Acute Toxicity of Sodium Fluorescein to Ashy Pebblesnails *Fluminicola fuscus*

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Abstract

Water resource agencies and groundwater scientists use fluorescein dyes to trace ground water flows that supply surface waters that may contain threatened or endangered mollusk species. Since little is known of the toxicity of sodium fluorescein to mollusks, we tested the toxicity of sodium fluorescein to the ashy pebblesnail *Fluminicola fuscus*. The pebblesnail was selected as a surrogate test species for the threatened Bliss Rapid snail *Taylorcocha serpenticola* that is endemic to the Snake River and its tributaries in the Hagerman Valley, Idaho. In laboratory tests, we expose replicated groups of snails to a series of concentrations of fluorescein in a static 24 h exposure at 15°C. Following the exposure, we removed snails, rinsed them, and allowed a 48 h recovery in clean water before recording mortality. We estimated 377 mg/L as the median lethal dose. Mortality to snails occurred at concentrations well above those expected in test wells during the monitoring efforts.

Introduction

Groundwater tracing especially through aquifers is very difficult to conduct without a dye tracer. Many dyes can be used to trace the flow of groundwater from areas of recharge to areas of discharge. Dye tracer tests can be used in a multitude of applications, such as: (1) determination of flow paths and residence times; (2) measurement of aquifer properties; (3) determination of site-specific hydrology; (4) determination of recharge areas for wellhead and springhead protections; and (5) planning for response to spills of hazardous materials (Field et al. 1995). A known amount of dye is added to the water source and detection stations are

established to monitor water discharge. Flow rates are measured by determining the concentration of the dye as it flows past the detection station.

Fluorescent dyes are often chosen for tracer studies because of low toxicity, availability, low cost, water solubility and stability, and ease of detection (Field et al. 1995). Sodium fluorescein ($C_{20}H_{10}Na_2O_5$; Acid Yellow 73; CAS number 518-47-8) also known as uranine, is used frequently for aquifer tracer studies (Figure 1). Sodium fluorescein is an orange powder that turns a bright green to yellow color when small quantities are diluted with water. Precaution is recommended when working with the powdered form because of skin and respiratory irritation, but aqueous solutions are considered very safe. The compound is sensitive to light and will decompose into pthalic acid and resorcinol (Smart 1984).

The Idaho Department of Water Resources (IDWR) and Idaho Power are conducting groundwater tracer studies within the Hagerman area of the Snake River Aquifer in Southern Idaho. The aquifer in this region has many springs that are interconnected, and used in fish farming and conservation aquaculture operations. In addition to concerns that the dye could affect fish physiology and endocrinology, the U. S. Fish and Wildlife Service has concerns that the fluorescein dye may be toxic to federally listed threatened or endangered invertebrate species that occupy the springs, such as the threatened hydrobiid Bliss Rapids snail *Taylorcocha serpenticola* (Hershler et al. 1994).

Fluorescein dye has been used in studies of animal health to localize areas of pathology. In fish health, fluorescein dye is used as a tool to detect skin lesions in propagated and free ranging fish (Noga and Udomkusonsri 2002). In studies of invertebrates, mytilid mollusks affected by heavy metal and other pollutants can be analyzed for pathology of gill and mantle

regions through use of fluorescein markers (Fishelsona at al. 1999) that only penetrate cell membranes that are damaged.

Few studies have been conducted to determine the toxicity of sodium fluorescein to aquatic organisms. Marking (1969) reported sodium fluorescein was of low toxicity to fish species and estimated LC_{50} following a 24 h exposure as 3,828 to 5,000 mg/L for rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and bluegill (*Lepomis macrochirus*), respectively. Information on the toxicology to invertebrates is scarce. Walthall and Stark (1999) conducted extensive experiments to determine the chronic and acute toxicity of sodium fluorescein and phloxine B to *Daphnia pulex*. They reported the 48 h LC_{50} of fluorescein was 337 mg/L. Smart (1984) reviewed the published literature on the toxicity of 12 fluorescent dyes to selected species and reported a 48 h LC_{50} of 10 mg/L for sea urchins (*Hemicentrotus pulcher-rimus*) and 1 mg/L for blue mussels (*Mytilus edulis*). Michelson (1964) exposed *Australorbis* and *Helisoma* sp snails to sodium fluorescein for 5 consecutive days at concentrations of 1, 10, and 100 mg/L, and reported mortality of 80 to 100% in concentrations of 10 and 100 mg/L.

