EFFECTS OF INSECTICIDE AND ADJUVANT MIXTURES ON CLADOCERANS

AND COHO SALMON

By

ANGELA DIANE DEARDORFF

A thesis submitted in partial fulfillment of

the requirements for the degree of

MASTER OF SCIENCE

WASHINGTON STATE UNIVERSITY

Department of Environmental Science

December 2007

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of

ANGELA DIANE DEARDORFF find it satisfactory and recommend that it be

accepted.

_____
Chair

_____

_____

ACKNOWLEDGEMENT

I would like to thank my Advisor, Dr. John Stark for all of his help and advice with my research project and thesis.

I would like to thank my committee members, Dr. Allan Felsot and Dr. Carol Anelli for their guidance in completing my thesis, although we were at a distance.

Thank you to Oriki and Grace Jack who trained me in the laboratory, helped me with my research on numerous occasions, and gave me a lot of advice.

There are many people in my life that have supported me throughout the years, particularly in graduate school. My friends and family members have been very understanding and I always appreciated the much needed phone calls and visits. My parents, Jim and Diane Deardorff have always supported my goals in life, no matter what my goals were. Thank you for the phone conversations, mailing me cookies and cards when I needed them, and for fixing my car on multiple occasions. Thank you to my boyfriend, Ryan Ancesty for pushing me when I needed it and for being so encouraging when I just wanted to quit. Even though my attending graduate school meant that we would be 3,000 miles apart from each other for a few years, you have never stopped believing in me and encouraging me to achieve my goals. Thank you so much.

EFFECTS OF INSECTICIDE AND ADJUVANT MIXTURES ON CLADOCERANS AND COHO SALMON

ABSTRACT

By Angela Diane Deardorff, M.S.

Washington State University

December 2007

Chair: John D. Stark

Acute lethal concentration estimates and instantaneous rates of increase (*ri*) were determined on the cladocerans, *Ceriodaphnia dubia* Richard, *Daphnia pulex* (Leydig), *Daphnia magna* Straus, and Coho salmon, *Oncorhynchus kisutch* (Walbaum) exposed to the agricultural adjuvant, R-11, and the insecticide, spinosad. Mixtures of R-11 and spinosad were also evaluated on *C. dubia* and *O. kisutch* in acute studies. Using acute data and life tables, the accuracy of using one species to predict effects on other species was determined using three species of *Daphnia*.

Acute mortality estimates for R-11 indicated that all LC50 estimates were higher than the Expected Environmental Concentration (EEC) of 0.79 mg/l, for all species

tested. The LC50 estimate for spinosad was much lower than EEC (0.068 mg/l) for *C. dubia* and *D. magna*. Spinosad was not hazardous to *D. pulex* and *O. kisutch* at EEC. The hazard assessments for *C. dubia* and *D. magna* indicated that at EEC, spinosad poses a significant threat.

When *C. dubia* was exposed to a binary mixture of spinosad and R-11 LC25s, it indicated that synergism may have occurred. The mixture of R-11 and spinosad resulted in bottom gilling and erratic swimming behavior in *O. kisutch*, although mortality did not differ from the controls.

The chronic 8-day studies of R-11 and spinosad and *C. dubia* were found to cause extinction at concentrations slightly higher than the acute LC75 and LC70, respectively, indicating that *C. dubia* cannot withstand high levels of mortality without population decline ($ri < 0$).

Although the three daphnid species had similar life history traits, modeling indicated that exposure to EEC resulted in population recovery times of 1, 5, and 12 weeks for *D. pulex*, *D. magna*, and *C. dubia* populations, respectively. Recovery times were equivalent to 0.31, 1.28, and 3.48 generation times for *D. pulex*, *D. magna*, and *C. dubia* populations, respectively. Recoveries > 1 generation time are indicative of damage at the population level. Even though these species had similar life history traits, exposure to the same pesticide concentration resulted in very different recovery times. Therefore, the use of surrogate species to make predictions about even closely related species may not be prudent.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

ACUTE TOXICITY OF SPINOSAD AND R-11 TO THREE CLADOCERAN SPECIES AND COHO SALMON

CHAPTER 4

MODELING THE EFFECTS OF SPINOSAD ON THREE CLADOCERAN SPECIES: CAN SURROGATE SPECIES BE USED TO ACCURATELY PREDICT EFFECTS ON OTHER SPECIES?

LIST OF FIGURES

CHAPTER 3

AN EVALUATION OF THE EFFECTS OF SPINOSAD AND R-11 ON
***CERIODAPHNIA DUBIA* POPULATIONS**

CHAPTER 4

MODELING THE EFFECTS OF SPINOSAD ON THREE CLADOCERAN

SPECIES: CAN SURROGATE SPECIES BE USED TO ACCURATELY

PREDICT EFFECTS ON OTHER SPECIES?

CHAPTER 1

INTRODUCTION

1.1 Pesticides

Pesticides have consistently ranked among the most concerning chemicals that enter the environment since Rachel Carson published the influential *Silent Spring* in 1962. Since then, the awareness of and trepidation about the use of pesticides has significantly increased. Because of the vast amount of pesticides that are manufactured each year, these concerns are not unwarranted. Globally, pesticides account for $32 billion dollars per year, with the United States accounting for nearly a third of global pesticide consumption (Kiely et al. 2004). Although pesticide use briefly declined in the 1980s, their use is increasing once more. The use of herbicides and insecticides on U.S. corn alone has increased by an order of magnitude since the early 1960s (Berenbaum et al. 2000). Although pesticides have been prevalent throughout most of modern time, little is still understood about the potential adverse effects to nontarget species. Types of pesticides include insecticides, herbicides, fungicides, and rodenticides and may be used singly or in combination.

According to a USGS study of pesticides in streams from 1992-2001, approximately 9.6% of agricultural streams, 6.7% of urban streams, and 12.5% of undeveloped streams that were sampled had concentrations of one or more pesticides that exceeded human health standards (Gilliom et al. 2006). In domestic wells and public supply wells, human health standards were only exceeded in 1% of the samples. Pesticide concentrations were often above water quality guidelines for wildlife. For example, in 57% of agricultural streams, 83% of urban streams, 42% of mixed-use streams, and 13% of undeveloped streams, the wildlife standards were exceeded. Bed sediment and fish tissue were also

tested in this study to determine the concentrations of organochlorine pesticides present. Most organochlorine pesticides were banned by 1990, but residues are still present in sediment and fish tissue. Approximately 70% of urban streams, 31% of agricultural streams, 36% of mixed streams, and 8% of streams in undeveloped areas had concentrations of pesticides in bed sediment that exceeded one or more aquatic life benchmarks.

Mixtures of pesticides were found 90% of the time in streams from agricultural, urban, or mixed-land used watersheds to have ≥ 2 types of pesticides or degradates. Approximately 20% of the time, mixtures of ≥ 10 pesticides were detected. Fish tissue and sediment generally contained mixtures of organochlorine pesticides and their degradates and by-products. Mixtures were less common in ground water, but 47% of agricultural shallow wells and 37% of shallow urban wells had ≥ 2 pesticides in them. Less than 1% of wells sampled had ≥ 10. Total combined toxicity of these pesticides is unknown. This study calls for additional research of mixture toxicity because existing water quality standards that use individual toxicities may be insufficient and are not representative of common exposures. Gilliom et al. (2006) also noted that some agricultural adjuvants are of toxicological importance, although they are not addressed in this report.

This thesis will examine if the acute toxicity of spinosad and R-11 to *Ceriodaphnia dubia* Richard, *Daphnia pulex* (Leydig), and *Daphnia magna* Straus, and to Coho salmon, *Oncorhynchus kisutch* (Walbaum), differ when applied in binary mixtures as compared to single chemicals. Also examined were potential differences in the instantaneous rates of increase (*ri*) when *C. dubia* is exposed to single chemicals as

compared to the controls in 8-day studies. Additionally, the use of different species of *Daphnia* as potential cohort species will be evaluated.

1.2 Test Organisms

The aquatic cladocerans *Ceriodaphnia dubia, Daphnia pulex,* and *Daphnia magna* were used in this research study. The lifecycle of these species are well documented and they are often studied as indicator species of environmental contaminants in research and used for regulatory purposes. *Daphnia* are primary consumers, and as such, are an integral part of the food web. *Daphnia* are ubiquitous in the freshwater environment and feed on algae and bacteria. These species are also food for some invertebrate and fish species. A loss of this species would harm higher trophic levels.

Coho salmon, *O. kisutch*, are on the threatened and endangered species list and as such, development of toxicity data for pesticides and adjuvants that they might be exposed to in the wild is essential. The large number of fish species that are listed as threatened or endangered is an indicator of the importance of aquatic toxicological studies. In the United States, well over a hundred species of fish are listed under the Endangered Species Act (ESA) as threatened or endangered (U.S. Fish and Wildlife Service 2007). As a growing number of fish are added to the ESA, it is important to understand how these organisms react to chemicals occurring in their environment. Very little is known about the interactions between pesticides and adjuvants with fish. Pesticides and agricultural adjuvants may pose a significant threat to the health of these fish species when considering mortality, reproduction, and behavior.

1.3 Adjuvants

Agricultural adjuvants are compounds that are added to a pesticide spray tank to modify a pesticide's performance and the physical properties of the spray mixture (Stark and Walthall 2003). The chemical compositions of adjuvants vary and include nonionic surfactants, organosilicone surfactants, and nonionic-organosilicone blends (Stark and Walthall 2003). These same agricultural adjuvants are also part of the formulation of certain pesticides and are designed to act as wetting agents, spreaders, stickers, emulsifiers, dispersing agents, drift control agents, foam suppressors and penetrants. Agricultural adjuvants are not registered under the Federal Insecticide, Fungicide, Rodenticide Act (1996) when used as additives to registered pesticides and, therefore, toxicity data is not required by the United States Environmental Protection Agency U.S.E.P.A. (1990). However, some states have a registration system for spray tank adjuvants that requires submission of toxicity data.

Little is known about the effects of agricultural adjuvants on aquatic organisms. However, some of these products have been found to be toxic to certain species (Sharma and Singh 2001, Henry et al. 1994, Abdelghani et al. 1997, Purcell and Schroeder 1996, Liu and Stansly 2000, Mangan and Moreno 2001, Stark and Walthall 2003). Some adjuvants, including R-11, act as xenoestrogens and have an effect on reproduction.

1.4 Pesticides and Adjuvants

Relatively little research has been conducted on the effects of mixtures of pesticides and adjuvants. Pesticides and adjuvants may be present together in surface water

systems. However, little is known about toxic interactions between these compounds. The actual effects of these chemicals in nature are, for the most part, undocumented and have not been quantified. Few investigations have been conducted regarding lethal and sublethal effects of these chemicals, and past publications have not generally investigated the effects of mixtures of these chemicals. Sublethal effects are of particular concern because symptoms do not necessarily become evident immediately. Sublethal effects for many of these chemicals have not yet been determined. These effects can be significant, especially when they alter an organism's fecundity.

Although pesticides and adjuvants may regularly exist in the environment at low levels, fluctuations of these chemicals may occur several times per year. Seasonal patterns in pesticide and adjuvant concentrations in the water correlated with timing and the amount used in application, frequency/amount of runoff, and timing/distribution of land-management practices (e.g. irrigation) (Gilliom et al. 2006). This fluctuation in chemical concentration may occur several times per year and may adversely impact aquatic organisms at certain times of the year more than others. Chemicals may also reach the water through atmospheric deposition and leaching through the soil.

Researching the interactions of chemical mixtures is important because chemicals usually exist in the water in combinations of two or greater (Gilliom et al. 2006). This will allow us to glean a more realistic representation of the effects of these chemicals on aquatic organisms in situ.

1.5 Chemical Mixtures

Pesticides occurring in nature are normally not present individually, but in complex mixtures (Gilliom et al. 2006). Mixtures of chemicals at low levels are constantly present in the aquatic environment. These mixtures may or may not interact with each other, causing a host of various outcomes in situ (Lydy et al. 2004). It is largely unknown how these mixtures impact aquatic life, although regulations on water quality only account for single chemicals. Mixtures of chemicals have the potential to exacerbate the living conditions of aquatic organisms, causing them to exhibit stress at relatively low concentrations of chemicals. Chemicals have various modes of action, and these differences may increase the potential for mortality and sublethal effects, such as a decline in fecundity. Chemicals may interact with each other, causing a greater than additive impact, which significantly increases the toxicity of the compounds (synergism). These chemicals may also have a cumulative or additive effect in which the toxicity of an individual compound is, essentially, added to each compound in the mixture. For example, chemicals with similar modes of action often exhibit additive toxicity (Lydy et al. 2004). Conversely, these mixtures also may interact antagonistically, which decreases the toxicity of the compounds as compared to additive toxicity.

Because of the possibility of these types of interactions, it is essential that we learn more about the interactions between mixtures of pesticides and adjuvants. Many papers have been written on the effects of mixtures of pesticides and adjuvants on aquatic organisms. Results from each paper vary depending on the mixtures of chemicals and test organisms used.

A literature review of 26 studies was conducted by Deneer (2000) in order to determine if mixtures of pesticides normally exhibit concentration addition. However,

little is known about toxic interactions between these chemicals. Of the 202 mixtures analyzed in this study, over 90% followed the concentration addition model. The remainder of the mixtures exhibited both synergistic and antagonistic effects. About half of the combinations of chemicals that exhibited greater than additive toxicity were organophosphorus esters, carbamates, and pyrethroids, which are commonly used pesticides.

1.5.1 Effects of Chemical Mixtures on Daphnia spp.

Barata et al. (2006) evaluated the effects of the metals cadmium and copper, and the pyrethroid insecticides λ-cyhalothrin and deltamethrin singly and in binary mixtures on *Daphnia magna*. When the chemicals were tested singly, cadmium was found to be more toxic to *D. magna* than copper. Both of the insecticides were found to be extremely toxic with EC50s ranging between 0.1 to 0.22 ng/l. All of the chemicals were found to exhibit the sublethal effect of feeding inhibition. The chemicals were combined in eight binary mixtures. The results showed that the chemicals exhibit additive toxicity. In three out of the eight tested mixtures, toxic interactions were observed among the chemicals. The ratio of the chemicals tested seemed to be important to the amount of toxicity observed. It appeared as though higher concentrations of the insecticides and lower concentrations of the metals corresponded to a mixture with higher toxicity. This is in accordance with the toxicity that was observed when the chemicals were tested singly.

An additive response of diazinon and chlorpyrifos on *Ceriodaphnia dubia* was found in a study by Bailey et al. (1997). Diazinon and chlorpyrifos were studied jointly because they are often found in the same waterways. In this study, tests were conducted in

laboratory water, natural water, and storm water. Values for all types of water were approximately the same and all exhibited additive behavior based on the toxic unit (TU) approach.

Diazinon, when combined with ammonia, was found to have less than additive (antagonistic) toxicity against *Ceriodaphnia dubia* (Bailey et al. 2001). Diazinon and ammonia are commonly found in the environment together. Ammonia is present because it is a byproduct of organic material degradation. The data that were developed suggested that both diazinon and ammonia have some toxic effects. The actual toxicity values for diazinon and ammonia combined were about 30% less than the predicted values for additive toxicity.

In a study by Olmstead and LeBlanc (2005), the effects of realistic concentrations of chemical mixtures involving nine chemicals, were determined for *Daphnia magna*. Of the nine chemicals tested, six are commonly used as pesticides or agricultural adjuvants (carbaryl, chlorpyrifos, N,N-Diethyl-m-toluamide, diazinon, 1,4-dichlorobenzene, and 4-nonylphenol). The other chemicals tested were bisphenol A, caffeine, and fluoranthene. When tested singly, carbaryl, chlorpyrifos, and diazinon all exhibited a concentration dependent reduction in lifespan. A reduction in lifespan was not observed in the 4-nonylphenol groups after exposure to concentrations less than 200 µg/l. However, exposure to 200 µg/l resulted in 100% mortality. All other chemicals did not reduce lifespan at the concentrations tested. It was found that when in combination, chemicals exerted a greater effect than when applied singly. In fact, a model accurately calculated (r^2=0.976) that if all chemicals were combined at concentrations less than 10 times that found in the water samples (median values), 100% of *D. magna* would perish after 24 h.

This differed significantly from the experiments in this study that tested only one chemical at a time.

A study by Shaw et al. (2006) investigated the effects of cadmium and zinc singly and in mixtures to four species of daphnids, *Ceriodaphnia dubia*, *Daphnia magna*, *Daphnia ambigua*, and *Daphnia pulex*. It was found that *D. magna* was more tolerant of single metals as compared to the responses of other daphnids. Mixtures of cadmium and zinc were tested in combinations of LC15, LC50, and LC85 values for each metal. When LC15 combinations of both metals were tested, all daphnids had similar responses. This also occurred with most samples tested at concentrations equivalent to the acute LC15 for cadmium mixed with LC50 and LC85 values of zinc. Less than additive effects were observed when LC15 concentrations of zinc were mixed with LC50 and LC85 concentrations of cadmium. Tests with *D. magna* showed that an additive model was followed for most combinations, which differed from the other species. This study showed that *D. magna* exhibited different responses to cadmium and zinc than other daphnids.

A study by Xie et al. (2006) examined the effects of copper, phenanthrene, and 9,10-phenanthrenequinone on *Daphnia magna*. The latter two chemicals are polycyclic aromatic hydrocarbons. Copper was found to be the most toxic substance of the three, although when copper was combined with phenanthrenequinone, the toxic effect was much greater. The combination was found to produce a significant amount of reactive oxygen species. When ascorbic acid was added to the mixture, the effects of reactive oxygen species decreased. The copper and phenanthrene mixture resulted in additive toxicity.

1.5.2 Effects of Chemical Mixtures on Fish

    The acute toxicity of the herbicides Roundup, Garlon-3A, and 2,4-D, and the chemical additive Syndets surfactant were evaluated singly and in binary mixtures to channel catfish, bluegill sunfish, and crawfish (Abdelghani et al. 1997). When evaluated singly, it was determined that the toxicity of Roundup was greater than Garlon-3A which is greater than 2,4-D for the two species of fish, however, this was reversed for the crawfish. This indicated that there was some variability in the toxicity of chemicals to vertebrates and invertebrates. Syndets surfactant was found to be significantly more toxic than the three herbicides tested in all species tested. Mixtures of herbicides were found to exhibit additive or slightly less than additive toxicity, although lethal concentrations of the mixtures were found to be less than the recommended application rate. The authors concluded that although mixtures were toxic to the tested organisms at concentrations lower than those recommended for field application, the mixtures would likely not enter waterways at those levels because they would be diluted.

    It was determined that when testing a mixture of a pyrethroid (esfenvalerate) and an organophosphate (chlorpyrifos), the organophosphate increased the toxicity of the pyrethroid on the fathead minnow (*Pimephales promelas*) (Belden and Lydy 2006). This synergistic effect was anticipated because organophosphate insecticides have been known to produce a similar effect on pyrethroid toxicity probably because both types of chemicals are neurotoxins and result in hypersensitivity of the nervous system. The toxicity of these pesticides on midge larvae (C*hironomus tentans*) was close to an

additive effect. Pyrethroids and organophosphates are the most common insecticides to be found in surface water, and are often used under similar circumstances.

