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Toxicological effects of the aquatic herbicide, fluridone, on male water mites (Hydrachnidiae: Arrenurus: Megaluracarus)

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Abstract The acute toxicities for technical grade fluridone (Sonar^{TM}) and the commercial formulation of fluridone (Sonar®AS) were assessed for male water mites (Hydrachnidiae: Arrenurus: Megaluracarus). Signs of toxicity were evaluated by detection of locomotor dysfunction or death after exposure to concentrations of 100,000, 10,000, 1,000, and 100 μg/L of Sonar^{TM} and 10,000, 5,000, 1,000, 100, and 10 μg/L of Sonar®AS in US EPA, moderately hard reconstituted water (MHRW). The median effective concentration (EC50) was 891 and 631 μg/L for Sonar^{TM} at 48 and 96 h and less than 10 μg/L for Sonar®AS at 96 h. Increased duration of exposure to Sonar®AS from 48 to 96 h had a significant effect on increasing the rate of combined morbidity and mortality. At the lowest concentration of Sonar®AS tested, which is half the concentration allowed within 400 m of any functioning potable water intake for human usage, 40% of the mites were adversely affected at 48 h and 70% were affected after 96 h of exposure. This study demonstrates that Sonar®AS is 60-fold more toxic to water mites than the active ingredient alone. At currently acceptable application rates of 90–150 μg/L fluridone, the addition of ingredients classified as inert, as in Sonar®AS, result in an increased risk of adverse effects on populations of male water mites (Arrenurus: Megaluracarus) in aquatic ecosystems.

Keywords Inert ingredients · Non-target species · Apex predators · Acute toxicity · Aquatic invertebrates · Dispersants

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Introduction

Invasive species of plants such as Eurasian watermilfoil (*Myriophyllum spicatum* L.) and curly leaf pondweed (*Potamogeton crispus* L.) have severely disrupted the ecology of many lakes in North America (Smith and Pullman 1997). They form dense monocultures that inhibit the normal growth of native species of plants and animals. Fluridone [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4(1H)-pyridinone] is an aquatic herbicide that is increasingly used in integrated pest management programs to control invasive plant species. It functions by inhibiting the synthesis of carotenoids involved in photoprotection of the photosynthetic apparatus (Dankov et al. 2009; Getsinger et al. 2002; Hamelink et al. 1986). Plants differ in their spectrum of light accessory pigments, allowing them to compete against each other for unique niches in aquatic ecosystems (Nelson and Cox 2005). Fluridone differentially inhibits photolysis depending on the proportion of carotenoids present in various plant species.

In earlier studies (Yi 2008), reduced abundance and diversity in water mite populations was observed in sites

treated with herbicides compared to sites that did not use herbicides as part of their management practices. This was the impetus for studying the acute toxicity of fluridone on water mites since fluridone was used at one site as a tool in an integrated pest management program for the reduction of exotic plant species.

Effective application rates for fluridone vary considerably in the literature. According to information reviewed by the Environmental Protection Agency (EPA) in 2004, fluridone requires 45 days of contact time to be effective (United States Environmental Protection Agency 2004). Because of its ability to disperse, the maximum application rate is 90 μg/L for a one-time treatment of an entire body of water, and for partial lake treatments, the maximum cumulative application rate is 150 μg/L per growth cycle (United States Environmental Protection Agency 2004). In field trials, commercial formulations of fluridone (Sonar® A.S., SePro SRTC, Whitakers, NC) have been shown to be selectively effective in controlling weeds at relatively low concentrations of 10–20 μg/L (Smith and Pullman 1997). Depending on the timing of application, the volume of the lake, and its associated thermocline, initial concentrations of 5 μg/L followed by repeated applications of 2 μg/L, every 21 days, will selectively and effectively reduce Eurasian watermilfoil populations (Getsinger et al. 2002). This is well below the maximum allowable application rates as outlined by the EPA. It is also well below the 20 μg/L maximum application rate allowed within 400 m of a functioning potable water intake source intended for human use (Sonar®A.S., SePro Corporation product insert; United States Environmental Protection Agency 2004).

