general esterases, some of which are presumed to be carboxylesterases (Siegfried and Young 1993). This may explain why malathion was the least toxic insecticide when tested individually.

The mechanism associated with increased toxicity in mixtures containing malathion at this point is speculative. There is strong support for the theory that malathion inhibits P-450 enzymes (De Matteis 1974, Uchiyama et al. 1975). Inhibition has been shown to be associated with the liberation of sulfur during P-450 activation of malathion to malaoxon. The liberated sulfur then covalently binds to the enzyme, leaving it inhibited. Consequently, metabolism of the additional compound present in the mixture is slowed, greatly increasing its toxicity. Inhibition of P-450 enzymes has also been shown to occur with other phosphorothioate insecticides such as parathion, diazinon, and fenitrothion (Halpert and Neal 1981, Uchiyama et al. 1975). However, our results showed that the mixture of chlorpyrifos, a phosphorothioate insecticide, and carbaryl was additive. This suggests that either chlorpyrifos does not inhibit P-450 enzymes as strongly as other phosphorothioates or that other mechanisms associated with this mixture and possibly the mixtures with malathion.

One alternative mechanism associated with the greater than additive toxicity observed in mixtures containing malathion may be related to increased activation of malathion to malaoxon. Johnston et al. (1989) attributed the potentiation of malathion toxicity in the presence of the fungicide prochloraz to an increase in malathion activation by P-450 enzymes induced by the fungicide. Enzyme induction studies with chlorpyrifos and carbaryl have shown chlorpyrifos to be an inducer of P-450 enzymes in chickens (Vodela and Dalvi 1995) whereas carbaryl did not increase P-450 enzyme activity in
mice (Robacker et al. 1981). However, administration of carbaryl and malathion in combination to Sprague-Dawley rats produced a significant increase in malaoxon in the urine as opposed to rats given malathion alone indicating activation of malathion (Lechner and Abdel-Rahman 1986).

Another alternative mechanism is the inhibition of carboxylesterases by the additional chemicals present in the mixture. Carbamate and organophosphate insecticides have been shown to inhibit carboxylesterases the same way they inhibit cholinesterases by binding to the active site and phosphorylating the enzyme (Thompson et al. 1991, Escartin and Porte 1997). Carboxylesterases, as previously mentioned, can greatly reduce the toxicity of malathion by cleaving the carboxyl esters associated with its structure. Inhibition of these enzymes may have increased the amount of malathion activated into malaoxon; greatly increasing its toxicity in the mixture.

Many toxicity tests are conducted using standardized procedures to produce repeatable results under specific controlled conditions. (ASTM 1993). However, these procedures and conditions do not simulate a natural environment for many test organisms, especially organisms of lotic habitats. Such abnormal conditions may provide additional stress to the organism and consequently alter its susceptibility to the test chemical. Because black fly larvae require flowing water to support their feeding mechanism and oxygen requirements, the orbital shaker toxicity test was developed to produce a current in the flask that simulates flowing water. *Simulium vittatum* IS-7 larvae are typically found attached to substrates in the water column of streams with their cephalic fans opened into the current for feeding. The orbital shaker toxicity test simulates these conditions: black fly larvae attach to the sides of the flasks and open their
fans into the current produced by the shaker. Although no food was added to the flasks in the toxicity tests for this study, similar toxicity experiments have shown through larval gut analysis that larvae do actively feed when food is added to the flask (Chapter 4).

Although this testing procedure is more representative of lotic systems that black flies inhabit, stability of the insecticides was greatly reduced over the 48 hr testing period due to the mixing and swirling of the water inside the flasks. Percent losses for chlorpyrifos, carbaryl and malathion were 69.10, 43.30, and 59.50, respectively. This also illustrates the importance of analytically determining chemical concentrations used in toxicity tests. Not all chemicals are stable in water for 48 or 96 hrs even under controlled laboratory conditions. Consequently, toxicity data analyzed through nominal concentrations alone may greatly overestimate the concentration of the toxicant causing the effect.

To compensate for the discrepancy between the initial and final concentrations detected in the water during the 48 hr toxicity tests, the geometric mean for each of the six initial and final concentrations was calculated and analyzed with the logistic model to produce an average LC50. By determining an average LC50 from the geometric mean of the highest and lowest concentrations detected in the test waters, the exponential loss of the insecticide can be accounted for along with the variation in loss among the test concentrations. Thus, a better estimate of the LC50 produced from the orbital shaker toxicity test data is achieved. Unfortunately, we were not able to conduct a similar analysis with the analytical data for the mixtures due to the lack of sensitivity in the detection of carbaryl. Only 50 ml of sample water was extracted for analysis. If a larger volume had been used, perhaps increased sensitivity would have been achieved and
carbaryl concentrations could have been quantified. Consequently the degree to which mixtures containing malathion were greater than additive may have been underestimated and are likely more toxic than our data supports.

From other orbital shaker toxicity tests conducted in our laboratory with bacterial insecticides, it has been shown that larvae may vary slightly from rearing to rearing based on comparison of LC50 values with a standard product. Thus, it is standard practice to repeat toxicity tests with separate rearings to account for any possible variation that may influence the LC50 estimate. In this study, larval rearings appeared to vary slightly through comparison of 95% confidence limits associated with the three LC50s generated for each insecticide and mixture. One repetition was significantly different in all sets of experiments except for carbaryl, malathion and chlorpyrifos-carbaryl-malathion. However, in all repetitions where one LC50 value was either significantly higher or lower than the other two, analytical concentrations of the insecticide(s) detected in the water samples were either lower or higher respectively accounting for some of the variation. If in fact all the variation is associated with error in the preparation of the insecticide concentrations, *S. vittatum* IS-7 colony larvae make excellent toxicity test organisms because of the ability to produce repeatable results from week to week.

Results of this study indicate that lawn-care insecticides commonly detected in urban and suburban streams elicit greater than additive toxicity when aquatic organisms are exposed to them as mixtures. The method for determining the joint toxicity of these chemicals used equivalent toxic units of the individual insecticides over a range of concentrations such that a mortality distribution was produced that could be statistically analyzed for estimating a LC50 value. This method was useful in determining the toxic
nature (i.e. greater than additive, additive, or less than additive) of the insecticides as mixtures. However, further research with environmentally realistic concentrations and proportions of these insecticides is needed to better assess the impacts these chemicals have on organisms inhabiting urban and suburban streams.
CHAPTER 4

INFLUENCES OF A LABORATORY DIET AND NATURAL SESTON ON THE BIOAVAILABILITY OF CARBARYL, CHLORPYRIFOS, AND MALATHION TO BLACK FLY LARVAE (DIPTERA: SIMULIIDAE) IN AN ACUTE TOXICITY TEST

Introduction

Filter-feeding organisms in lotic habitats utilize suspended particulate organic matter as a food source. Insecticides entering waterways through runoff and spray-drift may adsorb to this and other suspended material making them potentially more or less available to these organisms. Several studies with filter-feeding organisms have shown either an increase or decrease in adverse effects related to insecticide exposure in the presence of suspended solids (Fredeen 1953, Hall et al. 1986, Ekelund et al. 1987, Hermsen et al. 1994, Shultz and Liess 2001). Likewise, the addition of food in laboratory toxicity tests with filter-feeding organisms may influence toxic effects measured due to chemical interactions with the food. Thus, it is important to determine what chemical bioavailability effects may be encountered when food is added.

