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Toxicity of Fluoride to the Endangered Unionid Mussel, *Alasmidonta raveneliana*, and Surrogate Species

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The Appalachian elktoe, *Alasmidonta raveneliana*, is a federally-listed endangered unionid mussel whose range once included the Cumberland and Tennessee River drainages, but is now limited to the Tennessee River and its tributaries, Nolichucky River system, Pigeon River system, Mills River, and Little River (Burch 1975, USFWS 1996, Bogan 2002). The decline in abundance of this species and other unionids is believed to have resulted from habitat destruction, competition with nonindigenous species, the loss of host fish species that are necessary for larval transformation to the juvenile stage, and contamination (Williams et al. 1993, USFWS 1994, The Nature Conservancy 1992). One of the healthiest remaining populations of the Appalachian elktoe mussel is found in North Carolina's North Toe River that now receives discharges from feldspar mining operations containing significant concentrations of fluoride. Fluoride is known to be toxic to fish, zooplankton, aquatic insects and some adult unionid mussels (Smith et al. 1985, Feiser 1986, Camargo and Tarazona 1990, Muley 1990). However, no fluoride toxicity data were available for early life stages of the endangered *A. raveneliana.* The present study was conducted primarily as a means of evaluating fluoride as a possible limiting factor in the recovery of this mussel species. A second goal was to test several fish species as potential hosts for the mussel larvae during transformation.

MATERIALS AND METHODS

Adult *A. raveneliana*, *Actinonais pectorosa*, *Utterbackia imbecillis* and *Lampsilis fasciola* mussels were collected from the Little Tennessee River, Franklin, North Carolina (*A. raveneliana*); Lake Chapman, Athens, Georgia (*U. imbecillis*); and the Clinch River, Virginia (*L. fasciola*, *A. pectorosa*), with the use of glass-bottomed buckets or by SCUBA diving (*U. imbecillis*). The mussels were transported to the USEPA Region 4 laboratory in Athens, GA in wet burlap bags held in an insulated container. Upon arrival at the laboratory, the mussels—except *U. imbecilis*, were placed in a recirculating water bath at 16°C, the same as water from which they were collected. *U. imbecillis* were held at 20°C, similar to the temperature of Lake Chapman. Collection and transport followed appropriate permit requirements.

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Unionid mussels have a parasitic stage during which their larvae (glochidia) encyst on gills or fins of host fish and transform into juvenile mussels. This process can take a week to several weeks to complete depending on the mussel species and temperature. To produce juvenile mussels in the laboratory, we collect glochidia from female mussels and place them on the gills of fish hosts, then maintain the fish until the juveniles drop off. They are ~0.25-0.35 mm in length at that time.

Glochidia for fish infections and toxicity tests were collected from adult *A. raveneliana* by puncturing marsupia (egg-filled gills) with a scalpel and washing larvae out with water from a squirt bottle. This mode of collection appeared to be non-lethal to the adult females that were returned to their point of collection within a week of glochidia harvest. Tagged elktoe mussels from which glochidia were removed were recollected in a healthy condition a year later. Glochidia from the other more common mussel species were more completely harvested, generally resulting in the death of the adult.

The banded sculpin, *Cottus carolinae,* a known host for *A. raveneliana* glochidia, (Gordon and Moorman 2001) were collected in north Georgia. The mottled sculpin, *Cottus bairdii*, were collected in South Carolina for testing as a host because its range is currently sympatric with *A. raveneliana* while *C. carolinae*'s is not (USFWS 1996). Largemouth bass, *Micropterus salmoides* and smallmouth bass, *M. dolomieu* were also infected with Appalachian elktoe glochidia because they were readily available and are widely distributed fish.