The Bliss Rapids snail of the Snake River is considered threatened by the U.S. Fish and Wildlife Service (U. S. Fish and Wildlife Service 1992). The snails are limited in their range to a small section of the Snake River and its tributaries in southern Idaho. Snails are small (2.0 - 4.0 mm), light to orange colored, with 3.5 to 4.5 whorls and a blunt apex. They prefer habitat with flowing water and stable cobble-boulder substratum or flowing springs. The snails do not burrow, and avoid habitats with fine sediments. The Bliss Rapids snails are moderately photophobic, residing on the lateral sides and undersides of rocks during daylight and they migrate to the uppermost surfaces of rocks at night (U. S. Fish and Wildlife Service 2009). The

snails are considered an annual species, are dioecious and can reproduce multiple times. Eggs are laid singly in small capsules attached to rocks from December through March (U. S. Fish and Wildlife Service 2009). After their initial listing in 1992, the status has been reviewed and listing retained in 2004, 2008, and 2009 (U.S. Fish and Wildlife Service 2009).

A more common hydrobiid snail, the ashy pebblesnail, *Fluminicola fuscus* (Haldeman, 1847) occupies the same springs of concern, and was selected as a surrogate organism for toxicity testing. The shell of the pebblesnail is tan, brown, or reddish, and the shape is subglobular to trochoid, with the apex eroded. The ashy pebblesnails can grow much larger than the Bliss Rapids snail, up to 11.2 mm with 4 - 4.5 whorls (Duncan 2008). Pebblesnails are dioecious, live from 1 - 2 years, and eggs are laid from spring to autumn on plants and stones. Most snails die following reproduction. Pebblesnails are considered a species of conservation concern, but within the Hagerman Valley, they are rather widely distributed and occur at high population densities.

The objectives of this study were to determine the toxicity of sodium fluorescein to the ashy pebblesnail following a 24 h static exposure. With these data we simulated worst-case scenarios to determine if use of a sodium fluorescein in aquifer trials could likely affect target hydrobiid snails. The threatened status of the Bliss Rapids Snails makes direct testing of sodium fluorescein toxicity difficult. The ashy pebblesnail was selected for testing because it inhabits the same springs as the Bliss Rapids Snail and has a similar life history. Differences in size or niche selection could result in differences in overall sodium fluorescein toxicity between the two species. However, the ashy pebblesnail was the best-identified surrogate species for the Bliss Rapids snail.

Methods

Experimental Animals –

Pebblesnails were collected from springs at Hagerman National Fish Hatchery (HNFH) on 16 February and 22 March 2010, packaged at the hatchery in moist towels, placed into plastic bags, and shipped in coolers to the University of Idaho College of Natural Resources Fisheries wet laboratory in Moscow. Upon arrival, the snails were washed on a 2.0 mm sieve, and snails between 2.0 and 4.0 mm were transferred into 2 L containers into dechlorinated, aged well-water equilibrated to 15˚C. Snail containers were placed into an aquarium with a lid to provide shading. The water in each container was changed every other day, and temperature in the test room was maintained at 15°C throughout trials. A natural photoperiod for the latitude was provided in the test room. Pebblesnails were retained in the lab no more than three weeks.

Test Substance and Test Concentrations

Sodium fluorescein for tests was provided by IDWR and shipped to the University of Idaho on 5 February. All solutions of sodium fluorescein were prepared in the dark using deionized water at 20°C. Sodium fluorescein powder was measured to the nearest mg, for a stock solution, and volumetric pipettes and flasks were used to create test concentrations. Initial tests were conducted as range finding with four concentrations: 0.2, 2.0, 20.0, and 200.0 mg/L. A more extensive study was conducted using seven target concentrations to estimate an LC_{50}. These concentrations were: 0.2; 20.0; 50.0; 71.0; 100.0; 141.0; and 200.0 mg/L. All solutions were equilibrated to 15˚C in the dark before testing. We report the concentrations of the test substance without adjusting for activity. Approximately 50 mL of each test solution was collected before and after each exposure trial and refrigerated (4˚C). These archived samples were shipped to IDWR for assurance verification, and some were tested by IDWR. However,

since most test concentrations were higher than those detectable with IDWR equipment, samples of test solutions from a range of concentrations were sent to Ozark Underground Laboratories, Protem, Missouri, for verification. Typical test solutions were low in conductivity (1 to 96 μS/ cm), and pH ranged from 7.75 to 9.1.