It is normally not recommended that test organisms be fed during short-term (≤ 96 h) toxicity tests in order to minimize dissolved oxygen depletion and loss of test chemical (Parish 1985). However, in long-term toxicity tests (≥ 96 h), organisms need to

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Overmyer, J.P. and R. Noblet. 2002. Submitted to Archives of Environmental Contamination and Toxicology. 07/31/02
feed in order to meet physiological demands and maintain health. Although loss of test chemical and dissolved oxygen depletion may be factors that influence the outcome of the test, the effect of starvation may have just as much, if not more influence on the outcome. In addition, experiments designed to study the influence of contaminants on life-history endpoints, such as growth, fecundity and reproductive success, may be compromised significantly when the test organisms are deprived of nutrients required for normal development.

The purpose of this study was to determine the effects of two food sources, a laboratory prepared diet and natural seston, on the bioavailability of the organophosphate insecticides chlorpyrifos (O,O-Diethyl O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate), and malathion (Diethyl mercaptosuccinate, O,O-dimethyl-dithiophosphate), and the carbamate insecticide carbaryl (1-Naphthyl-N-methylcarbamate) to filter-feeding black fly larvae, *Simulium vittatum* Zetterstedt cytospecies IS-7, in a 24 hr orbital shaker toxicity test. Insecticides were chosen based on their widespread use for controlling insect pest species and presence in aquatic systems. Data obtained from this study will aid in determining what concentration of food can be added in long-term experiments without altering (i.e. increase or decrease) the bioavailability of the insecticides. In addition, data obtained may aid in determining if insecticide bioavailability is altered in the presence of seston in streams and rivers commonly inhabited by filter-feeding organisms.
Materials and Methods

Organisms

Black fly larvae (Diptera: Simuliidae) are filter-feeding insects found in streams and rivers throughout the world. They are able to filter out suspended material from the water column ranging in size from 0.09-350 µm with their labral fans (Wotton 1976, Chance 1977). Their primary role in aquatic ecosystems is converting ultra-fine particulate organic matter into fine particulate organic matter that can be utilized by other organisms and as a food source for fish and other invertebrates (Cummins 1987). Larvae for this investigation were obtained from the University of Georgia (UGA) Simulium vittatum IS-7 colony being reared under the methods outlined by Gray and Noblet (1999).

Chemicals

Analytical-grade standards of chlorpyrifos, malathion and carbaryl were purchased from Chemservice (Westchester, PA). Standards were certified to be ≥ 98% pure. Stock solutions of all standards were prepared by dissolving a weighed amount of insecticide into 100 ml of pesticide-grade acetone and stored at 4°C in amber bottles with Teflon®-lined caps.

Test Water

Moderately-hard reconstituted test water was prepared using the method of Weber (1993). Temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness were measured before and after each test except alkalinity and hardness, which were measured pre-test only (Table 4.1). These parameters, excluding alkalinity and hardness, were
Table 4.1. Mean values with standard error for water quality parameters measured before and after acute orbital shaker toxicity tests. All parameters except alkalinity and hardness were measured for each treatment level. Line 1 represents pre-testing values and line 2 represents post-testing values.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DO (mg/L)</th>
<th>pH</th>
<th>Conductivity (μS/cm)</th>
<th>Temperature (°C)</th>
<th>Alkalinity (mg/L as CaCO₃)</th>
<th>Hardness (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABORATORY DIET TRIALS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 8.76(0.11)</td>
<td>8.21(0.02)</td>
<td>276.30(1.45)</td>
<td>21.11(0.24)</td>
<td>65.00(1.00)</td>
<td>90.00(1.00)</td>
</tr>
<tr>
<td></td>
<td>2 8.80(0.14)</td>
<td>8.10(0.03)</td>
<td>285.36(2.88)</td>
<td>20.81(0.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Control (300mg/L)</td>
<td>1 8.53(0.09)</td>
<td>8.02(0.02)</td>
<td>317.96(1.24)</td>
<td>21.11(0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 5.23(0.21)</td>
<td>7.15(0.06)</td>
<td>340.40(2.39)</td>
<td>21.23(0.17)</td>
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<td></td>
</tr>
<tr>
<td>75mg/L</td>
<td>1 8.60(0.09)</td>
<td>8.13(0.03)</td>
<td>285.90(1.61)</td>
<td>21.11(0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 7.74(0.14)</td>
<td>7.64(0.08)</td>
<td>293.02(2.79)</td>
<td>21.18(0.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150mg/L</td>
<td>1 8.57(0.09)</td>
<td>8.08(0.02)</td>
<td>295.41(1.77)</td>
<td>21.11(0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 7.13(0.12)</td>
<td>7.50(0.08)</td>
<td>311.56(2.38)</td>
<td>21.32(0.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>225mg/L</td>
<td>1 8.56(0.09)</td>
<td>8.05(0.02)</td>
<td>306.63(1.13)</td>
<td>21.11(0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 6.25(0.15)</td>
<td>7.29(0.06)</td>
<td>327.27(2.19)</td>
<td>21.63(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300mg/L</td>
<td>1 8.53(0.09)</td>
<td>8.02(0.02)</td>
<td>317.96(1.24)</td>
<td>21.11(0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 5.38(0.32)</td>
<td>7.20(0.05)</td>
<td>342.48(2.78)</td>
<td>21.72(0.17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SESTON TRIALS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 9.43(0.20)</td>
<td>8.13(0.02)</td>
<td>278.53(1.57)</td>
<td>21.58(0.18)</td>
<td>67.00(1.00)</td>
<td>93.00(1.00)</td>
</tr>
<tr>
<td></td>
<td>2 8.89(0.14)</td>
<td>8.20(0.02)</td>
<td>287.52(1.23)</td>
<td>21.16(0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seston Control (150mg/L)</td>
<td>1 8.72(0.08)</td>
<td>8.08(0.02)</td>
<td>274.37(1.48)</td>
<td>21.58(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 8.75(0.14)</td>
<td>8.20(0.02)</td>
<td>283.39(1.22)</td>
<td>21.40(0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5mg/L</td>
<td>1 8.74(0.07)</td>
<td>8.15(0.03)</td>
<td>277.96(1.78)</td>
<td>21.58(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 8.69(0.13)</td>
<td>7.64(0.08)</td>
<td>287.56(1.11)</td>
<td>21.56(0.16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15mg/L</td>
<td>1 8.73(0.09)</td>
<td>8.16(0.03)</td>
<td>276.79(1.36)</td>
<td>21.58(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 8.73(0.13)</td>
<td>8.20(0.01)</td>
<td>287.84(1.57)</td>
<td>21.60(0.12)</td>
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<td></td>
</tr>
<tr>
<td>75mg/L</td>
<td>1 8.71(0.08)</td>
<td>8.11(0.03)</td>
<td>274.76(1.66)</td>
<td>21.58(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 8.64(0.13)</td>
<td>8.20(0.01)</td>
<td>288.09(1.57)</td>
<td>21.79(0.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150mg/L</td>
<td>1 8.72(0.08)</td>
<td>8.08(0.02)</td>
<td>274.37(1.48)</td>
<td>21.58(0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 8.62(0.13)</td>
<td>8.20(0.01)</td>
<td>287.63(1.34)</td>
<td>21.91(0.12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
measured for each treatment level of food and seston to determine their respective effects on water quality. Temperature, dissolved oxygen, and conductivity were measured using a YSI 85 water quality meter and pH was measured with a Corning 440 pH meter. Alkalinity and hardness concentrations were determined through titration with 0.2 N H₂SO₄ and 0.1 M EDTA respectively (APHA, AWWA, and WPCF 1995).