The combined effects of five known estrogenic chemicals were evaluated on fathead minnows (*Pimephales promelas*) (Brian et al. 2005). Estradiol, ethynyl estradiol, nonylphenol, octylphenol, and bisphenol A were evaluated singly and in equipotent mixtures in order to see if the chemicals, when in a mixture, deviated from their predicted toxicity. It was determined that these chemicals exhibited additive toxicity, as their toxicities did not differ from the additive prediction. It was concluded in this paper that estrogenic chemicals, because of their similar modes of action, exhibit predictable additive toxicity.

Mixtures of chemicals at low concentrations that included toxicants with narcosis I, narcosis II, and oxidative phosphorylation uncoupler modes of action were tested on the fathead minnow (*Pimephales promelas*) were found to follow the concentration addition model, even at very low concentrations (Broderius et al. 2005). Lethal and sublethal effects were also observed at very low concentrations. The concentrations of chemicals tested were close to the NOEL and previously were not believed to have an adverse effect at those concentrations. Some of the mixtures tested were composed of chemicals that had similar modes of action, which normally will follow a concentration addition model. When chemical mixtures of dissimilarly acting compounds were tested, the results were less predictable. The results indicated that the effect was less than additive as compared to the concentration addition model, but the independent action/response addition model showed that the combinations were more toxic than anticipated. Toxicity curves were similar for chemical mixtures that had similar modes of action.

A study on the effects of a mixture of methyl tert-butyl ether (MTBE) and fluoranthene on the fathead minnow (*Pimephales promelas*) found that the presence of MTBE exacerbated the effects of fluoranthene (Cho et al. 2003). MTBE is used as an additive in reformulated gasoline and fluoranthene is one of the most commonly found polycyclic aromatic hydrocarbons found in exhaust from an internal combustion engine. MTBE and fluoranthene are commonly found together in the aquatic environment. The mortality of fathead minnows that were exposed to MTBE by itself did not differ from the controls.

Esfenvalerate, a pyrethroid, and diazinon, an organophosphate, were found to act synergistically with larval fathead minnows (Denton et al. 2003). This synergistic effect was found to be possible because diazinon first inhibits carboxylesterases and carboxylesterases would normally detoxify esfenvalerate. This leaves the organism as a more receptive target to the pesticide mixture. This mode of action appears to be true for mixtures of organophosphates and pyrethroids.

Acute and long-term effects of mixtures of 3,4-dichloroaniline and lindane were observed on zebrafish (*Brachydanio rerio*) (Ensenbach and Nagel 1995). Chemicals tested singly were conducted in tap water and water from the Rhine River. Mixture toxicity was only tested in tap water. When tested singly and in water from the Rhine River, lindane affected the early lifecycle of the zebrafish at 80 µg/l, whereas 3,4-dichloroaniline did not have any significant effects. Water origin did not appear to affect the results of the tests. In an acute test, it was observed that the mixture exhibited additive toxicity. When zebrafish were tested in a mixture containing concentrations as low as 2 µg/l 3,4-dichloroaniline and 40 µg/l lindane, growth and development of their

early stages were affected, indicating that a mixture of these compounds may increase the toxicity of each respective chemical.

The effects of mixtures of 3,4-dichloroaniline and lindane were observed on the reproduction of the zebrafish (*Brachydanio rerio*) (Ensenbach and Nagel 1997). The lengths and weights of the F_1- and F_2- generation zebrafish were reduced as compared to controls even at the lowest concentrations tested (2 μg/l 3,4-dichloroaniline and 40 μg/l lindane). If the zebrafish were exposed for their entire life to the mixture and the concentration of the mixture increased over time, at 100 μg/l 3,4-dichloroaniline and 40 μg/l lindane, zebrafish irreversibly stopped spawning. Zebrafish that had not been exposed to the mixture throughout their lifetime were also exposed to a mixture of 100 μg/l 3,4-dichloroaniline and 40 μg/l lindane and resulted in a reduction in egg production. Egg production completely ceased in zebrafish after exposure to concentrations of 200 μg/l 3,4-dichloroaniline and 40 μg/l lindane. These reproductive effects may be observed at concentrations that are present in the environment and may result in extinction over time.

It was discovered that when tributyltin (TBT) and polychlorinated biphenyls (PCBs) were introduced into the environment of the Japanese medaka (*Oryzias latipes)*, they exhibited a greater toxicity than would be achieved if the fish were exposed to each chemical alone (Nakayama et al. 2005). When used alone and administered to fertilized eggs, TBT caused abnormal development of the eyes, decreased the number of eggs that hatch, and caused an increase in swim-up failure. PCBs can increase the amount of time it takes for the medaka to hatch. When PCBs were combined with TBT in this study, the resulting toxicity was synergistic as compared to the effects of the chemicals tested

14

singly. For example, when the developing embryos were given a dose of 1 μg TBT g^{-1}, no toxic effects were observed. However, after adding PCB to 1 μg TBT g^{-1}, abnormal eye development resulted. The use of PCBs alone did not cause abnormal eye development, which indicates that PCBs increase the toxicity of TBT. The addition of 5 or 25 μg TBT g^{-1} to PCBs also increased the time to hatch, indicating that TBT may also interact with PCBs and increase their toxicity.

An additive relationship was discovered between organophosphate (chlorpyrifos oxon, diazinon-O-analog, and malaoxon) and carbamate insecticides (carbaryl and carbofuran) on Chinook salmon (Scholz et al. 2006). The oxon form of a pesticide is a byproduct of degradation and is often found to be more toxic to living organisms than its parent compound. All tested pesticides function as acetylcholinesterase (AChE) inhibitors. The additive effect was caused by their common mode of action, which acted as a combined stronger dose of an AChE inhibitor. These compounds are commonly found together in waterways, so these effects may be observed in situ.

Teuschler et al. (2005) investigated the effects of binary mixtures of benzene and toluene on the development of the medaka (*Oryzias latipes*) heart. The endpoints in the embryonic medaka included heart rate, heart rate progression, and lethality. The results were compared to an additive response model. A synergistic relationship was observed with the heart rates of the exposed medaka after 72 h, meaning that their heart rates were greatly reduced as compared to the additive model. An additive or antagonistic relationship was seen with heart rates at 96 h. An antagonistic association was observed for all concentrations on the heart rate progression and for lethality.

1.5.3 Effects of Chemical Mixtures on Other Species

   The effects of atrazine in binary combination with the organophosphates chlorpyrifos, methyl parathion, and diazinon were observed in *Hyalella azteca* (aquatic amphipod) and *Musca domestica* (domestic housefly) (Anderson and Lydy 2002). The organophosphates were tested in concentrations associated with their respective lethal concentrations 1, 5, 15, and 50. Atrazine concentrations tested were 0, 10, 40, 80, and 200 µg/l for *H. azteca* and 0, 200, and 2,000 ng/mg for *M. domestica*. When testing concentrations of atrazine that were ≥ 40 µg/l in combination with organophosphates, the toxicity in *H. azteca* was greatly increased. The mixtures also caused a decrease in acetylcholinesterase activity as compared to the effects of single chemicals. *H. azteca* were also pretreated with atrazine in order to determine if a significant difference would occur in the toxicity of the organophosphates. *H. azteca* did appear to be more sensitive to the organophosphates after pretreatment to at least 40 µg/l atrazine. *M. domestica* did not exhibit any differences when tested with single chemicals and mixtures.

   Belden and Lydy (2000) studied the effects of the herbicide atrazine on the insecticides chlorpyrifos, methyl parathion, diazinon, and malathion in binary mixtures on midge larvae (*Chironomus tentans*). Atrazine was added to four concentrations of the other insecticides at the environmentally relevant concentration of 40 µg/l. This concentration of atrazine is much lower than would be expected to cause acute toxicity. In fact, even at 10,000 µg/l, atrazine does not cause acute toxicity. Atrazine interacted with all of the insecticides at all concentrations tested, except for malathion, and caused

16

synergistic reactions. Atrazine increased the toxicity of chlorpyrifos fourfold, while it doubled the toxicity of methyl parathion and diazinon. It is hypothesized that atrazine may have a sublethal effect which may influence the toxicity of some chemicals. All of the chemicals, except for malathion, are of the same class of chemical compounds. They are phosphorodithioates with aromatic side chains. Malathion is a phosphorodithioate with an aliphatic side chain. This difference may prevent atrazine from modifying its toxicity and may be a potential reason for the lack of a synergistic reaction between atrazine and malathion.

When the herbicide Fomesafen was applied to a freshwater pond along with the adjuvant Agral 90, phytoplankton and zooplankton ratios were altered (Caquet et al. 2005). Overall, phytoplankton growth increased. Of 65 phytoplankton taxa present, only one (*Chlorophyceae*) was inhibited by the mixture. Less sensitive species of phytoplankton increased in numbers because of the decline of *Chlorophyceae*. Of the 20 zooplankton species that were identified in the experiment, calanoid copepods were the only zooplankton species that were detrimentally affected by the mixture. The zooplankton community predominantly consisted of nonpelagic rotifers. Normally, rotifers do not compete well with other species, but after the decrease in calanoid copepod species, they were able to proliferate. The toxicity of a mixture of Fomesafen and Agral 90 significantly increased as compared to the toxicity of individual use. A change to the ratios of phytoplankton and zooplankton in their respective communities was observed. An alteration in phytoplankton and zooplankton species may change the overall species infrastructure of aquatic environments because of the change in species availability at the bottom of the food web.

The toxicity of three pesticides (atrazine, chlorpyrifos, and chlorothalonil) individually and in mixtures (atrazine-chloryrifos and atrazine-chlorothalonil) was evaluated in the phytoplankton species *Dunaliella tertiolecta* (DeLorenzo and Serrano 2003). Atrazine and chlorothalonil decreased the *D. tertiolecta* populations at concentrations of ≥ 25 µg/l and 33.3 µg/l, respectively. Chlorpyrifos toxicity is not expected to be observed naturally because only at concentrations ≥ 400 µg/l did significant toxicity occur to *D. tertiolecta*. The mixture of atrazine and chlorpyrifos exhibited additive toxicity and the mixture of atrazine and chlorothalonil exhibited synergistic toxicity. The EC50 of the mixture of atrazine and chlorpyrifos occurred when concentrations were 27 and 438 µg/l, respectively. The EC50s of atrazine and chlorothalonil were 18 µg/l for each chemical, making the combination 1.83 times more toxic than if the chemicals were tested individually. Atrazine is a common contaminant in the water and the occasional addition of cholorothalonil to water may prove toxic for *D. tertiolecta*. This would ultimately impact higher trophic levels if a significant portion of the phytoplankton succumbed to the toxic effects of the mixture.

Joint toxicity of binary mixtures of arsenic, copper, or cadmium with carbofuran, dichlorvos, or malathion was determined on the copepod, *Tigriopus brevicornis* (Forget et al. 1999). Nine mixtures were studied and 96 h LC50 tests were employed along with a measure of acetylcholinesterase inhibition. Mixtures included binary combinations of copper, dichlorvos, carbofuran, arsenic, malathion, and cadmium. After using a toxic unit approach, it was determined that in all samples, the combined chemicals interacted synergistically. Metals and pesticides have been found to exhibit synergistic effects in

other studies. Mixtures of copper-malathion and cadmium-malathion were found to be the most toxic mixtures as compared to all other mixtures tested.

The effects of 17β-estradiol (E_2) in combination with cadmium on the embryos of the amphibian *Bufo arenarum* were observed in Fridman et al. (2004). E_2 itself can be lethal to *B. arenarum* embryos. The LC50 after 48 h of exposure to E_2 was 36.5 μmol/l and the NOEC was 10 μmol/l. Three concentrations of E_2 (0.1, 1, and 10 μmol/l) interacted with 1 mg/l cadmium and increased the toxicity of cadmium. For each concentration of E_2, the lethality of the mixture to the embryos was 76.7, 80, and 83.3%, respectively. This level of toxicity was observed for the first three days of the experiment, after which the toxicity of the mixture decreased. At seven days, the embryos exhibited no difference between the samples treated with E_2 plus cadmium and those treated with only cadmium. Estradiol is a common contaminant in the water, as is cadmium. If the presence of estradiol can significantly increase the toxicity of cadmium on *B. arenarum*, the toxicity may be greater than would be predicted based on data from each individual chemical.

Mixtures containing an organophosphate and a carbamate were tested on adult Long-Evans rats in order to determine if there is a relationship between hypothermia and cholinesterase inhibition (Gordon et al. 2006). Organophosphates and carbamates are anticholinesterase chemicals and hypothermia may be used as an endpoint to exposure of this class of chemicals. The ratios of chemicals were given in 2:1 and 1:1 ratios (chlorpyrifos:carbaryl). The other endpoints used in this study are the inhibition of cholinesterase activity in the brain and plasma. Brain and plasma cholinesterase levels resulting from the 2:1 mixture showed that this ratio of concentrations exerted additive properties. The temperature study indicated that the mixture of pesticides exerted an

antagonistic relationship as compared to chemicals tested singly. The 1:1 ratio resulted in a significant antagonistic relationship between the chemicals in the brain and plasma. The 1:1 ratio also resulted in an additive relationship regarding body temperature. These inconsistencies denote a problem with associating body temperature with amount of cholinesterase inhibition.

Howe et al. (1998) reported a synergistic action after exposing the northern leopard frog (*Rana pipiens*) American toad (*Bufo americanus*) to a mixture of alachlor and atrazine. Both early and late larval stages were tested. Tests were also conducted on rainbow trout and channel catfish in order to make a comparison in levels of toxicity between the species. A 1:1 mixture of the two pesticides was found to exhibit greater than additive toxicity to most species and life stages of organisms tested. The older amphibian larvae tended to be more sensitive than the younger larvae. The older larvae of the American toad had a 96 h LC50 value of 1.5 mg/l and the younger larvae of the species had a 96 h LC50 of 1.8 mg/l. The late larvae of the northern leopard frog had a 96 h LC50 of 2.1 mg/l and the early larvae had an LC50 of 6.5 mg/l. The rainbow trout and channel catfish were generally more tolerant of the chemicals than either stage of amphibian larvae, with LC50 values of 6.5 mg/l and 7.5 mg/l respectively.

The effects of mixtures of metals were determined on various aquatic organisms naturally present in the water in a mesocosm experiment (Jak et al. 1996). It was determined that *D. magna* was more tolerant of mixtures of metals than other species based on published toxicity values. The metals evaluated were added as salts and included As_2O_5, $CdCl_2$, $CrCl_3$, $CuCl_2$, $Hg(NO_3)_2$, $NiCl_2$, $Pb(NO_3)_2$, and $ZnCl_2$. *Ceriodaphnia dubia* that were present in the study were severely affected by the addition

of metals and their populations dramatically decreased. *Daphnia cucullata* that were present in the experiment did not appear to be as sensitive as some other species with increasing metal concentrations. *Bosmina* species were not present in large numbers throughout most of the experiment, probably due to their low intrinsic rate of increase. Calanoid copepods and rotifer *Brachionus* species also were not abundant throughout the experiment. This experiment examined naturally occurring species in their own environment with the addition of mixtures of metals that can be present in the environment. Mesocosm experiments are important because they do not require extrapolation from the laboratory to real life conditions.

Jin-Clark et al. (2002) studied the effects of atrazine and cyanazine on chlorpyrifos in binary mixtures. Tests were conducted on the aquatic midge, *Chironomus tentans*. When applied singly, none of the chemicals tested exhibited a high level of toxicity. When applied in binary mixtures, atrazine increased the toxicity of chlorpyrifos by 1.8-fold. Cyanazine increased the toxicity of chlorpyrifos by 2.2-fold. It does not appear that the addition of atrazine and cyanazine altered the mechanism of action of chlorpyrifos, but instead increased the toxicity of chlorpyrifos. Mixtures of atrazine or cyanazine and chlorpyrifos in vivo inhibited acetylcholinesterase activity. Both mixtures exhibited synergistic activity against acetylcholinesterase functioning. When this study was conducted on *C. tentans* acetylcholinesterase in vitro, the results were different and did not show a synergistic reaction.

The effects of mixtures of chemicals in the soil were examined on the nematode *Caenorhabditis elegans* (Jonker et al. 2004). Chemicals were studied individually and in binary mixtures that include copper-zinc, copper-cadmium, cadmium-lead, copper-

carbendazim, and copper-carbendazimiprodione. Carbendazim and carbendazimiprodione are both fungicides. Metals and fungicides are often found together in the soil. An antagonistic relationship between the chemicals and *C. elegans* was found at low concentrations and synergism was detected at concentrations higher than the EC50. Conclusive observations regarding interactions in the soil and toxicity were not found in this study.

Binary mixtures of nine common pesticides found in the Sacramento-San Joaquin Delta were tested on the midge, *Chironomus tentans* (Lydy and Austin 2004). Chemicals tested included the organophosphate insecticides azinphos methyl, chlorpyrifos, diazinon, and methidathion, the triazine herbicides cyanazine and simazine, the triazinone herbicide hexazinone, the organochlorine DDE, and the substituted urea herbicide diuron. Binary tests were conducted over a 96 h time period. Most of the mixtures tested resulted in additive responses from *C. tentans*, however when some organophosphate insecticides were mixed with some herbicides, synergistic toxicity was observed. For example, even low concentrations of hexazinone (25 µg/l) greatly increased the toxicity of methidathion. However, the toxicity of azinphos methyl was not increased by any of the herbicides tested and cyanazine was the only herbicide that increased the toxicity of diazinon. Midges that were pre-exposed to DDE did not exhibit a more toxic response upon exposure to organophosphate insecticides than midges that were not pre-exposed to DDE.

The effects of mixtures of the organophosphates chlorpyrifos and malathion and the carbamate carbaryl were determined on the black fly larvae, *Simulium vettatum* IS-7 (Overmyer et al. 2003). When all three insecticides were combined, the effects were found to be greater than additive. The tertiary mixture exhibited greater toxicity (lower

LC50 measurements) than binary mixtures. The increased mortality as observed in the binary mixtures followed an additive model. This indicates that if all of these compounds are present in the same waterway, aquatic invertebrates may experience the effects of synergistic toxicity.

Sublethal concentrations of atrazine, in combination with organophosphate insecticides (chlorpyrifos, malathion, methoxychlor, methyl-parathion, mevinophos, and trichlofon) were tested to evaluate the toxicity on the midge *Chironomus riparius* (Pape-Lindstrom and Lydy 1997). The combinations of atrazine-methoxychlor and atrazine-mevinophos produced less than additive results. A combination of atrazine, methyl-parathion, and methoxychlor resulted in weakly synergistic effects. All other binary combinations resulted in statistically significant synergistic reactions.