To date, there is only one published laboratory study from 1986 that has investigated the acute and chronic effects of the technical formulation of fluridone (98–99% active ingredient) and the field formulation containing 48% active ingredient (479 g/L) and 52% inert ingredients (dispersing and suspending agents in addition to humectants in water). This was a collaborative study between four separate laboratories over the course of 5 years that primarily assessed the effects of technical grade fluridone on 4 species of freshwater invertebrates, 3 species of marine invertebrates and 5 species of freshwater fish (Hamelink et al. 1986). All chronic toxicity studies were performed with technical grade fluridone. The acute toxicity studies were a combination of technical and commercial formulation exposure and the results were combined for statistical analysis. Thus, all further references to fluridone included both the technical grade and commercial formulation (Hamelink et al. 1986). For the acute toxicity studies the half maximal effective concentration at 48 h (EC50) for technical grade fluridone ranged from 3.0 to 7.4 mg/L in *Daphnia* and 0.8–2.2 mg/L in midge larvae. Crayfish had a 14-day half maximal lethal concentration (LC50) of greater than 16.9 mg/L. Amphipods had an LC50 (96 h) of greater than 32 mg/L. To assess toxicity in fish, technical grade fluridone was again the major toxicant used (24 studies using the technical grade versus 7 using the commercial formulation). As with invertebrate studies, 96 h LC50 s were well above the recommended application rates for use of the herbicide. Based on these studies the authors concluded that, "fluridone is not expected to have adverse effects on the species tested or on similar non-target aquatic organisms" (Hamelink et al. 1986).

Aquatic mites are found within all freshwater aquatic ecosystems and they are the only known group of holometabolic mites (Smith et al. 2001). Adult and deutonymphal stages are considered apex predators in aquatic ecosystems. The larval stages are parasitic and can infect upwards of 20–50% of natural populations of aquatic insects including flies (Diptera) that vector zoonotic diseases (Abro 1982; Jalil and Mitchell 1972; Smith 1988; Wetzel 2001). The protonymph and tritonymph stages are quiescent transitional periods of significant developmental change in the mites' morphology and physiology. During this time the mites do not move or feed but are found developing within the cuticle of the previous stage, attached by their chelicerae to aquatic plants. In this stage they are vulnerable to changes in the environment (Smith et al. 2001; Smith 1988).

Different species of mites are exposed to herbicides during varying stages of development throughout the year. We hypothesized that fluridone, at environmentally relevant application rates, would interfere with the ability of aquatic mites to adequately complete their life cycle. We also hypothesized that the addition of "inert" ingredients would significantly increase the toxicity of fluridone to aquatic mites by enhancing the bioavailability and bioaccumulation of the herbicide, similar to what has been found in studies on the differential toxicity of technical grade glyphosphate versus the commercial formulation (Roundup®) (Folmar et al. 1979; Mann and Bidwell 1999). Inert ingredients such as surfactants and humectants can change the fluidity of the structure of plant and invertebrate surfaces leading to increased penetration of herbicides such as glyphosphate and fluridone (Monsanto Company, St. Louis, MO) (Freeman and Rayburn 2006; Haefs et al. 2002; Ramsey et al. 2005).

Materials and methods

Collection of water mite test organisms

Mites were collected from Lake Mingo in Kennekuk Cove County Park, located in the Vermilion County Conservation District, approximately five miles west of Danville, Illinois (N:40.20776° W:087.73576°, elevation 560′).

Pesticides are not used within the district's holdings. Male mites of the genus *Arrenurus,* sub-genus *Megaluracarus,* referred to as *Megaluracarus,* were used because of their abundance and stable taxonomic identification.

The mites were collected using an aquatic net that was modified by replacing the original netting with 250 μm mesh netting. Using the net, the top layer of sediment was gently disrupted. The net was then moved through the water in a back-and-forth, figure-eight motion, to collect mites distributed throughout the water column and among aquatic plants. Moss, detritus, and sediment surrounding macrophytes where mites may rest or hide were vigorously shaken and rinsed inside the nets.

