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Abstract

Groundwater scientists use fluorescein dyes to trace ground water resources that supply springs which may contain threatened or endangered mollusk species. To estimate risks of a commonly used groundwater tracer to the threatened Bliss Rapids snail *Taylorcocha serpenticola* we tested the toxicity of sodium fluorescein solutions to a surrogate species, the ashy pebblesnail *Fluminicola fuscus.* Trials were conducted in static 24-h exposures to several levels of treatment as well as controls with no sodium fluorescein at temperatures similar to those in the habitat of concern. We estimated 377 mg L^{-1} as the median lethal concentration for the ashy pebblesnails. Using these data, we concluded that risks to mollusks from the proposed groundwater testing were likely low.

keywords: mollusk toxicology; species extrapolation; risk assessment; aquifer monitoring

Introduction

Accurate ground water tracing is increasingly important for water management, especially in arid regions of the globe where conflicts of water use and allocation intersect with demands for agriculture and other human uses in light of climate change (Llamas and Martinez-Santos 2005, Schaible et al. 2010). Monitoring of spring discharge and river base flows is necessary for irrigators, hydropower generators, and other water users to allow for planning and water allocation decisions in western North America (Palmer et al. 2009, Boggs et al. 2010).

Dye tracer tests with fluorescent dyes are used in groundwater studies (e.g., to determine flow paths, residence times, site-specific hydrology, recharge areas, springhead protections) and assist in planning for response to spills of hazardous materials because of their reported low toxicity, cost, solubility, and ease of detection (Field et al. 1995). Sodium fluorescein ($C_{20}H_{10}Na_2O_5$; Acid Yellow 73) is used frequently for aquifer tracer studies. Sodium fluorescein is an orange powder that turns a bright green to yellow color when small quantities are diluted with water, and the compound undergoes UV degradation (Smart 1984, Walthall and Stark 1999).

In the Snake River plain of southern Idaho (Smith 2004), water allocation conflicts and environmental compliance concerns have led to increased efforts to model and understand the groundwater systems (Johnson et al. 1999; Miller et al. 2003; Cosgrove and Johnson 2004, 2005). The Thousand Springs area near Hagerman, Idaho is the primary discharge point for the Eastern Snake Plain Aquifer (Boggs et al. 2010). These springs and associated rivers and streams contain several aquatic species of concern including three ESA listed species: Utah valvata (*Valvata utahensis*); Snake River Physa (*Physa natricina*); and Bliss Rapids snail (*Taylorconcha serpenticola*) (U. S. Fish and Wildlife Service 1992, 2009). Of these species, the Bliss Rapids snail of the Snake River has a distribution limited to a small section of

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the Snake River and its tributaries in southern Idaho (Hershler et al. 1994, US Fish and Wildlife Service 2009). This small section of the Snake River was the focus of proposed aquifer testing. Bliss Rapids snails are small (2.0 - 4.0 mm), light brown to orange colored, with 3.5 to 4.5 whorls and a blunt apex. The snails prefer habitat with flowing water or springs and stable cobble-boulder substratum. The Bliss Rapids snails are moderately photophobic, reside on the lateral sides and undersides of rocks during daylight and migrate to the uppermost surfaces of rocks at night (U S. Fish and Wildlife Service 2009).

Information on the toxicity of fluorescein dye to mollusks is especially scarce. Michelson (1964) exposed *Australorbis* and *Helisoma* sp. snails to sodium fluorescein for 5 consecutive days at concentrations of 1, 10, and 100 mg L^{-1}, and reported mortality of 80 to 100% in concentrations of 10 and 100 mg L^{-1}. Smart (1984) reviewed the published literature on the toxicity of 12 fluorescent dyes to selected species and reported exposures of blue mussel eggs (*Mytilus edulis*) to 1 mg L^{-1} for 48 h had no effect. Other invertebrates show a range of sensitivity to fluorescein. Walthall and Stark (1999) conducted extensive experiments to determine the chronic and acute toxicity of sodium fluorescein and phloxine B to *Daphnia pulex,* and reported that the 48 h LC_{50} of fluorescein was 337 mg L^{-1}.