Laboratory Diet

Laboratory diet was prepared by blending equal portions of soybean meal and Advanced Nutrition™ rabbit feed (Purina Mills, St. Louis, MO) in a blender for two minutes. This is the standard diet used to feed the black fly colony at UGA. Before each test, approximately 9.5 g of dry diet was weighed out on an AB204 Mettler Toledo balance and added to 2 L of moderately hard water. Contents were stirred for several minutes and poured through a 53 µm sieve to remove large particles that would not stay suspended. Because some of the diet material was filtered out during the sieving process, concentration of the stock diet solution was determined by measuring turbidity on a Hach 18900 Ratio Turbidimeter. Diet solutions of 30, 60, 90, and 120 NTUs were prepared through appropriate dilutions of the stock diet. Actual concentrations of diet in the water were determined gravimetrically by drying a 50 ml aliquot of each of the prepared diet solutions in a Thelco oven at 60°C for 24 hrs. A 50 ml aliquot was used because this was the volume transferred to each flask to make the appropriate concentration. Based on preliminary data, it was determined that for every 10 NTUs approximately 75 mg/L of diet was present. Thus, nominal concentrations of 75, 150, 225, and 300 mg/L were used to represent turbidity measurements of 10, 20, 30, and 40 NTUs in the test flasks. Actual
turbidity measurements and gravimetrically determined concentrations of diet in test flasks are listed in Table 4.2.

Table 4.2. Nominal and actual concentrations ± standard error of laboratory diet in test flasks with associated turbidity measurements. Actual concentrations were determined through gravimetric analysis of a 50 ml aliquot of the appropriate diet solution.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Nominal Concentration (mg/L)</th>
<th>Nominal Turbidity (NTU)</th>
<th>Actual Concentration (mg/L)</th>
<th>Actual Turbidity (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>75</td>
<td>10</td>
<td>81.21 ± 2.55</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>20</td>
<td>154.43 ± 8.80</td>
<td>18.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>30</td>
<td>247.01 ± 2.16</td>
<td>28.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>40</td>
<td>316.47 ± 9.43</td>
<td>37.7 ± 0.3</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>75</td>
<td>10</td>
<td>77.33 ± 2.45</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>20</td>
<td>138.00 ± 7.92</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>30</td>
<td>204.77 ± 4.15</td>
<td>29.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>40</td>
<td>290.20 ± 5.66</td>
<td>38.3 ± 0.3</td>
</tr>
<tr>
<td>Malathion</td>
<td>75</td>
<td>10</td>
<td>81.33 ± 5.15</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>20</td>
<td>133.10 ± 17.03</td>
<td>19.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>30</td>
<td>210.03 ± 2.03</td>
<td>29.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>40</td>
<td>285.33 ± 15.05</td>
<td>38.0 ± 0.6</td>
</tr>
</tbody>
</table>

Seston

Seston was obtained from an uncontaminated first order stream at the Coweeta Hydrologic Laboratory (Macon County, North Carolina) using a 0.6 m Coshocton wheel proportional runoff sampler (Parsons 1954) mounted below a weir (Cuffney and Wallace 1988). The sampler delivered 0.6% of the stream flow into three covered 125 L settling barrels. After one week, the barrels were removed from the sampler. Seston collected in
the last two barrels was allowed to settle for 12 hrs. After settling, the seston at the bottom of the barrels was siphoned into a container and allowed to dry.

**Testing Procedure (Diet experiments)**

For each test, larvae were removed from the rearing tank and placed into enamel pans with 1L of rearing-medium water. Fifteen 4<sup>th</sup>-5<sup>th</sup> instar larvae were transferred with larval forceps into 250 ml Pyrex® flat-bottom extraction flasks containing 95 ml of moderately-hard reconstituted water. Instar number was determined post-testing through measurement of the cephalic apotome width (Hudson 1994) using an ocular micrometer at 45X on a Meiji Techno dissecting scope. After the larvae were added to the flasks, 50 ml of the appropriate diet concentration were added to each flask. Controls received 50 ml of moderately hard water. Flasks were loaded onto a New Brunswick Scientific G-10 Gyratory® shaker and allowed to acclimate for 10 min at 100 rpm, 10 min at 125 rpm and 40 min at 150 rpm, the final speed for the duration of the experiment, for a total 1 hr acclimation period. The G-10 shaker produces a current in the flasks similar to natural stream flow so that the larvae can feed. This acclimation period allows the larvae to position themselves in the optimal flow pattern within the flasks.

Prior to dosing the flasks, insecticide solutions were prepared in 200 ml volumetric flasks by spiking reconstituted water with insecticide stock solution. The contents were then emptied into 250 ml amber bottles until dosing. After the acclimation period, the flasks were dosed with 5 ml of the appropriate insecticide concentration with a 5 ml glass pipet using a Drummond pipet-aid® to bring the final volume in each flask to 150 ml. Carrier (acetone) and diet controls were dosed with 5 ml of moderately hard
water containing an equivalent concentration of the acetone carrier used. Immediately after dosing, flasks were capped with aluminum foil to minimize evaporative water loss and prohibit external contamination. After 24 hrs, larvae were removed from the flasks and mortality was assessed. Any larva that did not respond to a gentle probing was considered dead.

Four diet concentrations and three controls, a carrier (acetone) control, a positive control containing no diet and dosed with the same concentration of insecticide, and a diet control containing the highest concentration of diet and dosed with acetone, were tested on one shaker with five flasks per diet concentration and control, bringing the final totals to 35 flasks and 525 larvae. Each of the three insecticides was tested on a separate shaker. Three orbital shaker toxicity tests were conducted for each insecticide. Testing was conducted with ambient air temperatures in the laboratory measuring 20°C (± 0.5) and a 16:8 light:dark photoperiod. Nominal concentrations of insecticide used were 0.7, 60, and 250 µg/L of chlorpyrifos, carbaryl, and malathion respectively.

Testing Procedure (Seston experiments)

Similar testing procedures were followed as in the diet experiments except for the addition of the seston to the flask. Dry seston was weighed out individually for each flask on an AB204 Mettler Toledo balance. The material was then added to 120 ml of moderately hard water in the flasks. Residual seston on the weighboat was rinsed into the flask with 25 ml of moderately hard water. Concentrations of seston tested were 5, 15, 75, and 150 mg/L. Seston concentrations of 5 and 15 mg/L were used to represent
conditions black fly larvae might encounter in natural systems while concentrations of 75 and 150 mg/L were used for comparison with the laboratory diet.

Data Analysis

All data were adjusted for control mortality before statistical analysis using Abbott’s formula (Abbott 1925). Experiments with control mortality >10% were not considered valid. Mortality data were arcsine transformed and analyzed for homogeneity of variances and normality using a Bartlett’s and Shapiro-Wilk’s test respectively. A one-way analysis of variance (ANOVA) with larval rearing date as a blocking factor, was used to determine statistical significance followed by a Tukey’s studentized range test to determine differences among treatment means ($\alpha = 0.05$).

Results

To determine that larvae had fed during the test, guts of randomly selected larvae were dissected and observed for the presence of laboratory diet or seston. All dissected guts were full of material in both the laboratory diet and seston experiments indicating that feeding was not impaired in the flasks. Laboratory diet at concentrations $\geq 150$ mg/L significantly altered the bioavailability of all three insecticides tested ($p < 0.001$). The effect however was specific to the insecticide, with malathion and carbaryl toxicity increasing and chlorpyrifos toxicity decreasing (Figure 4.1). The most profound effect of laboratory diet on bioavailability was observed with carbaryl-dosed larvae, where mortality was increased by 41, 134, 200 and 313 % at diet concentrations of 75, 150, 225 and 300 mg/L respectively. Natural seston had little effect on the bioavailability of the
three insecticides except in carbaryl-dosed larvae where mortality was significantly increased \((p = 0.042)\) when the seston concentration was 150 mg/L in the flasks (Figure 4.2).

