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FINAL REPORT

LABORATORY INVESTIGATION OF THE EFFECTS OF CONDUCTIVITY ON A SUITE OF STREAM ORGANISMS FOUND IN THE COAL MINING REGIONS OF THE UNITED STATES: AMPHIBIANS, BENTHIC MACROINVERTEBRATES, AND FISH.

Submitted to: Cecil Slaughter, Hydrologist
U.S. Office of Surface Mining and Reclamation and Enforcement

Submitted by: Dr. Mindy Yeager-Armstead
Associate Professor
Integrated Science and Technology
Marshall University
Huntington, WV

Dr. Sara Sweeten
Research Scientist
Conservation Management Institute
Virginia Polytechnic Institute and State University
Blacksburg, VA

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ABSTRACT

In order to remove the variability associated with field studies, laboratory toxicity testing was employed to demonstrate the potential link between elevated specific conductivity in mining-influenced streams and the impairment of native aquatic taxa. With standard toxicity testing methods and United States Environmental Protection Agency surrogate organisms failing to demonstrate toxicity at the level of conductivity implicated in field studies, this research sought more sensitive organisms and endpoints than the traditional toxicity testing. Sensitivity data were generated for eight native taxa including four amphibian and four fish species. Non-traditional endpoints (hatch rates) for the fathead minnow provide additional information on the effects of elevated conductivity discharges in mining influenced streams. Wood frogs were the most sensitive organisms with a No Observed Effect Concentration (NOEC) for growth impairment at 300 µS/cm in the 7-day testing; all test concentrations were significantly reduced with respect to the control. Wood frog survival was also affected by conductivity with a NOEC of 1,800 µS/cm. Differences were seen between life stages tested. Hatch rates of both fathead minnow and rainbow trout eggs tended to decrease at higher conductivities. Fathead minnow larvae and rainbow trout larvae were less sensitive to conductivity than eggs. Fathead minnow hatch rates were slightly more tolerant of conductivity than rainbow trout. In the fantail darter, Jefferson salamander, brook trout and rainbow trout larval testing, survival, not growth, were the most sensitive endpoints. This could be that differences in organism weights among concentrations were too minimal to be an effective sub-lethal endpoint.

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ACRONYMS

Acronym	Definition
LOEC	Lowest Observed Effect Concentration
NOEC	No Observed Effect Concentration
IC25	Inhibition Concentration 25%
EPA	Environmental Protection Agency
MS-222	Tricaine mesylate
C	Celsius
L	Liter
mg	Milligram
cm	Centimeter
h	Hour
µS/cm	Micro Siemens per centimeter
WET	Whole Effluent Testing
MSD	Minimum significant difference
PMSD	Percent minimum significant difference

INTRODUCTION

For many years the link between land disturbance and conductivity has been documented (Dow and Zampella 2000), particularly in relation to surface coal mining (Lindberg et al. 2011). However, it hasn't been until recently that researchers have suggested a direct causal link between conductivity and biological stream health (US EPA 2011). Multiple field studies have shown declines in macroinvertebrate assemblages in streams with elevated conductivity due to large scale land use such as surface coal mining (e.g. Pond et al. 2008, Cormier et al. 2013, Timpano et al. 2015). Additionally, several studies have found correlations between conductivity and fish communities (Freund and Petty 2007, Hitt and Chambers 2014, Hitt et al. 2016). However, none of these studies have fully accounted for all other potential variables associated with large scale disturbances that might cause biological declines. Without taking into account these factors, it is difficult to determine a causal link between conductivity and biological impairment. For example, Pond et al. (2008) stated, "elevated specific conductance might simply be an indicator of mining disturbance, and other mining-related variables (e.g., metal concentrations) might be causing or contributing to the impairment".

Surface coal mining can alter stream hydrology, physical stream habitat, and water chemistry. All of these factors can have both acute and chronic effects on specific aquatic biota in mining-influenced streams and can lead to the stream becoming biologically impaired (Northingham et al. 2011, Pond 2012). Additionally, these factors can have additive effects. For example, Cook et al. (2015) found that benthic macroinvertebrates were more sensitive to water quality degradation when physical stream habitat was impaired. Distinguishing which factors (i.e. stream habitat, conductivity, suspended sediment, heavy metals) are directly affecting stream biota can be difficult. Because of the complicated natural of determining causation through field

studies, it is perhaps best to begin to examine a potential causal link between conductivity and stream biota in a laboratory setting.

Limited laboratory work has examined the effects of conductivity on freshwater organisms, and much of this work has focused on surrogate organisms such as water fleas (*Ceriodaphnia dubia*), water scuds (*Hyalella azteca*), fathead minnows (*Pimephales promelas*), and fingernail clams (*Sphaerium simile*) (Kennedy et al. 2005, Soucek and Kennedy 2005, Elphick et al. 2011, Soucek et al. 2011). Overall, the results from this research showed variable responses depending on the ionic composition of the conductivity and the study organism. Surrogate organisms do not always accurately represent native stream life and does not adequately demonstrate impairment at the level of that determined in field studies. Only a few studies have begun to use native stream biota to examine the effects of conductivity (Armstead et al. 2013, Kunz et al. 2013, Ciparis et al. 2015). One recent study by Kunz et al. (2013) demonstrated levels of conductivity from simulated mining discharges influencing freshwater invertebrates, including one representative mayfly taxa. This study had control conditions for the test organisms at 275 microsiemens per centimeter (µS/cm) which is very close to the target field threshold of 300 µS/cm clearly demonstrating what the authors point out, that our general understanding of total dissolved solids toxicity is currently inadequate (Kunz et al. 2013).

There is a critical need to better understand the effect of conductivity on stream organisms native to the coal mining regions of the United States. Our research has attempted to provide better insight on how conductivity influences a variety of stream organisms in a laboratory setting. We have utilized native taxa, and surrogate taxa with traditional and non-traditional endpoints, to seek, through the most sensitive responses, the levels of conductivity expected to cause impairment in stream organisms. Specific objectives include:

1. Examining the effects of mining-related conductivity on multiple stream taxa native to the Appalachian mining region.

2. Comparing the sensitivity of different life-stages of native organisms to mining-related conductivity.

METHODS

At the Virginia Tech facility, toxicity testing was conducted utilizing wood frogs, Jefferson salamanders, spotted salamanders, American toads, and fantail darters. At Marshall University the sensitive life stages of brook trout, rainbow trout, fathead minnows, and mayfly taxa were exposed to the simulated mine effluent. Testing included multiple life stages, including sensitive egg and larval stages with lethal and sub-lethal endpoints.

Reconstituted Stream Water

Toxicity testing generally followed the US EPA's "Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms" (US EPA 2002b). A reconstituted stream water indicative of streams in the surface coal-mining region receiving alkaline discharges has been developed and utilized previously in mesocosm studies (Armstead et al. 2013). This reconstituted water was developed using the ionic ratios and concentrations of major ions found in mining influenced streams (Figure 1). Conductivities in the dilution series tested ranged from approximately 2,400 µS/cm to 100 µS/cm and included both US EPA reconstituted moderately hard water and site waters for controls as noted. The six testing concentrations are listed in Table 1.

Virginia Tech Toxicological Testing Methods

The testing at Virginia Tech was done in the Department of Fish and Wildlife Conservation's Aquaculture Center in Blacksburg, Virginia. Four replicates were used for each of the six test

concentrations for a total of 24 units. For each unit, 10 liters of test water was placed in a 5-gallon bucket. Each bucket was randomly placed in one of four 50-gallon water baths for a total of six buckets per water bath (Figure 2 and Figure 3). Each bucket had air-line for aeration during the test. Water baths were not heated during the testing. Each test ran for seven days. At the end of the test, all living animals were counted. To quantify growth, organisms were euthanized using MS-222, dried in a drying oven, and weighed. Virginia Tech ran toxicity tests on spotted salamander larvae (*Ambystoma maculatum*), Jefferson salamander larvae (*Ambystoma jeffersonianum*), wood frog tadpoles (*Lithobates sylvaticus*), American toad tadpoles (*Anaxyrus americanus*), and fantail darter larvae (*Etheostoma flabellare*).

Marshall University Toxicological Testing Methods

Toxicity testing at Marshall University was conducted in laboratory space in the Weisberg Applied Engineering Complex and included chronic toxicity testing on fathead minnows (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), and brook trout (*Salvelinus fontinalis*). Three Ephemeroptera taxa were utilized in acute toxicity testing; the giant mayfly, *Hexagenia limbata* (Ephemeridae), the minnow mayfly *Acentrella sp.* (Baetidae), and *Epeorus sp.* (Heptageniidae).

The toxicity of the simulated mining discharge was evaluated using the US EPA's standard toxicity test organism, the fathead minnow. To evaluate the effects of low-level chronic exposure to elevated conductivity and to reduce the influence of osmoregulatory change on embryos and larvae utilized in testing, hatch rates of eggs laid in high conductivity discharge was analyzed. Ten-gallon tanks were filled with the six aforementioned concentrations of simulated mining influenced stream water with four females and one male in each breeding tank (Figure 4). Water temperature was set at 21 ±3 degree Celsius (ºC) and placed on a 16 hour day/8 hour night light schedule, with "daylight" receiving 250 ±10 lux. Eggs were removed from the breeding

tanks each day and moved to 2-liter (L) aerated beakers with test concentration conductivity maintained in the incubation tanks. Eggs laid and eggs hatched were counted daily. US EPA's Short-term Methods (EPA 2002a) for conducting embryo/larval evaluations provided the basis for study design, however, methods were varied for the experiment with spawning, fertilization and development actually taking place in the reconstituted high conductivity stream water to reduce osmoregulatory stress during introduction of organisms to the test solutions. Endpoints evaluated include egg survival or percent hatch. Fathead minnow larval toxicity testing methods also followed the US EPA's standard methods. Less than 24-hour old larvae were placed in 6-well cell culture plates for exposure to the dilution series. Ten larvae were placed in each well with 4 replicates of each concentration. The well plates were stored under 250 ± 10 lux florescent lighting with a 16 hour light/8 hour dark photoperiod. Organisms were fed *Artemia sp*. daily per the methods and water was changed daily.