Because of concerns for the small population size of the threatened Bliss Rapids snail, we chose a more common hydrobiid snail, the ashy pebblesnail, *Fluminicola fuscus* (Haldeman 1847) that occupies the same springs of concern as a surrogate organism for our toxicity testing. Pebblesnails are considered a species of conservation concern (Duncan and Huff 2009), but within the Hagerman Valley they are rather widely distributed and occur at high population densities. 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 because it inhabits the same springs as the Bliss Rapids snail and has a similar life history.

The objectives of our study were to determine the toxicity of sodium fluorescein to the ashy pebblesnail in a 24 h static exposure.

Methods

Experimental Animals and Test Substance

Pebblesnails were collected from springs at Hagerman National Fish Hatchery from February to March 2010, packaged in moist towels, placed in plastic bags, and shipped in coolers to the University of Idaho College of Natural Resources Fisheries wet laboratory in Moscow. Upon arrival, snails were washed with de-chlorinated, aged and aerated well water (pH 7.8; conductivity 320 µs cm^{-1}) and individuals between 2.0 mm and 4.0 mm were transferred into containers with 2 L of well water. Snails were maintained in an incubation room at 15 °C and provided a natural photoperiod for the latitude. We changed the water in each container every other day, and individual test organisms were retained in the lab < 3 weeks.

Test sodium fluorescein solutions were prepared in the dark using deionized water. The sodium fluorescein (Kingscote Chemicals, Miamisburg, OH) was the same substance to be used for groundwater tests to be conducted by Idaho Department of Water Resources. Concentrations were prepared by weight to volume dilutions, a stock solution (200 mg L^{-1}) was prepared, and volumetric pipettes and flasks were used to measure the 0.2, 2.0, 20 mg L^{-1} test concentrations. Initial tests were conducted as range finding with four concentrations (0.2, 2.0, 20, and 200 mg L^{-1}) and a full study was conducted using seven target concentrations to estimate an LC_{50}: 0.2; 20; 50; 71; 100; 141; and 200mg L^{-1}. All solutions were equilibrated to 15 °C in the dark before testing. We report the concentrations of the test substance without adjusting for photo-activity of the dye. Approximately 50 mL of each test solution was collected before and after each exposure trial and refrigerated (4 °C), and archived samples were shipped to Ozark Underground Laboratories (Protem, MO) for verification with spectrophotometry. Typical test solutions were low in conductivity (1 to 96 µS/ cm), and pH ranged from 7.5 to 8.9. Water used in test solutions was > 90% saturated with oxygen, and was monitored before and after trials.

Experimental Design

We placed 10 snails in each of three 150 mL beakers for a given test concentration for each trial (three replicates per concentration). To begin a test, 100 mL of a test solution equilibrated to 15 °C was poured into each beaker. Beakers with snails were kept in a darkened aquarium in a cold room (15 °C) to limit light degradation to the test compounds. Snails in three beakers with deionized water without dye served as controls for each test.

After a 24 h exposure, we poured the test solution and snails into a small sieve to recover all snails, and rinsed snails 3X with aerated aged well water and placed them into a new beaker with approximately 50 mL aged well water. We inspected each beaker with the aid of a dissecting microscope and watched for snail movement, and probed individual snails that were not active to elicit movement or tactile response. Snail mortality was assessed immediately, at 24 and 48 h. We provided this time for recovery to ensure that stressed individuals had a chance to recover, as some snails appear dead inside closed opercula, but are actually still alive. The mortality after 48 h was used as the response variable. Each trial was replicated three times over two weeks for a total of 9 beakers tested at each concentration.

Statistical Analysis

Since mortality is time dependent and log increases in concentrations were used, we explored models using generalized linear models in SAS with PROC GENMOD (SAS Institute Inc. 2002-2008) to test across the replicate dates of testing and potential interactions using in the model: Dead/Total = Concentration + Date + (Concentration*Date), 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^{-1}); Date = Test of toxicity repeated over three different dates

We modeled the response using concentration as linear and log transformed variables using logit, probit, and complimentary log-log link distribution functions to explore model fits. We detected significant Concentration*Date interactions and inspected the data for outliers by comparing predicted values to the observed response variable. As a result, we censored from our data one replicate (3 beakers) testing the highest concentration because of abnormally low mortality observed in this group (likely from avoidance behavior), thus increasing the conservative nature of our estimates. When these outlier data were excluded, we had no significant date or interaction effects (all $P >$ 0.2). In all models, residual plots were examined to determine best model fit, to reduce auto and cross-correlation. To estimate the LC_{50} we used a reduced model without the date and interaction term as they were not significant. We compared the log likelihood scores for each distribution (probit normal, logit, and log log distribution) to select the lowest score. Using coefficients from the selected model, we calculated the LC_{50} and 95% confidence intervals and used predicted solutions to plot the response and CI for a range of concentrations.