![Figure 4.1. Mean mortality ± standard error of *S. vittatum* IS-7 exposed to carbaryl, malathion and chlorpyrifos in the presence of a laboratory diet. Mortality data were arcsine transformed before statistical analysis. Asterisks (*) represent mortality significantly different from the respective controls for each insecticide.]
Figure 4.2. Mean mortality ± standard error of *S. vittatum* IS-7 exposed to carbaryl, malathion and chlorpyrifos in the presence of seston. Mortality data were arcsine transformed before statistical analysis. Asterisks (*) represent mortality significantly different from the respective controls for each insecticide.

Because of the differences in insecticide bioavailability effects between the two food sources, post-experiment statistical analysis of particle size and percent total organic carbon (TOC) and dissolved organic carbon (DOC, particle size < 0.45 µm) were conducted on the two materials using a Student’s t-test. Particles of laboratory diet were shown to be significantly smaller than the seston (n = 300, p < 0.001, α = 0.05). Average particle size ± standard error of the laboratory diet and seston were 11.65 ± 0.47 µm and 30.93 ± 3.25 µm respectively. TOC and DOC contents were significantly higher in the laboratory diet compared to seston (n = 3, p < 0.001, α = 0.05). The average percent TOC and DOC was 26.4% and 19.1% for the laboratory diet and 1.1% and 0.6% for the seston respectively (Figure 4.3).
Figure 4.3. Total organic carbon (TOC) and dissolved organic carbon (DOC) contents of laboratory diet and seston used in the orbital shaker toxicity tests.

Discussion

It is believed that organic chemicals, such as insecticides, become less bioavailable to aquatic organisms through adsorption to suspended solids or organic material in the water column and sediments (Hall et al. 1986, Landrum et al. 1987, Muir et al. 1994, Kadlec and Benson 1995, Kusk 1996). However, filter-feeding organisms, which ingest suspended material and utilize the organic component as a food source, may actually accumulate more of the chemical leading to possible increases in adverse effects (Fredeen 1953, Ekelund et al. 1987, Fisher and Clarke 1990, Hermsen et al. 1994, Schultz and Liess 2001). Results of this study with filter-feeding black fly larvae, *S. vittatum* IS-7, indicate that both increases and decreases in insecticide bioavailability may occur when organic material is present and ingested.

The primary objective of this study was to determine if the addition of food would influence the toxicity of insecticides by either increasing or decreasing their availability.
to the organism. Our results indicated that bioavailability was altered when a laboratory diet was added at concentrations ≥ 150 mg/L. Thus, it was determined that concentrations of laboratory diet < 150 mg/L could be added to toxicity tests without affecting insecticide bioavailability and subsequent toxicity. In addition, it was determined that the effects of food on insecticide bioavailability were chemical specific with carbaryl and malathion toxicity increasing and chlorpyrifos toxicity decreasing. From the results obtained from the laboratory diet study, it was of interest to determine if similar alterations in insecticide bioavailability may occur in natural systems with seston as the food source. Results indicated that insecticide bioavailability was not altered at seston concentrations filter-feeding organisms may naturally encounter (5 and 15 mg/L). Bioavailability was only affected in flasks dosed with carbaryl at the highest seston concentration tested (150 mg/L) in which larval mortality was significantly higher than in the positive controls.

The difference in bioavailability effects between the two food sources in our laboratory tests was most likely due to the composition and particle sizes of the materials. Both TOC and DOC contents of the laboratory diet were significantly greater than the natural seston. Organic carbon, especially the dissolved fraction, has been shown to adsorb organic compounds and influence bioavailability (Landrum et al. 1985, McCarthy et al. 1985, Kukkonen and Oikari 1987, Servos et al. 1989, Day 1991). In addition, the source and nutritional quality of organic carbon has been shown to affect chemical partitioning (Landrum et al. 1984) and uptake rate (Gunnarsson et al. 1999) respectively. Because the organic carbon of the two food materials are from different sources (laboratory diet = forage and grain by-products, seston = leaf and wood material) it is
likely that the insecticides partitioned to the organic carbon and were taken up by the larvae differently.

Particle size may have also influenced the results obtained from the two food sources. In the orbital shaker toxicity tests, large particles of seston had a tendency to fall out of suspension and very small particles would adhere to the silk produced by the larvae for attachment sites on the sides of flasks. Consequently, a certain percentage of the seston was unavailable for ingestion. Although some of the laboratory diet also adhered to the sides of the flasks, very little settled out on the bottom due to the uniform size of the particles as a result of sieving. Seston was not sieved in these experiments because black fly larvae in natural systems would encounter a wide range of particle sizes, some of which may be too large for ingestion. Thus, in order to make the exposure as realistic as possible for the seston experiments, particle size was not manipulated through sieving.

The addition of laboratory diet in the toxicity tests affected the bioavailability of the three insecticides differently with carbaryl and malathion toxicity increasing and chlorpyrifos toxicity decreasing. This may be explained in part by the organic carbon partitioning coefficient ($K_{oc}$) for each insecticide. Chlorpyrifos, the insecticide most negatively affected by the laboratory diet (i.e. decreased bioavailability), has the highest affinity for organic carbon ($\text{Log } K_{oc} = 3.78$) among the three. As the organic carbon content increased in relation to increased laboratory diet, more chlorpyrifos may have become bound resulting in a reduction in the amount of free chemical available for uptake and a decrease in toxicity. Bound chlorpyrifos may have been too large to cross the biological membranes necessary to reach the active sites. Other studies have alluded
to the fact that chemicals bound to organic carbon may become too large to cross
membranes leading to decreased bioavailability (Landrum et al. 1987, Servos et al. 1989,
Day 1991). Carbaryl, the insecticide most positively affected by the laboratory diet (i.e.
increased bioavailability), has the lowest affinity for organic carbon (Log $K_{oc} = 2.36$).
However, even if carbaryl’s affinity for organic carbon was less than chlorpyrifos
resulting in more of the free chemical available for uptake, there was still more chemical
reaching the active site than in flasks with no food added. Thus, in addition to
integumental uptake of the free chemical, uptake must have taken place in the gut during
ingestion of the laboratory diet.

Black fly larvae not only capture and ingest particulate organic matter but also
dissolved organic matter (particles < 0.45 μm) (Ciborowski et al. 1997) which, as
previously stated, binds organic chemicals. Consequently, utilization of dissolved organic
material as a food source could potentially increase the amount of insecticide available
for uptake by the larvae if the chemical is in a form that can be readily absorbed. The
high pH of the black fly midgut, pH ≅ 9.8 (Undeen 1979), may have aided in dissociating
the bound insecticide allowing the chemical to be absorbed.

Similar increases in toxicity were seen with malathion as with carbaryl but to a
lesser extent. The affinity of malathion for organic carbon (Log $K_{oc} = 3.25$) is greater
than carbaryl. Thus, some of the chemical may have been bound and unavailable, but
presumably some malathion was taken up in the midgut leading to increased toxicity.