The collected sediment at the bottom of the net was then put into a water-filled, clear plastic bag and the material was agitated. The resulting mixture of mites, sediment, and other debris (and other invertebrates) was poured off into nested 1.4 mm and 250 μm, 8 inch (203.2 mm), brass, W.S. Tyler sieves (W.S. Tyler, Mentor, OH), leaving the heavier material such as sand, stones and gravel in the bag. The nested sieves were then gently washed 2–3 times. This separated the larger plant material and debris, leaving the mites in the 250 μm sieve. The resultant mixture of washed fine sediment and mites was then put into a large mouth Nalgene bottle filled with water for transport back to the laboratory. At the laboratory, the contents of the Nalgene bottle were poured into a white tray of shallow water. The mites were then collected by hand aspiration with a bulb pipette.

Laboratory protocol for acclimation, care, and usage of water mites

Acclimation of the mites to their new environment occurred for a minimum of one week before the start of the first series of studies. During the period of acclimation the newly collected mites were divided into two 1 L beakers containing moderately hard reconstituted water (MHRW) (United States Environmental Protection Agency 2002). MHRW does not contain micronutrients and is used for freshwater toxicity testing of invertebrates that are normally found in freshwater ecosystems in the Midwestern Great Lakes region of the United States. Throughout the course of the study, the average pH, conductivity, alkalinity, and hardness of the MHRW used was 8.1 ± 0.1, 301 ± 4 μmhos/cm, 67 ± 3 mg/L $CaCO_3$, and 91 ± 2 mg/L as $CaCO_3$.

During the period of acclimation in the laboratory prior to testing, the mites were fed ostracods, mosquito eggs/larvae (when available), *Daphnia magna*, and *Ceriodaphnia dubia*. Mites readily ate newly emerged culicid larvae and cladocerans. Food was provided for prey items in the form of 1 mL of a 1:1 (vol:vol) mixture of algae (*Pseudokirchneriella subcapitata*, Aquatic Biosystems, Fort Collins, Colorado) and a yeast/cereal leaves/trout chow mix (YCT), (Aquatic Research Organisms, New Hampshire), added to each one liter beaker. The mites were held in environmental chambers, at 23–25°C, 16 h of light and 8 h of dark. Water was oxygenated using an oxygen stone and pump system during the period of acclimation. Nylon mesh netting (1,000 μm) was used as a substrate for the mites to rest and hide.

To prevent contamination by other environmental chemicals in the laboratory, all glassware was cleaned in a non-surfactant, biodegradable, phosphate-free solution of Liquinox (Alconox, Inc., Jersey City, NJ). The glassware was then soaked in a solution of 10% nitric acid for 24 h and rinsed four times with double de-ionized water.

Fluridone preparation

Fluridone was purchased as 99.5% fluridone (Sonar™, Chem Service, West Chester, PA) and as the formulated Sonar®AS (SePro SRTC, Whitakers, NC). Sonar®AS includes 58.3% herbicidally-inert ingredients, whose identities are proprietary information. Because of the possibility of chemical interactions with the inert ingredients and the possible enhancement of bioavailability and/or toxicity of active and inert ingredients to the mites (Ramsey et al. 2005), acetone was not used to bring fluridone into solution. This would not be the protocol for preparation in the field, and thus we did not want to introduce this in the laboratory.

A suspension was made of 100,000 μg/L Sonar™ in MHRW water. From this, serial dilutions were made using volumetric flasks to obtain nominal solutions containing 100,000, 10,000, 1,000, and 100 μg/L concentrations of fluridone diluted with MHRW.

Preparation of the stock solution of Sonar®AS was based by weight on an equivalent amount of active fluridone in solution. Sonar®AS contains 0.48 kg active ingredient (fluridone) per liter. This translates to a concentration of 1,000 μg active fluridone per 2.1 μL Sonar®AS. In order to make an initial, nominal, stock solution of 10,000 μg/L fluridone, 21 μL of Sonar®AS were diluted in 1 L MHRW. Serial dilutions of 5,000 and 1,000 μg/L were also made from the 10,000 μg/L fluridone in stock solution of Sonar®AS using volumetric flasks. The initial range finding concentrations for testing of 10,000, 5,000 and 1,000 μg/L of fluridone in Sonar®AS were chosen in order to bracket the 6,300 μg/L acute 48 h LC50 given for *Daphnia magna* and the average 48 or 96 h LC50 or EC50 for aquatic invertebrates of 4,300 ± 3,700 μg/L (Hamelink et al. 1986; http://www.mass.gov/agr/pesticides/water/Aquatic/Aquatic/Fluridone.doc).