Results

We estimated the median lethal concentration of fluorescein to ashy pebblesnails as 377 mg L^{-1} ($\pm$ 95% C.I. = 243 – 939). Target concentrations for the model development were selected in 0.1 log intervals to assure a range of response. We measured minimal mortality in the control beakers (1.1 % ± 0.03) and no mortality in tests of 0.2 mg L^{-1} fluorescein (Table 1). One test replicate exposed to 200 mg L^{-1} had low mortality. The higher survival in this replicate was due to avoidance behavior of the snails that was observed in other circumstances as well. When the sodium fluorescein solution was introduced in the test beakers, snails often moved up the sides of the glass to avoid exposure. We controlled for this avoidance behavior by moving the test snails back into the solution. This particular replicate had active snails that repeated avoidance, and were observed along the sides of the beaker at the end of the trial. By censoring the data from test replicate 3 (concentration 200 mg L^{-1}) we removed a significant interaction term that appeared in statistical models used to predict median lethal dose. The elimination of these points provided a model with better confidence and a more conservative estimate of the LD_{50} (Figure 1).

TABLE 1. Summary of mortality data used in models, including mean percent mortality (± SD) for ashy pebblesnails by test concentration and replicate date of testing. The data for replicate 3 at 200 mg L^{-1} were censored from the final models, and are indicated in bold font. Final mortality was determined after 48 h of recovery in fresh well water. Trials were conducted from 25 March to 1 April. a =general statistics with censored data.

Concentration		Replicate 1		Replicate 2		Replicate 3	
(mg L^{-1}) and rep	Mean ±SD mortality	Dead	Total tested	Dead	Total tested	Dead	Total tested
0	1.1±0.03	0	10	0	10	1	10
0		0	10	0	10	0	10
0		0	10	0	10	0	10
0.2	0±0.00	0	10	0	10	0	10
0.2		0	10	0	10	0	10
0.2		0	10	0	10	0	10
20	7.8±0.11	1	10	3	10	0	10
20		0	10	0	9	0	10
20		0	10	2	10	1	10
50	13.3±0.09	1	10	1	10	1	10
50		0	10	3	10	1	10
50		2	10	1	10	2	10
71	19.0±0.10	1	10	1	9	2	10
71		2	10	3	10	4	10
71		1	10	2	10	1	10
100	21.1±0.12	0	11	3	10	2	10
100		3	10	4	10	2	10
100		1	10	2	10	2	10
141	21.1±0.23	1	10	6	10	1	10
141		1	10	2	10	0	10
141		1	10	6	10	1	10
200	38.2±0.22	4	10	5	10	***1***	***9***
200		6	10	8	11	***2***	***10***
200	50.5±0.14[a]	3	10	5	10	***1***	***10***

The model residuals were normally distributed for all distributions used to assess model fit, and the log likelihood values estimated for the several distributions were similar (Table 2). For clarity, we choose a probit model and normal distribution for estimating the probability of mortality. Our model had decreasing precision as probability of mortality increased because we never achieved high mortality in test concentrations (Table 1).

Comparisons between the data provided by spectrophotometric analysis of selected test concentrations and our target prepared solutions showed a highly significant linear relationship ($r^2 = 0.99$) between our target test concentrations and those reported in the random sample of solutions analyzed by the laboratory (Figure 2). However, the predicted concentration by spectrophotometry was higher than that of the test samples we prepared. These differences could be attributed to errors in the dilution of test substances for the spectrophotometer.

The addition of dye to the deionized water increased the conductivity of test solutions from that of 0 for controls to 96 mg L^{-1} in the highest test solution. Although mollusks and most organisms cannot live for extended periods in deionized water, short exposures were not harmful. Since mortality in control containers and in concentrations of fluorescein was low, we did not consider low conductivity a factor in the responses measured.