Experimental Design –

All tests were conducted in 150 mL acid washed glass beakers. We placed 10 healthy snails in each of three beakers for a given test concentration and test replicate. To begin a test, 100 mL of a test solution equilibrated to 15°C was poured into each beaker. We tested snails in three beakers with pure deionized water without dye to serve as controls. Test beakers with snails were kept in a darkened aquarium in a cold room (15°C) to limit deterioration of the test compounds.

We observed snail behavior every 15 minutes during the first hour of exposure, and then every hour for the next 4 h. At the end of a 24h exposure, we poured the test system into small sieve to recover all snails, and then rinsed the snail three times with clean, aged laboratory water. We then placed snails into small cups with aged water, assessed mortality immediately, then again at 24 and 48 h. We inspected each beaker with the aid of a dissecting microscope and watched for snail movement, and touched individual snails that were not active with a probe to elicit movement or tactile response. Each trial was replicated three times over two weeks for a total of 9 beakers tested at each concentration. All data from these trails are summarized in Appendix A1 - A4.

Statistical Analysis –

All analyses were conducted using SAS 9.2 (SAS Institute Inc. 2002-2008). We explored variable interaction using PROC GENMOD with replicate as the class variable in the model:

Dead/Total = Concentration +Replicate + (Concentration*Replicate), where

Dead = the number of dead snails in beakers 1 - 3;

Total = the total number snails tested at each concentration, generally 30;

Concentration = test concentration of sodium fluorescein (mg/L);

Replicate = Test of toxicity repeated over three different dates

We modeled the response using concentration as a linear and log transformed variable using logit, probit, and complimentary log-log link functions. When significant interactions were detected, we inspected data for outliers by comparing predicted values to the observed response variable. Residual plots were examined to determine best model fit. After fitting the best model, we tested distributions to obtain the lowest log likelihood score to estimate the LC_{50}.

Results

The samples of the test substance sent to Ozark Underground Laboratories for verification produced a robust linear relationship of predicted to observed concentrations, with reported concentrations observed to be somewhat higher than predicted (Table 1; Figure 2).

Snail Behavior at Exposure –

When the sodium fluorescein solution was introduced into the beakers, the snails were observed crawling up the sides of the test containers. Some snails would also float on top of the water and then eventually close up and sink to the bottom, and if they landed shell down, they were observed sometimes having difficulty righting themselves. We detected no increase in this response with increasing test substance concentration, and observed this behavior in controls. Some of the mortality recorded in our tests and control containers was attributed to snails

crawling above the water level and drying out. After 48 h of recovery, dried dead snails could not be separated from snails that died in the test solution and all were recorded as mortality.

Model Fit and Median Lethal Concentration

We found the interaction of Concentration X Replicate was significant in models when all data were included. Through inspection, we identified responses in one replicate of test concentration of 200 mg/L to be abnormally low in mortality. By removing these data points we reduced the significance of the interaction term for concentration (without log transformation), and estimated a more conservative LD_{50}. The model residuals were normally distributed for all models, and log likelihood values estimated in all models were similar (Table 2). For clarity, we choose a probit model and normal distribution for estimating the probability of mortality. We estimated the median lethal concentration to be 377 mg/L. Our model had decreasing precision as probability of mortality increased, because we never achieved high mortality in trials, even at the highest tested concentrations (Figure 3; Table A6).