Another possible explanation for the difference in effects of laboratory diet on the
toxicity of the three insecticides is the behavioral response of the larvae to the individual
insecticides. *Simulium vittatum* IS-7 larvae, when exposed to carbaryl, detached from the
sides of the flasks and sank to the bottom. Black flies in nature will release themselves from their attachment site and drift downstream when unfavorable conditions are detected. This detachment behavior has been documented in laboratory studies testing carbaryl for use in black fly control programs (Jamnback and Frempong-Boadu 1966). In our closed system experiments, larvae cannot release and find a more favorable site. Their only option is to sink to the bottom of the flask, which may be more unfavorable than the original attachment site. The combination of insecticide exposure, unfavorable attachment site, and reduced water quality conditions with increasing diet concentration (Table 1) may have increased the susceptibility of *S. vittatum* IS-7 to carbaryl. Some larvae exposed to malathion also detached from the sides of the flasks. However, the onset of this behavior was much slower and not as common. The majority of the larvae exposed to chlorpyrifos released only after a lethal concentration had been acquired.

From the results of this study, it was determined that laboratory diet could be added to the water in toxicity tests with black flies at concentrations < 150 mg/L without affecting the bioavailability of the insecticide being tested. In addition, we discovered that the effects of food on the bioavailability and subsequent toxicity of insecticides is chemical dependent and the quality and source of the food material is a factor in determining these effects. The ability of *S. vittatum* IS-7 to capture and ingest dissolved organic material may have been a factor in the increased bioavailability seen with carbaryl and malathion. Thus, black flies may be more susceptible to insecticides that weakly bind to organic material than other filter-feeding organisms.
CHAPTER 5

EFFECTS OF CARBARYL, CHLORPYRIFOS AND MALATHION PULSE-EXPOSURES INDIVIDUALLY AND AS MIXTURES ON GROWTH, DEVELOPMENT AND SURVIVAL OF BLACK FLY LARVAE (DIPTERA: SIMULIIDAE)

Introduction

Organisms in aquatic systems are often continuously exposed to low concentrations of insecticides and/or episodically exposed to high concentrations for short periods of time. These exposures may not cause the organism to perish. However, many life history endpoints such as growth (McKenney 1986, Fairchild et al. 1992, Barry et al. 1995, Blockwell et al. 1996, Ferrando et al. 1996, McKenney et al. 1998, Nebeker et al. 1998, Stuijfzand et al. 1999, Hamm and Hinton 2000, Sanchez et al. 2000), generation time (Ferrando et al. 1996) and reproduction (McKenney 1986, Holdway and Dixon 1986, Fairchild et al. 1992, Wong 1996, Sanchez et al. 2000) become compromised due to sublethal insecticide exposure. Thus, the overall fitness of the organism may be reduced leading to possible population decreases and subsequent alteration of ecosystem function. Reduced organismal fitness may result from allocating assimilated energy towards detoxification and homeostatic mechanisms required for survival and away from activities that support growth and reproductive processes (Blockwell et al. 1996).
The organophosphate insecticides chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate), and malathion (diethyl mercaptosuccinate, O,O-dimethyl-dithiophosphate), and the carbamate insecticide carbaryl (1-naphthyl-N-methylcarbamate) are widely used for control of insect pests in the U.S.A. These chemicals are commonly detected in aquatic systems, especially urban and suburban waterways where they have been used to control insect pests on turf, ornamental plants, and in home gardens, due to runoff from rain events (Hippe et al. 1994, USGS 1998, USGS 1999). Chlorpyrifos, malathion and carbaryl have been shown to affect life history endpoints as individual chemicals (Marian et al. 1983, Seuge and Bluzat 1983, Hermanutz et al. 1985, Muniandy 1987, Khillare and Wagh 1988, James and Sampath 1994, Green and Chandler 1996, Thankamoni Amma and Konar 1996, Ramakrishnan et al. 1997, Chandler and Green 2001, Callaghan et al. 2001, Fordham et al. 2001, Nguyen and Janssen 2002). However, little is known about how mixtures of these chemicals may alter these endpoints. Previous studies have shown that insecticide mixtures containing malathion possess greater than additive toxicity in 48 hr acute toxicity tests with black fly larvae, *Simulium vittatum* IS-7 (Chapter 3). Thus, it is important to determine the possible impacts these insecticides may have on the life histories of biota inhabiting these waterways in individual and mixture exposure scenarios.

In this study, the effects of chlorpyrifos, malathion and carbaryl individually and as mixtures on growth, development and survival of the black fly, *Simulium vittatum* cytospecies IS-7 Zetterstedt, in a pulsed-exposure scenario will be investigated using a trough flow-through dosing system. This system provides flowing water simulating conditions naturally encountered by black fly larvae and allows insecticides to be added
and collected without disrupting the test organisms. Preliminary studies have shown that
*S. vittatum* IS-7 larvae undergo normal development in this system. Thus, any
abnormalities detected can be attributed to insecticide exposure.

**Materials and Methods**

**Organisms**

Black fly larvae (Diptera: Simuliidae) are often dominant species in stream
ecosystems. They are holometabolous, filter-feeding insects commonly found on rocks,
trailing vegetation, snags, and other substrates in streams and rivers. Their primary role
in aquatic ecosystems is converting ultra fine particulate organic matter into fine
particulate organic matter that can be utilized by other organisms. Larval simuliids also
serve as a food source for fish and other invertebrates (Cummins 1987). Larvae for this
investigation were obtained from the University of Georgia (UGA) *Simulium vittatum*
IS-7 colony being reared under the methods outlined by Gray and Noblet (1999).

**Chemicals**

Analytical-grade standards of chlorpyrifos, malathion and carbaryl were
purchased from Chemservice (Westchester, PA). Standards were certified to be ≥ 98%
pure. Stock solutions of all standards were prepared by dissolving a weighed amount of
insecticide into 100 ml of pesticide-grade acetone and stored at 4°C in amber bottles with
Teflon®-lined caps.
Test Water

Water used throughout the experiments was filtered through a 1 µm pre-filter followed by activated carbon filtration (U.S. Filter, Broadview, IL). Filtered water was then run through an ultraviolet sterilizing unit (Aquafine Corp., Valencia, CA) before entering the trough system. Temperature, pH, dissolved oxygen, conductivity, turbidity, alkalinity, and hardness were measured throughout the test (Table 5.1). All parameters, except turbidity, alkalinity and hardness, were measured hourly using a Hydrolab Datasonde® 4a water quality multiprobe. Turbidity was measured daily with a Hach 2100P portable turbidimeter. Alkalinity and hardness concentrations were measured every two days and determined through titration with 0.2 N H₂SO₄ and 0.1 M EDTA respectively (APHA, AWWA, and WPCF 1995). Testing was conducted at ambient air temperatures in the laboratory and a 16:8 light:dark photoperiod with crepuscular timing to simulate dawn and dusk.

Table 5.1. Mean values ± standard error for water quality parameters measured during experiments. Line 1 represents values measured during experiments with individual insecticides; line 2 represents values measured during experiments with mixtures of the insecticides.

