Bull Environ Contam Toxicol (2009) 82:549–553
DOI 10.1007/s00128-009-9643-6

Acute Toxicity and Hazard Assessment of Spinosad and R-11 to Three Cladoceran Species and Coho Salmon

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Received: 26 March 2008 / Accepted: 7 January 2009 / Published online: 22 January 2009

Abstract Acute toxicity data and hazard assessments were developed for three cladoceran species, *Ceriodaphnia dubia*, *Daphnia pulex*, and *Daphnia magna*, and juvenile Coho salmon, *Oncorhynchus kisutch* after exposure to the insecticide, spinosad and adjuvant R-11. The effect of a mixture of these compounds was also determined with *C. dubia* and *O. kisutch*. Spinosad was virtually non-toxic to *O. kisutch*. Hazard assessments indicated that R-11 posed no hazard to any of the species tested while spinosad only posed a hazard to *C. dubia*. Mixture studies indicated that spinosad and R-11 may interact synergistically in *C. dubia*.

Keywords Spinosad · R-11 · *Daphnia* · Salmon · Mixture

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. Agricultural adjuvants are also 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). Some agricultural adjuvants are nonylphenol polyethoxylates which have been shown to be toxic to various aquatic organisms (Bakke 2003).

Spinosad is one of the new pesticides that is being marketed for control of a range of pest species (DowElanco 1996; Crouse et al. 2001). Spinosad is being used more frequently since becoming registered for use as an organic insecticide. R-11 is a commonly used adjuvant that is applied with pesticides to improve performance (Stark and Walthall 2003). R-11 is also a component of certain pesticide formulations. Thus, spinosad and R-11 may be entering surface water systems together in areas where they are used for pest control via drift and/or runoff.

The objective of this study was to determine the acute toxicity of R-11 and spinosad to *Ceriodaphnia dubia* Richard, *Daphnia pulex* (Leydig), *Daphnia magna* Straus and Coho salmon, *Oncorhynchus kisutch* (Walbaum). These species were chosen for study because they are representative of invertebrate and vertebrate species inhabiting aquatic ecosystems. Acute LC50 estimates were compared to expected environmental concentrations (EEC). 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.

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Materials and Methods

Three Cladocerans, *D. pulex*, *D. magna*, and *C. dubia*, were reared in reconstituted dilution water (RDW). All RDW used in this study was prepared according to a method modified from a USEPA protocol (2002) resulting in a RDW with pH 7.4–7.8, conductivity 260–320 μS, dissolved oxygen (DO) >8.0 mg/L, alkalinity of 60–70 mg/L and a hardness of 80–100 mg/L. Cultures were housed in an environmental chamber with a photoperiod of 18:6 h light:dark, 25.0 ± 0.1°C, and 50.0 ± 0.1% relative humidity (RH). The feeding solution for cladocerans 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 cladoceran cultures, transferred to new cups containing RDW, and fed 0.3 mL of feeding solution.

Juvenile Coho salmon, *O. kisutch*, were obtained from the University of Washington (UW) hatchery on March 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. The Coho salmon were housed in 757 L free standing circular recirculating tanks of dechlorinated municipal water (x ± SEM – temperature 10.80 ± 0.03°C, pH 6.40 ± 0.01, dissolved oxygen 40.23 ± 0.09 mg/L, salinity 0.02 ± 0, total dissolved solids 0.26 ± 0.02 g/L, total hardness as $CaCO_3$ 110–120 mg/L, and alkalinity 74 mg/L) on a 12 h light-dark schedule. Coho used in the experiments were 6–9 months old, with an average length (±SEM) of 7.96 ± 0.12 cm and average weight of 5.03 ± 0.28 g. Coho were fed Bio Vita starter #1 Crum (Bio-Oregon, Inc.) once daily.

Spinosad (Success), 240 g active ingredient (ai)/L, was obtained from Dow AgroSciences LLC9330 Zionsville Rd., Indianapolis, Indiana, 46268, USA). R-11 is a nonionic surfactant whose active ingredient is a mixture of nonylphenol polyethoxylates (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.