Mixtures of chemicals are inherently difficult to regulate, as shown in Pounds et al. (2004). The effects of binary mixtures consisting of cadmium, mercury, supermercury (Hg_2), methylmercury, and trimethyltin were studied on cultured murine renal cortical cells because metals target these types of cells. The results were juxtaposed with three models, Carter's statistical isobologram, Barton's nonlinear, and Kodell and Pounds' linear models. After testing, some combinations of metals had similar readings from each of the models, although others exhibited significant differences. Differences in models may create the formation of improper conclusions about the interactions between chemicals, even if all models exhibit a good fit to the data. In order to classify the toxicity of chemical combinations, models are used to determine if the mixture exhibits antagonistic, additive, or synergistic behavior. If models are not appropriate, incorrect conclusions may be formed. All models indicated that the mixture of supermercury-

mercury interacted synergistically. The mixtures of mercury-cadmium and methyl mercury-trimethyltin both exhibit additive or synergistic toxicity, depending on the model used.

The toxicity of sublethal concentrations of the herbicide atrazine and nitrate on the African clawed frog (*Xenopus laevis*) was examined in Sullivan and Spence (2003). Toxicity was evaluated singly and in binary combinations. At higher concentrations of atrazine, the mean weight of tadpoles at metamorphosis increased, as did the time it took to reach metamorphosis. Increasing levels of nitrate correlated positively with the mean weight of the tadpoles at metamorphosis. At sublethal levels, atrazine appears to have a significant negative impact on the tadpole's development. The combination of atrazine and nitrate had a significant impact on tadpole weight and snout-vent length at metamorphosis, although the effect was variable. At lower concentrations of the chemical mixture, the effect appeared to be synergistic. At the highest concentrations, an antagonistic relationship was observed.

Polycyclic aromatic hydrocarbons (PAHs) were studied individually and in binary mixtures in order to determine their neurotoxicity to SY5Y human neuroblastoma cells and C6 rat glioma cells (Tang et al. 2003). Both of these types of cells have been found to be sensitive markers of neurotoxicity. The chemicals tested included benzo[a]pyrene, chrysene, anthracene, and pentachlorophenol. The mixtures of benzo[a]pyrene plus chrysene and benzo[a]pyrene plus pentachlorophenol did not exhibit a greater toxicity to the SY5Y cells than from benzo[a]pyrene alone. In C6 cells, an additive cytotoxic response for both mixtures was observed at concentrations less than those from the individual chemicals. Anthracene did not exhibit toxicity at any concentration tested and

was not included in the mixture study. It was found that PAHs probably only cause neurotoxicity when their concentrations are near their solubility limits.

Van der Geest et al. (2000) studied the effects of mixtures of copper and diazinon to mayfly (*Ephoron virgo*) larvae in order to determine if the result exhibited additive toxicity. The mortality of the larvae was used as the endpoint in this 96 h study. The concentration of copper was 77 μg/l and for diazinon it was 11.8 μg/l. The ratio of diazinon:copper in this study, according to toxic units, was 3:2. It was determined that the result of the mixture is significantly antagonistic because the toxic unit value was 1.3. A toxic unit of 1 indicates additivity. In studies using similar chemicals, differing outcomes have been observed, including synergistic toxicity. This difference may be due to the chemicals and/or species evaluated.

1.6 Research Objectives

The objectives of this thesis were to:

1. determine the acute toxicity of R-11 and spinosad with 48 h mortality studies on *C. dubia*, *D. pulex*, and *D. magna* and through 96 h mortality studies on Coho salmon, *O. kisutch*. The acute LC50 estimates were also compared to the expected environmental concentrations (EEC) to determine the potential hazard that these chemicals might pose to each species.

2. determine if R-11 and spinosad cause population level effects on *C. dubia* through a series of 8-day experiments. The acute LC50 estimates and instantaneous rate of increase (*ri*) were also compared for each chemical tested in order to see how closely the acute and chronic toxicity data correlate with each other.

3. determine how accurately one species could be used to predict what happens to another species after exposure to toxicants. This was accomplished by evaluating the effects of spinosad on populations of daphnids using life table data and a population modeling approach.

1.7 References

Abdelghani AA, Tchounwou PB, Anderson AC, Sujono H, Heyer LR, and Monkiedje A. 1997. Toxicity evaluation of single and chemical mixtures of Roundup, Garlon-3A, 2,4-D, and Syndets Surfactant to Channel Catfish (*Ictalurus punctatus*), Bluegill Sunfish (*Lepomis microchirus*), and Crawfish (*Procambarus* spp.). *Environmental Toxicology and Water Quality* 12(3): 237-243.

Anderson TD, Lydy MJ. 2002. Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. *Environmental Toxicology and Chemistry* 21(7): 1507-1514.

Bailey HC, James RK, Elphick R, Mulhall AM, Hunt P, Tedmanson L, and Lovell A. 2000. Whole effluent toxicity of sewage treatment plants in the Hawkesbury-Nepean watershed, New South Wales, Australia, to *Ceriodaphnia dubia* and *Selenastrum capricornutum*. *Environmental Toxicolology and Chemistry* 19:72– 81.

Bailey HC, Miller JL, Miller MJ, Wiborg LC, Deanovic L, and Shed T. 1997. Joint acute toxicity of diazinon and chlorpyrifos to *Ceriodaphnia dubia*. *Environmental Toxicology and Chemistry* 16(11): 2304-2308.

Barata C, Baird DJ, Nogueira AJA, Soares AMVM, and Riva MC. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. *Aquatic Toxicology* 78(1): 1-14.

Belden JB, Lydy MJ. 2000. Impact of atrazine on organophosphate insecticide toxicity. *Environmental Toxicology and Chemistry* 19(9): 2266-2274.

Belden JB, Lydy MJ. 2006. Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. *Environmental Toxicology and Chemistry* 25(2): 623-629.

Berenbaum M, Brusseau M, Dipietro J, Good R, Gould F, Gunsolus J, Hammock BD, Hartung R, Marrone P, Maxwell B, Raffa K, Ryals J, Shaner D, Seiber J, and Zilberman D. 2000. *The Future Role of Pesticides in U.S. Agriculture*. National Research Council. Washington, D.C.

Brian JV, Harris CA, Scholze M, Backhaus T, Booy Petra, Lamoree M, Pojana G, Jonkers N, Runnalls T, Bonfà A, Marcomini A, and Sumpter JP. 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environmental Health Perspectives* 113(6): 721-728.

Broderius SJ, Kahl MD, Elonen GE, Hammermeister DE, and Hoglund MD. 2005. A comparison of the lethal and sublethal toxicity of organic chemical mixtures to the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 24(12): 3117-3127.

Caquet T, Deydier-Stephan L, Lacroix G, Le Rouzic B, and Lescher-Moutoué F. 2005. Effects of fomesafen, alone and in combination with an adjuvant, on plankton communities in freshwater outdoor pond mesocosms. *Environmental Toxicology and Chemistry* 24(5): 116-1124.

Carson R. 1962. *Silent Spring*. Boston: Houghton Mifflin.

Cho E, Bailer J, and Oris JT. 2003. Effect of methyl *tert*-butyl ether on the bioconcentration and photoinduced toxicity of fluoranthene in fathead minnow larvae (*Pimephales promelas*). *Environmental Science and Technology* 37(7): 1306-1310.

DeLorenzo ME, Serrano L. 2003. Individual and mixture toxicity of three pesticides: atrazine, chlorpyrifos, and chlorothalonil to the marine phytoplankton species *Dunaliella tertiolecta*. Part B—Pesticides, Food Contaminants, and Agricultural Wastes. B38(5): 529-538.

Deneer JW. 2000. Toxicity of mixtures of pesticides in aquatic systems. *Pest Management Science* 56(6): 516-520.

Denton DL, Wheelock CE, Murray SA, Deanovic LA, Hammock BD, and Hinton DE. 2003. Joint acute toxicity of esfenvalerate and diazinon to larval fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 22(2): 336-341.

Ensenbach U, Nagel R. 1995. Toxicity of complex chemical mixtures: acute and long-term effects on different life stages of zebrafish (*Brachydanio rerio*). *Ecotoxicology and Environmental Safety* 30(2): 151-157.

Ensenbach U, Nagel R. 1997. Toxicity of binary chemical mixtures: effects on reproduction of zebrafish (*Brachydanio rerio*). *Archives of Environmental Contamination and Toxicology* 32(2): 204-210.

Forget J, Pavillon J-F, Beliaeff B, and Bocquene G. 1999. Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepoda, Harpacticoida). *Environmental Toxicology and Chemistry* 18(5): 912-918.

Fridman O, Corró L, and Herkovits J. 2004. Estradiol uptake, toxicity, metabolism, and adverse effects on cadmium-treated amphibian embryos. *Environmental Health Perspectives* 112(8): 862-866.

Gilliom RJ, Barbash JE, Crawford CG, Hamilton PA, Martin JD, Nakagaki N, Nowell LH, Scott JC, Stackelberg PE, Thelin GP, and Wolock DM. 2006. The Quality of

Our Nation's Waters—Pesticides in the Nation's Streams and Ground Water, 1992-2001: U.S. Geological Survey Circular 1291. 172 p.

Gordon CJ, Herr DW, Gennings C, Graff JE, McMurray M, Stork L, Coffey T, Hamm A, and Mack CM. 2006. Thermoregulatory response to an organophosphate and carbamate insecticide mixture: Testing the assumption of dose-additivity. *Toxicology* 217(1): 1-13.

Henry CJ, Higgins KF, and Buhl KJ. 1994. Acute toxicity and hazard assessment of Rodeo, X-77 Spreader, and Chem-Trol to aquatic invertebrates. *Archives of Environmental Contamination and Toxicology* 27(3): 392-399.

Howe GE, Gillis R, and Mowbray RC. 1998. Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environmental Toxicology and Chemistry* 17(3): 519-525.

Jak RG, Maas JL, and Scholten MCTh. 1996. Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh water plankton communities in enclosures. *Water Research* 30(5): 1215-1227.

Jin-Clark Y, Lydy MJ, and Zhu KY. 2002. Effects of atrazine and cyanazine on chlorpyrifos toxicity in *Chironomus tentans* (Diptera: Chironomidae). *Environmental Toxicology and Chemistry* 21(3): 598-603.

Jonker MJ, Sweijen RAJC, and Kammenga JE. 2004. Toxicity of simple mixtures to the nematode *Caenorhabditis elegans* in relation to soil sorption. *Environmental Toxicology and Chemistry* 23(2): 480-488.

Kiely T, Donaldson D, and Grube A. 2004. Pesticides Industry Sales and Usage: 2000 and 2001 Market Estimates. EPA-733-R-04-001. Washington, DC: Office of Pesticide Programs, U.S. Environmental Protection Agency.

Liu TX, Stansly PA. 2000. Insecticidal activity of surfactants and oils against silverleaf whitefly (*Bemisia argentifolii*) nymphs (Homoptera: Aleyrodidae) on collards and tomato. *Pest Management Science* 56: 861-866.

Lydy MJ, Austin KR. 2004. Toxicity assessment of pesticide mixtures typical of the Sacramento-San Joaquin Delta using *Chironomus tentans*. A*rchives of Environmental Contamination and Toxicology* 48(1): 49-55.

Lydy M, Belden J, Wheelock C, Hammock B, and Denton D. 2004. Challenges in Regulating Pesticide Mixtures. *Ecology and Society*. 9(6): 1. [online] URL: http://www.ecologyandsociety.org/vol9/iss6/art1.

Mangan RL, Moreno DS. 2001. Photoactive dye insecticide formulations: adjuvants increase toxicity to Mexican fruit fly (Diptera: Tephritidae). *Journal of Economic Entomology* 94: 150-156.

Nakayama K, Oshima Y, Nagafuchi K, Hano T, Shimasaki Y, and Honjo T. 2005. Early-life-stage toxicity in offspring from exposed parent medaka, Oryzias latipes, to mixtures of tributyltin and polychlorinated biphenyls. *Environmental Toxicology and Chemistry* 24(3): 591-596.

Olmstead AW, LeBlanc GA. 2005. Toxicity assessment of environmentally relevant pollutant mixtures using a heuristic model. *Integrated Environmental Assessment and Management* 1(2): 114-122.

Overmyer JP, Armbrust KL, and Noblet R. 2003. Susceptibility of black fly larvae (Diptera: *Simuliidae*) to lawn-care insecticides individually and as mixtures. *Environmental Toxicology and Chemistry* 22(7): 1582-1588

Pape-Lindstrom PA, Lydy MJ. 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. *Environmental Toxicology and Chemistry* 16(11): 2415-2420.

Pounds JG, Haider J, Chen DG, and Mumtaz M. 2004. Interactive toxicity of chemical mixtures of cadmium, mercury, methylmercury, and trimethyltin: model-dependent responses. *Environmental Toxciology and Pharmacology* 18(2): 101-113.

Purcell MF, Schroeder WJ. 1996. Effects of Silwet L-77 and diazinon on three tephritid fruit flies (Diptera: Tephritidae) and associated endoparasitoids. *Journal of Economic Entomology* 89: 1566-1570.

Scholz NL, Truelove NK, Labenia JS, Baldwin DH, and Collier TK. 2006. Dose-additive inhibition of Chinook salmon acetylcholinesterase activity by mixtures of organophosphate and carbamate insecticides. *Environmental Toxicology and Chemistry* 25(5): 1200-1207.

Sharma SD, Singh M. 2001. Surfactants increase toxicity of glyphosate and 2,4-D to Brazil pusley. *HortScience* 36: 726-728.

Shaw JR, Dempsey TD, Chen CY, Hamilton JW, and Folt CL. 2006. *Environmental Toxicology and Chemistry* 25(1): 182-189.

Stark JD, Walthall WK. 2003. Agricultural adjuvants: acute mortality and effects on population growth rate of *Daphnia pulex* after chronic exposure. *Environmental Toxicology and Chemistry* 22(12): 3056-3061.

Sullivan KB, Spence KM. 2003. Effects of sublethal concentrations of atrazine and

nitrate on metamorphosis of the African clawed frog. *Environmental Toxicology and*

Chemistry 22(3): 627-635.

Tang Y, Donnelly KC, Tiffany-Castiglioni E, and Mumtaz MM. 2003. Neurotoxicity of

polycyclic aromatic hydrocarbons and simple chemical mixtures. *Journal of*

Toxicology and Environmental Health, Part A 66(12): 919-940.

Teuschler LK, Gennings C, Hartley WR, Carter H, Thiyagarajah A, Schoeny R, and

Cubbison C. 2005. The interaction effects of binary mixtures of benzene and toluene

on the developing heart of medaka (*Oryzias latipes*). *Chemosphere* 58(9): 1283-

1291.

US Environmental Protection Agency. 1990. Code of Federal Regulations. 40: Parts 150-

189. Washington, DC.

USFWS Threatened and Endangered Species System (TESS). 2007.

<http://ecos.fws.gov/tess_public/SpeciesReport.do?groups=E&listingType=L&mapst

atus=1>.

van der Geest HG, Greve GD, Boivin ME, Kraak MHS, and Van Gestel CAM. 2000.

Mixture toxicity of copper and diazinon to larvae of the mayfly (*Ephoron virgo*)

judging additivity at different effect levels. *Environmental Toxicology and Chemistry* 19(12): 2900-2905.

Xie F, Koziar SA, Lampi MA, Dixon DG, Norwood WP, Borgmann U, Huang XD, and Greenberg BM. 2006. Assessment of the toxicity of mixtures of copper, 9,10-phenanthrenequinone, and phenanthrene to *Daphnia magna*: evidence for a reactive oxygen mechanism. *Environmental Toxicology and Chemistry* 25(2): 613-622.

CHAPTER 2

ACUTE TOXICITY OF SPINOSAD AND R-11 TO THREE CLADOCERAN

SPECIES AND COHO SALMON

2.1 Abstract

Acute toxicity data and hazard assessments were developed for three cladoceran species, *Ceriodaphnia dubia* Richard, *Daphnia pulex* (Leydig), and *Daphnia magna* Straus, and juvenile Coho salmon, *Oncorhynchus kisutch* (Walbaum). Hazard assessments were based on acute mortality estimates and were compared to expected environmental concentrations (EEC) that had been previously calculated for two chemicals, the insecticide, spinosad, and the agricultural adjuvant R-11. The effect of a mixture of both of these compounds was also determined with *C. dubia* and *O. kisutch*. Acute mortality estimates and 95% CL for *C. dubia* exposed to R-11 and spinosad were 9.07 mg/l (8.81—9.30) and 0.00178 mg/l (0.0013—0.0025), respectively. The acute mortality estimates and 95% CL for *D. pulex* exposed to R-11 and spinosad were 13.16 mg/l (12.45-14) and 0.129 mg/l (0.07725-0.181), respectively. For *D. magna* exposed to R-11 and spinosad, the values were 17.65 mg/l (16.55-18.74) and 0.00482 mg/l (0.00191-0.00998), respectively. No mortality was observed when the Coho salmon were exposed to concentrations as high as 500 mg/l. The LC50 estimate and 95% CL of R-11 to *O. kisutch* was 11.014 mg/l (10.631—11.623), which was higher than the EEC reported above. Sublethal effects were observed in *O. kisutch* exposed to R-11, but not to spinosad. Bottom gilling and erratic swimming behavior occurred when *O. kisutch* was exposed to R-11. The effects of a mixture of 10 mg/l R-11 and 10 mg/l spinosad did not result in a difference in mortality as compared to each chemical singly, although there were sublethal effects that appeared to be more severe than in either of the positive controls. The mixture of R-11 and spinosad also resulted in bottom gilling and erratic

swimming behavior of *O. kisutch*. The LC50 estimates for R-11 were higher than the EEC of 0.79 mg/l for all species tested. The LC50 estimate for spinosad was much lower than EEC of 0.068 mg/l for *C. dubia* and *D. magna*. Hazard assessments for *C. dubia* and *D. magna* also indicated that at EEC, spinosad poses a significant threat as their hazard assessment values were 38.20 and 14.11, respectively. At EEC, spinosad does not seem to pose a hazard to *D. pulex* and *O. kisutch*.

When the LC25 of spinosad and the LC25 of R-11 were combined in an experiment with *C. dubia*, it was found that approximately 88% of the *Daphnia* died. The estimated mortality should have been approximately 61%, indicating that spinosad and R-11 may interact synergistically in *C. dubia*.

2.2 Introduction

The potential effects that pesticides might have on the environment and human health are still being debated. The Food Quality Protection Act, an act of Congress passed in 1996, mandated a severe reduction in the use of many of the traditional broad-spectrum pesticides for a wide range of agricultural uses (FQPA 1996). The purpose of this act was to protect consumers, especially children, who may be particularly susceptible to the effects of pesticides (NRC 1993, Goldman 1998). In response to the FQPA, pesticide producers have developed new pesticides designed to be more toxic to pest species than to nontarget organisms (Stark and Banks 2001). Although these new pesticides appear to be less damaging to biological controls of pests than to pest species, less work has been conducted on their potential effects on aquatic organisms (Stark and Vargas 2003). Additionally, agricultural adjuvants are used as tank mixes with pesticides to improve their performance (Stark and Walthall 2003). Agricultural adjuvants are often part of the formulations of pesticides. Even less work has been conducted on the effects of adjuvants on aquatic organisms (Stark and Walthall 2003).