<table>
<thead>
<tr>
<th>Dissolved Oxygen (mg/L)</th>
<th>pH (NTUs)</th>
<th>Turbidity (µS/cm)</th>
<th>Specific Conductance (°C)</th>
<th>Temperature (mg/L as CaCO₃)</th>
<th>Alkalinity (mg/L as CaCO₃)</th>
<th>Hardness (mg/L as CaCO₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.2 (0.4)</td>
<td>7.5 (0.1)</td>
<td>4.3 (0.2)</td>
<td>116.6 (7.5)</td>
<td>20.5 (0.5)</td>
<td>36.3 (0.3)</td>
</tr>
<tr>
<td>2</td>
<td>6.5 (0.5)</td>
<td>7.4 (0.1)</td>
<td>3.6 (0.3)</td>
<td>122.4 (4.5)</td>
<td>22.0 (0.5)</td>
<td>40.4 (1.3)</td>
</tr>
</tbody>
</table>
Food Solution

Food was prepared by blending equal portions of soybean meal and Advanced Nutrition™ rabbit feed (Purina Mills, St. Louis, MO) in a blender for one minute. Fifty ml of the blended food was then blended with 1 L of filtered tap water for 1 min and poured through a 53 µm sieve to remove large particles not utilized by the larvae. The food solution was stored in the refrigerator at 4°C until needed.

Trough Flow-Through Dosing System

The trough flow-through dosing system operates in two modes, circulation and flow-through. The system operates in circulation mode throughout the experiment except during dosing when it is switched to the flow-through mode. In this system, water is supplied to five 76.2 X 2.5 X 2.3 cm³ glass troughs from a 208 L Nalgene® polyethylene tank (head tank) fitted with a PVC ball valve and a 1.9 cm PVC pipe manifold (Figure 5.1). A polypropylene stopcock is attached to each of the five exits on the manifold to regulate water flow to each trough. Water from the manifold empties into modified 250 ml Pyrex® beakers that functioned as spillways for the troughs and as mixing chambers for the addition of insecticide. Troughs are mounted on a 121 X 58.4 cm² plywood platform supported by zinc shelving braces positioned at a slight angle producing a slope of –0.03 for proper water flow and depth in the troughs. Baffles made from sections of 0.8 cm ID polypropylene tubing cut in half were placed at 12.7 cm intervals in the trough bottom to increase the depth and turbulence of the water. Water from the troughs empties into a 246 L stainless steel tank (catch tank) and is circulated back to the head tank with a Teel® 4RH 11 circulating pump and 2.54 cm ID Tygon® tubing. An overflow valve connected to 2.54 cm ID Tygon® tubing leading back to the catch tank has been placed at
the 170 L mark on the head tank so that a constant volume would be maintained in the head tank for stabilizing flow rates. Stir-Pak® mixers with a stainless steel shaft and propeller are mounted on both the head and catch tank to keep food particles in suspension throughout the experiments. A 19 X 91.4 cm², 500 µm mesh, polypropylene screen is mounted below the troughs to prevent larvae from entering the catch tank.

**Figure 5.1.** Diagram of trough-flow-through dosing system.

Prior to dosing, the system is converted from a circulating system into a flow-through system. The screen (Figure 5.1 G) below the troughs is removed and replaced with individual screens attached to the end of each trough to catch any drifting larvae during insecticide exposure. A 209.6 X 10.8 X 6.4 cm³ section of PVC rain gutter is attached to the top of the catch tank to collect the exposure water so that no insecticide
enters the catch tank. The circulating pump is turned off and filtered water is added to replace the water being collected by the gutter. The rate of water addition is regulated such that 170 L of water is maintained in the head tank at all times. Excess water entering the overflow valve is periodically drained from the catch tank during dosing. Food solution is pumped into the head tank with a Meco-O-Matic® liquid solution feeder pump to maintain water turbidity between 3 and 7 NTUs during dosing. Previous studies have shown that this turbidity level does not affect the bioavailability of the insecticides (Chapter 4). For delivering insecticides to the troughs, five Meco-O-Matic® liquid solution feeder pumps are housed under the plywood platform. Insecticide solutions are delivered from a polypropylene carboy to the modified spillway beakers through 0.95 cm OD polypropylene tubing at a rate of 50 ml/min. Addition of insecticide to the beakers ensures mixing of the chemical with tank water to produce the desired concentration before entering the troughs. After dosing, the gutter and individual trough screens are removed, the large screen is reattached over the catch tank and the circulation pump turned on to resume normal system operation.

Testing Procedure

Prior to the addition of larvae, the system was filled with approximately 260 L of filtered water with 250-300 ml of food solution added to the head tank giving the water a turbidity of 3-5 NTUs. Sections of nylon screen containing 10-11 day old S. vittatum IS-7 larvae (2\textsuperscript{nd}-3\textsuperscript{rd} instar) were removed from the rearing tanks and placed into an enamel pan with 1 L of rearing-medium water. Two hundred uniform-sized larvae were added to each trough using a plastic, disposable pipet. After all larvae were attached to the troughs,
the flow was gradually increased to a final rate of approximately 25 ml/sec/trough. Water was allowed to circulate through the system until the time of insecticide dosing.

Prior to insecticide dosing, flow rates for each trough were quantified and insecticide pumps calibrated in order to determine the concentration of the dosing solution needed for a 1 µg/L insecticide exposure. Dosing solutions were prepared by spiking 7 L of filtered water contained in 10 L polypropylene carboys with the appropriate concentration of the respective insecticide stock solutions. On days 1, 2, 3, 5, 7 and 9 of the experiment, larvae in three of the troughs were exposed to a 2 hr pulse of either chlorpyrifos, malathion or carbaryl at 1 µg/L or combinations of these insecticide totaling 1 µg/L (eg. 0.5 µg/L chlorpyrifos + 0.5 µg/L malathion or 0.33 µg/L chlorpyrifos + 0.33 µg/L malathion + 0.33 µg/L carbaryl). One trough served as a control receiving filtered water during dosing and a second trough served as an acetone control receiving a 2 hr acetone pulse of 1 µg/L. During dosing, approximately 300 ml of water were collected from the respective troughs in amber glass bottles with Teflon® lids and stored at -20°C for insecticide concentration determinations. Water temperature and turbidity were monitored during dosing to ensure that these respective parameters remained steady. Larvae in the troughs were counted daily to assess mortality over the experiment. The time of pupation and number of pupae in each trough were also recorded during the daily observations to assess development.

On days 0, 2, 4, 6, 8 and 10 of the experiment, 10 larvae were removed from each trough with soft metal forceps and preserved in 95% ethanol for head capsule width measurements to determine growth rates. Head capsule width, as opposed to total body length, was used because of the defined, rigid structure of the capsule and its correlation
with overall growth (Adler 2002, personal communication). Total body length, which incorporates soft body tissue, may be influenced by muscle contractions or relaxation leading to biased measurements especially when exposed to neurotoxic chemicals (Adler 2002, personal communication). Head capsules were measured using an ocular micrometer at 45X on a Meiji Techno dissecting scope.

Three repetitions of the individual insecticide and insecticide mixture exposure experiments were conducted. Repetitions were conducted on separate dates with separate black fly rearings. All experiments were terminated on day 14.

### Chemical Analysis

Concentrations for all three insecticides, singly and as mixtures, were analytically measured to determine the actual concentration exposed to *S. vittatum* IS-7 larvae. For all water samples, a 200 ml volume of water was pulled under vacuum through a Bakerbond C-18 solid-phase extraction cartridge. Insecticides were eluted from the cartridge with one 0.5 ml aliquot of ethylacetate, two 0.5 ml aliquots of a 50:50 v/v ethylacetate-diethyl ether mixture and one 0.5 ml aliquot of diethyl ether. Eluates were dried over anhydrous sodium sulfate and the volume adjusted to 2 ml.