Discussion

Our 24 h static exposure tests were designed to evaluate acute toxicity to the tracer. We did not conduct trials to determine chronic levels or effects on reproduction or other sub lethal endpoints. Our

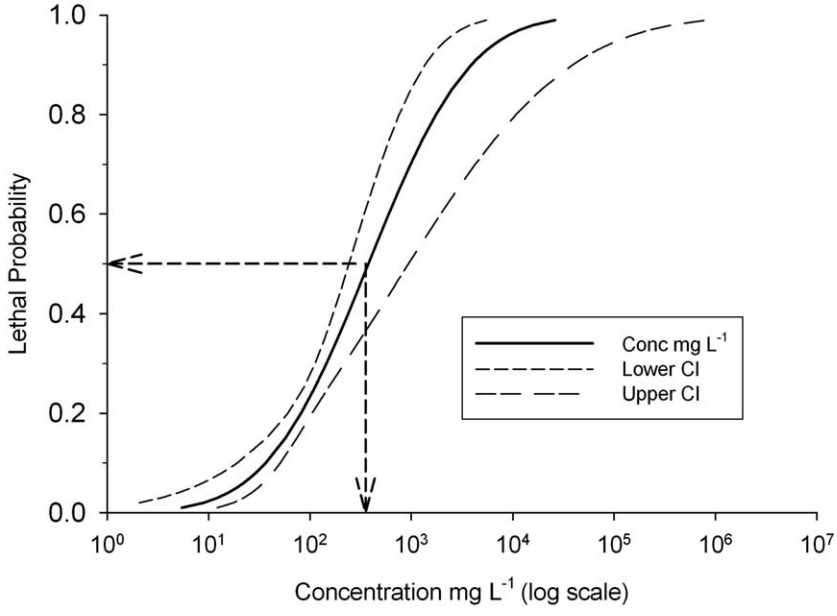

Figure 1. Plot of probability of mortality versus concentration (mg L^{-1}) of sodium fluorescein using predicted model coefficients for normal distribution probit regression model (solid line) with 95% confidence intervals (dashed lines). Note, width of confidence intervals above LC_{60} indicates high uncertainty in predictions. Arrows indicate the LC_{50} solution for this model.

TABLE 2. Summary of log likelihood statistics for simple model without day and day*concentration factors.

Test	Log likelihood
probit	-253.46
logit	-254.37
log-log	-254.77

LC_{50} estimate for pebblesnails is similar to that reported for *Daphnia pulex* (Walthall and Stark 1999) of 337 mg L^{-1} after 48 h exposure. Smart (1964) reported no effect of fluorescein on the development of eggs of sea urchins (*Hemicentrotus pulcherrimus*) and blue mussels exposed for 48 h to concentrations of 10 and 1 mg L^{-1}, respectively. Our choice of 24 h exposures represented the longest time dye would likely reside in the aquifer springs during testing. Our estimate of median lethal concentration was higher than those reported by Michelson (1964) for *Australorbis glabratus* and *Helisoma anceps anceps.* Michelson found concentrations of 10 and 100 mg L^{-1} caused 80 to 100% mortality in 5-day exposure, however few details of the methods of exposure were provided in the published study.

We found estimates of lethal concentration were higher than the concentrations likely to occur in dye tracer studies. The estimated worst-case scenario concentration for water exiting the springs was estimated at 0.2 mg L^{-1} (Farmer and Blew 2011). 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^{-1} (Farmer and Blew 2011) when fluorescein was injected into the wells at approximately 75,000 mg L^{-1}. Even when the total discharge was collected in activated charcoal packs (Smart and Simpson 2002) over 15 d, the maximum total eluted concentration was reported as 8.160 mg L^{-1} (Farmer and Blew 2011).