Spinosad is one of the new pesticides that is being marketed for control of a range of pest species (DowElanco 1996). Spinosad was chosen as a test chemical because it is a natural product derived from a fermentation byproduct of the soil bacterium *Saccaropolyspora spinosa* (Actinomycetes) whose use is becoming more frequent since becoming registered for use as an organic insecticide. R-11 was chosen as a test chemical because it is a commonly used agricultural adjuvant and is a component of certain pesticide formulations. Its main component is nonylphenol polyethoxylate (NPE)

which degrades into nonylphenol, a chemical which has been found to mimic estrogen (Trumbo 2005).

Little is known about the toxicity of the agricultural adjuvant R-11 and the insecticide spinosad on aquatic organisms. Additionally, both of these chemicals could end up in freshwater ecosystems together as mixtures. An evaluation of the toxicity of each chemical separately and as a mixture (with two of the species tested) was conducted in this study to develop information about the toxicity and potential hazard these chemicals might pose to aquatic organisms. Because pesticides are often found in mixtures of two or greater in the environment (Gilliom et al. 2006), it is important to test the effects of chemical mixtures as well as individual chemicals. Of particular concern is the toxicity of the combination of pesticides and adjuvants in the environment because they are often used together.

The objective of this study was to determine the acute toxicity of R-11 and spinosad, determined through 48 h studies on *C. dubia* Richard, *D. pulex* (Leydig), and *D. magna* Straus and through 96 h studies on Coho salmon, *O. kisutch* (Walbaum). The acute LC50 values were compared to the expected environmental concentrations (EEC). The EEC is defined as the concentration of pesticide in 150 l of water after a direct spraying over a forest at an average rate (Stark and Banks 2001). This corresponds to the highest possible concentration in the environment and would pose the highest risk to aquatic organisms. These experiments allow us to find out how susceptible each species is to each chemical. The two chemicals were also evaluated as a mixture with *C. dubia* and *O. kisutch* in order to determine if antagonism, synergism, or additive toxicity occurred.

2.3 Materials and Methods

2.3.1 Test Organisms

Cladocerans were obtained from cultures maintained at Washington State University, Puyallup. Each culture was maintained in ground water collected from a shallow aquifer located at WSU Puyallup. Cultures were housed in an environmental chamber with a photoperiod of 18h: 6h light: dark, $25.0 \pm 0.1°C$, and $50.0 \pm 0.1\%$ relative humidity (RH). The feeding solution for daphnids consisted of a 1:1.5 mixture of yeast-cereal leaves-trout chow (YCT) and the algal species *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) (Charles River Co., Wilmington, MA). Neonates were removed daily from daphnid cultures, transferred to new cups containing ground water, and fed a 0.3 ml feeding solution. The water was refrigerated when not in use and aerated at room temperature for at least 30 minutes before use. The water had a pH range of 7.4-7.8, conductivity of 260-320 μS, dissolved oxygen (DO) content greater than 8.0 mg/L, alkalinity of 60-70 mg/L, and a hardness of 80-100 mg/L. This corresponded to a classification of "moderately hard" according to EPA (1989) standard methods.

Juvenile Coho salmon, *O. kisutch*, were obtained from the University of Washington (UW) hatchery on 3/21/2007. These salmon were spawned from adult Coho salmon that had returned to the UW hatchery in Fall 2006 and were hatched in January 2007. They were entirely hatchery raised on the shore of Portage Bay, which is connected to Lake Union. Lake Union connects to the Puget Sound through the Ballard Locks (For more information, see http://www.fish.washington.edu/hatchery/research.html#facilities). The Coho salmon were housed in 757 L free standing circular containers with free flowing

water that had been filtered, de-chlorinated, UV-sterilized, and chilled to 12°C. The fish were maintained in a 16h: 8h light to dark cycle. The frye were fed Bio Vita starter #1 Crum (Bio-Oregon, Inc.) once daily.

2.3.2 Chemicals evaluated

2.3.2.1 Spinosad

Spinosad (Success, 240 g active ingredient (ai)/l, Dow AgroSciences LLC9330 Zionsville Rd., Indianapolis, Indiana, 46268, USA) is a naturally derived pesticide that has been found to be more toxic to pest insect species than to beneficial insects (DowElanco 1996). Spinosad has been listed as an organic insecticide. Its active ingredient is a mixture of spinosyns A and D, which are fermentation products of the soil bacterium *Saccharopolyspora spinosa* (Actinomycetes) (Crouse et al. 2001). Spinosad is a neurotoxin and acts as a contact and stomach poison (DowElanco 1996, Salgado 1998). The effects of this insecticide are consistent with the activation of nicotinic acetylcholine receptors and thus acts in a similar manner to nicotine in the nervous systems of animals, characterized by the excitation of the nervous system. Spinosad also has effects on GABA receptor function which may add to its toxicity to insects (Salgado 1998). Spinosad was chosen as a test chemical because it is a newer compound that is being used more and more frequently since gaining classification as an organic insecticide.

Spinosyns A and D have similar structures. They are relatively non-volatile and nonpolar. They sorb to sediment and soil readily and degrade quickly in the soil, having half-lives of 9.4-17.3 days when in the dark at 25°C. The spinosyns are degraded more

quickly through photolysis with half-lives of approximately one day at pH 7 and 25ºC (DowElanco 1996). In water, spinosad is relatively stable and degrades slowly from pH 5 to 7. However, at pH 9, half lives for spinosyns A and D are 200 and 259 days, respectively.

2.3.2.2 R-11

R-11 is a nonionic surfactant whose active ingredient includes nonylphenol polyethoxylate (NPE) (Wilbur-Ellis Co. 2007). NPE metabolizes into the more toxic and biologically active nonylphenol (NP), which is a known estrogen mimic (Trumbo 2005). NPE metabolizes into NP almost immediately after application in water (<1 h), although the concentrations of NP were approximately 1/50 the initial concentration of NPE (Trumbo 2005). R-11 was chosen as a test chemical because it is a commonly used agricultural adjuvant and is a component of certain pesticide formulations.

2.3.3 Development of Acute Toxicity Data for Daphnia

The toxicity of spinosad and R-11 were evaluated with acute mortality studies. Adjuvant and pesticide concentrations were prepared by serial dilution from newly prepared stock solutions in 100 ml ground water. Batches of five *C. dubia*, *D. pulex*, and *D. magna* neonates (<24 h old) at least in the third filial generation (F_3) were transferred into 30 ml plastic cups containing 25 ml of sample solution for each concentration tested. Four cups were used for each concentration. Daphnids were fed at least two hours before introduction to the test solutions. All tests consisted of several concentrations and a

control. Daphnia were starved for the duration of the experiments and adjuvant/pesticide solutions were not renewed. Mortality was assessed 48 hours after starting the experiment. Test organisms were kept at the same conditions listed above for rearing. *Daphnia* were considered dead if no movement occurred in the external and thoracic appendages or the heart following gentle prodding with a glass pipette following observation under microscopic magnification. This experiment was replicated at least 3 times on different days with different generations of daphnids.

2.3.4 Development of Acute Toxicity Data on O. kisutch (Coho salmon)

Spinosad and R-11 were evaluated in 96 h acute toxicity tests for *O. kisutch*. Adjuvant and pesticide concentrations were prepared by dilution from newly prepared stock solutions in 20 l system water (filtered, de-chlorinated, UV-sterilized, and chilled).

The juvenile Coho were acclimated to flowing tap water for at least 7 days prior to test initiation. Prior to each experiment, aquaria were rinsed and scrubbed with tap water and air-dried. 38 l aquaria were filled with 20 l tap water the day of the addition of chemicals. The experimental tanks were placed into a 757 l water bath of flowing system water to maintain temperature near 12°C. The approximate water volume to fish ratio was 4 l: 1 fish. Test amounts of pesticides and adjuvants were weighed out and added to the 20 l of tap water in the aquaria separately and in binary mixtures. Batches of five *O. kisutch* frye were transferred into the aquaria after the chemicals had been mixed into the water. Fish were not fed for the duration of their 96 h exposure. The solutions were aerated constantly throughout the experiment to ensure proper mixing. Fish that were in

the controls were treated the same as the test organisms, although they were not exposed to any chemicals.

The state of the Coho salmon was recorded at 6, 24, 48, 72, and 96 h. At these time intervals, mortality, gilling, and erratic swimming was recorded and any dead salmon were removed from the tanks. Salmon were considered dead when gill movement ceased. At the end of the experiment, fish were removed, weighed, and measured lengthwise.

2.3.5 Statistical Analysis

Acute concentration-mortality regressions were estimated with Probit analysis (Finney 1971) (SAS Institute 1999) after correction for control mortality using Abbott's formula (1925). If control mortality exceeded 5%, the tests were not used for the final analysis. Data from the mixture study was evaluated with analysis of variance (ANOVA) (SAS Institute 1999). Prior to analysis, percent mortality data was transformed with arcsine square root of proportion. Means were separated with the Student-Newman-Keuls test ($p = 0.05$).

Acute mixture toxicity on *C. dubia* was evaluated by combining the LC25 estimates for R-11 and spinosad (8.21 mg/l and 0.00394 mg/l, respectively). The LC25s were determined with Probit analysis of each chemical separately with a 95% confidence level. The acute mixture toxicity on *O. kisutch* was evaluated by using 10 mg/l R-11 and 10 mg/l spinosad. The R-11 concentration that was chosen because it approximated the acute LC25 estimate determined by Probit analysis. The 10mg/l spinosad concentration

was chosen because it was found that no mortality was observed even at concentrations up to 500 mg/l and, therefore, this concentration should not cause mortality by itself.

2.3.6 Hazard Assessment

Hazard assessments were developed by using the quotient method. This method entails using the expected environmental concentration (EEC) and dividing it by the toxicity endpoint, the LC50. Numbers greater than 1 indicate that the chemical poses a hazard to the tested species.

2.4 Results

2.4.1 Acute Toxicity Results for R-11

Acute mortality varied among the test species that were exposed to R-11 (Table 1). Based on overlap of the 95% CL, *C. dubia* was significantly more susceptible than the other species. Additionally, *O. kisutch* was more susceptible than *D. pulex* which was more susceptible to R-11 than *D. magna*. The order of susceptibility to R-11 from most susceptible to least was: *C. dubia* > *O. kisutch* > *D. pulex* > *D. magna*. The susceptibility of daphnids seemed to correspond to size; *C. dubia* is the smallest species, *D. pulex* is intermediate and *D. magna* is the largest species.

2.4.2 Acute Toxicity Results for Spinosad

Acute mortality varied among the tested species that were exposed to spinosad (Table 2). Based on overlap of the 95% CL, *C. dubia* and *D. magna* were equally susceptible to spinosad and both species were significantly more susceptible than *D. pulex* at LC50. For example, at LC50, *C. dubia* was approximately 72 times more susceptible to spinosad than *D. pulex*, and *D. magna* was approximately 27 times more susceptible than *D. pulex*.

Spinosad was found to be virtually non-toxic to *O. kisutch*. No mortality was observed even after exposure to 500 mg/l. The order of susceptibility to spinosad from most susceptible to least was: *C. dubia* = *D. magna* > *D. pulex* > *O. kisutch*.

2.4.3 Acute Toxicity Results for R-11 and Spinosad Mixtures to C. dubia and O. kisutch

When the LC25 of spinosad and the LC25 of R-11 were combined, it was found that approximately 88% of the *Daphnia* died. The estimated mortality should have been approximately 61%, indicating that spinosad and R-11 may interact synergistically in *C. dubia* (Table 3). There was no significant difference in mortality between *C. dubia* exposed to the LC25 of R-11 or spinosad. However, mortality in the mixture was significantly higher than mortality due to each chemical alone ($P = 0.001$; df = 2,9; $F = 21.83$).

When *O. kisutch* was exposed to 10 mg/l R-11 and 10 mg/l spinosad, the mortality of the salmon did not differ from results of either chemical tested singly. There were sublethal effects observed, however, that differed from either of the positive controls. *O. kisutch* that were exposed to mixtures appeared to be immobilized after only 6 h of exposure. The salmon exposed to 10 mg/l R-11 were actively swimming, albeit, erratically. The salmon exposed to 10 mg/l spinosad were actively swimming and

48

exhibiting normal behavior. After 72 h of exposure, *O. kisutch* exposed to the mixture appeared to be recovering and were all swimming erratically. Although the mixture of 10 mg/l R-11 and 10 mg/l spinosad did not cause significant mortality in the test organisms, sublethal effects were evident.

2.4.5 Hazard Assessments for Acute Toxicity

Hazard assessments for *C. dubia*, *D. magna*, *D. pulex*, and *O. kisutch* were based on LC50 estimates and previously calculated EEC values. Hazard assessments indicated that spinosad posed a hazard to *C. dubia* and *D. magna* only (Table 4). R-11 did not pose a hazard to any of the species because all of the hazard assessments were ≤ 1. A hazard assessment could not be developed for *O. kisutch* because an LC50 value could not be determined.

2.4.4 Weights and lengths of O. kisutch

There was no significant difference in the weights and lengths of *O. kisutch* exposed to R-11 (Table 5). The weights were evaluated with one way Analysis of Variance (ANOVA) ($F = 0.876$; df = 7, 19; $P = 0.543$) and the lengths were calculated with the Kruskal-Wallis one way ANOVA on Ranks ($P = 0.151$).

2.5 Discussion

The results of the acute toxicity study indicate that the LC50 of spinosad to two cladoceran species is lower than the EEC and the LC50 of R-11 is well above the EEC

for all species tested. The LC50 estimate for R-11 was higher than the EEC of 0.79 mg/l (Stark and Walthall 2003) and hazard assessments were all far below 1 for all species tested. Therefore, R-11 does not pose a hazard to *C. dubia*, *D. pulex*, *D. magna*, and *O. kisutch* at EEC. The LC50 estimate for spinosad was 0.00178 mg/l for *C. dubia* and 0.00482 mg/l for *D. magna*, which were both much lower than the EEC of 0.068 mg/l (Stark and Banks 2001). Spinosad hazard assessment values for *C. dubia* and *D. magna* were much higher than 1, which is another indication that spinosad poses a hazard to *C. dubia* and *D. magna* at EEC. Spinosad did not pose a hazard to *D. pulex* or *O. kisutch* at EEC. Spinosad was not acutely toxic to Coho salmon at concentrations up to 500 mg/l, although R-11 was more toxic to the salmon than *D. magna*. The mixture of R-11 and spinosad to *C. dubia* indicated that the chemicals combined may cause a synergistic reaction at a significance level of $p \leq 0.05$. The mixture of R-11 and spinosad to *O. kisutch* indicated that the mixture caused sublethal effects that were greater than either chemical tested singly. Therefore, mixtures of spinosad and R-11, which may occur in freshwater ecosystems, may cause more damage to aquatic organisms than either product alone.

2.6 References

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18: 265-267.

Crouse GD, Sparks TC, Schoonover J, Gifford J, Dripps J, Bruce T, Larson L, Garlich J, Hatton C, Hill RL, Worden TV, and Martynow JG. 2001. Recent advances in the chemistry of spinosyns. *Pest Management Science* 57: 177–185.

DowElanco. 1996. Spinosad Technical Guide. Form No. 200-03-001 (4/96).

Finney DJ. Probit analysis (3rd ed.). Cambridge: Cambridge University Press, 1971.

FQPA (Food Quality Protection Act). Congress 1996. U.S. Public Law 104-170. 104th Congress, August 1996.

Gilliom RJ, Barbash JE, Crawford CG, Hamilton PA, Martin JD, Nakagaki N, Nowell LH, Scott JC, Stackelberg PE, Thelin GP, and Wolock DM. 2006. The Quality of Our Nation's Waters—Pesticides in the Nation's Streams and Ground Water, 1992-2001: U.S. Geological Survey Circular 1291. 172 p.

Goldman LR. 1998. Linking research and policy to ensure children's environmental health. *Environmental Health Perspectives* 106 (Suppl. 3): 857- 862.

NRC (National Research Council). 1993. Pesticides in the diets of infants and children. Washington, D.C. National Academy Press.

Salgado VL. 1998. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pesticide Biochemistry and Physiology* 60: 91–102.

SAS Institute. 1999. SAS/STAT User's guide, Version 6. Institute, Cary, NC.

Stark JD, Banks JE. 2001. Selective pesticides: are they less hazardous to the environment? *Bioscience* 51(11): 980-982.

Stark JD, Vargas RI 2003. Demographic changes in *Daphnia pulex* (Leydig) after exposure to the insecticides spinosad and diazinon. *Ecotoxicology and Environmental Safety* 56: 334-338.

Stark JD, Walthall WK. 2003. Agricultural adjuvants: acute mortality and effects on population growth rate of *Daphnia pulex* after chronic exposure. *Environmental Toxicology and Chemistry* 22(12): 3056-3061.

Trumbo J. 2005. An assessment of the hazard of a mixture of the herbicide Rodeo and the non-ionic surfactant R-11 to aquatic invertebrates and larval amphibians. California Fish and Game. 91(1): 38-46.

Wilbur-Ellis (2007). R-11 Material Safety Data Sheet.

<http://www.cdms.net/ldat/mp956000.pdf>

Table 1. Acute (48 h) concentration-mortality estimates for daphnids and *O. kisutch* exposed to R-11.

Species	No. tested	Slope $\pm$ SE	LC50 (95% CL) (mg/l)
C. dubia	416	15.61 $\pm$ 1.62	9.07 (8.81—9.30)
D. magna	330	12.21 $\pm$ 2.22	17.65 (16.55—18.74)
D. pulex	319	6.55 $\pm$ 1.11	13.16 (12.45—14.01)
Oncorhynchus kisutch	205	20.2887 $\pm$ 5.2768	11.014 (10.631—11.623)

Table 2. Acute (48 h) concentration-mortality estimates for daphnids and *O. kisutch* exposed to spinosad.

Species	No. tested	Slope $\pm$ SE	LC50 (95% CL) (mg/l)
C. dubia	541	1.03 $\pm$ 0.081	0.00178 (0.00128—0.00247)
D. magna	180	0.79 $\pm$ 0.123	0.00482 (0.00191—0.00998)
D. pulex	381	1.01 $\pm$ 0.17	0.129 (0.077—0.181)
Oncorhynchus kisutch	50	N/A[1]	> 500

1/ Not applicable. An LC50 could not be generated based on the data.

Table 3. Mixture toxicity of R-11 and spinosad to *C. dubia*.

Chemicals Tested	No. tested	Mean % Mortality[1]	Standard Error
R-11 LC25	443	21.488a	3.008
Spinosad LC25	443	39.715a	9.005
Mixture of R-11 and Spinosad LC25s	443	88.313b	5.490

1/ Means within the column followed by different letters are significantly different ($p \leq$ 0.05; Student-Newman-Keuls test).