Chlorpyrifos and malathion concentrations were quantified using a Varian Saturn 2000 mass spectrometer with wave-board technology coupled with a Varian 3400 gas chromatograph with a split/splitless injector inlet. The gas chromatograph was outfitted with a 30 m x 0.25 mm Chrompack CP-5 column with a 0.25 micro-film loading. The column was set initially at 50°C and ramped to 120°C at 25°C/min after an initial 2 min hold time. Temperature continued to increase to 290°C at a rate of 8°C/min with a 1.7
min hold. The mass spectrometer was operated in the electron impact ionization mode with a single ion recording setting of m/z 173 for malathion and m/z 314 for chlorpyrifos.

Carbaryl residues in eluates were quantified using a Micromass, Quattro-Micro triple quadrupole mass spectrometer with a Waters Alliance 2690 solvent inlet delivery system. The liquid chromatograph was outfitted with a 250 x 2 mm Phenomenex Luna 5 µm Phenyl-Hexyl C18 column with a 20 µl injection volume. The column was held at 35°C with samples held at 4°C in the autosampler. Residues of carbaryl were eluted from the column with an acetonitrile-water gradient ramped from 10 – 95% acetonitrile over 13 min and held for 5 min. The mobile phase had 0.04% formic acid added to aid in the electrospray process. The flow rate used was 0.2 ml/min. The mass spectrometer was operated with electrospray ionization in the positive ion mode with a single ion recording method using ions of m/z 201.6 and 223.6 with a dwell time of 0.2 sec each. The source temperature was held at 80°C with a desolvation temperature of 140°C.

Data Analysis

Data for survival, onset of pupation, percent pupation, and total growth were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey’s studentized range test to determine differences among treatment means. Percent survival data were arcsine transformed before analysis. Data were analyzed for normality and homogeneity of variance with Shapiro-Wilk’s and Bartlett’s tests respectively. Data found not to be normally distributed or with unequal variances were subjected to a nonparametric Kruskal-Wallis test to determine significance. Growth rate data were analyzed with a quadratic polynomial model to determine differences in growth rates.
among treatment (Freund et al. 1986). An $\alpha$ value of 0.05 was used to determine statistical significance.

**Results**

**Chemical Analysis**

Results of the chemical analysis showed that the insecticide concentrations detected in the water samples were higher than predicted from the nominal values (Table 5.2). Carbaryl concentrations detected were approximately 30% higher than expected. There was much more discrepancy with the organophosphates chlorpyrifos and malathion with measured concentration approximately 10 fold higher than the nominal.

**Table 5.2.** Concentrations of chlorpyrifos (Chp), carbaryl (Carb) and malathion (Mal) individually and as mixtures detected in water samples from the trough flow-through dosing system. Measured concentrations are reported as the average ± standard error of the three repetitions. Concentrations were analytically determined using GC/MS for chlorpyrifos and malathion and LC/MS for carbaryl.

<table>
<thead>
<tr>
<th>Insecticide(s)</th>
<th>Nominal Concentration (µg/L)</th>
<th>Measured Concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chp</td>
<td>1.00</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>Carb</td>
<td>1.00</td>
<td>1.17 ± 0.11</td>
</tr>
<tr>
<td>Mal</td>
<td>1.00</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>Chp-Carb</td>
<td>0.50, 0.50</td>
<td>0.37 ± 0.10, 0.79 ± 0.08</td>
</tr>
<tr>
<td>Chp-Mal</td>
<td>0.50, 0.50</td>
<td>0.28 ± 0.01, 0.30 ± 0.02</td>
</tr>
<tr>
<td>Carb-Mal</td>
<td>0.50, 0.50</td>
<td>0.77 ± 0.06, 0.33 ± 0.00</td>
</tr>
<tr>
<td>Chp-Carb-Mal</td>
<td>0.33, 0.33, 0.33</td>
<td>0.20 ± 0.01, 0.53 ± 0.03, 0.18 ± 0.03</td>
</tr>
</tbody>
</table>
Individual Insecticides

Multiple 2 hr pulse exposures of chlorpyrifos, malathion or carbaryl had little effect on survival, growth and development of *S. vittatum* IS-7. Chlorpyrifos was the most toxic to the black fly larvae over the 14 d experiments with an average percent survival of 88.6% which was significantly lower (p ≤ 0.0203) than survival of larvae exposed to carbaryl (96.5%) and malathion (96.4%) exposed larvae (Figure 5.2). However, larval survival was not significantly lower (p > 0.05) than either the acetone controls (95.3%) or water controls (95.7%).

The quadratic term in the regression model proved to be significant in all treatments and controls (p ≤ 0.0395). Thus, the quadratic polynomial model was appropriate for analysis of larval growth rates and accounted for > 93% of the variation in the data (R² ≥ 0.9302). No significant differences were detected in growth rates among insecticide exposed and control larvae (Figure 5.3). Total larval growth during the experiment (day10 – day 0) was also not affected by insecticide exposure (Figure 5.4). There were no significant effects of insecticide exposure on development. The onset of pupation as well as the overall number of *S. vittatum* IS-7 pupating over the 14 d experiment were similar among controls and treated insects (Figures 5.5, 5.6).
Figure 5.2. Mean survival (n = 3) of *S. vittatum* IS-7 larvae after multiple pulse exposures to chlorpyrifos (chp), carbaryl (carb) and malathion (mal) individually (A) and as mixtures (B). Error bars represent ±1 standard error. Columns with different letters are statistically different (α = 0.05).
Figure 5.3. Mean growth rates (n = 3) of *S. vittatum* IS-7 larvae after multiple pulse exposures to chlorpyrifos (chp), carbaryl (carb) and malathion (mal) individually (A) and as mixtures (B). Error bars represent ± 1 standard error.
**Figures 5.4.** Mean total growth (n = 3) of *S. vittatum* IS-7 larvae after multiple pulse exposures to chlorpyrifos (chp), carbaryl (carb) and malathion (mal) individually (A) and as mixtures (B). Error bars represent ±1 standard error. Columns with different letters are statistically different (α = 0.05).
Figure 5.5. Mean time to onset of pupation (n = 3) of S. vittatum IS-7 larvae after multiple pulse exposures to chlorpyrifos (chp), carbaryl (carb) and malathion (mal) individually (A) and as mixtures (B). Error bars represent ± 1 standard error. Columns with different letters are statistically different (α = 0.05).
Insecticide Mixtures

Binary and ternary mixtures of the insecticides had no effect on the life history endpoints of *S. vittatum* IS-7 in this study. There were no significant differences in survival, onset of pupation or overall number of larvae pupating among controls and insects exposed to insecticide mixtures (Figures 5.2, 5.5, 5.6).

The quadratic term was determined to be significant in all rates analyzed (p ≤ 0.0008). Thus, the quadratic polynomial model was appropriate for analysis of larval
growth rates in the insecticide mixture experiments. The model accounted for > 93% of the variation in the data ($R^2 \geq 0.9321$). Larval growth rates were not significantly different among treatments or controls (Figure 5.3). Total growth during the experimental period was also shown not to be affected by exposure to the insecticide mixtures (Figure 5.4).

**Discussion**

The purpose of this study was to investigate possible effects of environmentally realistic concentrations of the insecticides chlorpyrifos, carbaryl and malathion in a pulsed exposure scenario on survival, growth and development of *S. vittatum* IS-7. Concentrations of these insecticides in urban watershed streams are typically < 1 µg/L (Hippe et al. 1994, USGS 1998, USGS 1999). However, detection of these insecticides, especially carbaryl, at sub-ppb levels can often be difficult to accurately quantify. Nominal insecticide concentrations ranging from 0.33 - 1 µg/L used in this study resulted in actual insecticide concentrations in the water that were low enough to be considered environmentally realistic and high enough that concentration quantification could be accomplished.