Discussion

We estimated the LC_{50} of fluorescein to ashy pebblesnails was 377 mg/L. Our tests of 24 h static exposure were designed to evaluate acute toxicity. We did not conduct any trials to determine chronic levels or effects on reproduction or other stress. This LC_{50} estimate of is within a similar range reported for some other vertebrate and invertebrate species. Walthall and Stark (1999) reported median lethal concentrations in 48 h exposures of *Daphnia pulex* as 337 mg/L. However, our tests were conducted for 24 h, as this was considered the longest time an exposure to the dye would occur. Smart (1984) reported that blue mussels and sea urchins were more sensitive. Our tests were conducted at 15°C for 24 h exposures, and temperature of testing and water quality during testing can likely affect the results. The water used for mixing test

solutions in our study was neutral pH, and was double-deionized. The fluorescein test solutions were low in conductivity, ranging from 1 to 96 µS/cm. The pH was difficult to measure with the low conductivity, and was recorded from 7.7 to 9.1. The conductivity of water in the springs at the HNFH ranges from 290 to 370 µS/cm; pH ranges from 7.8 to 8.4 (Hagerman Hatchery Evaluation Team 2010).

Our estimate of the lethal concentration of sodium fluorescein solution was higher than the concentrations proposed for use in dye tracer studies. The IDWR estimated a worst-case scenario concentration for water exiting the springs of 0.2 mg/L. According to our probit model, these concentrations would not show any acute toxicity in the ashy pebblesnail. The results of this study coupled with existing published data indicate that the potential toxicity to aquatic organisms from sodium fluorescein used for aquifer tracer studies is minimal. Data from tracer tests conducted in the Hagerman area aquifer near Malad Gorge State Park reported maximum resurgent concentrations of fluorescein at 0.0011 mg/L (Farmer and Blew 2011). In those studies, fluorescein was injected dye into the wells at approximately 75,000 mg/L. Even when the total discharge was collected in charcoal packs over 15 d, the maximum total eluted concentration was reported as 8.160 mg/L (Farmer and Blew 2011).

The ashy pebblesnail was selected as a surrogate for our studies since both the Bliss Rapids snail and the pebblesnail co-occur in the same springs, and are both hydroboiid snails. Only by conducting tests with Bliss Rapids snails would we validate these assumptions. In addition, our tests were conducted in static not flow through conditions. Flowing water conditions similar to the springs could affect the results of exposure and increase or decrease the toxicity, depending on the circumstances. Preston et al. (2001) found that increased toxicant sensitivity occurred at ecologically relevant levels of fluid motion in studies with rotifers. It is

possible that fluid motion-toxicant interactions can affect toxicant uptake, and alter the bioavailability of food resources. The snails have much different microhabitat requirements, and we can only speculate that flow through conditions in the natural setting could likely alter the outcome.

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References

Duncan, N., and R. Huff 2009. Fluminicola fuscus species fact sheet. Interagency Special Status/Sensitive Species Program. U.S. Department of Interior, Bureau of Land Management, Oregon/Washington and U.S. Department of Agriculture, Forest Service, Region 6. 3 pp. http://www.fs.fed.us/r6/sfpnw/issssp/planning-documents/species-guides.shtml. Accessed October 2010.

Farmer, N., and C. D. Farmer. 2011. Fluorescent dye traces tests and hydrogeology near the Malad Gorge State Park (Hopper well test). Idaho Department of Water Resources Open File Report. January 28, 2011.

Field, M.S., R.G. Wilhelm, J.F. Quinlan, and T.J. Aley. 1995. An assessment of the potential adverse properties of fluorescent tracer dyes used for groundwater tracing. Environmental Monitoring and Assessment 38:75-96.

Fishelson L., V. Bresler, R. Manelisa, Z. Zuk-Rimonb, A. Dotanb, H. Hornungc, A. Yawetz. 1999. Toxicological aspects associated with the ecology of *Donax trunculus* (Bivalvia, Mollusca) in a polluted environment. The Science of the Total Environment 226:121-131.

Frest, T. J., and E. J. Johannes. 2000. An annotated checklist of Idaho land and freshwater mollusks. Journal of the Idaho Academy of Science, December 2000

Hagerman Hatchery Evaluation Team. 2010. Water quality monitoring of springs used for fish production at the Hagerman National Fish Hatchery; relating nitrate levels to specific conductance. Lower Snake River Compensation Plan Report. U.S. Fish and Wildlife Service. Boise, Idaho.

Hershler, R., T. J. Frest, E. J. Johannes, P. A.Bowler and F. G. Thompson. 1994. Two new genera of Hydrobiid snails (Prosobranchia: Rissooidea) from the northwestern United States. Veliger 37: 221 -243.