Table 4. Hazard assessments for R-11 and spinosad on all species tested

Species	Hazard Assessment[1] for R-11	Hazard Assessment[1] for Spinosad
C. dubia	0.0871	38.20
D. magna	0.04476	14.11
D. pulex	0.060	0.527
Oncorhynchus kisutch	0.0764	N/A[2]

1/ Hazard assessment values were calculated with the equation: EEC/LC50. Values greater than 1 indicate that the chemical poses a hazard to the tested species.
2/ Value could not be calculated because the LC50 could not be determined.

Table 5. Average lengths and weights of juvenile *O. kisutch* tested with R-11 with 95% standard error.

Concentration mg/l	No. tested	Weight (g)[1]	Length (cm)[2]
0	25	4.190 $\pm$ 0.676	7.50 $\pm$ 0.249
9	20	4.840 $\pm$ 0.568	7.945 $\pm$ 0.211
9.5	15	4.453 $\pm$ 0.417	7.836 $\pm$ 0.225
10	25	5.091 $\pm$ 0.634	8.025 $\pm$ 0.226
10.5	15	5.979 $\pm$ 1.120	8.427 $\pm$ 0.276
11	20	4.725 $\pm$ 0.390	7.935 $\pm$ 0.201
11.5	10	6.459 $\pm$ 2.211	8.450 $\pm$ 0.378
12	15	4.511 $\pm$ 0.330	7.553 $\pm$ 0.188

1/ Means for weights were not significantly different ($P > 0.05$; One Way ANOVA, SAS Institute [1999]).

2/ Means for lengths were not significantly different ($P > 0.05$; Kruskal-Wallis One Way ANOVA; SAS Institute [1999]).

CHAPTER 3

AN EVALUATION OF THE EFFECTS OF SPINOSAD AND R-11 ON

CERIODAPHNIA DUBIA POPULATIONS

3.1 Abstract

The effects of the insecticide, spinosad and the agricultural adjuvant, R-11 were evaluated on populations of the water flea, *Ceriodaphnia dubia* Richard with 8-day population growth studies. Populations of *C. dubia* declined rapidly after exposure to spinosad. Extinction occurred (defined as negative population growth rate) after exposure to 0.01 mg/l which was below the expected environmental concentration of 0.068 mg/l. Population size was significantly reduced after exposure to 0.0005 mg/l spinosad. The number of offspring/surviving female was significantly reduced compared to the control after exposure to spinosad concentrations ≥ 0.0025 mg/l.

R-11 caused *C. dubia* populations to decline, approaching a growth rate of 0 after exposure to 10 mg/l. Population growth rate was not affected after exposure to the EEC of 0.79 mg/l. Population size was significantly reduced after exposure to ≥ 5 mg/l R-11. The number of offspring per surviving female was significantly reduced compared to the control after exposure to R-11 concentrations ≥ 5 mg/l. These results indicate that sublethal effects of R-11 against *C. dubia* negatively affected their fecundity even if it did not significantly affect the mortality of the original population.

3.2 Introduction

Evaluation of the effects of toxicants is often initially done with acute mortality estimates. Although exposure of populations to toxicants may result in extirpation, for example when pest species are controlled in crops, more often toxicants can have negative effects on populations resulting in temporary declines. Exposure to pesticides and agricultural adjuvants, for example, can result in both lethal and sublethal effects causing population declines (Stark et al. 1997, Stark and Vargas 2005, Stark and Walthall 2003). Therefore, it is important to evaluate toxicants at the population level so that a measure of the total effect can be gained (Stark and Banks 2003). In this study, population-level effects of the insecticide, spinosad, and the agricultural adjuvant, R-11, were evaluated with a population-level study on *Ceriodaphnia dubia* Richard.

Spinosad is one of the new pesticides that is being marketed for control of a range of pest species (DowElanco 1996). Spinosad was chosen as a test chemical because it is a natural product derived from a fermentation byproduct of the soil bacterium *Saccaropolyspora spinosa* (Actinomycetes) whose use is becoming more frequent since becoming registered for use as an organic insecticide. R-11 was chosen as a test chemical because it is a commonly used agricultural adjuvant and is a component of certain pesticide formulations. Its main component is nonylphenol polyethoxylate (NPE) which degrades into nonylphenol, a chemical which has been found to mimic estrogen (Trumbo 2005).

The LC50 values that were generated in Chapter 2 indicated at which concentrations R-11 and spinosad cause 50% of their populations to die in a 48 h period. Chronic 8 d studies have the ability to determine mortality in the original daphnids tested and can also

determine if the chemicals affected their fecundity because 8 days allows enough time for *C. dubia* to produce multiple broods of progeny under ideal circumstances. Studies that last for 8 days give more insight into potential population level effects when the population is exposed to R-11 and spinosad separately. This was measured by calculating the instantaneous rates of increase (*ri*).

To determine if R-11 and spinosad had population level effects on *C. dubia*, 8-day studies were conducted to assess mortality and fecundity differences as compared to a control. The instantaneous rate of increase (*ri*) was calculated to determine if there was a difference between control and experimental values. Determining population level effects through chronic studies enables us to determine if the chronic exposure to R-11 and spinosad can cause mortality and/or alter the fecundity of the organisms tested. If no mortality is observed in the original *Daphnia* and significantly fewer offspring are observed, this indicates that sublethal reproductive effects have occurred.

The objective of this study was to determine if R-11 and spinosad can cause population level effects on *C. dubia* through a series of 8-day experiments. The acute LC50 values and instantaneous rate of increase (*ri*) were also compared for each chemical tested in order to see how closely the acute and chronic toxicity data correlate with each other.

3.3 Materials and Methods

3.3.1 Test Organisms

Daphnia cultures were obtained from cultures maintained at Washington State University, Puyallup. Each culture is maintained in ground water collected from a shallow aquifer located at WSU Puyallup. They were housed in an environmental chamber with a photoperiod of 18h: 6h light: dark, $25.0 \pm 0.1°C$, and $50.0 \pm 0.1\%$ relative humidity (RH). The feeding solution for *Daphnia* contains a 1: 1.5 mixture of yeast-cereal leaves-trout chow (YCT) and the algal species *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) (Charles River Co., Wilmington, MA). Neonates were removed daily from *Daphnia* cultures, transferred to new cups containing ground water, and were fed 0.3 ml feeding solution. The water was refrigerated when not in use and aerated at room temperature for at least 30 minutes before use. The water had a pH of 7.4-7.8, conductivity of 260-320 µS, dissolved oxygen (DO) content greater than 8.0 mg/l, alkalinity of 60-70 mg/l, and a hardness of 80-100 mg/l. This corresponded to a classification of "moderately hard" according to EPA (1989) standard methods.

3.3.2   Chemicals evaluated

3.3.2.1 Spinosad

Spinosad (Success, 240 g active ingredient (ai)/l, Dow AgroSciences LLC9330 Zionsville Rd., Indianapolis, Indiana, 46268, USA) is a naturally derived pesticide that has been found to be more toxic to pest insect species than to beneficial insects (DowElanco 1996). Spinosad has been listed as an organic insecticide. Its active ingredient is a mixture of spinosyns A and D, which are fermentation products of the soil bacterium *Saccharopolyspora spinosa*, (Actinomycetes) (Crouse et al. 2001).

Spinosad is a neurotoxin and acts as a contact and stomach poison (DowElanco 1996, Salgado 1998). The effects of this insecticide are consistent with the activation of nicotinic acetylcholine receptors and thus acts in a similar manner to nicotine in the nervous systems of animals, characterized by the excitation of the nervous system. Spinosad also has effects on GABA receptor function which may add to its toxicity to insects. Spinosad was chosen as a test chemical because it is a newer compound that is being used more and more frequently since gaining classification as an organic insecticide.

Spinosyns A and D have similar structures. They are relatively non-volatile and nonpolar. They sorb to sediment and soil readily and are readily degraded in the soil. They have half-lives of 9.4-17.3 days when in the dark at 25ºC. The spinosyns are degraded more quickly through photolysis with half-lives of only a few days. In water, spinosad is relatively stable and degrades slowly at pH 5 and 7. Half lives for spinosyns A and D, respectively, are 200 and 259 days at pH 9. Photolysis occurs quickly, with half lives at approximately one day at pH 7 and 25ºC (DowElanco 1996).

3.3.2.2 R-11

R-11 is a nonionic surfactant whose active ingredient includes nonylphenol polyethoxylate (NPE) (Wilbur-Ellis Co. 2007). NPE metabolizes into the more toxic and biologically active nonylphenol, which is a known estrogen mimic (Trumbo 2005). R-11 was chosen as a test chemical because it is a commonly used agricultural adjuvant and is a component of several pesticide formulations.

3.3.3 Population Studies with C. dubia

The effects of spinosad and R-11 on survival, fecundity, population size, and population growth rate of *C. dubia* was determined by exposing < 24 h old *Daphnia* to each chemical at various concentrations. Each chemical was evaluated separately. Ten neonates were be added to 100 ml solutions of each test solution in 296 ml SOLO® clear plastic cups. The *Daphnia* were added to the cups by using a disposable small-bore glass pipette. After the addition of the *Daphnia* to the pesticide solutions, 1.0 ml of the feeding solution, as detailed above, was added to each cup. The samples were then transferred into a freestanding environmental chamber, as detailed above, and covered with Plexiglas® to avoid evaporation. *Daphnia* were fed 1.0 ml feeding solution daily. After 8 days, the cups were removed from the environmental chamber and all *Daphnia* were counted. The protocol for chronic toxicity studies on *C. dubia* had to be slightly altered from the protocol for *Daphnia pulex*. A chronic study for *D. pulex* lasts 10 days because after 10 days of exposure, the *Daphnia* have had time to produce multiple broods of progeny and it can be determined if the chemicals used have decreased fecundity or caused mortality (Walthall and Stark 1997). *C. dubia* has been found to begin reproduction at 4 days, instead of 5 days like *D. pulex*, so the protocol was modified in order to make sure that only the original 10 daphnids per cup were reproducing and not their F_1 offspring. Therefore, this study was evaluated after 8 days. *Daphnia* were considered alive if they moved following gentle prodding with a glass pipette. The survival of the original *Daphnia* and the number of offspring produced were determined.

The effects of spinosad and R-11 on population growth rate of *C. dubia* were determined by developing the instantaneous rate of increase (*ri*), a measure of population growth rate (Stark et al. 1997; Walthall and Stark 1997; Banken and Stark 1998; Sibly 1999; Stark and Banken 1999; Stark and Banks 2001; Herbert et al. 2004). The purpose of the instantaneous rate of population growth is to measure population increase and it is calculated with the following equation:

$$ri = \ln (N_f / N_o) / \Delta t,$$

where N_f is the final number of animals, N_o is the initial number of animals, and Δt is the change in time (number of days the experiment was run). Solving for *ri* yields a rate of population increase or decline similar to that obtained by the intrinsic rate of increase (r_m) (Walthall and Stark 1997). Values of $ri > 0$ indicate a growing population, $ri = 0$ indicates a stable population, *ri* values < 0 indicate that a population is in decline and is headed toward extinction.

The experiment was conducted at least three times on different days with different generations of *Daphnia*. The instantaneous rate of increase was developed as the measure of population growth rate. Survival of the original individuals used to start the population studies, the number of offspring per surviving female and final population number were recorded 8 d after the start of the study

3.3.4   Statistical Analysis

Data on the number of final number of individuals (population size) at the end of the study and the number of offspring/surviving female were analyzed with analysis of variance (ANOVA) (SAS Institute 1999). Means were separated with the Student-Newman-Keuls method ($p = 0.05$). These data did not meet the assumptions of normality and/or equal variances necessary for ANOVA. Therefore, the data was transformed with a square root transformation prior to statistical analysis. Data presented in this paper are untransformed. For the offspring/female data, the highest concentration tested (0.05 mg/l) was dropped from the statistical analysis because all values were 0.

3.4 Results

3.4.1 Spinosad

The instantaneous rate of increase (*ri*) of *C. dubia* declined as the concentrations of spinosad increased (Fig. 1). The population declined rapidly after exposure to spinosad. Populations of *C. dubia* were headed towards extinction (defined as negative *ri*) after exposure to 0.0052 mg/l spinosad (Fig. 1). This value is significantly lower than the EEC of 0.068 mg/l (Stark and Banks 2001).

In chapter 2, acute-mortality estimates were developed for *C. dubia* exposed to spinosad. A comparison of the acute concentration-mortality curve to the 8 d population growth rate curve revealed that stable population growth rate (*ri* =0) occurred after exposure to 0.0052 mg/l, which was equivalent to the acute LC70 (Fig. 2). This means that a population of *C. dubia* cannot withstand relatively high concentrations of spinosad without becoming extinct.

Chronic exposure of *C. dubia* to spinosad resulted in high levels of mortality in the individuals used to start the populations ($F = 23.67$; df = 5,27; $p < 0.001$) (Fig. 3). This means that the declines in population growth rate observed were due in part to mortality in the original individuals, although it also shows that the remaining individuals produced fewer offspring than the controls.

The final number of individual *C. dubia* (population size) was significantly reduced by the lowest concentration evaluated (0.0005 mg/l) ($F = 98.72$; df = 5,27; $p < 0.001$) (Fig. 4). All of the spinosad concentrations evaluated significantly reduced population size of *C. dubia*.

The number of offspring per surviving female were significantly ($F = 44.38$; df = 4,19; $p < 0.001$) reduced after exposure to 0.0025 mg/l spinosad and higher (Fig. 5).

3.4.2 R-11

The instantaneous rate of increase (*ri*) of *C. dubia* declined as the concentrations of R-11 increased (Fig. 6). The population declined rapidly after exposure to R-11 concentrations higher than 5 mg/l (Fig. 6), although this concentration was significantly higher than the EEC of 0.79 mg/l (Stark and Walthall 2003).

In chapter 2, acute-mortality estimates were developed for *C. dubia* exposed to R-11. A comparison of the acute concentration-mortality curve to the 8 d population growth rate curve revealed that stable population growth rate (*ri* =0) occurred at concentrations slightly higher than 10.12 mg/l, which was equivalent to the acute LC75 (Fig. 7). This means that a population of *C. dubia* cannot withstand high concentrations of R-11 relative to the acute LC50 without becoming extinct.

Chronic exposure of *C. dubia* to R-11 did not result in high levels of mortality in the individuals used to start the populations ($F = 13.36$; df $= 7,53$; $p < 0.001$) (Fig. 8). This means that the declines in population growth rate observed were due mainly to effects on reproduction (see below).

The final number of individual *C. dubia* (population size) was significantly reduced after exposure to ≥ 5 mg/l ($F = 50.038$; df $= 7,57$; $p < 0.001$) (Fig. 9).

The numbers of offspring/surviving female were significantly ($F = 47.23$; df $= 7.61$; $p < 0.001$) reduced after exposure to ≥ 5 mg/l (Fig. 10). These data indicate that the majority of the effects on population growth and growth rate were due to sublethal effects on reproduction.

3.5 Discussion

Results of this study indicate that spinosad and R-11 caused different effects on populations of *C. dubia*. Spinosad caused high levels of mortality in the individuals used to start the populations of *C. dubia* while R-11 caused little mortality in these individuals. Spinosad also affected reproduction and the combination of mortality in the individuals used to start the population and reductions in offspring accounted for population declines. However, with R-11, the majority of the population declines were due to reductions in offspring per surviving female. Thus, R-11 and spinosad reduced populations of *C. dubia* with different mechanisms.

Stark and Walthall (2003) report that although the mode of action of agricultural adjuvants, such as R-11, is widely regarded to be suffocation, it appears that it has a negative impact on reproduction in *Daphnia pulex*. A significant reduction in the

fecundity of *Daphnia magna* after exposure to 4-nonylphenol was observed in Baldwin et al. (1997). This also seems to be the case in this study with R-11 and *C. dubia*. This may be due to the fact that the primary ingredient of R-11 is nonylphenol polyethoxylate, which degrades into the known estrogen mimic nonylphenol (Trumbo 2005). Spinosad, in comparison, has similar mortality curves for both the original population and offspring, indicating that the reduction in offspring is due to both a reduction in the amount of adults and a decrease in fecundity of the individuals that survived.

3.6 References

Baldwin WS, Graham SE, Shea D, and LeBlanc GA. 1997. Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol. *Environmental Toxicology and Chemistry* 16 (9): 1905-1911.

Banken JAO, Stark JD. 1998. Multiple routes of pesticide exposure and the risk of pesticides to biological controls: A study of neem and the sevenspotted lady beetle (Coleoptera: Coccinellidae). *Journal of Economic Entomology* 91(1): 1-6.

Crouse GD, Sparks TC, Schoonover J, Gifford J, Dripps J, Bruce T, Larson L, Garlich L, Hatton C, Hill RL, Worden TV, and Martynow JG. 2001. Recent advances in the chemistry of spinosyns. *Pest Management Science* 57: 177-185.

DowElanco. 1996. Spinosad Technical Guide. Form No. 200-03-001 (4/96).

Herbert IN, Svendsen C, Hankard PK, and Spurgeon DJ. 2004. Comparison of instantaneous rate of population increase and critical-effect estimates in *Folsomia candida* exposed to four toxicants. *Ecotoxicology and Environmental Safety* 57 (2): 175-183.

Salgado VL. 1998. Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pesticide Biochemistry and Physiology* 60: 91–102.

SAS Institute. 1999. SAS/STAT User's guide, Version 6. Institute, Cary, NC.

Sibley RM. 1999. Efficient experimental designs for studying stress and population density in animal populations. *Ecological Applications* 9(2): 496-503.

Stark JD, Banken JAO. 1999. Importance of population structure at the time of toxicant exposure. *Ecotoxicology and Environmental Safety* 42(3): 282-287.

Stark JD, Banks JE. 2003. Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology* 48: 505-519.

Stark JD, Banks JE. 2001. Selective pesticides: are they less hazardous to the environment? *Bioscience* 51(11): 980-982.

Stark JD, Tanigoshi L, Bounfour M, and Antonelli A. 1997. Reproductive potential: its influence on the susceptibility of a species to pesticides. *Ecotoxicology and Environmental Safety* 37(3): 273-279.

Stark JD, Vargas RI. 2005. Toxicity and hazard assessment of fipronil to *Daphnia pulex*. *Ecotoxicology and Environmental Safety* 62 (1): 11-16.

Stark JD, Walthall WK. 2003. Agricultural adjuvants: acute mortality and effects on

population growth rate of *Daphnia pulex* after chronic exposure. *Environmental

Toxicolology and Chemistry* 22: 3056-3061.

Trumbo J. 2005. An assessment of the hazard of a mixture of the herbicide Rodeo and

the non-ionic surfactant R-11 to aquatic invertebrates and larval amphibians.

California Fish and Game. 91(1): 38-46.

Walthall WK, Stark JD. 1997. Comparison of two population-level ecotoxicological

endpoints: the intrinsic (r_m) and instantaneous rates (ri) of increase. *Environmental

Toxicology and Chemistry* 16(5): 1068-1073.