Trout species testing also followed US EPA methods with the following variation to accommodate the coldwater species: lower temperature, decreased light, increased test chamber size, and use of a recirculating system so the water flowed over the eggs for aeration. Testing was conducted in a 13±3 °C recirculating bath which housed the 6 concentrations with 4 replicates in each effluent concentration (Figure 5). Fifteen, 2-day post fertilization, eggs were placed in chambers and were evaluated daily for fungus, dissipation, and hatch. The eggs were housed in covered, recirculating glass containers in dark conditions that only received 20 ± 10 lux for approximately 20 hours a day via ambient light from a window. Once hatched, larvae were preserved for teratogenic evaluations. Similar methods adjustments were implemented for the coldwater toxicity testing. Ten 24-48-hour old larvae were placed in 2-L beakers with 6 concentrations and 4 replicates of each high conductivity concentration. Test chambers were

stored in a cool water bath (13±3°C) and test water was renewed every 24 hours. The beakers were stored under 250 ± 10 lux lighting with a 16 hour light/8 hour dark photoperiod. Modifications to the US EPA's standard methods were consistent with rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) 7-Day Survival and Growth Test Method developed by Lazorchak and Smith (2007).

Statistical Analysis

Statistical analysis of the toxicity testing was conducted in accordance with the aforementioned methods using the US EPA's WET Analysis Spreadsheet V1.6.1 and are presented herein. The appropriate statistical procedure to apply to each dataset is selected on the individual datasets ability to meet necessary assumptions for different statistical tests. This is why similar endpoints may be described by different statistical tests. Graphs representing the endpoints depict the average condition (mortality, weight, reproductive output) of the replicates for each concentration shown as the group mean. A dose response (i.e. less survival, growth or reproduction at higher concentrations) is frequently seen in toxicity testing and a necessary statistical assumption for calculation of endpoints determined from the Linear Interpolation Method. When the dose response is not maintained a statistical smoothing process is initiated to assure the integrity of the interpolation methods. The outcome of this process is presented on the graphs as the "smooth mean". Significant statistical differences can be determined by selected probability factors, such as 0.05 or 0.01, or by distance from the control condition. While the former are dependent on normality, the latter are determined by the minimum significant difference (MSD) or the percent minimum significant difference (PMSD) from the control which indicates statistical significance. This number is determined based on control variability and is considered the "trigger" for where statistically significant impairment is demonstrated. Specific methods for each species are described below.

Test Species

Spotted Salamanders (*Ambystoma maculatum*)

Eight adult spotted salamanders (5 males, 3 females) were caught in pitfall traps in Price's Fork Park, Price's Fork, Virginia on February 28, 2017. The salamanders were brought back to the lab where they were all placed in a single container with about 10 cm of water with a substrate of fist-sized rocks and gravel (Figure 6 and Figure 7). On March 2, 2017, approximately 12 spermatophores were in the bottom of the container. Between March 3 and 7, eight small egg masses were produced. Eggs were kept in a 10-gallon aquarium with an aerator. On March 22, the eggs began to hatch. Toxicology tests were ran March 27-April 3 (seven days) using 10 spotted salamander larvae per bucket with four replicates of the six concentrations of sulfate-dominated synthetic mine effluent.

Jefferson Salamanders (*Ambystoma jeffersonianum*)

Three large Jefferson salamander egg masses were collected from an ephemeral pool at the Price's Fork Park, Price's Fork, Virginia on March 5, 2017 (Figure 8). Eggs began to hatch on March 18, 2017. A seven-day toxicity test was ran March 21-28, 2017 using ten larvae per bucket with four replicates of the six concentrations of sulfate-dominated synthetic mine effluent. All larvae were less than 24-hours old at the start of the test.

American Toads (*Anaxyrus americanus*)

American toad eggs were collected from Pandapas Pond, Virginia on April 14, 2017 (Figure 9). Eggs began hatching on April 16, 2017. A seven-day toxicity test was ran April 17-24, 2017. Forty tadpoles were used in each bucket with four replicates of the six concentrations of sulfate-dominated synthetic mine effluent. All tadpoles used were less than 24-hours old.

Wood Frogs (*Lithobates sylvaticus*)

Wood frog egg masses were collected from a small ephemeral pool at the Price's Fork Park, Price's Fork, Virginia on February 27, 2018 (Figure 10). Eggs began hatching March 4, 2018

and a seven-day toxicity test began on March 6. Forty tadpoles, less than 24-hours old, were used in each bucket with four replicates of the six concentrations of sulfate-dominated synthetic mine effluent.

Fantail Darters (*Etheostoma flabellare*)
Fantail darter eggs were collected from Tom's Creek, Montgomery County, Virginia on May 4, 2017. Eggs began to hatch on May 5, and a seven-day toxicity test was ran May 6-13, 2017. Eighteen larval fish (<24-hours old) were used in each bucket with four replicates of the six concentrations of sulfate-dominated synthetic mine effluent.

Fathead Minnows (Pimephales promelas)
Breeding-age fathead minnows were obtained from Aquatic Bio Systems, Inc. Fathead minnow testing was conducted in February and March, 2017. Replicate testing was conducted in June 2018.

Brook Trout (*Salvelinus fontinalis*)
Brook trout eggs were obtained from Paint Bank Hatchery, Paint Bank, VA. The brook trout larval toxicity test was initiated November 3, 2017 when the larvae were 10-14 days old.

Rainbow Trout (*Oncorhynchus mykiss*)
Rainbow trout eggs were purchased from Cold Springs Trout Farm in North Ogdon, Utah. On April 7, 2017, the rainbow trout embryo testing commenced (Figure 11). Larval tests were initiated on June 13, 2017 using larvae which were less than 24 hours old.

Attempted Species--Mountain redbelly dace (*Chrosomus oreas),* Two-lined salamander (*Eurycea bislineata),* Dusky salamander (*Desmognathus* fuscus), giant mayfly (*Hexagenia limbata),* and Spring Peepers (*Pseudacris crucifer*).
We also attempted to breed mountain redbelly dace (*Chrosomus oreas)* in the laboratory, but were largely unsuccessful. We only had one mountain redbelly larvae produced in 2017 and 15 larvae in 2018, not enough to run a toxicity test. We were unable to produce any two-lined

salamander (*Eurycea bislineata*) eggs in the lab. We also could not find enough two-lined salamander eggs in the field to run a test.

We attempted to breed spring peepers *(Pseudacris crucifer)* in the lab with some success using Luteinizing Hormone-Releasing Hormone analog (Figure 12 and Figure 13). However, we only had one gravid female, and she did not produce enough eggs to run a toxicology test.

In August 2017, we had one Dusky salamander (*Desmognathus* fuscus) lay a clutch of approximately 20 eggs in the lab. To our knowledge, producing Dusky salamander eggs in captivity using hormone injections has never been done before. Eggs were moved to a development chamber and were closely monitored (Figures 14-18). In the beginning of October, the eggs hatched over a 4-day period. Eleven of the approximate 20 eggs successfully hatched (Figure 19). However, in the first 14 days we had 100% mortality of the Dusky salamander larvae.

Hatch rate testing was attempted using the giant mayfly, *Hexagenia limbata*. Adult mayflies were collected from the Kanawha River, in Kanawha County, West Virginia and placed in breeding chambers overnight to facilitate fertilization. We collected eggs from the adult females via dissection or oviposition. Each individual egg clutch was divided and placed into test concentrations and incubated at 20°C until hatched; water changes were conducted every other day. Kanawha River water was used as a control to demonstrate the differences of egg/larval development between native water and US EPA's reconstituted moderately hard synthetic water. Because hatch rates were very low and because all test concentrations (in reconstituted water) were significantly different from the natural water control, these tests are being replicated; thus data for the giant mayfly were not included in this publication.

RESULTS

Amphibians and Fish

The seven-day test on wood frog tadpole survival showed a significant decrease in survival with a NOEC of 1,800 µS/cm of conductivity and a LOEC of 2,400 µS/cm (Figure 20). Additionally, wood frog tadpole growth exhibited a significant decrease with a NOEC of 300 µS/cm of conductivity and a lowest observed effects concentration (LOEC) of 600 µS/cm (Figure 21; Table 2).

Results for the 7-day conductivity toxicology test ran on Jefferson salamander larvae showed a significant decrease in survival with a NOEC of 1,800 µS/cm of conductivity and a LOEC of 2,400 µS/cm (Figure 22). Growth in milligrams (mg) of Jefferson salamander larvae was not effected by increasing concentrations of conductivity with a NOEC of 2,400 µS/cm and a LOEC >2,400 µS/cm (Figure 23 and Table 2).

Fantail darter larvae showed a significant decrease in survival with a NOEC of 1,800 µS/cm of conductivity and a LOEC of 2,400 µS/cm (Figure 24). Fantail darter larval growth did not exhibit any effects with a NOEC of 2,400 µS/cm and a LOEC >2,400 µS/cm (Figure 25).