Marking, L. L. 1969. Toxicity of Rhodamine B and fluorescein sodium to fish and their compatibility with Antimycin A. Progressive Fish Culturist 139-142.

Michelson, E.H. 1964. Shell fluorescence in *Australorbis glabratus* and other aquatic snails exposed to tetracyclines. Journal of Parasitology 50:743-747.

Noga, E. J. and P. Udomkusonsri. 2002. Fluorescein: A rapid, sensitive, nonlethal method for detecting skin ulceration in fish. Veterinary Pathology 39:726–731.

Preston B. L., T. W. Snell, D. M. Fields,, and M. J. Weissburget. 2001. The effects of fluid motion on toxicant sensitivity of the rotifer *Brachionus calyciflorus* Aquatic Toxicology 52:117–131.

SAS Institute Inc. 2002-2008. SAS/STAT Software Version 9.2. SAS Institute Inc. Cary, N.C.

Smart, P.L. 1984. A review of the toxicity of twelve fluorescent dyes used for water tracing. National Speleological Society Bulletin 4:21-33.

U. S. Fish and Wildlife Service 1992. Endangered and threatened wildlife and plants; determination of endangered or threatened status for five aquatic snails in south central Idaho. Federal Register 57(240): 59244-59257. 14 December.

U.S. Fish and Wildlife Service. 2009. Endangered and threatened wildlife and plants; 12-month finding on a petition to remove the Bliss Rapids snail (*Taylorconcha serpenticola*) from the list of endangered and threatened wildlife. Federal Register 74 (178):47536–47545, 16 September.

Walthall, W.K. and J.D. Stark. 1999. The acute and chronic toxicity of two xanthene dyes, fluorescein sodium salt and phloxine B, to *Daphnia pulex*. Environmental Pollution 104: 207-215.

Table 1: Summary of results of sodium fluoroscein concentration validation at Ozark Underground Laboratory in 2010. Fluorescein concentration and peak absorptions are reported. Only samples taken before tests were conducted were analyzed. Test 7512 was a calibration test.

Sample ID	Concentration mg/L	Date collected	Fluoroscein	
			Peak (mm)	mg/L
T7512	0.1	23 April	507.8	0.127
T7513	0.2	30 March	507.6	0.223
T7514	20.0	30 March	507.7	22.600
T7515	50.0	1 April	507.9	56.500
T7516	71.0	25 March	507.9	81.100
T7138	100.0	1 April	507.8	128.000
T7517	141.0	30 March	507.9	161.000
T7518	200.0	25 March	507.8	232.000

Table 2: Summary of log likelihood statistics for models tested for the full range study mortality.

Test	Log likelihood
logit	-254.37
probit	-253.46
Complimentary log-log	-254.77
probit-normal	-249.52
probit-Gompertz	-248.84

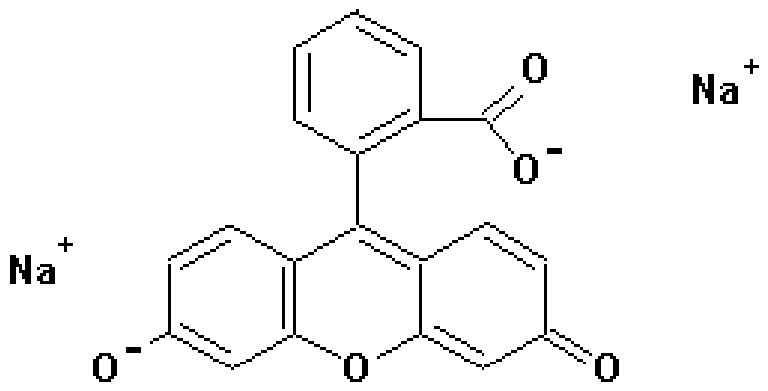

Figure 1. Chemical structure of sodium fluorescein.