Wilbur-Ellis (2007). R-11 Material Safety Data Sheet.

http://www.cdms.net/ldat/mp956000.pdf

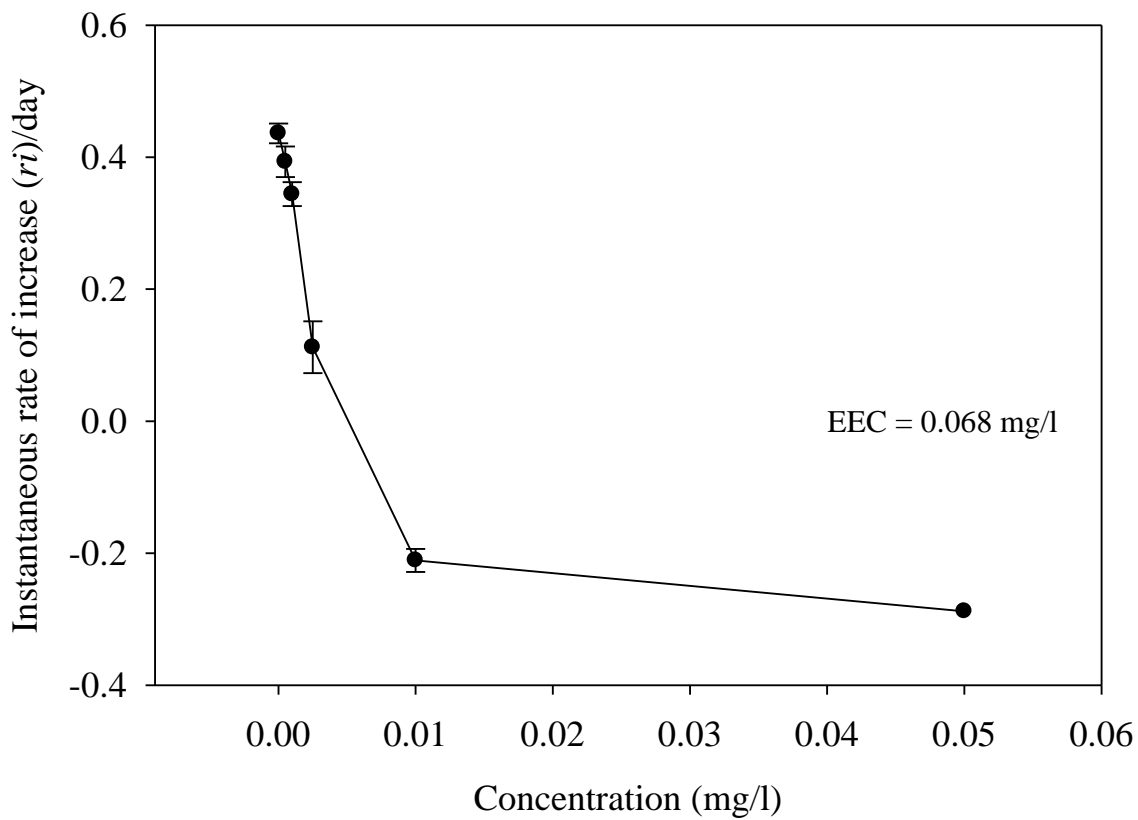**Figure 1.** Instantaneous rate of increase (*ri*) of *C. dubia* after exposure to spinosad with 95% CL.

Figure 2. Relationship between acute mortality (with 95% CL) and instantaneous rate of increase (*ri*) of *C. dubia* exposed to spinosad.

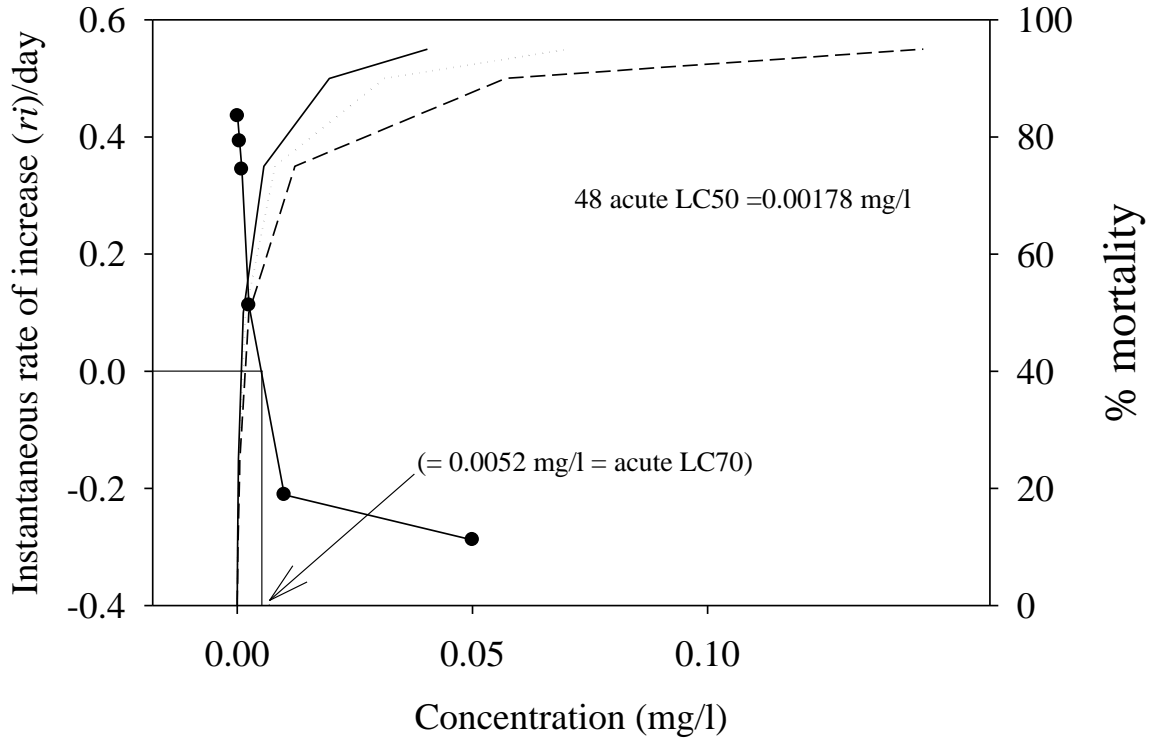48 acute LC50 =0.00178 mg/l

(= 0.0052 mg/l = acute LC70)

Fig. 3. Mean number of original individuals used to start the population after exposure to spinosad.
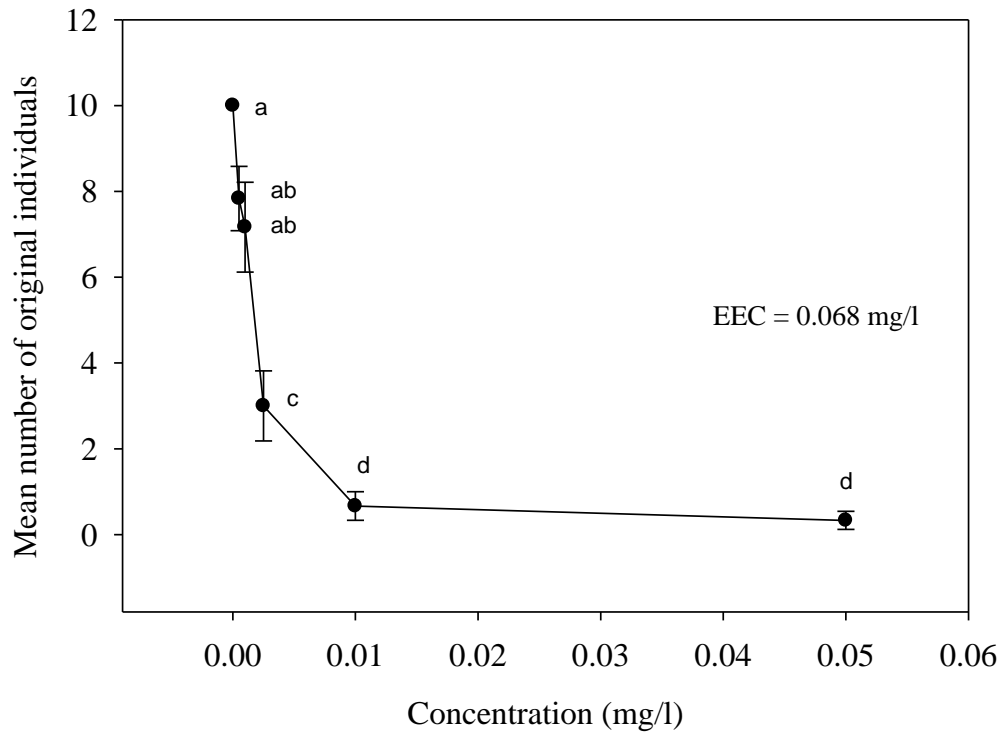
Fig. 4. Mean number of *C. dubia* offspring per surviving female after 8 d exposure to spinosad. Data points marked with different letters are significantly different from each other with a 95% CL.
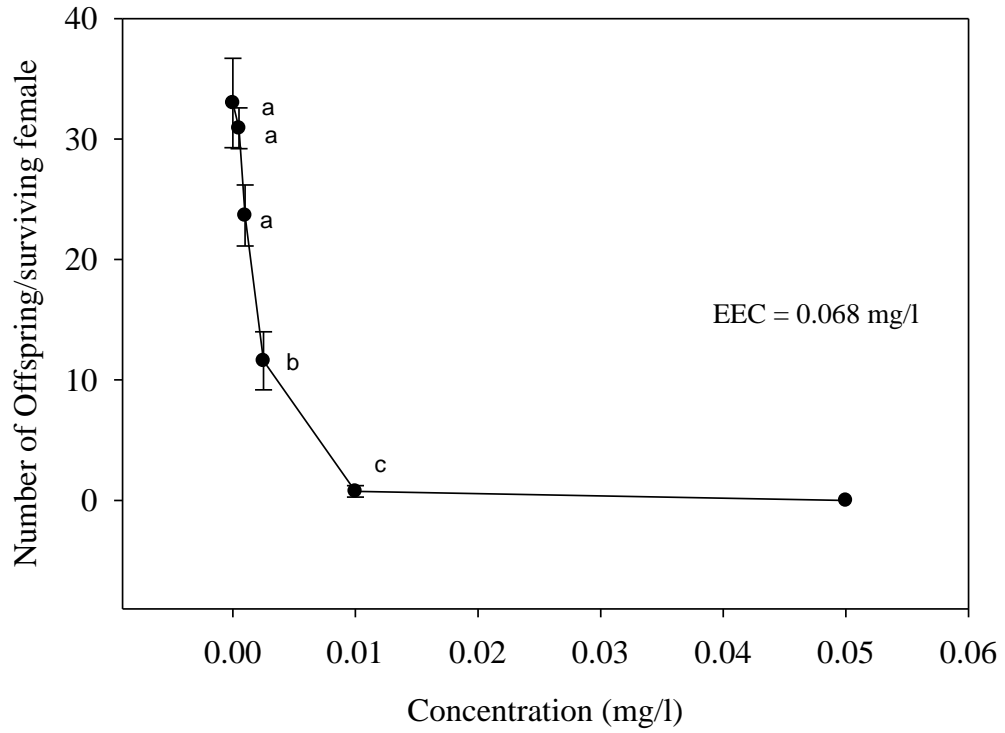
Fig. 5. Final number of individuals (original individuals and offspring) of *C. dubia* after exposure to spinosad. Data points marked with different letters are significantly different from each other with a 95% CL.
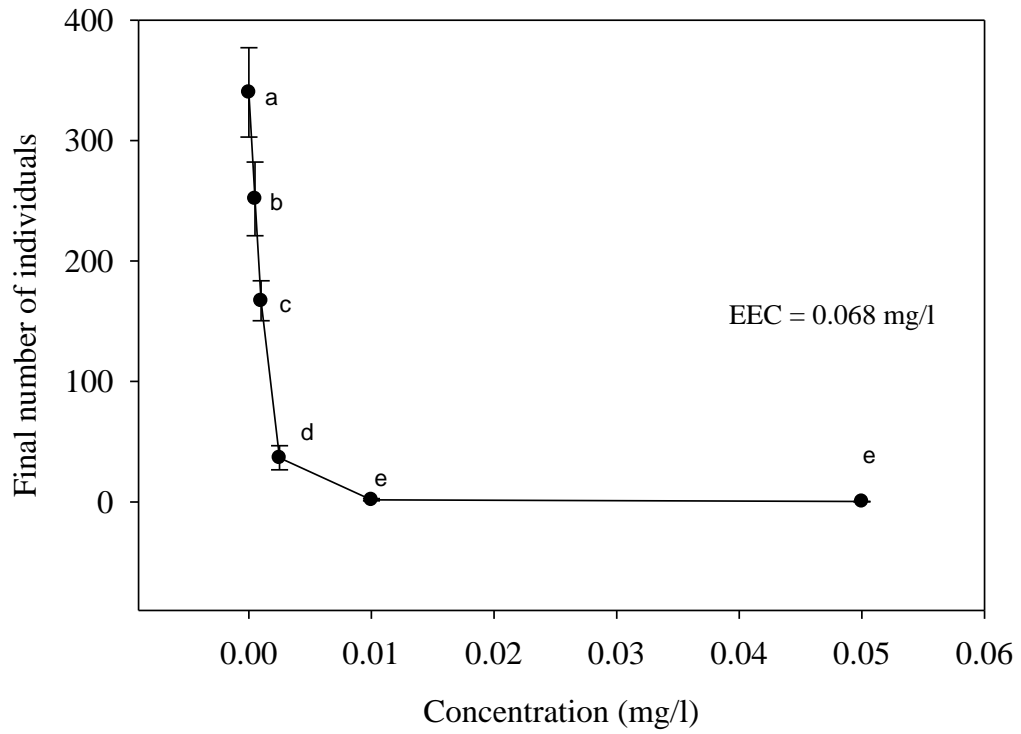
Figure 6. Instantaneous rate of increase (*ri*) of *C. dubia* after exposure to R-11 with 95% CL.
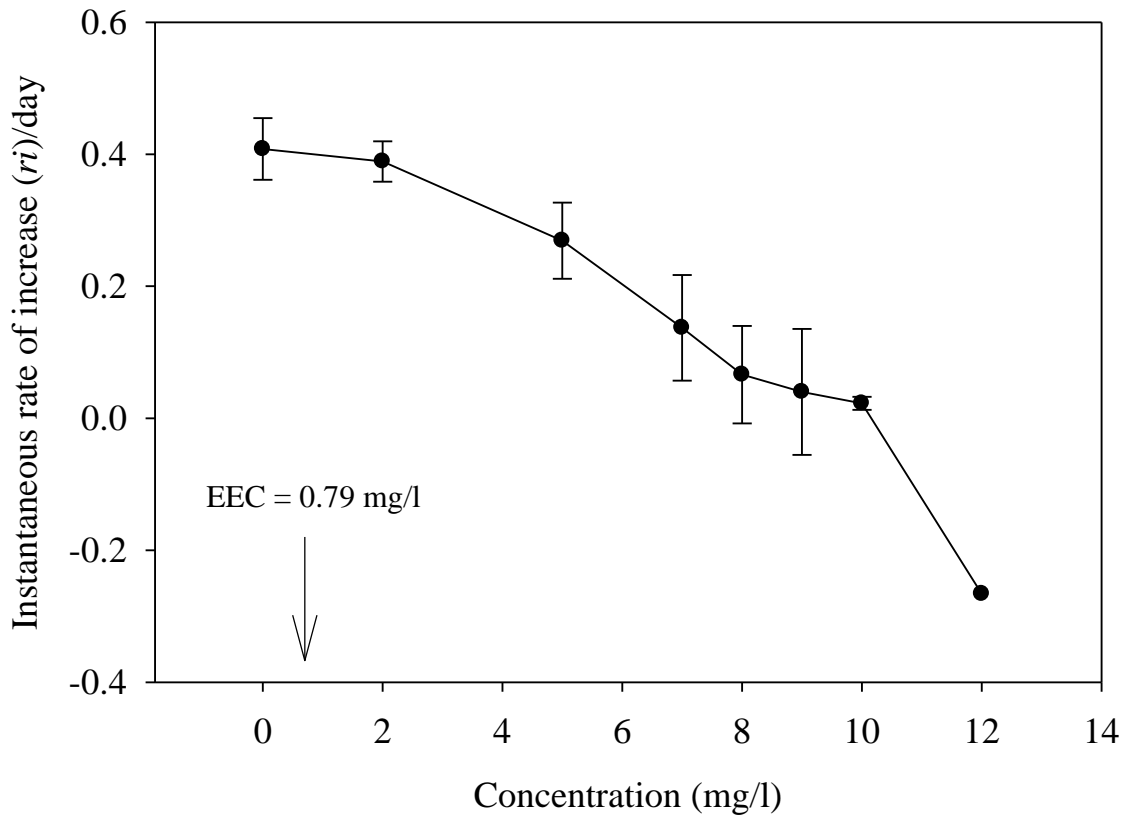
Figure 7. Relationship between acute mortality and instantaneous rate of increase (*ri*) for *C. dubia* exposed to R-11. The solid lines represents the instantaneous rate of increase (*ri*)/day. The dotted line represents the percent mortality of the acute toxicity tests involving R-11 and *C. dubia* flanked by the 95% CL.
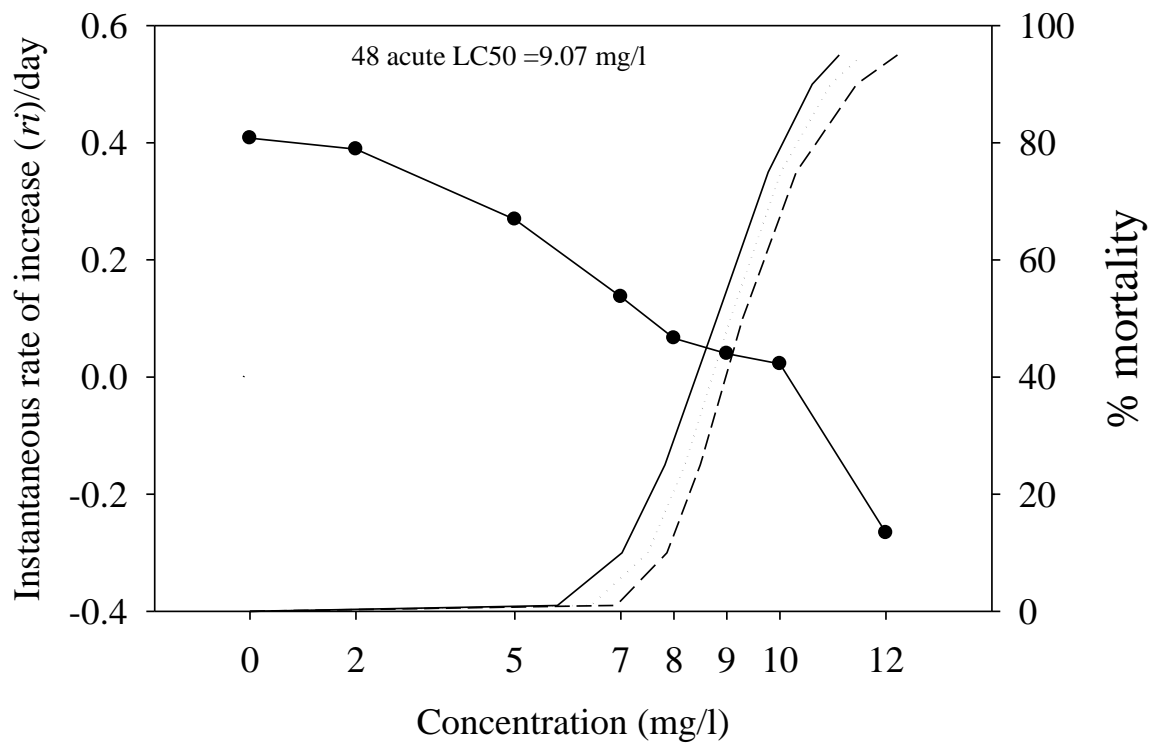

48 acute LC50 =9.07 mg/l

Fig. 8. Mean number of original individuals of *C. dubia* used to start population after exposure to R-11 with 95% CL.

