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Acute Toxic Effects of Neem-Based Insecticides on Crustaceans

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Azadirachtin (AZA), extracted from the seeds of the neem tree (*Azadirachta indica*), is considered to be the most biologically active component of neem-based insecticides. These natural pesticides are known to have strong antifeedant, growth regulatory, and sterility effects on insects (Schmutterer *et al.* 1981; Jacobson, 1989). Insects are classified in the phylum of Arthopoda and are characterized by the presence of an exoskeleton that must be shed in order to grow (Lachaise *et al.* 1993). The animal will undergo discontinuous growth through molting and this process is regulated by the hormone ecdysone (Brown and Cunningham 1939). Many studies have shown that 20-β-hydroxyecdysone is the physiologically active form of the insect-molting hormone (Börst and Engelmann 1974). It has been found that AZA may adhere to insect proteins through hydrogen bonding similar to that formed by 20-β-hydroxyecdysone and that the 20-hydroxy and 3-hydroxy positions of ecdysone are critical for biological activity (Schumutterer, 1995).

Like insects, crustaceans are classified as Arthopoda. There is little information regarding the toxic effects of AZA and neem-based pesticides on aquatic crustaceans. Most of the available data are from standard toxicity tests on water fleas and fish that are part of toxicological screening process for the commercial development of pesticides (Kreutzweiser 1997). Zebitz (1987) studied the toxicity of the commercial neem-based insecticide Margosan-O® on rainbow trout and bluegills and found 96-h LC_{50} values of 8.8 mg Margosan-O®/L for rainbow trout and 37 mg Margosan-O®/L for bluegills. When compared with other commonly used synthetic insecticides using the same species (*D. pulex*), neem-based insecticides have relatively low LC_{50} values. These relatively low LC_{50} values suggest that toxicity studies are necessary to understand the impact of these pesticides (Neemix™ and Bioneem™) on aquatic species in areas where agricultural runoff waters may carry significant amounts of pesticide to the environment. In this study, we conducted *in vivo* acute toxicity tests on selected species of crustaceans (*Procambarus clarkii*, crayfish; *Palaemonetes pugio*, grass shrimp; *Penaeus setiferus*, white shrimp; *Callinectes sapidus,* blue crab; and *Daphnia pulex*, water fleas) to provide information about the direct toxic effects of these insecticides, find the most sensitive species to these insecticides, and compare the results of acute toxicity tests to an insect species (*Culex quinquefasciatus,* mosquito).

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MATERIALS AND METHODS

Neemix™ (0.25% AZA or 2,500 μg/mL AZA, active compound, and 99.75% inert ingredients) was provided by Thermo Trilogy Corp., Columbia, MD. Bioneem™ (0.09% AZA or 900 μg/mL AZA and 99.91% inert ingredients, Safer® Inc., Bloomington, MN) was purchased from a local plant nursery in Baton Rouge, LA. Azadirachtin (~95% purity) was purchased from the Sigma Chemical Co., St. Louis, MO. Pure AZA was dissolved in ethanol (100% purity, Aaper Alcohol and Chemical Co., Shelbyville, KY) prior to making dilutions for desired concentrations. AZA content in Neemix™ and Bioneem™ was expressed as weight per volume (μg/mL) and the concentrations of Neemix™ and Bioneem™ were calculated based on their AZA equivalence.

Juvenile crayfish (~3-4 weeks old) were obtained from the University of Louisiana at Lafayette (Lafayette, LA). Blue crab megalopes (~2 months old) and juvenile grass shrimp (~1-1,5 months old) were collected from the Port Fourchon, LA wetland area near the shore using a light trap. Juvenile white shrimp (~2 months old) were collected from the Grand Chenier, LA, wetland area using a dip net. Mosquito eggs were collected with a dipper from a runoff canal in Denham Springs, LA. Water fleas were provided by C.K. Associates (Baton Rouge, LA). Environmental conditions for each species were adjusted according to their natural environmental conditions, e.g. temperature, salinity, dissolved oxygen (DO), and pH, with a photoperiod of 16 h light:8 h dark. These species were chosen because of the following reasons: a) their representation of different orders and classes of aquatic crustaceans, b) their representation of different functional feeding groups, and c) their varying sensitivities to environmental pollution.