Sculpins were infected by collecting glochidia from the female mussels, placing them in a beaker containing well water with a bubbling airstone for 30 minutes to allow the glochidia to attach to gills. The bass species were large enough that glochidia could be directly pipetted onto their gills. Sculpin were then held under low-light conditions in aquaria with recirculating water at 16°C for approximately six weeks until juvenile mussels excysted and dropped to the bottom of the tank. They were then siphoned from the tank and used in toxicity tests. Juvenile *U. imbecillis* mussels were produced on *Lepomis macrochirus* (bluegill), *A. pectorosa* on *M. dolomieu*, and *L. fasciola* on *M. salmoides* in five gal aquaria containing flow-through water at 20°C.

Test solutions were prepared by dilution of a stock of NaF dissolved in dilution water, measured with the fluoride probe to determine concentrations, and distributed to six-well plates that were used as test chambers. Once collected, glochidia were rinsed with clean test dilution water, soft or moderately hard (USEPA 1994), tested for viability, and randomly distributed into triplicate chambers per test concentration in six-well plates containing one of five concentrations of the fluoride solution (NaF; Fisher Scientific, 99.3% pure), or the dilution water control (Table 1). Lethality was assessed at the end of 24 hr and 48 hr using separate sets of glochidia (Keller and Ruessler 1997).

Table 1. Test conditions for 96-hr and 9-d toxicity tests with glochidia and juvenile mussels.

	Glochidia tests	**96-hr tests**	**9-d tests**
Renewal	None	None	Daily
Feeding regime	None	None	Algae
Lighting regime	16L:8D	16L:8D	16L:8D
Test temperature	25±1°C	25±1°C	25±1°C
Light intensity	500-1000 Lux	500-1000 Lux	500-1000 Lux
Test chamber	6-well plates (Polyethylene)	60x15 mm glass petri dish	60x15 mm glass petri dishes
Test solution volume	5 ml	10 ml	10 ml
Test concentrations	31, 62, 125, 250 and 500 mg F/L	31, 62, 125, 250 and 500 mg F/L	31, 62, 125, 250 and 500 mg F/L
Aeration	None	None	None
Number of replicates	3	3	3
Number of mussels per replicate	100	10	15
Test water	EPA moderately hard reconstituted water	EPA moderately hard reconstituted water	EPA moderately hard reconstituted water

Acute toxicity tests with the juveniles were conducted as described in Keller and Ruessler (1997). Test solutions were prepared by dilution of a stock, measured with the fluoride probe to determine concentrations, and distributed to 60x15 mm diameter glass petri dishes that were used as test chambers. The dishes were then covered with their glass lids. Dilution water consisted of USEPA (1994) moderately hard reconstituted freshwater at a hardness of 84 mg/L as $CaCO_3$. For some toxicity tests, dilution water hardness was reduced to 28-68 mg/L as $CaCO_3$ by addition of deionized water to better match conditions in streams containing the Appalachian elktoe. Tests were conducted for 96 hr, with daily determinations of juvenile survival, in static conditions with no feeding. LC_{50}s were calculated for all toxicity tests using Trimmed Spearman-Karber.

Nine-day (216 hr) tests were conducted using the same methods as the acute tests, except that the mussels were fed algae to promote growth. At the end of nine days, remaining live *A. raveneliana* mussels were placed in vials containing 90% ethanol preservative until their shell lengths could be measured. Shell length (anterior to posterior) was measured to the nearest hundredth millimeter using a microscope and ocular micrometer at 100X. These data were then analyzed via ANOVA and Duncan's test to determine if there were significant differences between the control and test concentrations. Fluoride concentrations were measured with an Orion ion selective probe #940900 having a detection limit of 0.02 mg/L at pH 5-7, and calibrated against a fluoride standard. Initial

measurements were also validated by separate analysis using USEPA Method 300, an ion chromatography method. Data are presented as the concentration of the F ion (mg F/L).

RESULTS AND DISCUSSION

Both species of sculpin produced juvenile *A. raveneliana*. This is significant given the fact that *C. bairdii* was not previously documented as a fish host. The *A. raveneliana* recovery plan (USFWS 1996) indicated that there was a need to identify additional host fish species. Our documentation of *C. bairdii* as a host, a fish that is sympatric with *A. raveneliana*, helps fulfill that stated need.