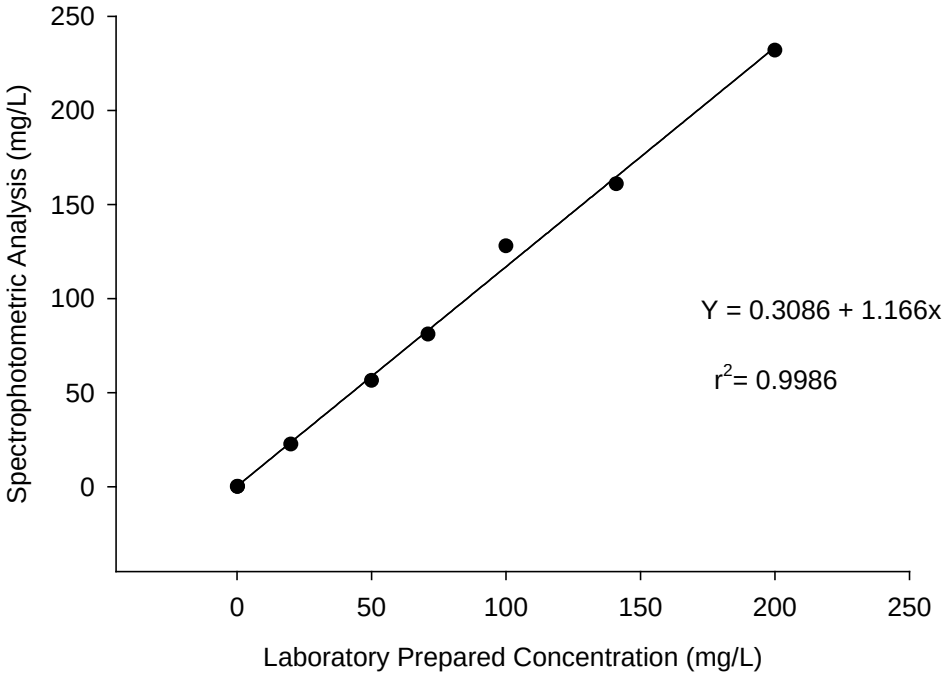

Figure 2. Plot of concentration of laboratory prepared sodium fluorescein versus concentrations determined via spectrophotometry at Ozark Underground Laboratory. Regression equation and r^2 of least squares fit are provided.

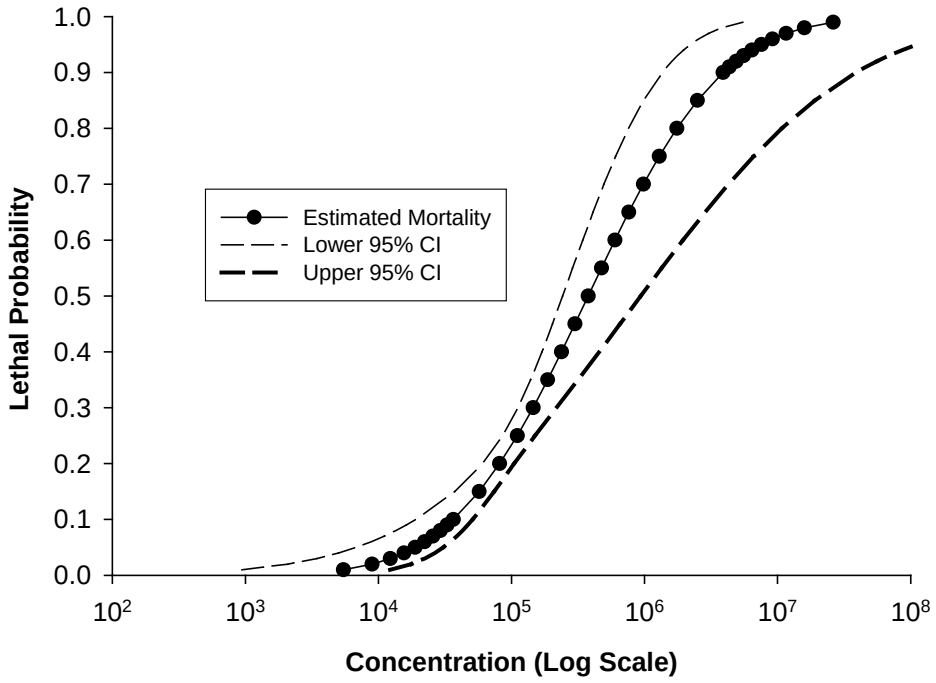

Figure 3: Summary of probability of mortality versus concentration (mg/L) of sodium fluorescein using predicted model solutions of normal distribution probit model (provided in Appendix Table A5). Note, width of confidence intervals above LC_{60} indicates high uncertainty in predictions.