Toxicity of fluridone (Sonar™) and Sonar®AS to water mites

In the normal state mites are very active, continually moving in a forward direction through the water column. Morbidity was defined as any deviation in normal, upright, forward locomotion. On exposure to either Sonar™ or Sonar®AS at toxic concentrations, the mites at first appeared weak, with little to no forward movement. They then became completely incapacitated, with their legs curled under and an accompanying loss of the ability to right themselves to a prone position. Slight tremors would start at the distal tarsi and progressively become more pronounced, culminating in severe tremors and seizures. At this point, if the mites were moved or touched with a probe, the whole process would begin again, starting with the very slight tremors of the distal tarsi.

Mortality in water mites was determined by absence of mobility, lack of movement of internal organs as seen through the cuticle, and fading or disappearance of their normal color.

Ten mites were assigned to each solution concentration tested. Each 50 ml test beaker used to hold the mites for testing contained only one male *Megaluracarus.* By limiting the study to males and separating the mites, stress from male competition, sexual reproductive behavior, and interspecies competition was minimized.

Each 50 mL test beaker contained 40 ml of either MHRW (control), or one of the test solutions. During the experimental phase mites were kept at 23–25°C. The beakers were covered in parafilm and wrapped in aluminum foil to minimize contamination by environmental microbes, exposure to light (photolysis) and evaporation. Fluridone is stable to hydrolysis, and its estimated half-life in the laboratory is estimated to be 7–33 days (Fox et al. 1996; MacDonald et al. 1996). Thus, we did not renew test solutions during the course of the exposure.

Morbidity and mortality of the mites in MHRW (control) and varying concentrations of Sonar™, Sonar®AS were assessed at 0, 24, 48, 72, and 96 h. Morbidity and mortality were combined for affected mites in determining the EC50 at 48 and 96 h.

Additional testing of the toxicity of Sonar®AS to water mites

Since a No Observable Adverse Effect Level (NOAEL) was not reached at 1,000 µg/L concentration of Sonar®AS, a third bioassay was performed with an additional ten replicates for the control (MHRW) and serial dilutions of 100 and 10 µg/L of fluridone in Sonar®AS. Morbidity and mortality were assessed at 0, 24, 48, 72, and 96 h. Mites cannot be successfully reared in the laboratory; this resulted in limited availability of test organisms and constrained a full repeat of test conditions with an adjusted concentration range. Again, preparation of the stock solution of Sonar®AS was based on an equal amount of active fluridone in solution. In order to make a solution that contains 1,000 µg/L fluridone, 2.1 µl of Sonar®AS was diluted with 1 l MHRW as a stock solution. This allowed for testing of the 100 µg/L recommended concentration for use in ponds and lakes by the manufacturer and for the study of 10 µg/L which is half the application rate of 20 µg/L permitted within 400 m of any functioning potable water intake destined for human usage.

Statistics

Data were analyzed between control and fluridone treated populations using the Chi square statistic (χ^2) with the null hypothesis of no difference in morbidity and mortality between mites used as controls and those treated with serial dilutions of Sonar™ and/or Sonar®AS (Glantz 2002). Degrees of freedom were equal to one. After testing for normality and homogeneity of variances, calculations of the EC50 were determined by the trimmed Spearman-Karber method of analysis using the binomial test to determine statistically conservative 95% confidence levels (http://www.epa.gov/nerleerd/stat2.htm; Sanathanan et al. 1987). Because combined mortality and morbidity proportions were not monotonically increasing, adjustments were made prior to the Spearman-Karber estimate. Probability levels that were greater than $p = 0.05$ were considered statistically non-significant.