Acute mortality studies with daphnids were conducted using the approach outlined in the EPA/600/4-90/027F manual "Methods for Measuring the Acute Toxicity of Effluent to Freshwater and Marine Organisms" (USEPA 1991). Adjuvant and pesticide concentrations were prepared by serial dilution from newly prepared stock solutions in 100 mL deionized-distilled 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 50 mL glass beakers containing 25 mL of sample solution for each concentration tested. Exposures were static non-renewal. Four beakers were used for each concentration. The following nominal concentrations of R-11 were evaluated in the acute toxicity test: *C. dubia* 0, 8, 9, 10, 11, 12 mg ai/L; *D. pulex* 0, 10, 11, 12, 13, 14, 15, 25 mg ai/L; *D. magna* 0, 12.5, 15, 17.5, 20, 22.5 mg ai/L. The following nominal concentrations of spinosad were evaluated in the acute toxicity test: *C. dubia* 0, 0.00005, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1 mg ai/L; *D. pulex* 0.1, 0.15, 0.25, 0.35, 0.5, 0.7 mg ai/L; *D. magna* 0, 0.0001, 0.001, 0.01, 0.02, 0.1, 0.2, 0.4 mg ai/L. Cladocerans were fed 2 h before introduction to the test solutions. Cladocerans were starved for the duration of the experiments and adjuvant/pesticide solutions were not renewed. Mortality was assessed 48 h after starting the experiment. Test organisms were kept at the same conditions listed above for rearing. Cladocerans 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 three times on different days with different generations of cladocerans.

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 of the system water (filtered, dechlorinated, UV-sterilized, and chilled) mentioned above. Prior to each experiment, aquaria were rinsed and scrubbed with tap water and air-dried. Aquaria (38 L) were filled with 20 L of the water described above for holding the Coho colony the day of the addition of chemicals. Aquaria were placed into a 757 L water bath of flowing water to maintain temperature at 10.8°C.

Test amounts of spinosad and R-11 were weighed out and added to the 20 L water in the aquaria separately and in binary mixtures. Exposures were static and non-renewal. Batches of five juvenile *O. kisutch* 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 control treatment were not exposed to R-11 or spinosad. The following nominal concentrations of R-11 were evaluated in this study: 0, 9.5, 10, 10.5, 12 mg ai/L. The following nominal concentrations of spinosad were evaluated in this study: 1, 10, 50, 100, 250, and 500 mg ai/L.

Observations in the Coho salmon studies were 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.

Acute mixture toxicity to one of the cladoceran species, *C. dubia*, was evaluated by combining the nominal LC25 estimates for R-11 and spinosad (8.21 and 0.00394 mg/L, respectively). The LC25s were determined with Probit analysis of each chemical separately with a 95% confidence level. The LC25 of each chemical was chosen because the mixture of each chemical should not result in 100% mortality of each species evaluated. However, exposure to the LC25 should result in a high enough level of mortality to determine whether mortality was additive, synergistic or antagonistic.

For *O. kisutch*, the mixture study was conducted with the R-11 LC25 (10 mg/L). However, because an LC50 estimate could not be determined for spinosad and *O. kisutch*, we chose the same concentration of spinosad (10 mg/L) to test in this study.

Hazard assessments were developed by using the quotient method. This method entails dividing the expected environmental concentration (EEC) by the LC50. Numbers greater than one indicate that the chemical poses a potential hazard to the tested species. The EEC for R-11 has been previously reported as 0.079 mg/L (Stark and Walthall 2003). The chronic exposure EEC for spinosad in surface water has been estimated to be 0.0023 mg/L (Federal Register 2005).

Acute concentration-mortality regressions were estimated with Probit analysis (Finney 1971; SAS Institute 1999) after correction for control mortality using Abbott's formula (1925). Control mortality never exceeded 5%. Data from the mixture study was evaluated with analysis of variance (ANOVA) (SAS Institute 1999). Means were separated with the Student–Newman–Keuls test ($p = 0.05$). Prior to analysis, percent mortality data were transformed with arcsine square root of proportion.

Results and Discussion

Acute mortality varied among the test species that were exposed to R-11 (Table 1).

Table 1 Acute concentration-mortality estimates for cladocerans (48 h) and *O. kisutch* (96 h) exposed to R-11

Species	Total no. tested	Slope ± SE	LC50 (95% CL) (mg/L)[a]
C. dubia	416	15.61 ± 1.62	9.07 (8.81–9.30)
D. magna	330	12.21 ± 2.22	17.65 (16.55–18.74)
D. pulex	319	6.55 ± 1.11	13.16 (12.45–14.01)
Oncorhynchus kisutch	205	20.29 ± 5.28	11.01 (10.63–11.62)

[a] Nominal, not measured concentrations were used to calculate the LC50 estimates presented

Based on a lack of 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 cladocerans seemed to correspond to size; *C. dubia* is the smallest species, *D. pulex* is intermediate and *D. magna* is the largest species. However, R-11 was not particularly acutely toxic to any of these species and the order of magnitude of susceptibility was very similar.

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*.