Fathead minnow reproductive and embryo toxicity testing found that no spawning occurred at the 100 µS/cm or 1,250 µS/cm concentrations (Figure 26). More eggs per clutch were laid in the 300 µS/cm concentration, and the most eggs hatched in this concentration as well. Hatch rates were lower at the 1,800 µS/cm and 2,400 µS/cm concentrations (Figure 26). Fathead minnow larvae showed no effects on survival or growth from the conductivity concentrations used in this study. Fathead minnow larvae had a NOEC of 2,400 µS/cm and a LOEC >2,400 µS/cm (Figure 27 and Figure 28; Table 2).

Results show the brook trout larvae 7-day survival having a NOEC of 600 µS/cm and a LOEC of 1,200 µS/cm, while their 7-day growth showed no statistical difference between the control and the effluent concentrations (NOEC = 2,400 µS/cm and LOEC > 2,400 µS/cm; Figure 29 and Figure 30; Table 2). Due to brook trout spawning season ending early in the hatchery, we were unable to conduct additional tests.

We examined Rainbow trout at several life stages. Embryo survival (or hatching success) had significant decreases with a NOEC of 600 µS/cm and a LOEC of 1,200 µS/cm (Figure 31; Table 2).

After hatching, two evaluators examined the larvae for teratogenic deformities which include spinal, cranio-facial, and fin deformities, as well as yolk sac edema anomalies. Any discrepancies between the two evaluators were re-examined by both parties together to come to a consensus. Overall the percentage of deformities and the percentage of spinal deformities was lowest at the 300 µS/cm concentrations (Figure 32).

Rainbow trout larvae less than 24 hours old at the start of the test had decreased survival with a NOEC of 1,200 µS/cm and LOEC of 1,800 µS/cm (Figure 33). Growth of rainbow trout larvae less than 24 hours old showed no differences among concentrations with a NOEC of 2,400 µS/cm and LOEC > 2,400 µS/cm (Figure 34; Table 2).

Spotted salamander larvae and American toad tadpoles showed no effects on survival or growth from the conductivity concentrations used in this study. Both of these species had a NOEC of 2,400 µS/cm and a LOEC > 2,400 µS/cm (Figures 35-38; Table 2)

CONCLUSIONS

Methods developed for rearing the non-traditional organisms were largely successful with field collected and laboratory fertilized eggs harvested from four amphibian and four fish species. Endpoints evaluated included survival in each test species, and growth (weight) and deformity in the larval and embryo/larval tests, respectively.

Overall, eight species were exposed to the simulated mining effluent for evaluation of the effects of mining-related conductivity on stream taxa native to the Appalachian mining region. Wood frogs were the most sensitive organisms with an NOEC for growth impairment at 300 µS/cm in the 7-day testing; all test concentrations were significantly reduced with respect to the control. Wood frog survival was also affected by conductivity with a NOEC of 1,800 µS/cm. Results among the four amphibian species varied greatly from the wood frog's sensitivity to no effect of our synthetic mine effluent on spotted salamanders or American toads. Jefferson salamanders and spotted salamanders are very closely related (in the same Genus), yet the Jefferson salamander was more sensitive to conductivity. It is unclear as to why the wood frog was the most sensitive species or why there are differences between two closely related species. More research is needed to examine the effects of conductivity on other amphibian species to give a better understanding of the mechanisms at work.

Life stage of the organism seems to have an effect on the sensitivity of an organism to increased conductivity. Results of the sensitivity of egg/embryo and larval stages for fathead minnows and rainbow trout resulted in hatch rates of both fathead minnow and rainbow trout eggs that tended to decrease at higher conductivities. Fathead minnow hatch rates were slightly more tolerant of conductivity than rainbow trout. Fathead minnow larvae and rainbow trout larvae were less

sensitive to conductivity than fathead minnow eggs and rainbow trout eggs; thus, emphasizing the importance of conducting toxicology tests on multiple life stages to ensure impacts of conductivity, or other toxicants, are identified when laboratory testing is utilized to protect aquatic communities.

In the fantail darter, Jefferson salamander, brook trout and rainbow trout larval testing, survival, not growth, were the most sensitive endpoints. This could be that differences in organism weights among concentrations was too minimal to be an effective sub-lethal endpoint. More research into alternative sub-lethal endpoints, such as length, developmental stage (amphibians), or brain development, is needed.

There were several species for which toxicity testing was attempted but was not completed, including mountain redbelly dace, two line salamanders, spring peepers, dusky salamanders and three Ephemeroptera taxa.

LIST OF PRESENTATIONS/PUBLICATIONS/PROPOSALS

Presentations

1. "Laboratory investigation on the effects of conductivity on a sensitive early lifestages of fish from the Appalachian region" by Logan Beach, Kyle Tasker, Mindy Armstead, and Mandee Wilson presented at Society of Environmental Toxicology and Chemistry November 2017 in Minneapolis, MN.
2. Poster presentation entitled "Comparative Analysis of Toxicity of Simulated High Sulfate Mine Effluent to Sensitive Life Stages of Native Ephemeroptera Taxa" by Geneve Edwards, Daniel Brady, Mindy Yeager-Armstead, Ph.D., and Mandee Wilson, M.S. presented at Society of Environmental Toxicology and Chemistry November 2017 in Minneapolis, MN.
3. "Laboratory Investigation of the Effects of Conductivity on a Suite of Stream Organisms" by invited speaker Sara Sweeten, Ph.D. presented at Virginia Cooperative Fish & Wildlife Research Unit Annual Meeting, September 2018, Blacksburg, VA.

Thesis

Logan Beach, MS student in College of Information Technology and Engineering, Thesis supported by the OSM grant, Will be submitted in December 2018.

Proposals

Armstead, M.M. Co-Principal Investigator with S.E. Sweeten. Effects of elevated conductivity from sulfate and chloride dominated mixtures on aquatic organisms from coal mining regions of the United States with evaluation of cumulative impacts from multiple stressors. Proposal for the Mine Drainage Technology Initiative Program – Funding Opportunity Number S17AS00005 – $200,000. Submitted August 31, 2017. Unsuccessful

Sweeten, S.E. Co-Principal investigator with C. Thompson and P. Angermeier. Effects of sulfate-dominated mining effluent on brain development of wood frog tadpoles. Proposal for the Rural Environmental Health Program, Virginia Tech. $5,000. Awarded March 2018.

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TABLES

Table 1. Conductivity levels of toxicity test concentrations. (us/cm, microsiemens per centimeter).

Test Water	**Conductivity**
100% Mine Effluent	2,400 µS/cm
75% Mine Effluent	1,800 µS/cm
50% Mine Effluent	1,250 µS/cm
25% Mine Effluent	600 µS/cm
US EPA Moderately Hard Water	300 µS/cm
Diluted US EPA Moderately Hard Water	100 µS/cm

Table 2. Summary of survival endpoints (mortality) in the 7-day toxicity tests of native taxa exposed to reconstituted mining influenced stream water with elevated conductivity. (NOEC, No Observed Effect Concentration; LOEC Lowest Observed Effect Concentration). Endpoints are reported as specific conductance (µS/cm) of the test solution.

	Survival endpoint	
Species	**NOEC (µs/cm)**	**LOEC (µs/cm)**
Spotted Salamander	2,400	>2,400
Fantail darter	1,800	2,400
American toad	2,400	>2,400
Wood frogs	1,800	2,400
Jefferson salamander	1,800	2,400
Fathead minnow hatch	600	1,200
Fathead minnow larval	2,400	>2,400
Brook trout larval	600	1,200
Rainbow trout hatch*	<600	600
Rainbow trout larval <24hrs	1,200	1,800
*Daphna pulex***	1,800	2,400

*Dilute EPA water was also significantly different from moderately hard water
**Standard toxicity testing organisms/endpoints with data generated in previous studies (Armstead, unpublished data).

Table 3. Summary of non-lethal endpoints (endpoints other than mortality) evaluated in 7-day toxicity tests with native taxa exposed to reconstituted mining influenced stream water with elevated conductivity. (NOEC, No Observed Effect Concentration; LOEC Lowest Observed Effect Concentration; µs/cm, microsiemens per centimeter; mg, milligram). Endpoints are reported as specific conductance (µs/cm) of the test solution.

	Non-lethal test endpoint		**Specific non-lethal test endpoint measured for each test**
Species	**NOEC (µs/cm)**	**LOEC (µs/cm)**	**Endpoint Criteria**
Spotted Salamander	2,400	>2,400	Weight (mg)
Fantail darter	2,400	>2,400	Weight (mg)
American toad	2,400	>2,400	Weight (mg)
Wood frogs	300	600	Weight (mg)
Jefferson salamander	2,400	>2,400	Weight (mg)
Fathead minnow larval	2,400	>2,400	Weight (mg)
Brook trout larval	2,400	>2,400	Weight (mg)
Rainbow trout hatch*	1,250	1,800	% of Eggs that Hatched Successfully % of Larval Fish with Deformities
Rainbow trout larval <24hrs	2,400	>2,400	Weight (mg)
*Daphna pulex***	1,200	1,800	Reproduction Rate

*Dilute EPA water was also significantly different from moderately hard water
**Standard toxicity testing organisms/endpoints with data generated in previous studies (Armstead, unpublished data).