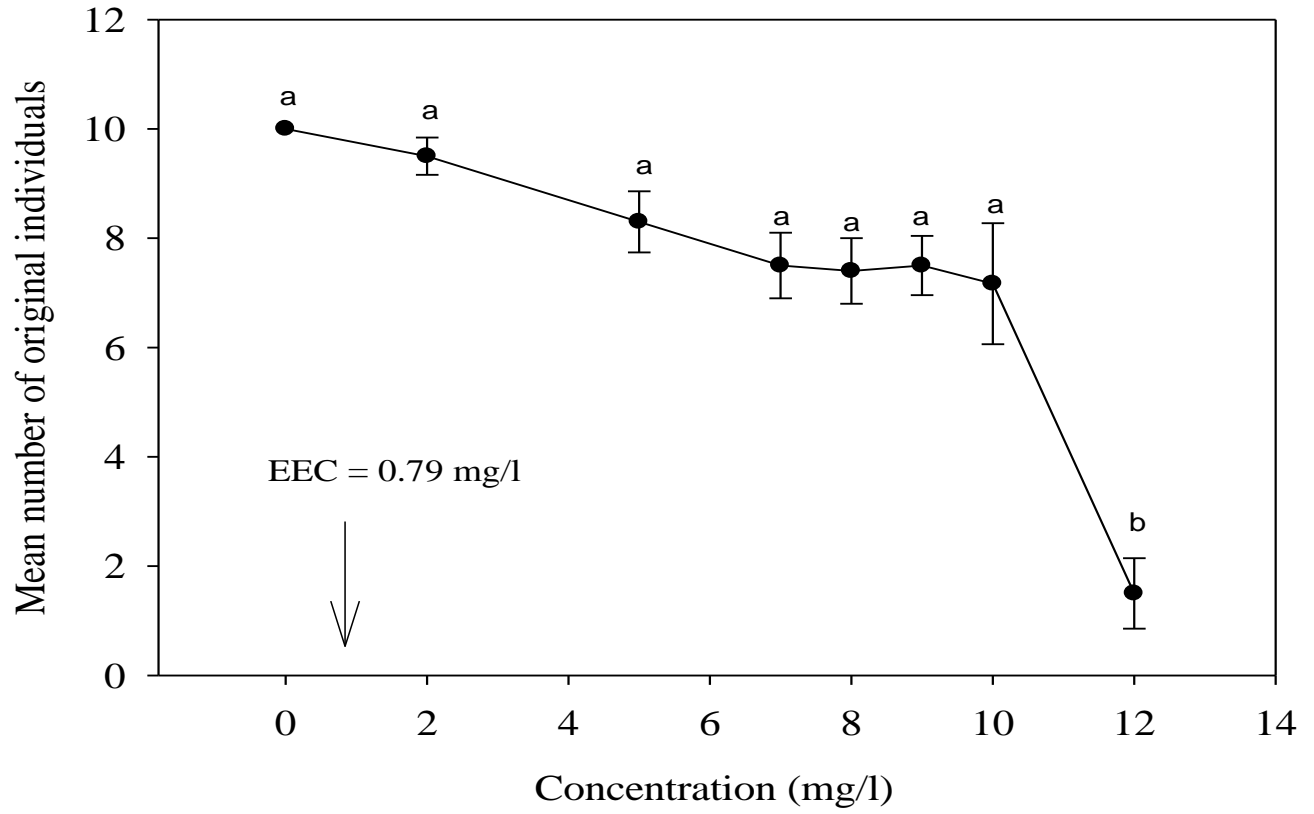**Fig. 9.** Mean number of *C. dubia* offspring per surviving female after 8 d exposure to R-11. Data points marked with different letters are significantly different from each other with a 95% CL.
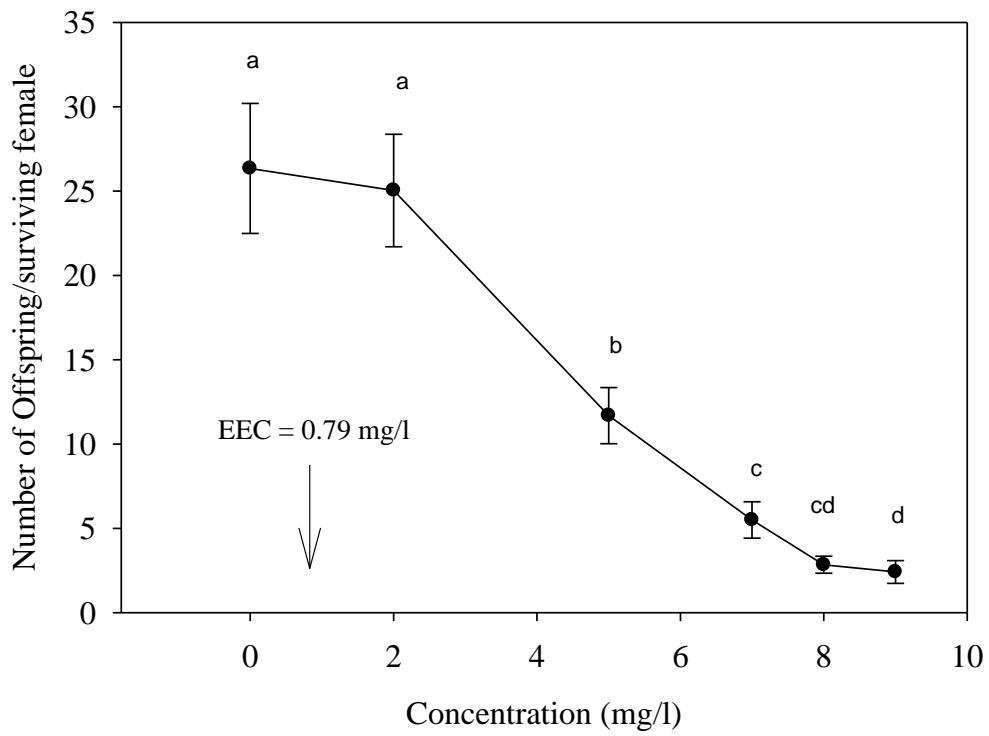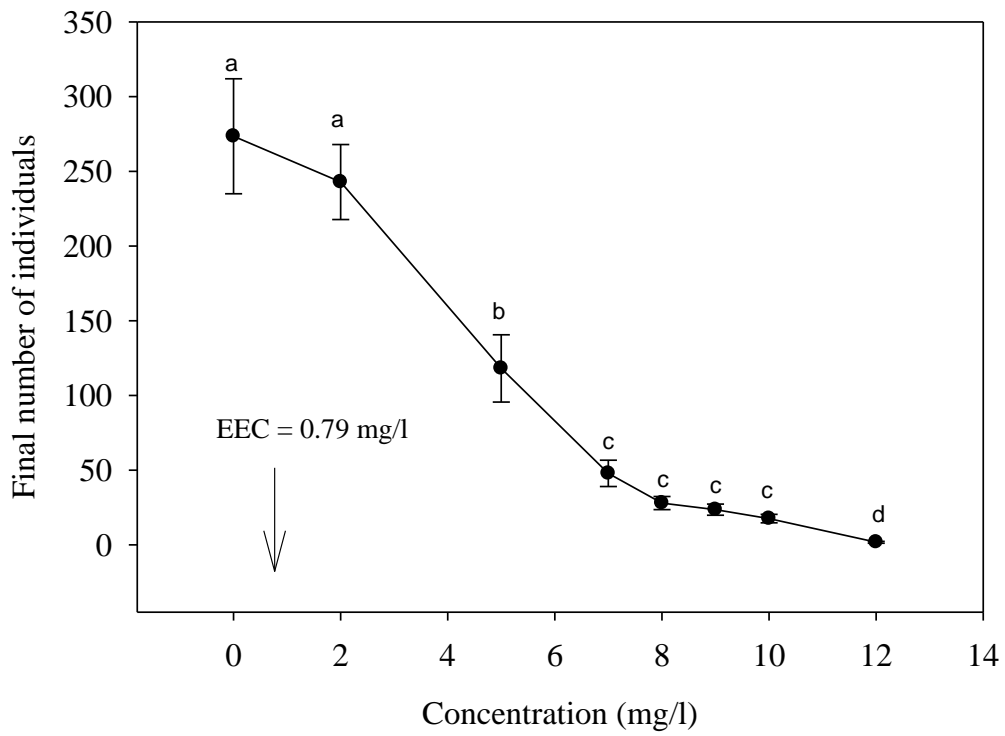
Fig. 10. Final number of individuals (original adults and offspring) of *C. dubia* after exposure to R-11. Data points marked with different letters are significantly different from each other with a 95% CL.

CHAPTER 4

MODELING THE EFFECTS OF SPINOSAD ON THREE CLADOCERAN SPECIES: CAN SURROGATE SPECIES BE USED TO ACCURATELY PREDICT EFFECTS ON OTHER SPECIES?

4.1 Abstract

In this study, the effects of spinosad, a natural, organic insecticide that is being used to control a wide-range of pest species, were evaluated using acute mortality and life table data in population models developed for three cladocerans, *Daphnia pulex* (Leydig), *D. magna* Straus, and *Ceriodaphnia dubia* Richard to determine how accurately one species could be used to estimate the effects on the other species. In a previous study, *D. pulex* was significantly less susceptible to spinosad than *C. dubia* and *D. magna* which were equally susceptible at LC50. Expected environmental concentrations (EEC) were compared to concentration-response curves to determine predicted mortality for each species. These predicted mortalities after exposure to the EEC were used in population models. At the EEC for spinosad (0.068 mg/l), predicted mortalities for *D. pulex*, *D. magna* and *C. dubia* were 38, 83 and 93%, respectively. Life tables were developed for each species without exposure to spinosad. Predicted mortalities at the EEC for spinosad were used in matrix population models developed with life table data. The endpoint of interest was recovery time after exposure to a stressor, in this case mortality. Recovery time was determined by comparing the time it took a control population to reach a predetermined number of individuals to the time it took the stressed population to reach the same number. Populations used in the simulations were started with 100 individuals in their respective stable age distribution. The model was run until 2 billion individuals were reached. Population modeling results indicated that exposure of each species to the expected environmental concentration (EEC) for spinosad would result in very different recovery times. The three species had similar life history traits. However, *D. pulex* had the highest population growth rate (λ) (1.2776/d) followed by *C. dubia* (1.2548/d) and *D.*

magna (1.2233/d). *D. pulex* had the shortest generation time (22.728 d) followed by *C. dubia* (24.128 d) and *D. magna* (27.281 d). Exposure to the EEC resulted in population recovery times of 1, 5, and 12 weeks for *D. pulex*, *D. magna* and *C. dubia* populations, respectively. These recovery times were equivalent to 0.31, 1.28, and 3.48 generation times for *D. pulex*, *D. magna* and *C. dubia* populations, respectively. Recoveries taking 1 generation time or more are indicative of damage at the population level. These results indicate that although *C. dubia* populations had an intermediate population growth rate, this species was the most at risk after exposure to spinosad at the EEC. Not only was *D. pulex* the least susceptible species in terms of acute mortality, this species also had the highest population growth rate, and was predicted to be the least impacted by spinosad at EEC. These results indicate that the use of even closely related species as surrogates for one another should be approached cautiously because of differences in susceptibility to toxicants and differences in life history traits among species.

4.2 Introduction

In ecological risk assessment, surrogate species are often used to make predictions about the fate of endangered species (http://www.epa.gov/oppfead1/endanger/consultation/ecorisk-overview.pdf, Stark 2006). Surrogate species are often used because species needing protection are threatened or endangered (T&E) and therefore their numbers are low and toxicological data cannot be developed for them. Two factors, however, may confound the use of surrogate species for determination of effects on another species; these are differential susceptibility to a specific chemical and/or differences in life history traits (Stark et al. 2004). Differential susceptibility has to do with differences in uptake, detoxification, and elimination of toxicants which vary even among closely related species. An important question is – how accurately can one species be used to predict what happens to another?

Cladocerans such as *Daphnia* spp. are an important component of aquatic ecosystems because they are primary consumers feeding on algae and bacteria and serve as a food source for other aquatic organisms including fish and invertebrates. Although *Daphnia* species are not threatened or endangered, they are often used as indicator species for estimating the effects of pollutants on aquatic ecosystems.

The most commonly generated toxicity data for *Daphnia* and other aquatic organisms are acute mortality estimates (LC50) or the concentration of a chemical that kills 50% of a population (Stark et al. 2004). The advantage of short-term toxicity data such as the LC50 is that it costs much less to produce than longer-term data. However, short-term toxicity data may not provide enough information about potential effects on populations

because chronic exposures can sometimes result in much higher mortality levels than predicted by acute exposures, and sublethal effects can occur which may impact populations at several levels, most notably by decreasing fecundity (Forbes and Calow 1999, Stark and Banks 2003, Stark 2005).

The objective of this study was to determine how accurately one species could be used to predict what happens to another species after exposure to toxicants. This was accomplished by evaluating the effects of spinosad on populations of daphnids and using a population modeling approach. Spinosad is a natural, organic insecticide that is being used to control a wide-range of pest species. Little is known about the effects of spinosad on aquatic organisms (Stark and Vargas 2003). Acute toxicity data after exposure to spinosad was developed for three cladoceran species, *Daphnia pulex* (Leydig), *D. magna* Straus, and *Ceriodaphnia dubia* Richard. Additionally, demographic data were developed for each species by developing life tables. These data were used to develop matrix population models whereby control populations could be compared to populations theoretically exposed to the chemicals. Population recovery was the endpoint of interest in the modeling study.

4.3 Materials and Methods

4.3.1 Chemicals evaluated

Spinosad (Success, 240 g active ingredient (ai)/l, Dow AgroSciences LLC9330 Zionsville Rd., Indianapolis, Indiana, 46268, USA) consists of a mixture of Spinosyns A and D which are fermentation products of the soil bacterium *Saccharopolyspora spinosa*

(Actinomycetes) (Crouse et al. 2001). Spinosad is a neurotoxin and acts as a contact and stomach poison (DowElanco 1996, Salgado 1998). The effects of this insecticide are consistent with the activation of nicotinic acetylcholine receptors and thus acts in a similar manner to nicotine in the nervous systems of animals, characterized by the excitation of the nervous system. Spinosad also has effects on GABA receptor function which may add to its toxicity to insects.

4.3.2 Acute mortality data used in the models

Acute mortality data used in the models in this study were developed in Chapter 2 for *D. magna* and *C. dubia*. Acute mortality data for spinosad and *D. pulex* was taken from Stark and Vargas (2003). A summary of the LC50 estimates are listed in Table 1.

4.3.3 Development of Demographic Data

For each species, 25 ml of water were transferred into a 30 ml plastic cup and one neonate was transferred into the test container using a disposable glass pipette. Daphnids used in this study were obtained from cultures at or beyond the third filial (F_3) generation. Thirty individuals were used for the development of each life table. Test containers were held in an environmental chamber at 25ºC. Daphnids were moved to fresh water every other day (Walthall and Stark 1997). Survival and reproduction were measured daily until all animals had died. The following demographic parameters were determined in this study: L_x, the proportion of females surviving at the start of the start of the age interval, M_x, the average number of female offspring produced per female by age x, birth

rate, death rate, intrinsic rate of increase, and the population growth rate (λ), the factor by which a population increases in size from time t to time $t + 1$ (Carey 1993). For a complete description of the calculation of these parameters see Carey (1993).

4.3.4 Model development

The population models developed for this study were age-based and age classes consisted of one-week intervals. Because of differences in longevity, the number of age classes varied among the species (Table 2). The model developed for *D. pulex* had 8 age classes, *D. magna* had 10 age classes and *C. dubia* had 9 age classes (Table 2). RAMAS Metapop (Akçakaya 2005) was used to develop age-structured models and took the form presented in Figure 1.

In these models, probabilities of survivorship and reproduction are organized into a transition matrix, which is then multiplied by a population vector containing information about the size of the population at each age *X* (Caswell 2001). Repeated iterations of the multiplication of the population vector and the transition matrix results in a stable population vector, so that further multiplication by the transition matrix results in multiplication of the population vector by a constant. Mathematically, this may be written as:

$$\lambda \mathbf{Z} \rfloor = \mathbf{L} \mathbf{Z} \rfloor$$

where L is the transition matrix Z is the population vector at equilibrium, and λ is the population growth rate. Once the population vector has reached equilibrium, this equation can easily be solved for λ.

Each model consisted of survival and fecundity values taken from the demographic study. Models developed to simulate pesticide exposure were developed by reducing the survival and fecundity values by the predicted mortality estimates. These mortality estimates were derived by comparing concentration-response curves for each species to the spinosad EEC (see next section below).

All models were run using exponential growth with 2 billion individuals set as the maximum population size (*K*). The starting population vector consisted of 100 individuals in the (deterministic) stable age distribution. Simulations had 1 replication and 52 time steps (weeks). Population recovery was determined by comparing the time it took each spinosad or R-11 exposed population to reach 2 billion individuals compared to the time it took the control to reach 2 billion individuals. Stark et al. (2004) have advocated the use of recovery time as an endpoint when modeling the effects of stressors on populations. Effects of toxicants can be compared by comparing recovery times of toxicant-exposed populations to control populations. Additionally, recovery time was compared to generation time by dividing recovery time by generation time. This results in a measure that Stark et al. (in press) have used to determine if populations are damaged. If recovery after a stressor is ≥ one generation time interval, then the population is damaged (Stark et al. 2007).

4.3.5 Expected Environmental Concentration

Expected environmental concentrations for spinosad and R-11 have been published previously (Stark and Banks 2001, Stark and Walthall 2003). The EEC for spinosad is 0.068 mg/l. This EEC value was compared to concentration-response curves (Fig. 1), to determine the corresponding predicted levels of mortality. At the EEC for spinosad (0.068 mg/l), predicted mortalities for *D. pulex*, *D. magna*, and *C. dubia* were 38, 83 and 93%, respectively (Fig. 1).