When the LC25 of spinosad and the LC25 of R-11 were combined, it was found that approximately 88% of the *Daphnia* died. Predicted mortality should have been 50%. The estimated mortality (adding the mortality for each chemical separately) should have been approximately 61% (21% + 40%), perhaps 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. Mortality in the mixture was significantly higher than mortality due to each chemical alone ($p = 0.001$; $df = 2.9$; $F = 21.83$). However, the differences observed (61% versus 88% mortality) may have been due to normal variability in the experiment.

Table 2 Acute concentration-mortality estimates for cladocerans (48 h) and *O. kisutch* (96 h) exposed to spinosad

Species	Total no. tested	Slope ± SE	LC50 (95% CL) (mg/L)[a]
C. dubia	541	1.03 ± 0.08	0.0018 (0.0013–0.0025)
D. magna	180	0.79 ± 0.12	0.0048 (0.0019–0.0100)
D. pulex	381	1.01 ± 0.17	0.1290 (0.0770–0.1810)
Oncorhynchus kisutch	50	NA[b]	>500

[a] Nominal, not measured concentrations were used to calculate the LC50 estimates presented

[b] 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	Total no. tested	Mean % mortality[a]	Standard error
R-11 LC25[b]	443	21.49a	3.01
Spinosad LC25	443	39.72a	9.01
Mixture of R-11 and Spinosad LC25s	443	88.31b	5.40

[a] Means within the column followed by different letters are significantly different ($p \leq 0.05$; Student–Newman–Keuls test)

[b] Nominal, not measured concentrations were used to calculate the LC25 estimates used in this experiment

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. However, sublethal effects were observed. *O. kisutch* exposed to mixtures appeared to be immobilized after only 6 h of exposure but not when exposed to each chemical separately. 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 exhibiting normal behavior. After 72 h of exposure, *O. kisutch* exposed to the mixture 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.

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 only to *C. dubia* (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* and spinosad because an LC50 value could not be determined, but because the LC50 was greater than 500 mg/L, spinosad does not pose a risk to *O. kisutch.*

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

Species	Hazard assessment[a] for R-11	Hazard assessment[a] for Spinosad
C. dubia	0.09	1.29
D. magna	0.04	0.48
D. pulex	0.06	0.018
Oncorhynchus kisutch	0.08	NA[b]

[a] Hazard assessment values were calculated with the equation: Hazard = EEC/LC50. Values greater than 1 indicate that the chemical poses a hazard to the tested species

[b] Value could not be calculated because the LC50 could not be determined

EEC for R-11 and spinosad are 0.079 and 0.0023 mg/L, respectively

EEC for R-11 from Stark and Walthall (2003). EEC for spinosad from Federal Register (2005)

In an earlier study, Curran et al. (2004) estimated an R-11 96 h acute LC50 for small juvenile rainbow trout (average length 35.69 mm; average weight, 0.39 g) of 5.18 mg/L and for large juveniles (average length 100 mm; average weight 15 g), 6.57 mg/L. These LC50 estimates fall below the low end of our 95% CL at LC50 (10.63 mg/L). Smith et al. (2004) reported an R-11 96 h acute LC50 of 6.0 (5.7–6.2) mg/L for juvenile rainbow trout. The 95% Cl from our study and the study by Smith et al. (2004) do not overlap.

The LC50 estimate for spinosad was 0.00178 mg/L for *C. dubia* which was lower than the EEC of 0.0023 mg/L reported in the Federal Register (2005). The spinosad hazard assessment value for *C. dubia* was higher than 1 which indicates that spinosad may pose a slight hazard to *C. dubia* but not to the other cladoceran species or *O. kisutch*. Spinosad was not acutely toxic to Coho salmon at concentrations up to 500 mg/L. 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 $p \leq 0.05$. Therefore, mixtures of spinosad and R-11, which may occur in freshwater ecosystems, may cause more damage to aquatic organisms than either product alone.

The results of this study indicate that spinosad may pose a slight hazard to only one of three cladoceran species evaluated, but not to Coho salmon. Although R-11 was found to be toxic to the cladoceran species and Coho salmon, it does not pose a hazard to any of these species at EEC. A mixture of R-11 and spinosad may react synergistically to *C. dubia* and therefore combinations of these two chemicals may be more damaging than either one alone.

Acknowledgments Special thanks to Maria Boroja, NOAA, Washington, DC for financial support. We are grateful to Nathaniel Scholz and Cathy Laetz, NOAA, Seattle, Washington for their help and advice with regard to Coho research. We thank Grace and Oriki Jack for technical support. We also thank Drs Allan Felsot and Carol Anelli who served as committee members for Angela Deardorff's Master Thesis project. This project was funded in part by NOAA.

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