FIGURES

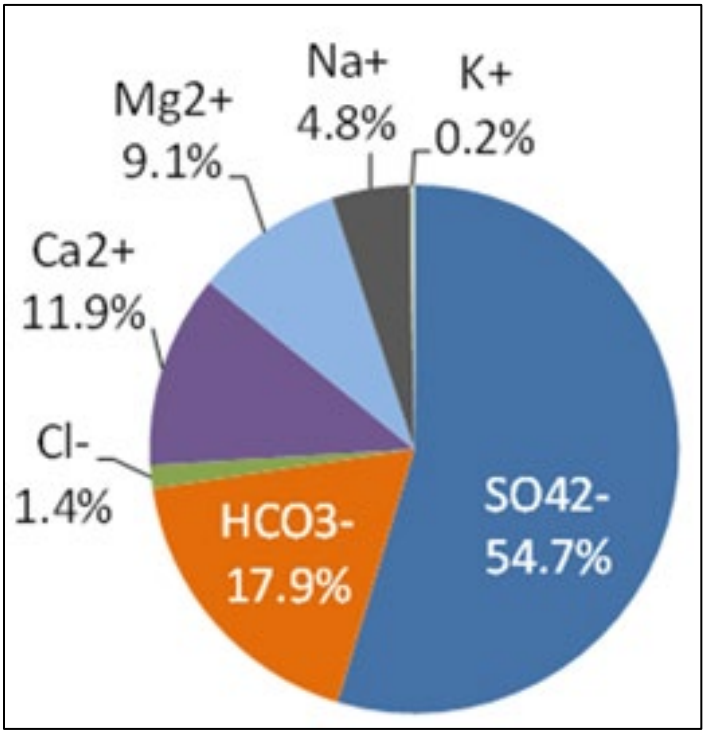

Figure 1. Ionic ratio of major ions for toxicity testing of stream taxa (Armstead et al. 2013).

Figure 2. Toxicological testing set-up at Virginia Tech. This picture shows eight 50-gallon water baths with six 5-gallon buckets in each water bath.

Figure 3. Each of the six randomly placed concentrations in one of the four water baths. Each bucket has 10 liters of test water and an airstone. This is a close-up picture of a water bath with six 5-gallon buckets filled with 10 liters of water.

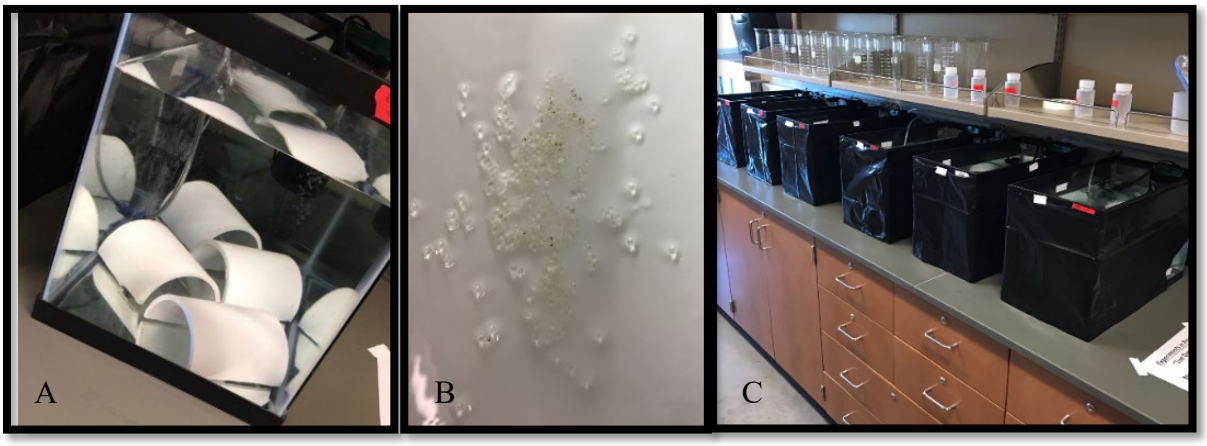

Figure 4. Fathead minnow breeding tanks (Panes A and C) and eggs laid on the inverted tile (Pane B).

Figure 5. Coldwater testing facilities. Test chambers for egg/embryo and larval tests were housed in coldwater baths to maintain test temperatures suitable for the coldwater species.

Figure 6. Adult spotted salamanders sitting on rocks in breeding chamber.

Figure 7. Adult spotted salamanders in the breeding chamber. This picture shows a 30 inch by 18 inch "under-the-bed" storage tub with approximately 4 inches of water, some gravel, some fist-sized rocks and four adult spotted salamanders.

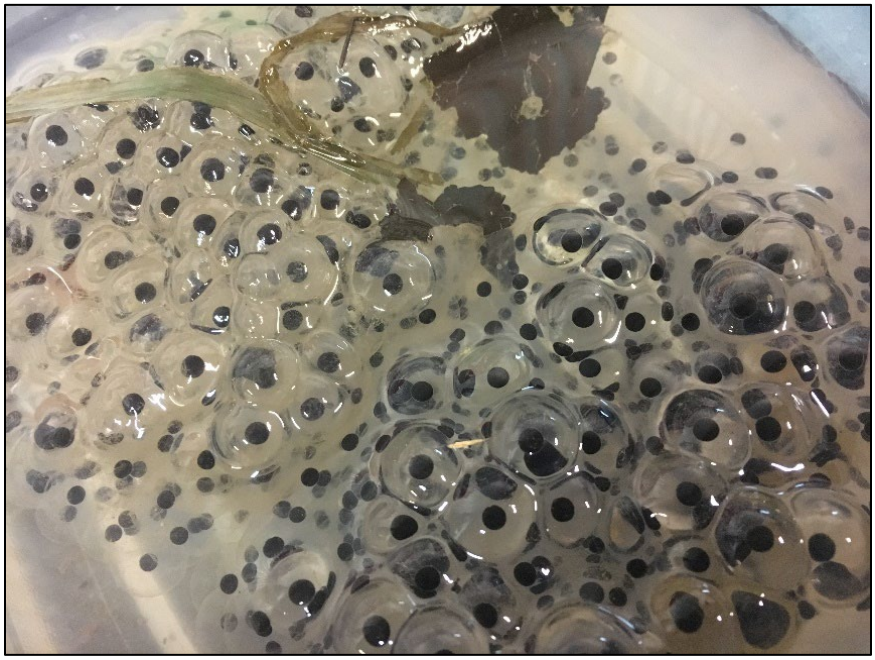

Figure 8. Jefferson salamander egg mass collected from Price's Fork, Virginia.

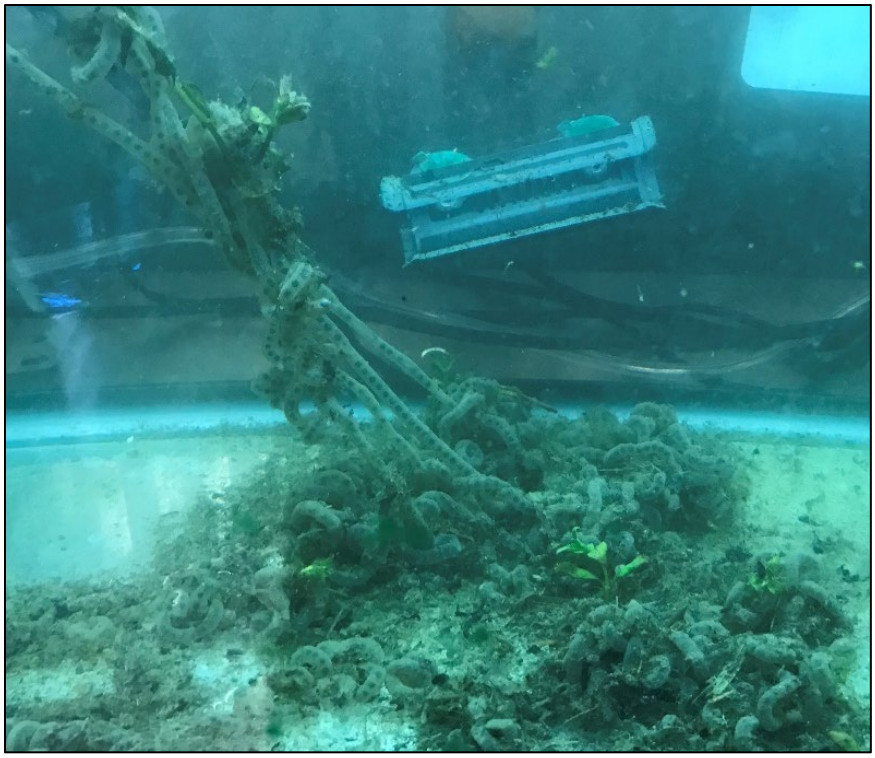

Figure 9. American toad eggs collected from Pandapas Pond, Virginia on April 14, 2017.

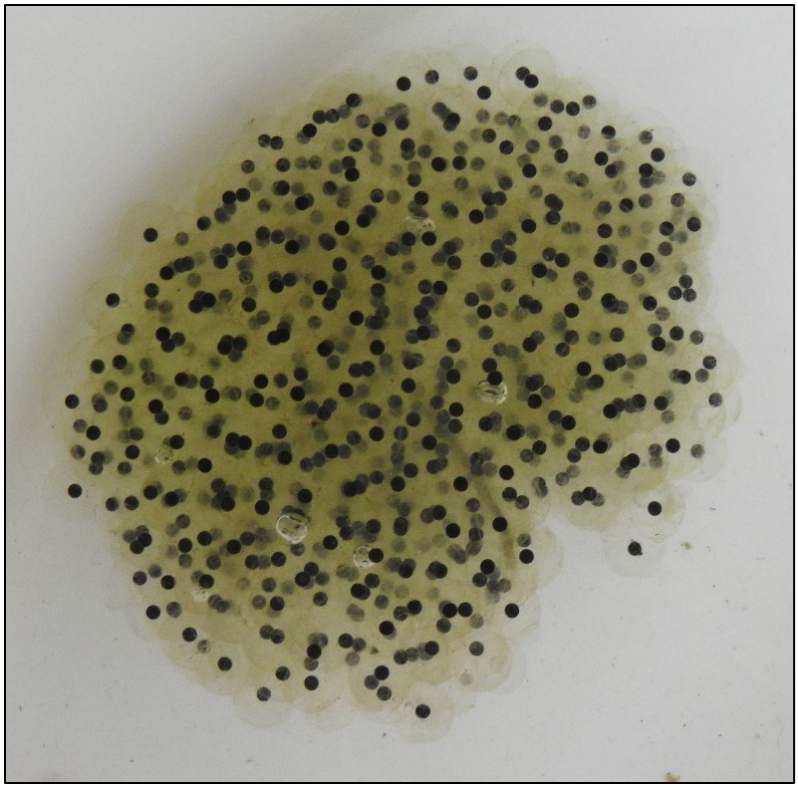

Figure 10. Wood frog eggs collected from Price's Fork, Virginia on February 27, 2018.