4.4 Results

In a previous study, *C. dubia* was approximately 72 times more susceptible to spinosad than *D. pulex*, and *D. magna* was approximately 27 times more susceptible than *D. pulex* at LC50 (Chapter 2). When the EEC for spinosad (0.068 mg/l) was compared to concentration-response curves for the three cladoceran species exposed to spinosad, predicted mortalities for *D. pulex*, *D. magna*, and *C. dubia* were 38, 83 and 93%, respectively. These mortality estimates were used in the population models.

4.4.1 Comparative demography and modeling of control populations

The demographic parameters for the cladoceran species were not extremely different (Table 3). However, *D. pulex* had the highest net reproductive rate (*Ro*), birth rate, intrinsic rate of increase (*ri*), population growth rate (λ), and the shortest generation

time, thus indicating that this species should grow at a faster rate than the other two species.

The population growth rate of *C. dubia* was higher than the growth rate for *D. magna* indicating that *C. dubia* populations should grow faster than *D. magna*. However, when population projections were developed from the population models, *C. dubia* initially increased faster than *D. magna* but both species reached the 2 billion individual level at the same time (Fig. 2). Populations of *D. pulex* grew faster than the other two species (Fig. 2). It took *D. pulex* 7 weeks to grow from 100 to 2 billion individuals. It took the other two species 8 weeks to reach the 2 billion level.

4.4.2 Population projections for populations exposed to the Spinosad EEC

Theoretical exposure to spinosad at the EEC resulted in 38% mortality in *D. pulex*. This level of mortality resulted in a population recovery period of 1 week for *D. pulex* (Fig.3). Exposure to the EEC for spinosad resulted in 83% mortality for *D. magna* (Fig. 4). This resulted in a 5 week recovery period for *D. magna*. Exposure to the spinosad EEC resulted in 93% mortality for *C. dubia* which caused a 12 week recovery period for this species (Fig. 5).

4.4.3 Comparison of population recovery to generation time

A comparison of recovery time interval to generation time showed that a recovery of 1 week for *D. pulex* was equivalent to 0.31 generation time. A recovery time interval of 5 weeks for *D. magna* was equivalent to 1.28 generation time. A recovery period of

12 weeks for *C. dubia* was equivalent to 3.48 generation time. These results indicate that both *D. magna* and *C. dubia* populations would be negatively impacted if they underwent these recovery periods.

4.5 Discussion

In this study, three closely related species were evaluated for the effects of spinosad on populations at a concentration likely to occur in the environment. Differences in susceptibility and differences in life history traits resulted in different recovery periods for each species, ranging from 1 to 12 weeks. Although these recovery periods seem short, a recovery period of 5 weeks for *D. magna* and 12 weeks for *C. dubia* were greater than a generation time and thus would cause damage to population of these species.

The question that was initially asked was – how accurately can one species be used to predict what happens to another? The results of this study indicate that very different outcomes can result in even closely related species when exposed to the same concentration. In some ecological risk assessments, data for closely related species is available. However, in other ecological risk assessments, toxicity data for closely related species are not available and thus species that are very distantly related, for example, using invertebrates to make predictions about vertebrates, must be used. Even though the species used in this study were closely related, large differences in recovery times were evident after exposure to the same concentration of spinosad. This was due to differences in demographic parameters and differences in susceptibility. These results indicate that the use of even closely related species as surrogates for one another should be approached

cautiously because of differences in susceptibility to toxicants and differences in life

history traits among species.

4.6 References

Akçakaya HR. 2005. RAMAS Metapop: Viability Analysis for Stage-Structured Metapopulations (version 5.0). Applied Biomathematics, Setauket, New York.

Carey JR. 1993. Applied demography for biologists with special emphasis on insects. Oxford University Press, New York.

Caswell H. 2001. Matrix Population Models: Construction, Analysis, and Interpretation. 2nd edn. Sinauer, Sunderland, MA.

Crouse GD, Sparks TC, Schoonover J, Gifford J, Dripps J, Bruce T, Larson L, Garlich L, Hatton C, Hill RL, Worden TV, and Martynow JG. 2001. Recent advances in the chemistry of spinosyns. *Pest Management Science* **57:** 177-185.

DowElanco. 1996. Spinosad Technical Guide. Form No. 200-03-001 (4/96).

Forbes VE, Calow P. 1999. Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environmental Toxicolology and Chemistry* 18: 1544-1556.

Salgado VL, Sheets JL, Watson GB, and Schmidt AL. 1998. Studies on the mode of action of spinosad: the internal effective concentration and the concentration

dependence of neural excitation. *Pesticide Biochemistry and Physiology* 60**:** 103-110.

Stark JD. 2005. How closely do acute lethal concentration estimates predict effects of toxicants on populations? *Integrated Environmental Assessment and Management* 1: 109-113.

Stark JD. 2006. Toxicity endpoints used in risk assessment: What do they really mean? SETAC Globe 7 (2): 29-30.

Stark JD, Banks JE. 2001. "Selective Pesticides": Are They Less Hazardous to the Environment? *BioScience* 51: 980-982.

Stark JD, Banks JE. 2003. Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology* 48: 505-19.

Stark JD, Walthall WK. 2003. Agricultural adjuvants: acute mortality and effects on population growth rate of *Daphnia pulex* after chronic exposure. *Environmental Toxicolology and Chemistry* 22: 3056-3061.

Stark JD, Banks JE, and Vargas RI. 2004. How risky is risk assessment? The Role that Life History Strategies Play in Susceptibility of Species to Stress. 2004. *Proceedings of the National Academy of Sciences* 101: 732-736.

Stark JD, Vargas RI. 2003. Demographic changes in *Daphnia pulex* (Leydig) after

     exposure to the insecticides spinosad and diazinon. *Ecotoxicology and*

     Environmental Safety 56: 334-338.

Stark JD, Vargas RI, and Banks JE. 2007. Incorporating Ecologically Relevant

     Measures of Pesticide Effect for Estimating the Compatibility of Pesticides and

     Biocontrol Agents. *Journal of Economic Entomology* 100 (4): 1027-1032.

www.epa.gov/oppfead1/endanger/consultation/ecorisk-overview.pdf. 2004. Overview

     of the Ecological Risk Assessment Process in the Office of Pesticide Programs,

     U.S. Environmental Protection Agency. Office of Prevention, Pesticides and

     Toxic Substances, Office of Pesticide Programs, Washington, D.C.

Walthall WK, Stark JD. 1997. Comparison of two population-level ecotoxicological

     endpoints: the intrinsic (rm) and instantaneous (*ri*) rates of increase.

     Environmental Toxicolology and Chemistry 16: 1068-1073.

Table 1. Acute (48 h) concentration-mortality estimates

for three cladoceran species exposed to spinosad.

Species	Spinosad LC50 (95% CL) mg/l
C. dubia	0.00178 (0.00128—0.00247)
D. magna	0.00482 (0.00191—0.00998)
D. pulex	0.129 (0.077—0.181)

Table 2. Age classes used in the RAMAS matrix models

	D. pulex	*D. magna*	*C. dubia*
Age class 1	1 to 7 days old	1 to 7 days old	1 to 7 days old
Age class 2	8 to 14 days old	8 to 14 days old	8 to 14 days old
Age class 3	15 to 21 days old	15 to 21 days old	15 to 21 days old
Age class 4	22 to 28 days old	22 to 28 days old	22 to 28 days old
Age class 5	29 to 35 days old	29 to 35 days old	29 to 35 days old
Age class 6	36 to 42 days old	36 to 42 days old	36 to 42 days old
Age class 7	43 to 49 days old	43 to 49 days old	43 to 49 days old
Age class 8	50 to 56 days old	50 to 56 days old	50 to 56 days old
Age class 9		57 to 63 days old	57 to 63 days old
Age class 10		64 to 70 days old	

Table 3. Life table parameters for three species of cladocerans.

Species	Ro	Birth rate $(b)^1$	Death rate $(d)^1$	Generation time (T) (days)	Doubling time (DT) (days)	Intrinsic rate of increase $(rm)^1$	λ^1
D. pulex	261.73	0.2458	0.0009	22.728	2.8297	0.2450	1.2776
D. magna	244.500	0.1826	-0.019	27.281	3.4386	0.2016	1.2233
C. dubia	239.133	0.2038	-0.023	24.128	3.0535	0.2270	1.2548

1/ These values are in units/day

Figure 1. Comparison of spinosad concentration-response curves and the EEC for spinosad
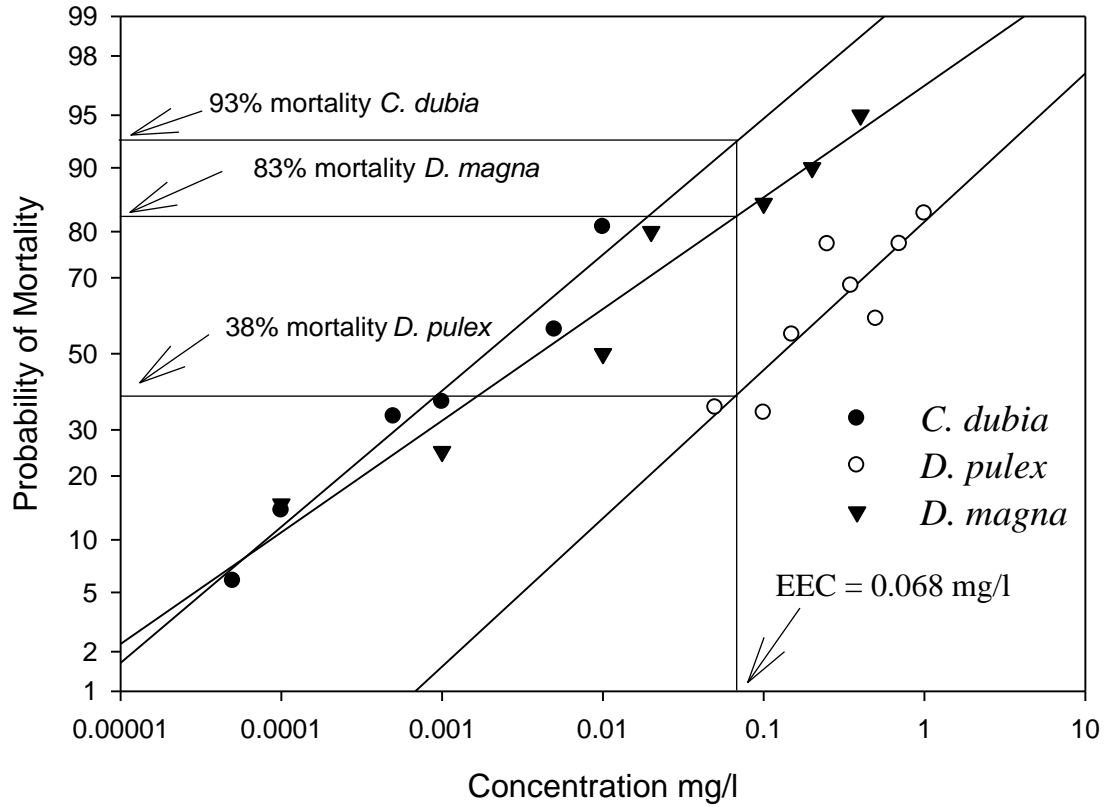
Figure 2. Population projections developed by matrix models for three daphnid species unexposed to chemical stressors.
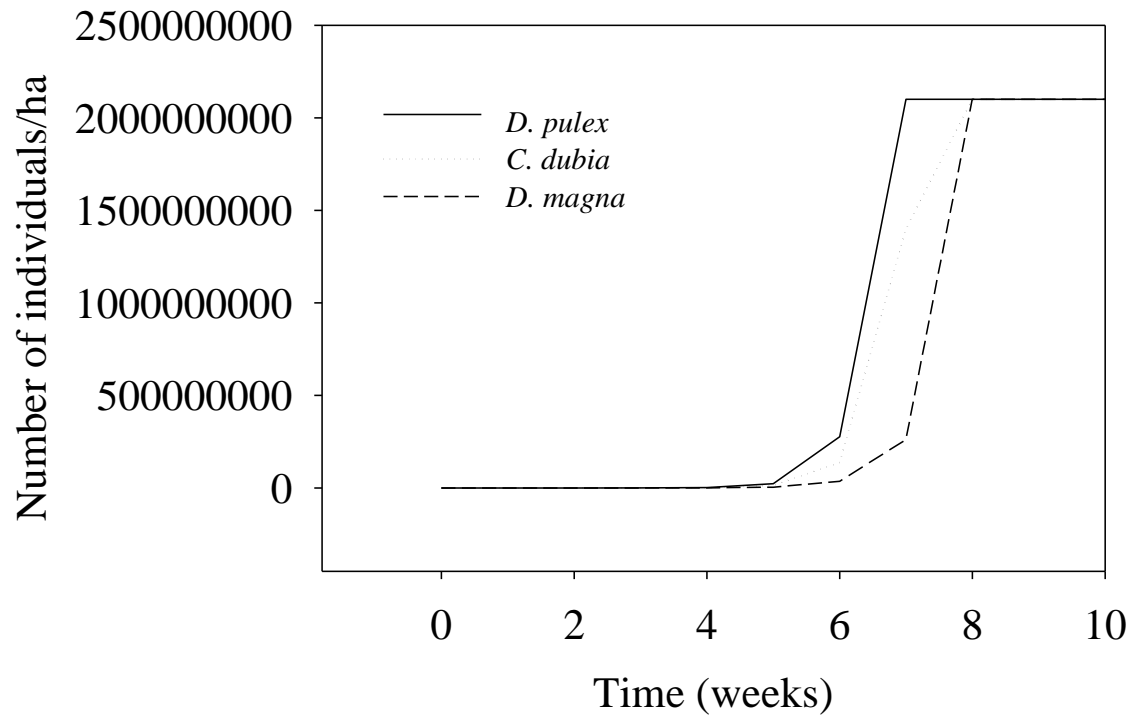
Figure 3. Population recovery of *D. pulex* after exposure to the EEC for spinosad.
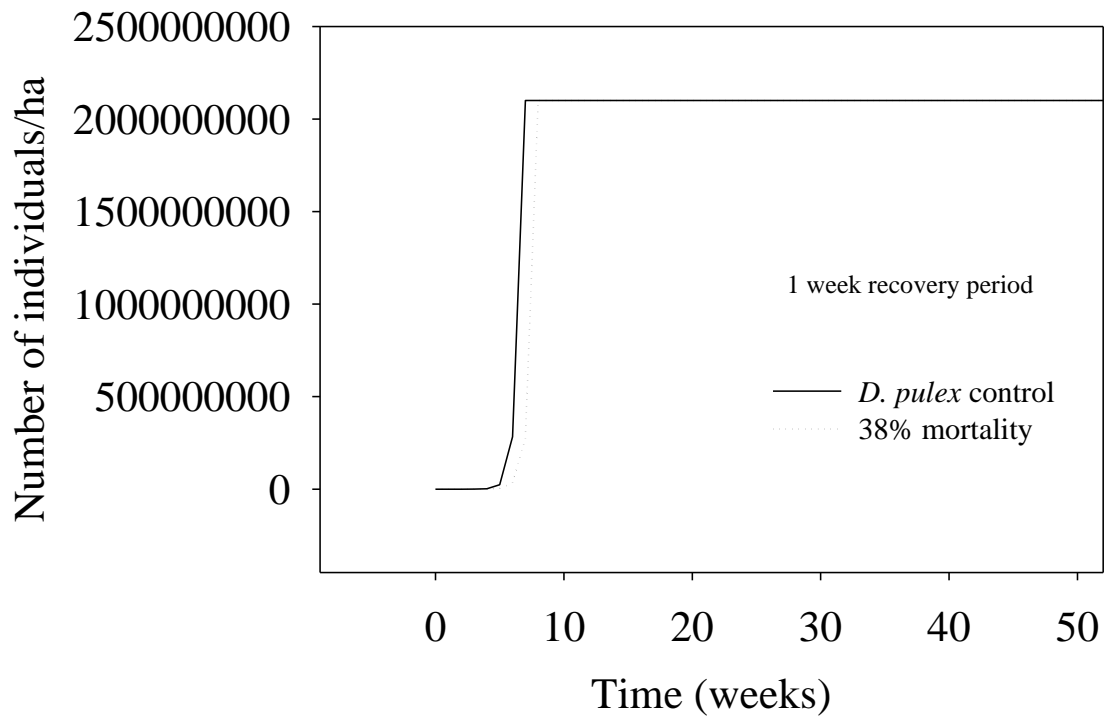
Figure 4. Population recovery of *D. magna* after exposure to the EEC for spinosad.
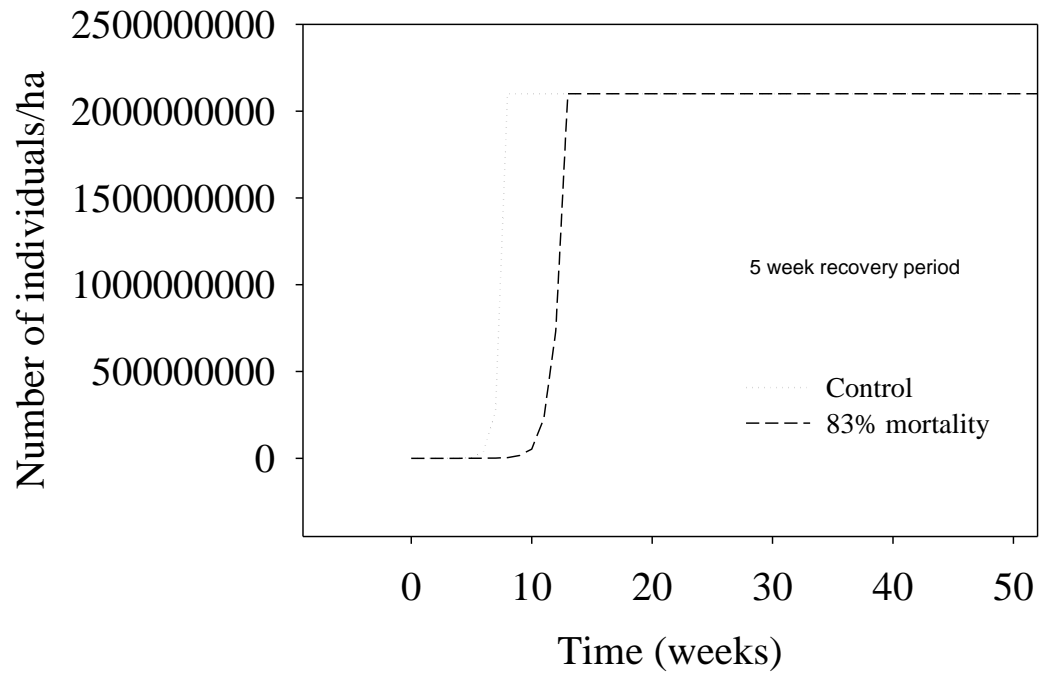
Figure 5. Population recovery of *C. dubia* after exposure to the EEC for spinosad.

Y-axis: Number of individuals/ha

2500000000
2000000000
1500000000
1000000000
500000000
0

X-axis: Time (weeks)

0   10   20   30   40   50

12 week recovery period

.... *C. dubia*
‐ ‐ ‐ ‐ 93% mortality