Acute lethal effects of the neem-based pesticides and pure AZA on the five crustacean species and the mosquito larvae were determined in static non-renewal acute toxicity tests (EPA 1993). Juvenile crayfish, white shrimp, grass shrimp, and blue crab megalopes were transported in water from their origin to the Louisiana State University (LSU), Department of Food Science Building (Baton Rouge, LA). Upon arrival, they were kept in an aquarium and fed with shrimp pellets for 3 days before the toxicity tests started. Blue crabs were fed with ground shrimp pellets. Mosquito eggs were carried in a small pail to LSU and kept in a glass container in deionized (DI) water until they hatched. The larvae were then fed with ground dry dog food until they reached the 3rd instar. Water fleas were kept in a white plastic container for 2 h before the toxicity tests started. During toxicity experiments, crayfish, grass shrimp, blue crab, and white shrimp were kept in separate glass containers (1½" in diameter, Carolina Biological Supply Co., Burlington, NC) to avoid cannibalism.

Shortly before starting exposure for the crayfish experiment, seven different concentrations ranging from 0 to 20 μg AZA/mL, were prepared in 20 mL of DI. The AZA concentrations were prepared in EtOH at 0.001, 0.01, 0.1, and 1 μg AZA/mL. Twenty crayfish were placed in glass containers (5 cm in diameter) at various pesticide dilutions. Observations, such as mortality and molting, were

made at 0, 24, 48, 72, and 96 h. Test animals were considered dead if they showed no movement after being agitated for 5 sec.

Salinity and temperature of water where blue crabs were collected were 30 ppt and 26°C. Saltwater at the same salinity was prepared using DI water and sea salt (Instant Ocean Salt, Aquarium Systems, Mentor, OH). The concentrations of Neemix™ were 0, 0.25, 0.5, 1, 2, and 4 µg AZA/mL (0, 0.1, 0.2, 0.4, 0.8, and 1.6 µL Neemix™/mL) in 10 mL of DI water. Bioneem™ and pure AZA were not used for this experiment. After preparing the solutions, twenty blue crabs per concentration were placed in separate glass containers (2 cm in diameter). The pH, temperature, salinity, and DO parameters were monitored and recorded at 0, 24, 48, 72 and 96 h. Animals were observed daily for mortality and molting.

The concentrations of Neemix™ used in grass and white shrimp experiments were 0, 0.625, 1.25, 2.5, 5, and 10 µg AZA/mL (0, 0.25, 0.5, 1.0, 2.0 and 4.0 µL Neemix™/mL). The concentrations of Bioneem™ were 0, 0.625, 1.25, 2.5, 5, and 10 µg AZA/mL (0, 0.69, 1.38, 2.76, 5.55, 11.11 µL Bioneem™/mL). Bioneem™ was not used in white shrimp toxicity tests, and pure AZA was not used in both grass and white shrimp experiments. Saltwater was prepared using DI water and sea salt (Instant Ocean Salt, Aquarium Systems, Mentor, OH) at 22 ppt for the grass shrimp experiment. Salinity of water where white shrimp were caught was 31 ppt. After preparing the desired concentrations in 31 ppt salt water, ten white shrimp or twenty grass shrimp were placed in separate glass containers with 5 cm in diameter.

For the water flea toxicity tests, seven dilutions of Neemix™, Bioneem™ or pure AZA were prepared at concentrations between 0 and 0.5 µg AZA/mL using DI water. Shortly after preparing the above dilutions, ten water fleas (less than 24 h old) per concentration were placed in glass vials. Water fleas were observed daily for mortality during 48 h of exposure.

The third instar larvae of mosquitoes were used to test acute toxicity of Neemix™, Bioneem™ or pure AZA. The concentrations of Neemix™,and Bioneem™ ranged from 0 to1 µg AZA/mL in 20 mL of DI water. Mosquito larvae were observed for their molting during 96-h exposure tests. The concentrations of pure AZA were prepared using 20 mL of DI water at concentrations of 0, 1.25, 2.5, 5, and 10 µg AZA/mL. For every concentration twenty larvae were placed in glass containers.