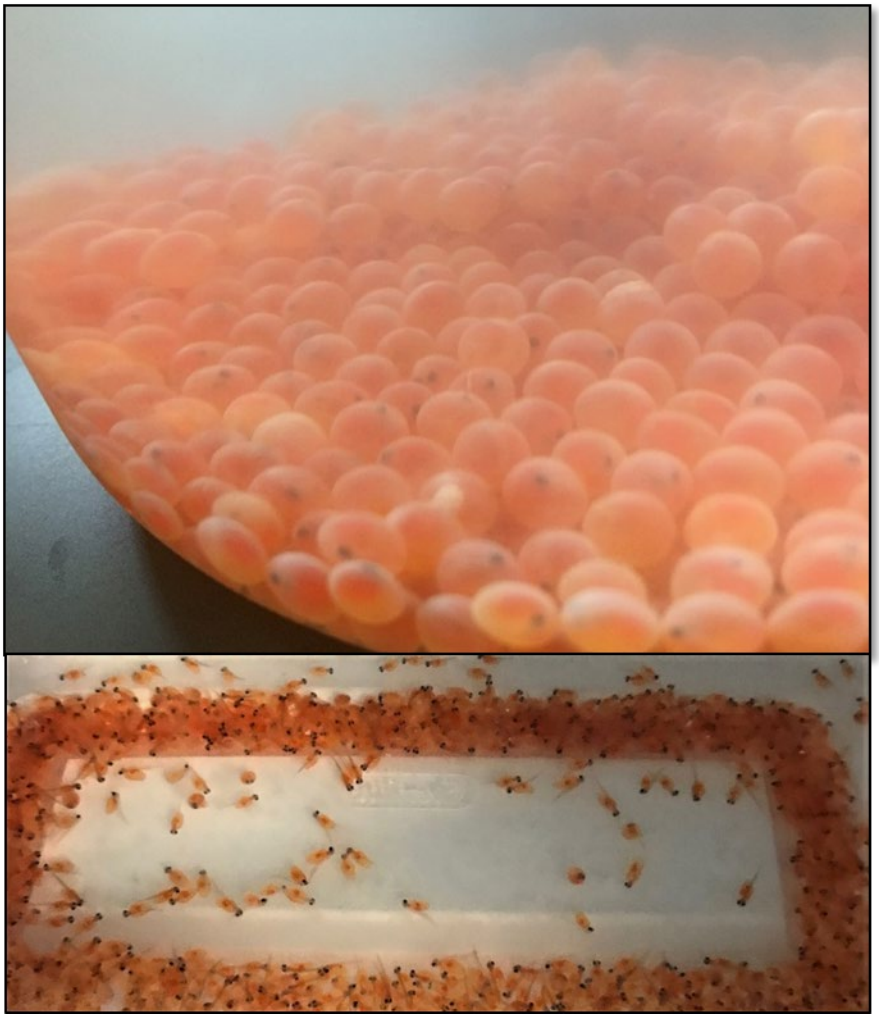

Figure 11. Rainbow trout eggs and hatching larvae used in toxicity testing.

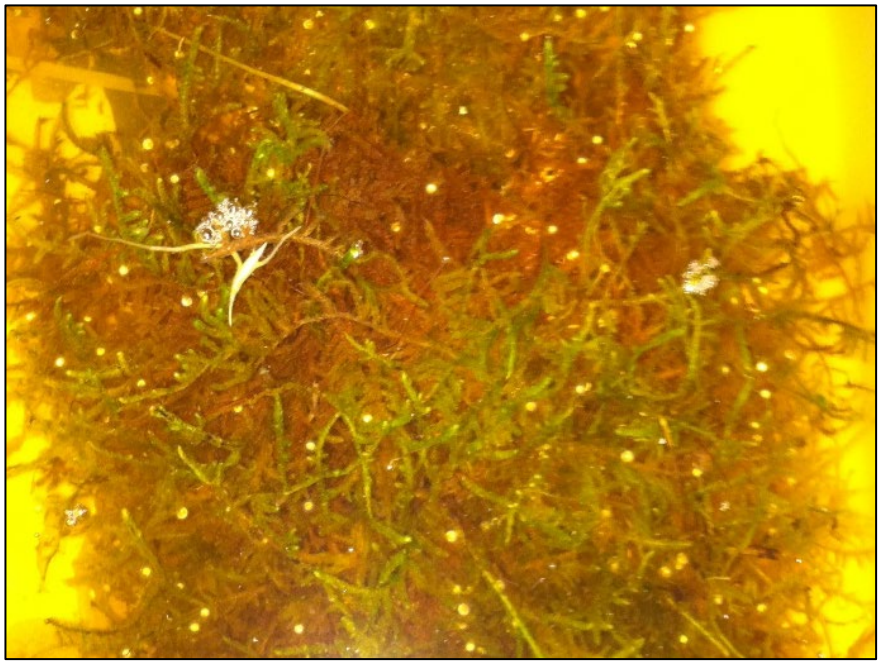

Figure 12. Spring peeper eggs laid in the lab on a clump of moss. This picture shows tiny white eggs on moss.

Figure 13. Single spring peeper egg. This is a picture of a single, tiny white spring peeper egg attached to a small piece of moss on the end of the researcher's thumb.

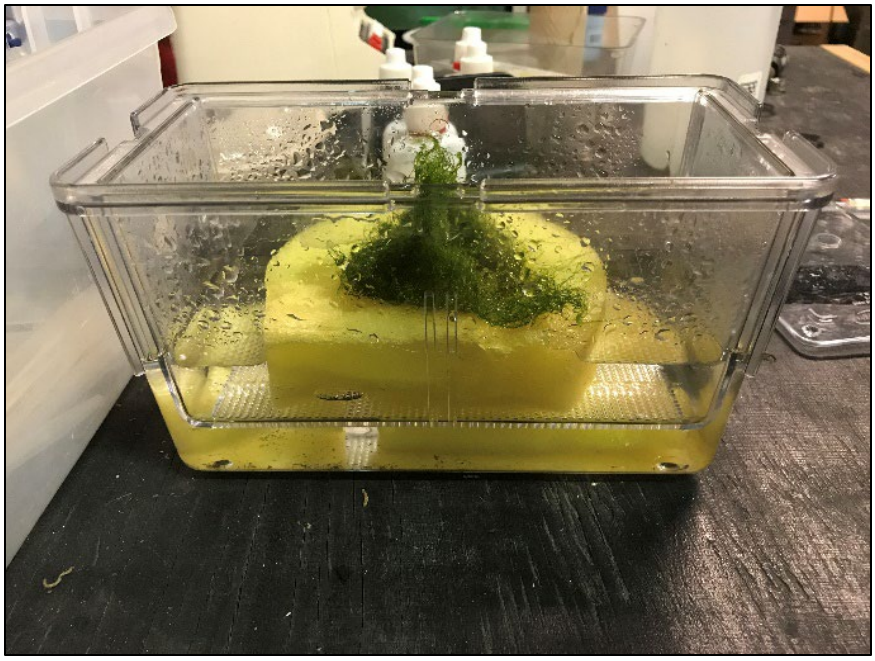

Figure 14. Dusky salamander egg chamber. Small clear plastic container with a large, yellow sponge and moss.

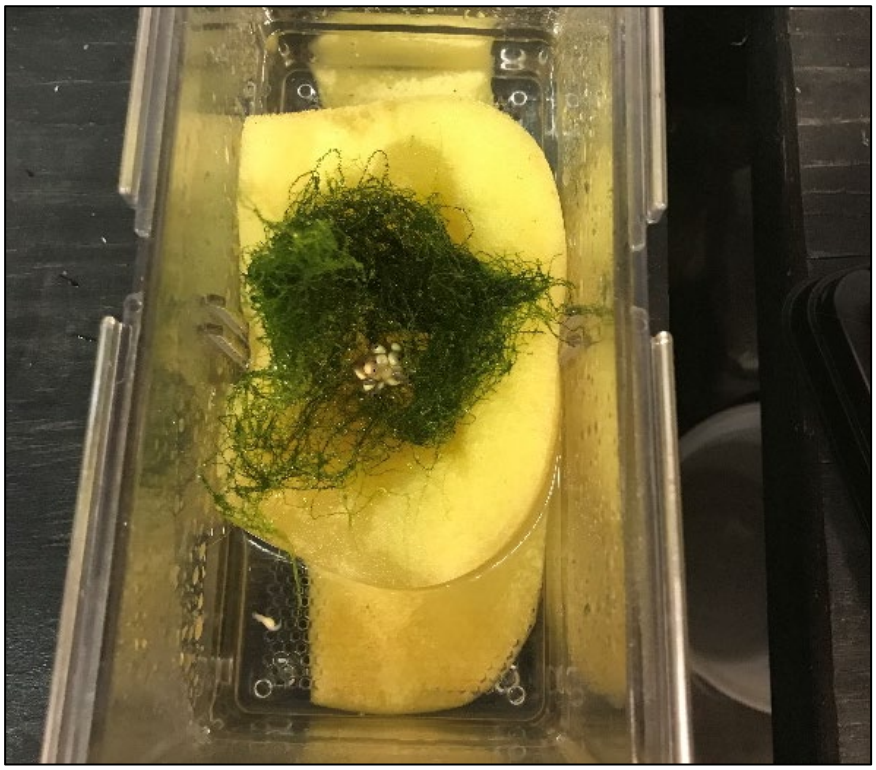

Figure 15. Dusky salamander egg chamber. A view of the egg chamber from the top with a small cluster of salamander eggs nestled in the moss.