Appendix A: Detailed data from all trials of toxicity.

Table A1. Data for range finding test, all three replicate days of testing are represented, and three replicate beakers for each day of testing. All trials were conducted from 21 February to 28 February NT: Not Tested

	Replicate 1			Replicate 2			Replicate 3		
Concentration (mg/L)	No. dead	Total tested	Percent mortality	No. dead	Total tested	Percent mortality	No. dead	Total tested	Percent mortality
0	1	11	9	0	9	0	0	10	0
0	1	10	10	0	10	0	0	10	0
0	0	10	0	0	10	0	0	10	0
0.2	0	10	0	0	11	0	2	10	20
0.2	0	10	0	1	10	10	0	10	0
0.2	0	10	0	0	10	0	0	10	0
2.0	0	10	0	0	10	0	0	10	0
2.0	1	10	10	2	11	18	0	10	0
2.0	1	10	10	0	10	0	0	10	0
20.0	0	10	0	1	10	10	0	10	0
20.0	0	10	0	1	10	10	0	9	0
20.0	0	10	0	1	10	10	0	10	0
200	NT	NT	NT	9	10	90	10	10	100
200	NT	NT	NT	9	10	90	8	10	80
200	NT	NT	NT	7	10	70	7	9	78

Table A2. Average percent and standard deviation mortality by concentration tested in the three replicate days of testing in the range finding tests.

Concentration (mg/L)	Mean $\pm$ SD percent mortality
0	2.1 $\pm$ 4.2
0.2	3.3 $\pm$ 7.1
2.0	4.2 $\pm$ 6.8
20.0	3.3 $\pm$ 5.0
200	84.6 $\pm$ 10.7

Table A3. Data for full range study, all three replicates represented. Bold and italicized numbers of replicate 3, 200 mg/L were censored from probit analysis as outlier values. All trials were conducted from 25 March to 1 April

	Replicate 1			Replicate 2			Replicate 3		
Concentration (mg/L)	No. dead	Total tested	Percent mortality	No. dead	Total tested	Percent mortality	No. dead	Total tested	Percent mortality
0	0	10	0	0	10	0	1	10	10
0	0	10	0	0	10	0	0	10	0
0	0	10	0	0	10	0	0	10	0
0.2	0	10	0	0	10	0	0	10	0
0.2	0	10	0	0	10	0	0	10	0
0.2	0	10	0	0	10	0	0	10	0
20.0	1	10	10	3	10	30	0	10	0
20.0	0	10	0	0	9	0	0	10	0
20.0	0	10	0	2	10	20	1	10	10
50.0	1	10	10	1	10	10	1	10	10
50.0	0	10	0	3	10	30	1	10	10
50.0	2	10	20	1	10	10	2	10	20
71.0	1	10	10	1	9	11	2	10	20
71.0	2	10	20	3	10	30	4	10	40
71.0	1	10	10	2	10	20	1	10	10
100.0	0	11	0	3	10	30	2	10	20
100.0	3	10	30	4	10	40	2	10	20
100.0	1	10	10	2	10	20	2	10	20
141.0	1	10	10	6	10	60	1	10	10
141.0	1	10	10	2	10	20	0	10	0
141.0	1	10	10	6	10	60	1	10	10
200.0	4	10	40	5	10	50	***1***	***9***	***11***
200.0	6	10	60	8	11	73	***2***	***10***	***20***
200.0	3	10	30	5	10	50	***1***	***10***	***10***

Table A4. Average percent and standard deviation mortality by concentration tested. [a] =General statistics with censored data.

Concentration (mg/L)	Mean ± SD percent mortality	
0	1.1	±0.033
0.2	0	±0.000
20.0	7.8	±0.109
50.0	13.3	±0.087
71.0	19.0	±0.104
100.0	21.1	±0.117
141.0	21.1	±0.226
200.0	38.2	±0.220
200.0[a]	50.5	±0.14

Table A5. Summary of predicted probability of mortality from probit model of response in eight concentrations of test substance and controls. The probit model was developed assuming a normal distribution. The 95% confidence intervals for each estimate are provided. These data were used in graphic presentation (Figure3).