After four weeks of incubation, eighteen juvenile *A. raveneliana* were collected from the tanks containing approximately ten *C. carolinae* at 16°C. The tanks were siphoned for juveniles weekly thereafter, however no more juveniles were found for another month, when 186 were collected from the tank containing ten *C. bairdii*. Gordon and Moorman (2001) collected juveniles after 19 days' incubation. Since they did not report their culture temperature, it is possible that Gordon and Moorman (2001) held their fish at a higher temperature (>16°C), which decreased the incubation period. A similar incubation period (~6 wk) was required to produce juveniles cultured in our first attempt. At that time, 100 juvenile *A. raveneliana* were collected from the tank containing *C. carolinae* five weeks after glochidia were placed on the sculpin. The *C. bairdii* died before any juveniles were produced. *Micropterus salmoides* and *M. dolomieu* were also infected with *A. raveneliana* glochidia, but they did not produce any juveniles.

Twenty-four hr glochidia toxicity tests performed with *A. raveneliana* and *U. imbecillis* mussels at 25°C produced LC_{50}s of 288 mg/L F and 351 mg/L F, respectively (Table 2). The NOEC for *A. raveneliana* glochidia was 250 mg/L F based on ANOVA and Dunnetts test. Forty-eight hr results were not reliable because of high control mortality (>25%) in both mussel species. Four species of mussels were used in juvenile toxicity tests--*L. fasciola*, *A. raveneliana*, *A. pectorosa*, and *U. imbecillis*. Ninety-six hr LC_{50}s for fluoride ranged from 172 mg/L F for *L. fasciola* to 347 mg/L F for *A. pectorosa*, in water with hardness ranging from 28 to 84 mg/L as $CaCO_3$ (Table 2). The NOEC for juvenile *A. raveneliana* mussels was 250 mg/L F, based on ANOVA and Dunnetts test. These values are similar to LC_{50}s (96- to 240-hr) for several species of fish, e.g., 124 mg/L F for brown trout, *Salmo trutta* (Wright 1977) and 315 mg/L F for fathead minnow, *Pimephales promelas* (Smith et al. 1985), and for adult *Indonaia caeruleus* mussels in 96-hr tests, LC_{50}=247 mg/L F (Muley 1990).

Two juvenile tests were conducted for nine days (216 hr) resulting in little decrease in LC_{50}s, i.e., *A. raveneliana*, LC_{50} of 223 mg/L F and *L. fasciola*, LC_{50} of 177 mg/L F, compared to those determined at 96 hr (Table 1). However, based on an ANOVA and Duncan's test, there were significant differences ($p \leq 0.05$) in growth (based on shell lengths) between controls and juvenile mussels exposed to fluoride (Table 3). Control mussels had a mean shell length ($\pm$ s.d.) of 0.362±

Table 2. Twenty-four hour to nine-day toxicity of fluoride to glochidia and juvenile mussels of several species at different water hardnesses.

Mussel Species	Life Stage[a]	Test Duration (h)	Hardness (mg/L as $CaCO_3$)	LC_{50} (mg F/L)	Lower 95% Confidence Limit (mg F/L)	Upper 95% Confidence Limit (mg F/L)
Alasmidonta raveneliana	j	96	28	303	287	320
A. raveneliana	j	168	28	240	213	271
A. raveneliana	j	216	28	223	195	255
A. raveneliana	g	24	30	288	278	299
Actinonaias pectorosa	j	96	84	298	266	334
A. pectorosa	j	96	68	347	n/c[b]	n/c
A. pectorosa	j	96	30	178	n/c	n/c
A. pectorosa	j	96	28	259	233	288
Utterbackia imbecillis	j	96	34	234	206	265
U. imbecillis	g	24	30	351	349	354
Lampsilis fasciola	j	96	32	172	164	180
L. fasciola	j	168	32	172	163	181
L. fasciola	j	216	32	177	n/c	n/c