Results

Toxicity of fluridone (Sonar™) to water mites

The EC50 at 48 and 96 h for Sonar™, 99.5% technical grade fluridone, was 891 and 631 µg/L, respectively. The binomial test showed that at 48 h, data points between 100 and 10,000 µg/L could be used as statistically sound conservative 95% confidence levels since the actual confidence level associated with these limits is 98.8281%. At 96 h the binomial test showed that data points between 0 and 10,000 µg/L could be used as statistically sound conservative 95% confidence limits since the actual confidence level associated with these limits was 99.9023%. At the lowest concentration of exposure, 100 µg/L, there was no significant difference between combined morbidity and mortality of the controls compared to Sonar™-exposed mites at either 48 ($\chi^2 = 1.06$, $p \geq 0.25$) or 96 h ($\chi^2 = 2.39$, $p \geq 0.10$), even though at 96 h 30% of the mites

Table 1 Effect of increasing concentrations of Sonar™ on the morbidity and mortality of water mites at 48 and 96 h of exposure

Sonar™ (μg/L)	Normal: 48 h (% mites)	Morbidity: 48 h (% mites)	Mortality: 48 h (% mites)	Normal: 96 h (% mites)	Morbidity: 96 h (% mites)	Mortality: 96 h (% mites)
0	100	0	0	90	0	10
100	90	0	10	60	30	10
1,000	50	30	20	50	10	40
10,000	0	90	10	0	70	30
100,000	0	50	50	0	40	60

Table 2 Percentage morbidity and mortality of water mites at 48 and 96 h after exposure to an equivalent concentration of fluridone present in Sonar®AS

Sonar®AS (μg/L)	Normal 48 h (% mites)	Morbidity 48 h (% mites)	Mortality 48 h (% mites)	Normal 96 h (% mites)	Morbidity 96 h (% mites)	Mortality 96 h (% mites)
0	80	20	0	80	10	10
10	60	30	10	30	60	10
100	70	20	10	10	80	10
0	100	0	0	90	10	0
1,000	80	20	0	30	60	10
5,000	0	100	0	0	100	0
10,000	0	90	10	0	90	10

Note: serial dilutions tested immediately follow the controls used in each separate assay

exposed to Sonar™ showed signs of morbidity and 10% were dead (Table 1).

Increased mortality was associated with increasing concentrations of Sonar™ for concentrations ≥1,000 μg/L. Combined morbidity and mortality from exposure to concentrations of 1,000 μg/L Sonar™ were significant at both 48 and 96 h post exposure ($\chi^2 = 10$, $p = 0.005$). At 10,000 μg/L and above, 100% of the mites were affected by exposure to Sonar™ at both 48 and 96 h. Mortality increased from 10% at 48 h to 30% at 96 h at 10,000 μg/L and from 50% at 48 h to 60% at 96 h after exposure to 100,000 μg/L (Table 1).

Toxicity of Sonar®AS to water mites

At 96 h 70% of the mites exhibited signs of toxicity at the lowest concentration tested, 10 μg/L (Table 2). This is significantly different from the combined morbidity and mortality of controls ($\chi^2 = 5.06$, $p = 0.025$) from attrition under laboratory conditions. Toxicity increased with increasing concentration of Sonar®AS with 100% of the mites affected at 5,000 μg/L. The 96 h EC50 is less than 10 μg/L. In calculating the 96 h EC50, the binomial test showed that data points between 0 and 5,000 μg/L could be used as statistically sound conservative 95% confidence limits since the actual confidence level associated with these limits was 99.9023%.

Duration of exposure had a significant effect on the rate of toxicity at concentrations less than 5,000 μg/L (Table 2). At 10 μg/L 40% of the mites were affected at 48 h versus 70% at 96 h of exposure. At 100 μg/L 30% were affected at 48 h versus 90% at 96 h and for 1,000 μg/L, 20% were affected at 48 h versus 70% at 96 h. At 5,000 μg/L and above, 100% of the mites were significantly affected at both 48 and 96 h ($\chi^2 = 20$, $p < 0.001$).

Discussion

Historically, most research on the effects of exposure of invertebrates to toxicants in aquatic ecosystems has been limited to a few model species (Colborn and Short 1999). The reason for our use of aquatic mites as a study model was to determine whether pesticides such as fluridone disrupt ecosystem diversity and stability despite relative harmlessness to the standard test species. In addition, the information on toxicity in standard test species such as *Daphnia* refers primarily to fluridone alone rather than to commercial formulations of the product for use in the aquatic environment.