	Sodium fluorescein concentration	
Probability	mg/L	95% confidence interval
0.01	5.45	0.94 – 12.21
0.02	8.96	2.08 – 17.61
0.03	12.28	3.44 – 22.25
0.04	15.56	5.02 – 26.55
0.05	18.87	6.81 – 30.69
0.06	22.23	8.82 – 34.75
0.07	25.67	11.06 – 38.78
0.08	29.20	13.52 – 42.83
0.09	32.83	16.22 – 46.94
0.10	36.57	19.16 – 51.12
0.15	57.15	37.42 – 74.24
0.20	81.50	60.67 – 104.85
0.25	110.50	86.13 – 150.36
0.30	145.25	112.22 – 218.49
0.35	187.13	139.70 – 317.10
0.40	237.98	169.82 – 457.26
0.45	300.30	203.80 – 655.85
0.50	377.54	242.93 – 938.90
0.55	474.65	288.89 – 1,347.34
0.60	598.95	343.92 – 1,948.00
0.65	761.72	411.34 – 2,855.03
0.70	981.34	496.25 – 4,275.56
0.75	1,289.90	607.17 – 6,616.22
0.80	1,748.94	759.54 – 10,766.40
0.85	2,493.97	985.35 – 19,004.53
0.90	3,897.67	1,366.12 – 38,877.81
0.91	4,341.51	1,478.16 – 46,219.24
0.92	4,881.15	1,610.25 – 55,775.98
0.93	5,552.25	1,769.08 – 68,582.75
0.94	6,411.39	1,964.97 – 86,395.44
0.95	7,554.66	2,214.88 – 112,429.05
0.96	9,160.92	2,549.24 – 153,213.46
0.97	11,610.95	3,029.98 – 224,172.81
0.98	15,910.86	3,811.76 – 371,838.42
0.99	26,142.54	5,471.97 – 825,733.80

Table A6. Summary of samples of sodium fluorescein test solutions collected before and after testing by replicate test number and concentration. Dates (2010) for each collection are provided. All samples were sent to IDWR for validation on 6 April, 2010. Representative samples of the pre-test solutions were selected by IDWR staff, coded, and sent to the Ozark Underground Laboratories (OUL), Protem, Missouri for spectrophotometric validation.

Replicate number	Pre test solution mg/L	Date collected	Sample code of pretest solution sent to OUL	Post test solution mg/L	Date collected
1	0	25 March		0	26 March
1	0.2	25 March		0.2	26 March
1	20.0	25 March		20.0	26 March
1	50.0	25 March		50.0	26 March
1	71.0	25 March	T7516	71.0	26 March
1	100.0	25 March		100.0	26 March
1	141.0	25 March		141.0	26 March
1	200.0	25 March	T7518	200.0	26 March
2	0	30 March		0	31 March
2	0.2	30 March	T7513	0.2	31 March
2	20.0	30 March	T7514	20.0	31 March
2	50.0	30 March		50.0	31 March
2	71.0	30 March		71.0	31 March
2	100.0	30 March		100.0	31 March
2	141.0	30 March	T7517	141.0	31 March
2	200.0	30 March		200.0	31 March
3	0	1 April		0	2 April
3	0.2	1 April		0.2	2 April
3	20.0	1 April		20.0	2 April
3	50.0	1 April	T7515	50.0	2 April
3	71.0	1 April		71.0	2 April
3	100.0	1 April	T7138	100.0	2 April
3	141.0	1 April		141.0	2 April
3	200.0	1 April		200.0	2 April

Table A7. Summary of water quality parameters in replicated readings of typical test solutions used in toxicity trials. All test solutions were made with double deionized water. Readings were made with a YSI 556 multiprobe System (YSI Inc., Yellow Springs, Ohio).

Concentration mg/L	Replicate	Temperature °C	Conductivity µS/cm	Salinity ppt	pH	pH mV
0.2	1	16.3	1	0	8.4	-44.0
0.2	2	16.2	1	0	8.2	-34.7
0.2	3	15.6	1	0	7.7	-12.6
200.0	1	16.0	96	0.04	9.2	-81.6
200.0	2	15.5	96	0.04	9.1	-76.0
200.0	3	15.3	96	0.04	9.1	-74.2