Statistical analyses included the calculation of lethal concentration for mortality of 50% (LC_{50}) of the bioassayed individuals, average, standard error of the parameters recorded daily, and 95% confidence intervals of LC_{50}. LC_{50} values on all mortality toxicological data and corresponding 95% confidence limits were calculated using a computer-based Sperman Kerber Procedure (SAS 1995). The differences among treatment levels in molting experiments were tested by two-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

LC_{50} values of Neemix™ for six aquatic species are listed in Table 1. Water fleas (*D. pulex)* had the lowest LC_{50} value, 0.07 μg AZA/mL (0.028 μL Neemix™/mL) for Neemix™ followed by mosquito and blue crab with LC_{50} of 0.57 μg AZA/mL (0.23 μL Neemix™/mL) and 1.15 μg AZA/mL (0.46 μL Neemix™/mL), respectively. Bioneem™ also showed the higher toxicity to *D. pulex* than all other species tested (Table 2). LC_{50} of Bioneem™ for *D. pulex* was 0.03 μg AZA/mL (0.033 μL Bioneem™/mL). In the present study, even though AZA concentrations were equivalent in the neem-based pesticides and pure AZA preparations, pure AZA was less toxic to *D. pulex* with an LC_{50} value of 0.382 μg AZA/mL. Comparison of the relative toxicity of Neemix™ or Bioneem™ with two commonly used organophosphate pesticides, namely Chlorpyrifos® and Malathion®, indicates that neem-based insecticides show similar toxicity to water fleas as organophosphate pesticides. LC_{50} values of Chlorpyrifos® and Malathion® for *D. magna* were reported by Leight and Dolah (1999) to be 0.001 and 0.033 μg/mL, respectively.

Mortality of blue crab megalopes exposed to Neemix™ for 96 h increased with increased concentrations of Neemix™. Megalopes that survived to juvenile at concentrations lower than 1 μg AZA/mL (0.4 μL Neemix™/mL) were able to molt and develop normally. This might be due to the fact that survivors were able to recover from the short-term toxic effects of Neemix™. Similar toxic effects have been observed with other pesticides such as Dimilin®. Blue crab larvae were exposed to 0.0005-0.006 μg/mL Dimilin® for 96 h and survival was always <5% at Dimilin® concentrations higher than 0.003μg/mL (Costlow 1979). These results suggest that blue crab is highly sensitive to exposure of any pesticide at megalope and larval stages, possibly because of the frequency of molting cycles (Heard 1982). Thus, in cases of spills or considerable agricultural run-off contaminated with neem-based insecticides, blue crabs may be one of the most susceptible crustacean species to direct exposure of these pesticides in the aquatic environment.

White shrimp were more sensitive to Neemix™ than grass shrimp at the same pesticide concentrations expressed as AZA equivalence (μg AZA/mL) (Table 1). During 96-h exposure, almost 100% of white shrimp died within the first 24 h of exposure to high concentrations (0.5, 1, and 2 μg AZA/mL) of Neemix™. Mortality did not occur as fast in grass shrimp exposed to high concentrations of the same pesticide. The reason for the different responses between the two shrimp species (white and grass) to Neemix™ is not known, however, previous studies indicated that the effectiveness of neem-based pesticides may vary among life stages, species, and formulations (Isman 1997).

Among all the species tested, crayfish were found to be the least sensitive to Neemix™ and Bioneem™. LC_{50} of the above pesticides for crayfish ranged from 4.71 to 6.60 μg AZA/mL (5.18 μL Bioneem™/mL or 2.64 μL Neemix™/mL). The LC_{50} for pure AZA was greater than the highest concentration used (>1 μg

Table 1. LC_{50} values of Neemix™ for six aquatic species[a].

Species	Estimated LC_{50}[b]	95% Confidence Intervals[b]	Exp. Dura. (h)
Water fleas	0.07 (0.028)	0.08 (0.032) - 0.06 (0.024)	48
Mosquitoes	0.57 (0.23)	0.65 (0.26) - 0.50 (0.20)	96
Blue crabs	1.15 (0.46)	1.39 (0.56) - 0.95 (0.38)	96
White shrimps	2.68 (1.07)	5.03 (2.01) - 1.43 (0.57)	96
Grass shrimps	3.81 (1.52)	4.75 (1.90) - 3.06 (1.22)	96
Crayfish	6.60 (2.64)	7.56 (3.02) - 5.75 (2.30)	96

[a] Data generated with Sperman Kerber Analysis (EPA , 1993)
[b] Concentrations based on AZA equivalence (μg AZA/ mL), values in parentheses represent the corresponding Neemix™ concentrations (μL/ mL)