[a]g=glochidia, j=juvenile [b]n/c=not calculable

0.019 mm, while mussels exposed to fluoride had mean lengths of 0.348±0.027 mm, 0.333±0.022 mm and 0.318±0.021 mm, for 31, 62 and 124 mg/L F, respectively. Using these results, the acute to chronic ratio (96 hr LC_{50} divided by the geometric mean of the chronic No Observed Adverse Effect Concentration [NOAEC] and Lowest Observed Adverse Effect Concentration [LOAEC], USEPA (1985) for F in these mussels is at least 10. Note that no NOAEC other than zero was defined.

These results suggest that glochidia and juvenile mussels are similarly sensitive to acute exposures to fluoride and that hardness has little or no effect on its toxicity. Acute toxicity occurred at concentrations that are two orders of magnitude greater than the North Carolina water quality standard of 1.8 mg F/L (NCDENR 2003). The North Toe River receives five point sources of mining waste containing fluoride. The maximum concentration of fluoride reported in these undiluted effluents is 70 mg F/L, less than half the 96 hr to 216 h LC_{50} determined for unionid mussels in the present study. Median instream concentrations of

Table 3. Shell lengths (anterior to posterior) for juvenile *Alasmidonta raveneliana* exposed to fluoride in soft water for nine days.

Fluoride Concentration (mg F/L)	Replicate	n	Mean (±stdev.) Shell Length by Replicate (mm)	Mean (±stdev.) Shell Length by Treatment (mm)
0	1	15	0.3663 ±0.0209	0.362 ±0.019
	2	15	0.3613 ±0.0187	
	3	14	0.3560 ±0.0150	
31	1	12	0.3429 ±0.0253	0.348 ±0.027*
	2	15	0.3522 ±0.0250	
	3	14	0.3558 ±0.0117	
62	1	14	0.3360 ±0.0230	0.333 ±0.022*
	2	10	0.3299 ±0.0249	
	3	12	0.3329 ±0.0214	
124	2	15	0.3251 ±0.0205	0.318 ±0.021*
	3	15	0.3118 ±0.0914	

*denotes a treatment that is significantly different from the control ($p \leq 0.05$)

fluoride (after mixing with receiving waters) downstream of the discharge pipes of these five facilities are typically less than the State standard. While maximum concentrations ranged from about 1.5 to 8.0 mg F/L over the past two years, exceeding the standards periodically, acute fluoride toxicity to mussels is unlikely.

Sublethal effects occurred at concentrations as low as one-tenth of the 96 hr LC_{50}s, and one-fifth of the 9-d LC_{50}s. Few studies have evaluated growth impacts in juvenile unionids resulting from short-term exposure to pollutants. The success of the current study is therefore of particular value. Results of the 9-day tests indicated that subchronic exposures of juvenile mussels to fluoride can impair their growth at 31 mg F/L, but this concentration is about 17 times that permitted by the State of North Carolina. Facilities are generally meeting this standard; the ambient concentration of fluoride in the North Toe River at Penland, NC (which is within a mile downstream of four of the five mine discharges) ranged from <0.1 to 3.0 mg/L F with a median of 0.8 mg F/L over the past decade. These concentrations are significantly higher than those from the North Toe River ambient monitoring station near Ingalls, NC (upstream of all five facilities) which ranged from <0.1 to 2.0 mg/L F with a median of 0.2 mg F/L over the past decade, but they are still well below the sublethal effects concentrations determined in this study. Because growth impacts were measurable after such a short time in this study and mussels live for decades, it would be worthwhile to perform more lengthy exposures at lower concentrations. Smaller adults are

known to suffer greater predation effects and reduced reproductive success compared to those of normal size and this could have a negative impact on populations in the long term.

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