Aquatic mite development occurs in both aquatic and terrestrial ecosystems. As apex predators and obligate parasites, they influence the life cycles of many orders of insects that rely on the stability of aquatic and surrounding

terrestrial ecosystems (Abro 1982; Jalil and Mitchell 1972; Smith et al. 2001; Smith 1988). The complexity of holometabolic development and the physiological mechanisms that are potentially disrupted by chemicals in the natural environment make water mites superb indicators of water quality (Gerecke and Schwoerbel 1991; Larimore and Bayley 1996).

This study demonstrates that at the currently recommended application rates of the commercial herbicide Sonar®AS, there is risk of toxicity to aquatic mites, a non-target species. The data suggest that both increasing concentrations of Sonar™ and Sonar®AS as well as the duration of exposure play an active role in toxicity. This is important since the effectiveness of commercial formulations of fluridone under field applications is based on exposure for periods greater than 45 days, with maximum cumulative concentrations of 150 μg/L of the active ingredient (United States Environmental Protection Agency 2004).

The difference in formulation between Sonar™ and Sonar®AS is the addition of "inert" ingredients to Sonar®AS. The reasons for the addition of inert ingredients (surfactants, humectants, and dispersants) to herbicides are to increase dispersion of the active ingredient throughout aquatic ecosystems and to enhance exposure and penetration of a chemical compound into aquatic plants. Controversy arises because of the broad nature of "inert" ingredients that may be present in pesticides and their ability in and of themselves to cause toxicity by disruption of normal physiological processes. This has been shown to occur in non-target species such as people and aquatic animals (fish and amphibians) (Colborn and Short 1999; Freeman and Rayburn 2006; Magnusson et al. 2001; Mann and Bidwell 1999; Partearroyo et al. 1991; Richard et al. 2005). In general "inert" ingredients are vehicles to enhance the bioactivity and absorption of the active ingredient: delivery systems that overcome an organism's natural defenses against penetration and activation from exposure to compounds in the environment. Thus, by making an herbicide 100 times more toxic to a plant species, theoretically the amount of toxicant applied to the plant could decrease 100-fold (Colborn and Short 1999). Based on our results, another conclusion is that "inert" ingredients also increase exposure of non-target organisms such as water mites to the toxic effects of herbicides.

A NOAEL could not be identified at the concentrations tested in this study for Sonar®AS. The 96 h EC50 for Sonar™ is 631 μg/L versus a 96 h EC50 less than 10 μg/L for Sonar®AS. At one half the maximum acceptable application rate for Sonar®AS in potable water (10 μg/L), 70% of the water mites were incapacitated by such severe locomotor deficits that they would be incapable of apprehending prey, reproducing, or defending themselves in their natural habitat. These values are considerably less than the values previously published in the literature for the acute toxicity of fluridone to invertebrates of 6,300 μg/L for the acute 48 h LC50 given for *Daphnia magna* (http://www.mass.gov/agr/pesticides/water/Aquatic/Aquatic/Fluridone.doc) and an average 48 or 96 h LC50 or EC50 for aquatic invertebrates of 4,300 ± 3,700 μg/L (Hamelink et al. 1986). Since two stages of the mite's life cycle, the tritonymph and protonymphal stages are intimately associated with aquatic plants, and since the mobile larval, adult, and deutonymphal stages are active within the water column and benthic zone, the mites will be exposed to these compounds at some point in their life-cycle. Thus, based on the data presented in this study, it could be concluded that exposure to sustained levels of Sonar®AS at levels of 10 μg/L for more than 96 h will have a detrimental effect on aquatic male mites in the genus *Arrenurus*. Further exploration into the effects on other non-target genera and species are clearly warranted.

Conclusion

This study demonstrates that at currently acceptable application rates of 90–150 μg/l, field formulations of fluridone, with the addition of ingredients found in Sonar®AS, have an increased risk for detrimental effects on populations of male water mites (Arrenurus: Megaluracarus) in aquatic ecosystems. Technical grade fluridone, however, appears to be relatively non-toxic at acceptable application rates, and allows survival of mites at ecologically sustainable levels. In conclusion, we believe that formulated fluridone has the potential for severely compromising the normal function and existence of populations of aquatic mites and that this information should be considered when incorporating herbicides into an integrated pest management program for the control of invasive plants in aquatic ecosystems.

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