AZA/mL). This result suggests that toxicity of Neemix™ and Bioneem™ to crayfish resulted from other formulation components, other components from neem, or from synergistic effects of the formulation components that enhanced the toxicity of AZA. Most crayfish that molted within 96 h died immediately after the molt (data not shown). This might be because of the greater sensitivity of post-molt animals to pesticide formulations. Similar results were found by Banken and Stark (1997), who studied the toxic effects of Neemix™ on aphid *Coccinella septempunctata* 1st and 4th instar. They reported that 4th instar (after 3 molt) aphids were more sensitive to the growth-disrupting effects of acute exposure to Neemix™ than the 1st instar (before molt). LC_{50} values of neem-based pesticides were higher than those of Endosulfan® (organochlorine pesticide, 0.009 μg/mL), Chlorpyrifos® (organophosphate (OP), 0.021 μg/mL), and Malathion® (OP, 5 μg/mL) (Cheah *et al.* 1980; Cebrian *et al.* 1992).

Both Neemix™ and Bioneem™ had strong toxic activities against mosquito larvae at concentration higher than 0.25 μg AZA/mL (0.100 μL Neemix™/mL) for Neemix™ and 0.0625 μg AZA/mL (0.069 μL Bioneem™/mL) for Bioneem™. The higher toxicity of Bioneem™ might be because of the presence of other compounds that are absent in Neemix™ or caused by synergistic effect of "inert ingredients" and pure AZA. The toxic effects of other neem compounds and pure AZA have been tested on different species of mosquito larvae. Sharma *et al.* (1993) tested neem oil on mosquito larvae at concentrations of 0.5, 1, and 2% mixed in coconut oil and reported that it produced strong antimolting and toxic action on mosquito larvae. Su and Mulla (1998) tested the ovicidal activity of Azad™ WP10 (10% AZA as an active agent) and Azad™ EC4.5 (4.5% AZA as an active agent) on two different mosquito egg colonies (*Culex tarsalis* and *Culex quinquefasciatus*) at 0, 0.1, 0.5, 1, 5, and 10 μg pesticide/mL. The egg colonies of *C. quinquefasciatus* were more susceptible to both formulations than *Culex tarsalis* egg rafts. The neem suspension at 1 μg/mL produced almost 100% mortality in eggs of *C. quinquefasciatus*, while 5 μg/mL neem suspension/mL showed 100% mortality in eggs of *C. tarsalis*. These results differ from those of Al-Sharook *et al.* (1991) who tested the toxicity and growth inhibitory activity of 96% pure AZA on *Culex*

Table 2. LC_{50} values of Bioneem™ for four aquatic species[a].

Species	Estimated LC_{50} [b]	95% Confidence Intervals [b]	Exp. Dura.(h)
Water fleas	0.03 (0.033)	0.04 (0.044) - 0.02 (0.022	48
Mosquitoes	0.14 (0.154)	0.15 (0.165) - 0.12 (0.120)	96
Grass shrimps	3.19 (3.51)	3.92 (4.31) - 2.59 (2.85)	96
Crayfish	4.71 (5.18)	5.75 (6.33) - 3.85 (4.24)	96

[a] Data generated with Sperman Kerber Analysis (EPA , 1993)
[b] Concentrations based on AZA equivalence (μg AZA/ mL), values in parentheses represent the corresponding Neemix™ concentrations (μL/ mL)

pipiens molestus 3rd instar larvae and found an LC_{50} value of 1-5 μg/mL for pure AZA on this species. As discussed above, different mosquito species (i.e. *Culex tarsalis* and *Culex quinquefasciatus*) have different sensitivities to AZA. Furthermore, the source and method of extraction used in AZA production may yield AZA with varying potency. Molting frequency decreased in mosquitoes as the concentrations of Bioneem™ and Neemix™ increased. This is expected since the number of deaths was also high at higher concentrations. There was no significant difference ($p>0.05$) between the molting rate at concentrations lower than 0.5 μg AZA/mL or 0.2 μL Neemix™/mL (data not shown). In this experiment, there was no significant difference ($p>0.05$) among treatments, except in 1% EtOH. EtOH at 1% concentration reduced molting of mosquito larvae by about 5%. Based on the results discussed above, AZA caused a sharper decrease in molting of mosquito larvae than the neem-based pesticides.

From the ecotoxicological point of view, it should be emphasized here that the LC_{50} values of Neemix™ and Bioneem™ in the present study were determined by exposing test animals to pesticides under laboratory conditions that would represent the worst case contamination scenario (e.g. accidental applications and spills). Under field conditions, soil microbial and chemical activities would influence the effect of these compounds.

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