Figure 16. Dusky salamander eggs Aug. 12, 2017. A small clump of white eggs sits on moss.

Figure 17. Dusky salamander eggs Sept. 5, 2017. The same small clump of eggs sits on moss. In this picture though, an eyespot is visible in each egg.

Figure 18. Dusky salamander eggs Sept. 22, 2017. The same small clump of eggs as in the previous two figures. A very pronounced eyespot is visible in each egg along with the salamander larvae body and egg sac.

Figure 19. Newly hatched Dusky salamander larvae on a plastic spoon, Sept. 27, 2017.

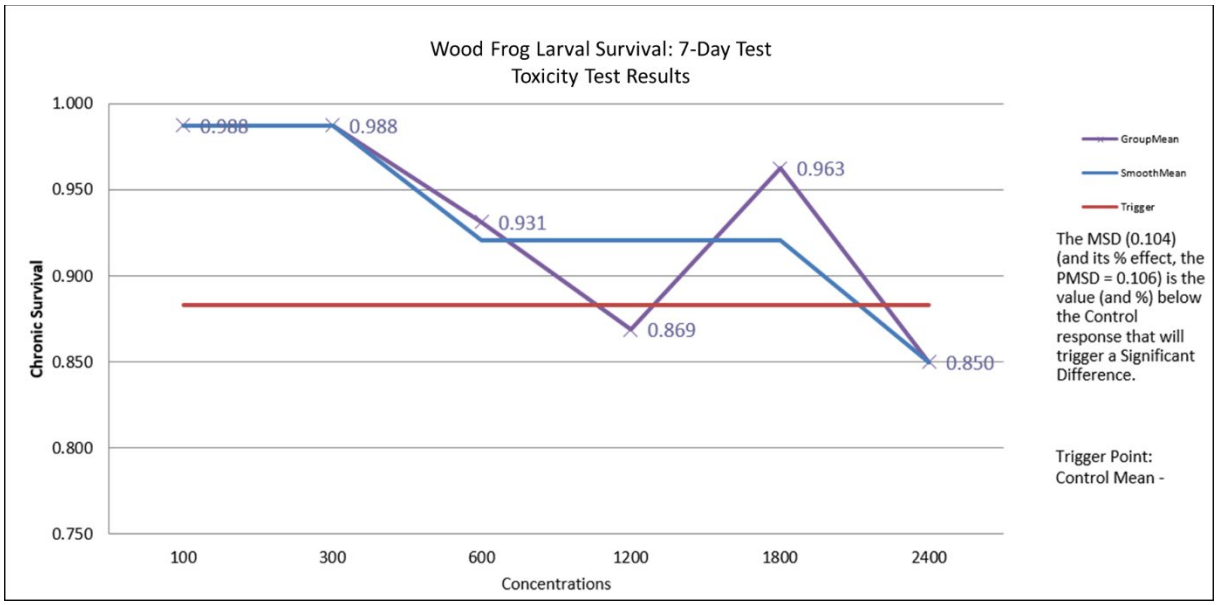

Figure 20. Wood frog larvae survival during a 7-day conductivity toxicity test (NOEC = 1,800 µS/cm; LOEC = 2,400 µS/cm).

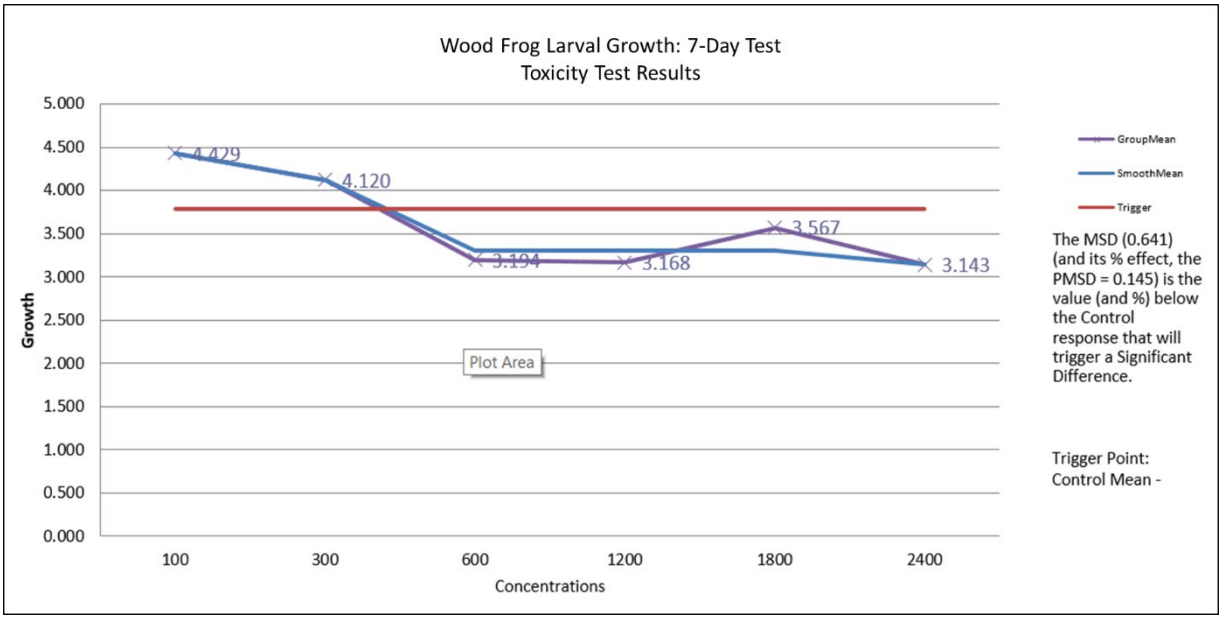

Figure 21. Wood frog larvae growth (in milligrams) during a 7-day conductivity toxicity test. Wood frog tadpole growth exhibited a significant decrease with a NOEC of 300 µS/cm and a LOEC of 600 µS/cm.

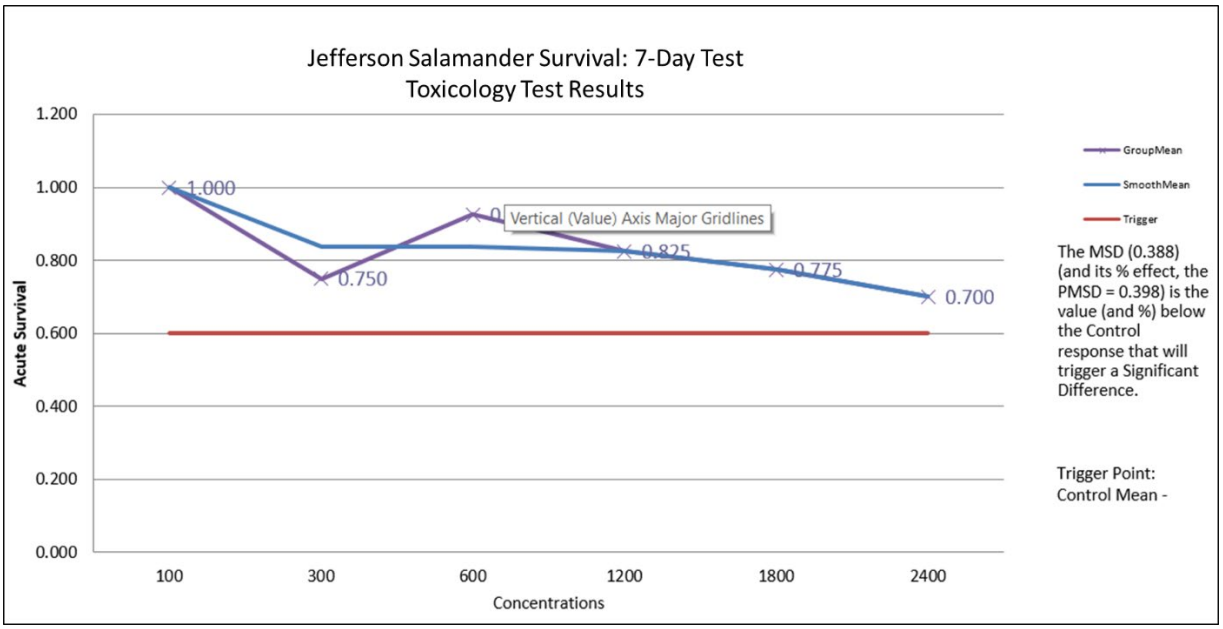

Figure 22. Jefferson salamander larvae survival during a 7-day conductivity toxicity test with a NOEC of 1,800 µS/cm and LOEC of 2,400 µS/cm.