Our tests were conducted at 15 °C, the temperature of the springs of interest. Clearly temperature and water quality during testing can affect the results. We used deionized water for mixing test solutions, and the resulting fluorescein test solutions were low in conductivity, ranging from 1 to 96 µS cm^{-1}. We speculate that low conductiv-

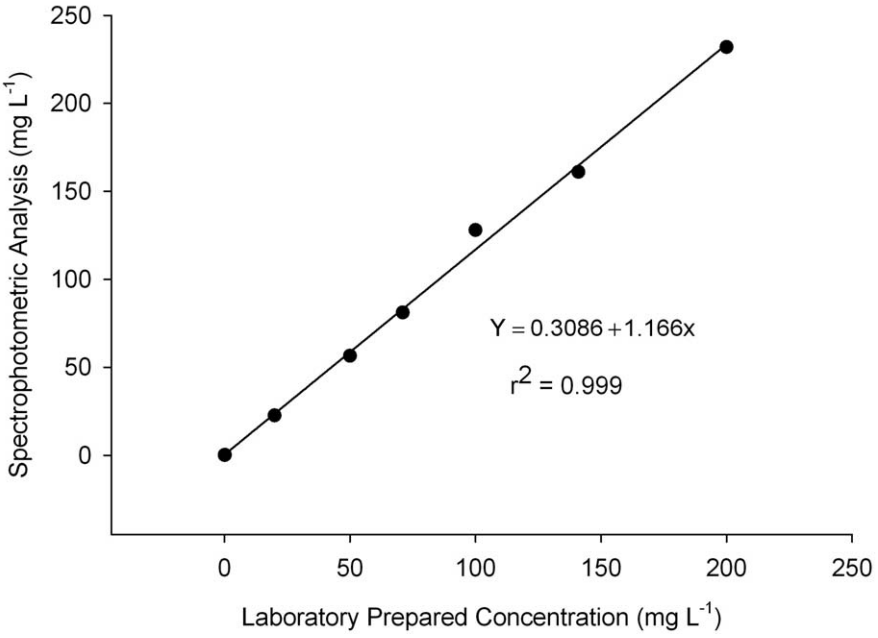

Figure 2. Plot of concentration of laboratory prepared sodium fluorescein versus concentrations determined via spectrophotometry at Ozark Underground Laboratory. Regression equation and r^2 of least squares fit are provided.

ity could likely increase the mortality from the test solution; however control samples had very low mortality. The conductivity of water in the springs that the snails were obtained from ranges from 290 to 370 µS L^{-1}; and pH ranges from 7.8 to 8.4 (Hagerman Hatchery Evaluation Team 2010). Our tests were conducted in static conditions, and 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 in rotifers when water movement increased at levels likely observed in the environment. It is possible that fluid motion-toxicant interactions can affect toxicant uptake, and alter the bioavailability of food resources.

The ashy pebblesnails we tested have distinct microhabitat requirements different from rotifers, but we speculate that flow through conditions in the natural setting could likely alter the outcome observed in our static tests. Ashy pebblesnails could also exhibit avoidance behavior and thus could move to a more preferred area with lower levels of dye. 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. We recognize that the two snails have similar life history and habitats, but only by conducting tests with Bliss Rapids snails could we validate these assumptions. Species sensitivity distributions have been used to compare the sensitivities of different test substances to groups of organisms, especially when endangered species are concerned (Brix et al. 2001, Hose 2005, Raimondo et al. 2009). The use of a surrogate species to determine the likely effects of fluorescein on the threatened Bliss Rapid snail must be considered with additional caution. Banks et al. (2010) used life history attributes for a suite of species to examine if traditional surrogate fish species accurately predicted the outcome on ESA listed salmonid populations. They cautioned that extrapolations from traditional test fish species failed to predict the population outcome for ESA listed species. Some of the additional factors that need to be considered in risk assessments include the risks from multiple stressors

from anthropogenic disturbances (Munns 2006, Awkerman et al. 2008).

Our estimated median lethal concentration is much higher than the expected worst-case scenario concentrations of 0.2 mg L^{-1} used in the ground water testing in the Snake River system. This higher LC_{50} provides some data to regulators concerned about the potential toxicity to threatened species. Clearly this small laboratory assessment needs to be further tested in field conditions with additional replication and monitoring of chronic responses. Increased use of several fluorescent dye tracers throughout the world should precipitate more quantitative and qualitative research on their effect on aquatic communities (Pouliquen et al 1996, Rowiński and Chrzanowski 2011).

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