Multiple pulse-exposures of chlorpyrifos, carbaryl and malathion had no effect on life history endpoints of *S. vittatum* IS-7 measured in this study. Although these insecticides have been shown in previous studies to affect life history parameters, higher concentrations as well as longer exposure periods were used (Marian et al. 1983, Seuge and Bluzat 1983, Hermanutz et al. 1985, Muniandy 1987, James and Sampath 1994, Green and Chandler 1996, Ramakrishnan et al. 1997, Chandler and Green 2001,
Callaghan et al. 2001, Fordham et al. 2001, Nguyen and Janssen 2002). It is well known that adverse or toxic effects are not produced by a contaminant unless it reaches the active site at a high enough concentration and for a sufficient amount of time to elicit a response (Eaton and Klaassen 1996). Thus it is likely that the 2 hr pulse exposures of these insecticides were not long enough and/or the exposure concentration not high enough to produce adverse effects in *S. vittatum* IS-7. Exposure time may have had more of an influence, especially in chlorpyrifos exposed larvae, considering the 48 hr LC50 of chlorpyrifos in *S. vittatum* IS-7 is 0.28 µg/L (Chapter 3).

Black fly larvae in this study were exposed to insecticides for 2 hrs followed by 24 or 48 hrs in insecticide-free water. This may have been ample time to fully recover from one exposure to the next, thus negating any cumulative effects. Kallander et al. (1997) reported that two 1 hr pulses of carbaryl were less toxic to the midge, *Chironomus riparius*, than one 2 hr pulse provided that they were held in clean water at least 2 – 6 hrs between doses. In addition, acetylcholinesterase activity in midges given two 1 hr pulses of carbaryl separated by 24 hrs in clean water was reactivated to control levels (Kallander et al. 1997). Likewise, Naddy et al. (2000) showed that *Daphnia magna* could survive two 6 hr pulses of chlorpyrifos at 0.5 µg/L with recovery time between pulses as opposed to one 12 hr pulse.

Experiments with mixtures of the insecticides also had no effect on the life history endpoints of *S. vittatum* IS-7 measured in this study. A previous study showed that the ternary mixture of chlorpyrifos-carbaryl-malathion and the binary mixtures of chlorpyrifos-malathion and carbaryl-malathion were greater than additive in toxicity during 48 hr acute toxicity tests (Chapter 3). However, these results were determined
from larvae exposed to a wide range of mixture concentrations of equitoxic proportion. Thus the length of exposure and concentrations used were quite different between the two studies. At lower concentrations and shorter exposure periods, the interactive toxicity of these insecticides appeared not to be significant.

Results from this study showed that environmentally realistic concentrations of insecticides, individually and as mixtures, had minimal effect on survival, growth and development of the black fly, *S. vittatum* IS-7. The 2 hr exposure period and the ability to recover between exposures may have aided the black flies in overcoming toxic effects of the insecticides. These results also illustrate the importance of concentration, exposure period, and recovery in determining the risks involved with insecticide exposure in aquatic systems.
CHAPTER 6

CONCLUSIONS

This study was designed to investigate the toxic effects of carbaryl, chlorpyrifos and malathion, three lawn-care insecticides commonly detected in urban and suburban watersheds, to Simulium vittatum IS-7 when exposed to the larvae singly and as mixtures. The first set of experiments was designed to determine the 48 hr LC50 values for the individual insecticides for S. vittatum IS-7 larvae using an orbital shaker toxicity test. Results showed chlorpyrifos to be the most toxic to the larvae followed by carbaryl and malathion. The LC50 values for the individual insecticide were then used to determine the concentrations needed to assess impacts of the insecticides as mixtures using the modified toxic unit approach of Marking (1985) as described by Pape-Lindstrom and Lydy (1997) in the orbital shaker testing system. The ternary mixture of chlorpyrifos-carbaryl-malathion and the binary mixtures of chlorpyrifos-malathion and carbaryl-malathion were shown to be greater than additive in toxicity. The mixture of chlorpyrifos-carbaryl, however, was shown to be additive.

In this experiment, the method for determining the joint toxicity of these chemicals used equivalent toxic units of the individual insecticides over a range of concentrations such that a mortality distribution was produced that could be statistically analyzed for estimating a LC50 value. Although this method was useful in determining the toxic nature (i.e. greater than additive, additive, or less than additive) of the insecticides as mixtures, experiments with environmentally realistic concentrations and proportions of these insecticides were needed to better assess the impacts these chemicals
may have on aquatic organisms, such as black flies, inhabiting urban and suburban streams.

The next experiment was designed to assess the influence of food material on the bioavailability of the insecticides in order to determine what concentrations of food could be added to long term experiments with *S. vittatum* IS-7 without altering insecticide toxicity. Results showed that the bioavailability of all three insecticides was significantly altered when laboratory diet concentrations in the flasks were $\geq 150$ mg/L. Chlorpyrifos bioavailability decreased whereas carbaryl and malathion bioavailability increased. Because significant results were observed with the laboratory diet, another experiment with natural seston was conducted to determine if seston could alter bioavailability in a similar manner. Results showed that seston had no effect on the bioavailability of chlorpyrifos or malathion whereas carbaryl toxicity was increased when seston concentrations reached 150 mg/L.

Differences in effects on insecticide bioavailability between the two food types may have been related to the particle size and organic carbon content of the two materials. Bioavailability differences among the three insecticides may have been related to the chemical’s affinity for organic carbon and/or the behavioral response of the larvae to the insecticide. These results indicate that quality and quantity of the food material, as well as the chemical characteristics of the insecticide being tested, are important factors in determining insecticide bioavailability.

The final experiment was designed to assess the effects of pulsed insecticide exposures at environmentally realistic concentrations on survival, growth and development of *S. vittatum* IS-7 larvae. Black fly larvae were exposed to multiple 2 hr
pulse exposures of chlorpyrifos, carbaryl and malathion singly and as mixtures in a trough flow-through dosing system. Results showed that these insecticides had little effect on black fly life history parameters individually or as mixtures. The only significant effect observed was in survival of larvae exposed to chlorpyrifos, which was significantly lower than in those exposed to malathion or carbaryl. However, survival was not significantly lower compared to controls indicating no overall effect of insecticide exposure. No significant effects were observed on growth or development time of the larvae as a result of exposure to these insecticides individually or as mixtures.

The lack of any significant effects from insecticide exposure on black fly life history may have been related to the short exposure period and ability of the larvae to recover from one exposure to the next. Future work with this system should include variation in the exposure time as well as the concentrations of insecticides tested. This would enable the calculation of No Observed Effects Concentrations (NOEC) often used in risk assessments. In addition, assessment of adults emerging from the troughs may provide useful and possibly more sensitive life history information. Parameters such as adult dry weight and energy reserves, and female egg production would provide additional insight into the effects of insecticide exposure on black fly life history.

The experiments conducted in this study have demonstrated the importance of concentration and exposure length in the toxicity of insecticides to aquatic organisms. In equitoxic proportions, certain mixtures of chlorpyrifos, carbaryl and malathion were shown to be greater than additive in toxicity. However at environmentally realistic and equivalent proportions, no effects were observed. While it is often desirable to conduct experiments at levels that produce observed effects, many times these concentrations are
unnaturally high and would rarely be encountered by the organism. Studies in which effects are observed over a range of chemical concentrations and exposure lengths encompassing environmentally realistic scenarios may provide the most useful information when assessing the toxicity of insecticides individually and as mixtures.
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