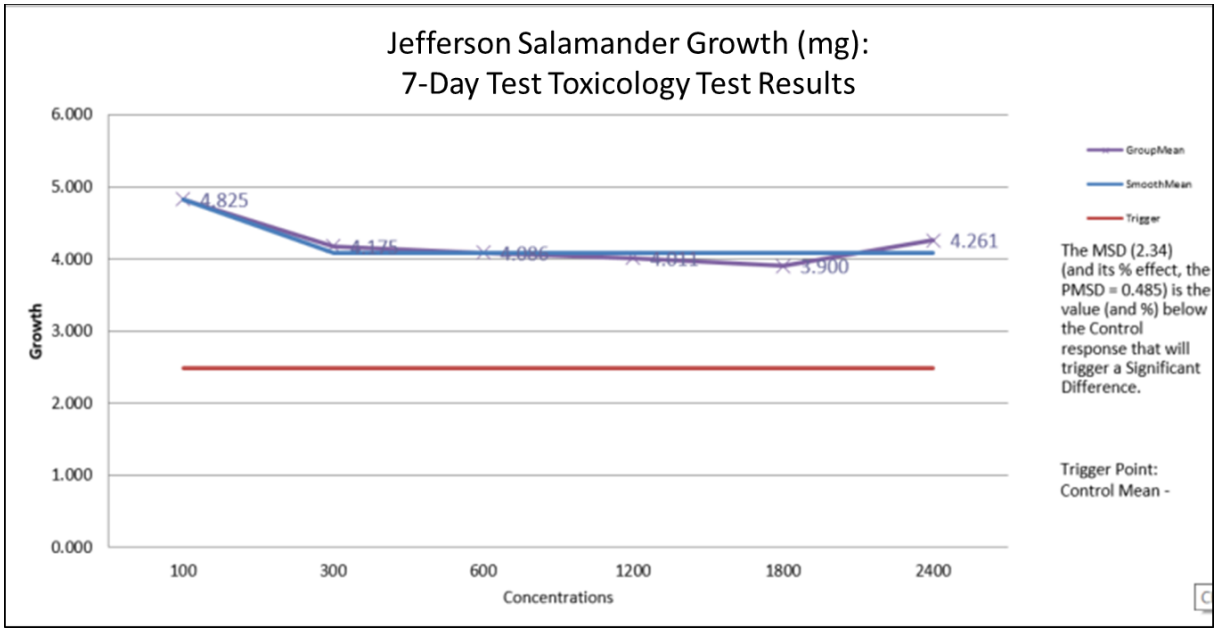

Figure 23. Jefferson salamander larval growth (mg) showed no significance with a LOEC of >2,400 µS/cm.

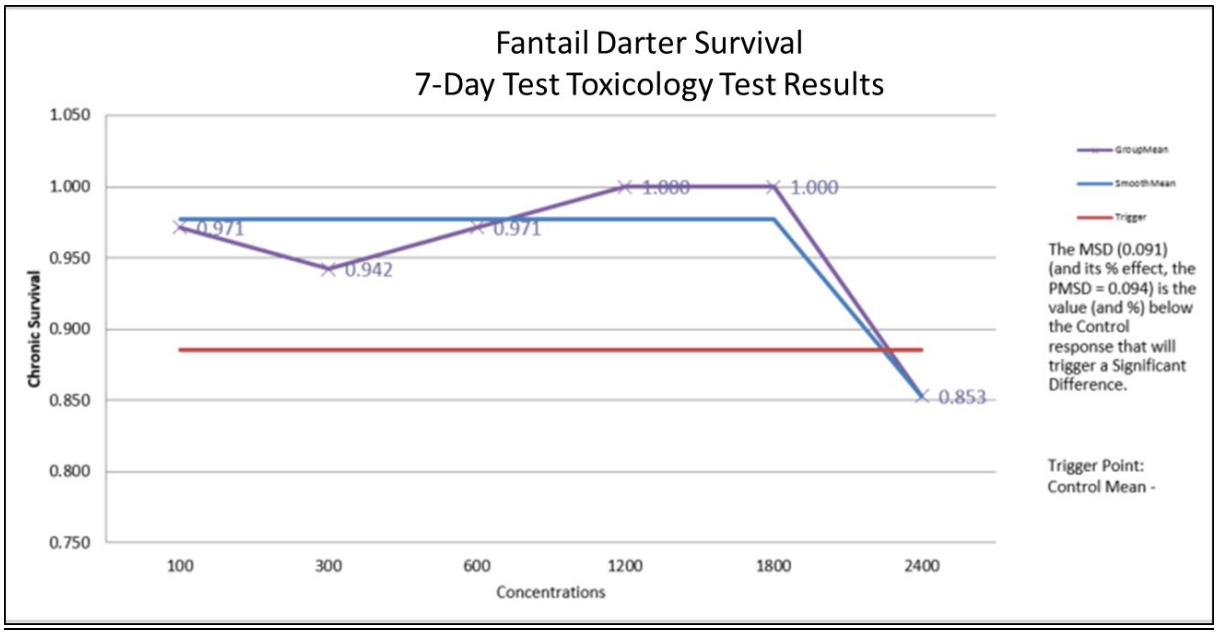

Figure 24. Fantail darter larvae survival with a NOEC of 1,800 µS/cm and LOEC of 2,400 µS/cm.

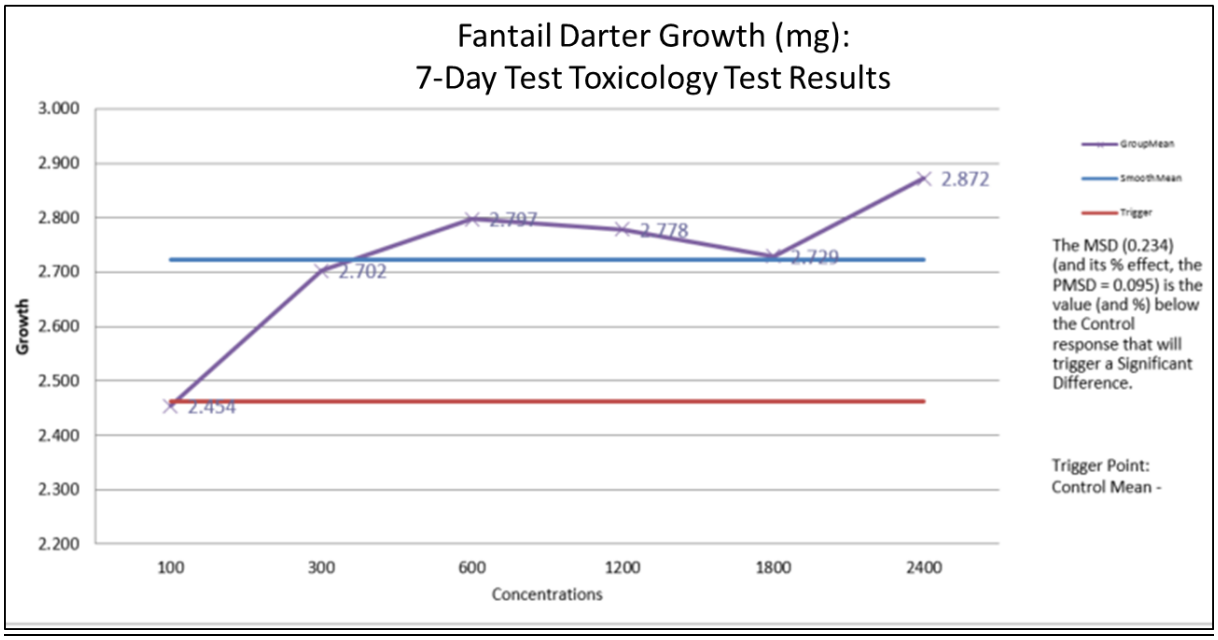

Figure 25. Fantail Darter larval growth (mg) of a 7-day conductivity toxicity test with a LOEC of > 2,400 µS/cm.

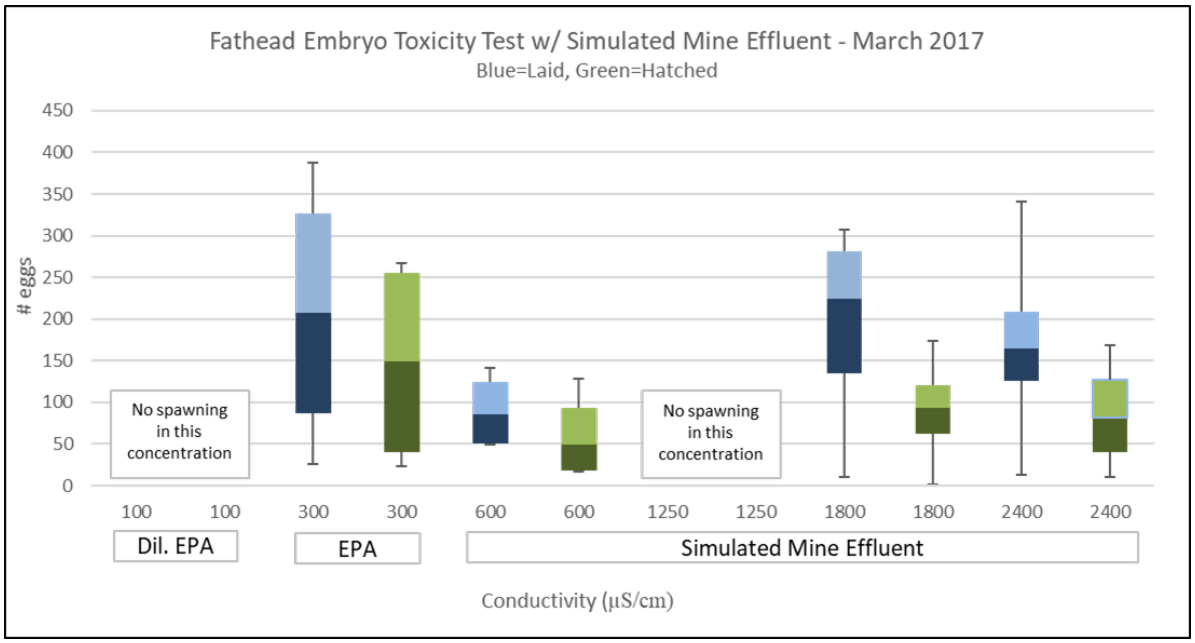

Figure 26. Fathead minnow egg and embryo toxicity testing of simulated mine effluent.

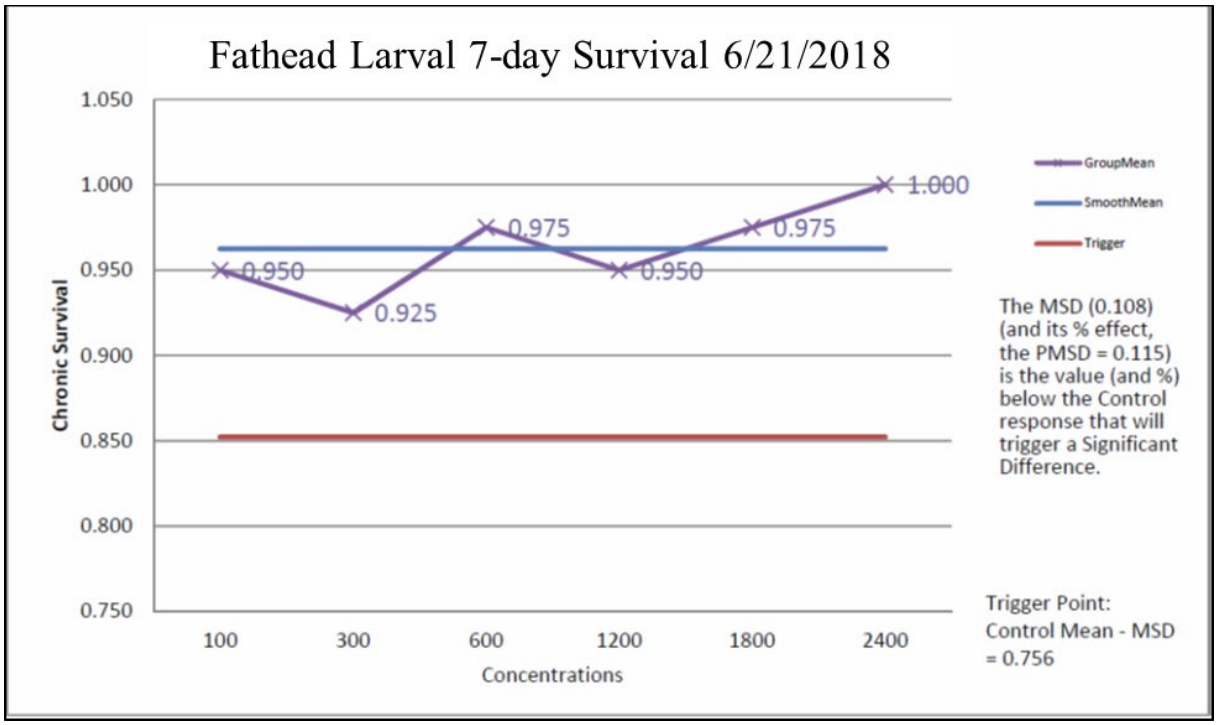

Figure 27. Results from the fathead minnow larval 7-day chronic survival testing showed an NOEC of 2,400 µS/cm, a LOEC >2,400 µS/cm and an EC25 >2,400 µS/cm.

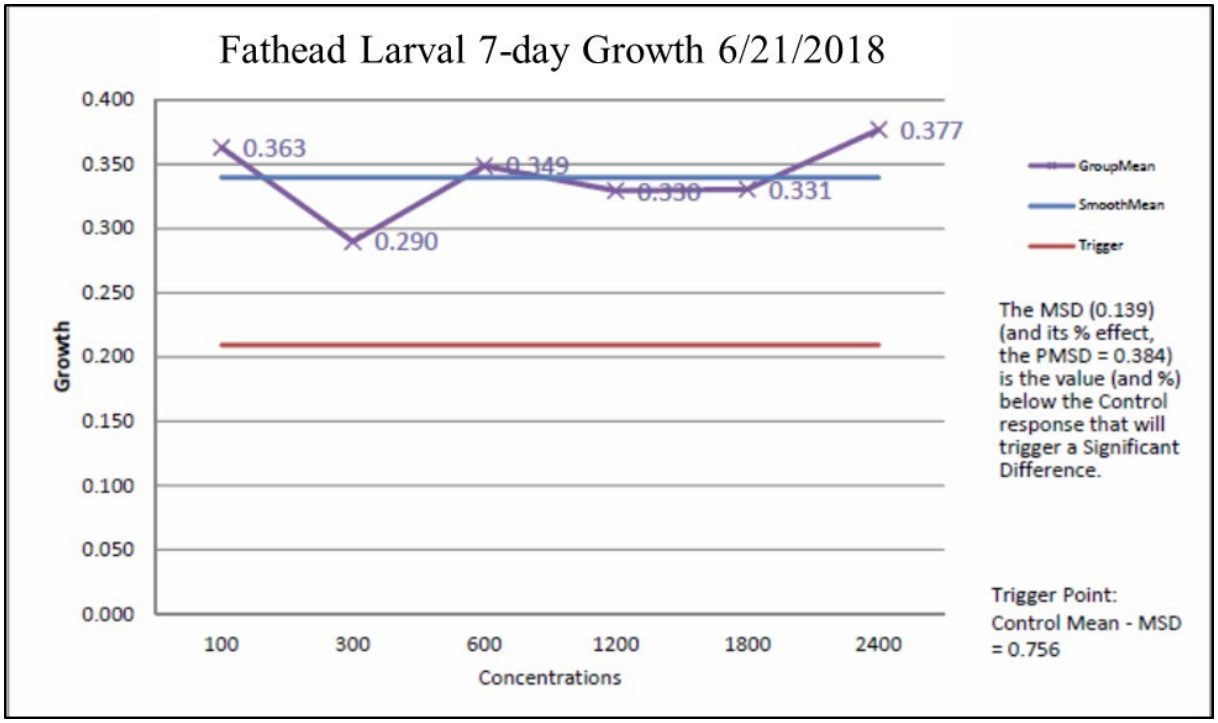

Figure 28. Fathead minnow growth was also analyzed during this 7-day test. Results showed an NOEC of 2,400 µS/cm, a LOEC >2,400 µS/cm and an IC25 >2,400 µS/cm.

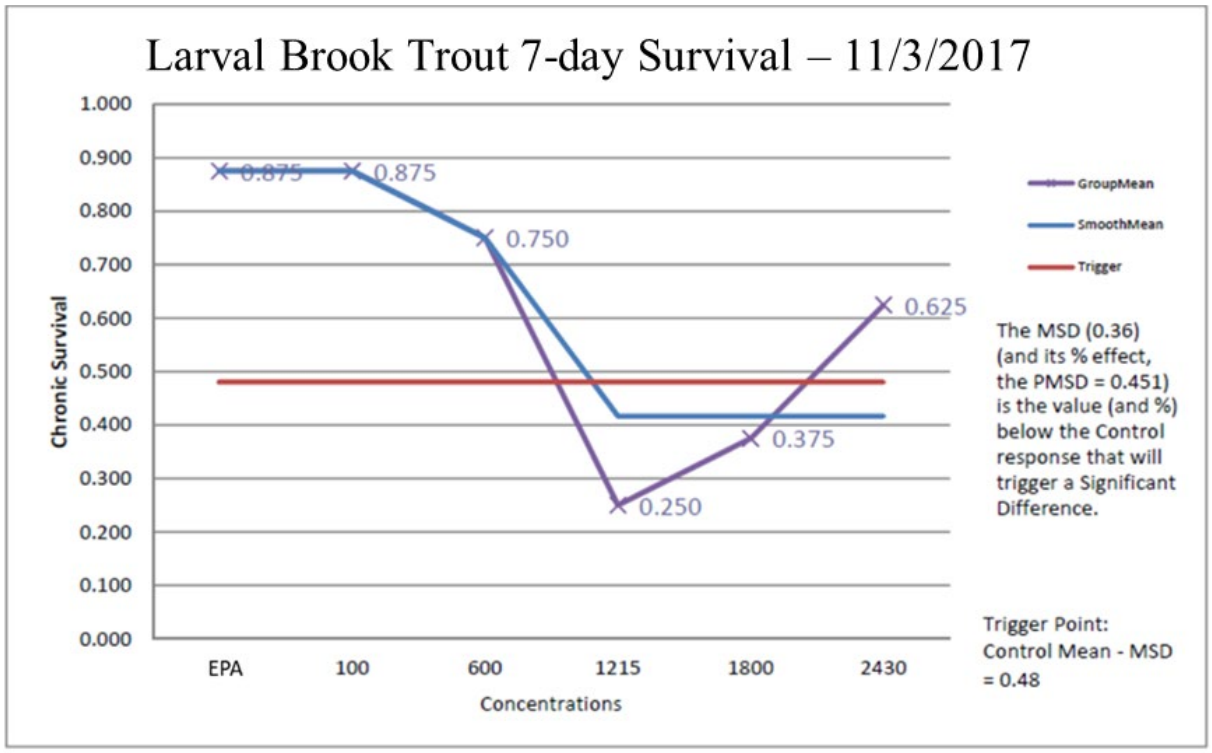

Figure 29. Larval Brook Trout survival showed significant decreases with an NOEC of 600 µS/cm and a LOEC of 1,200 µS/cm.

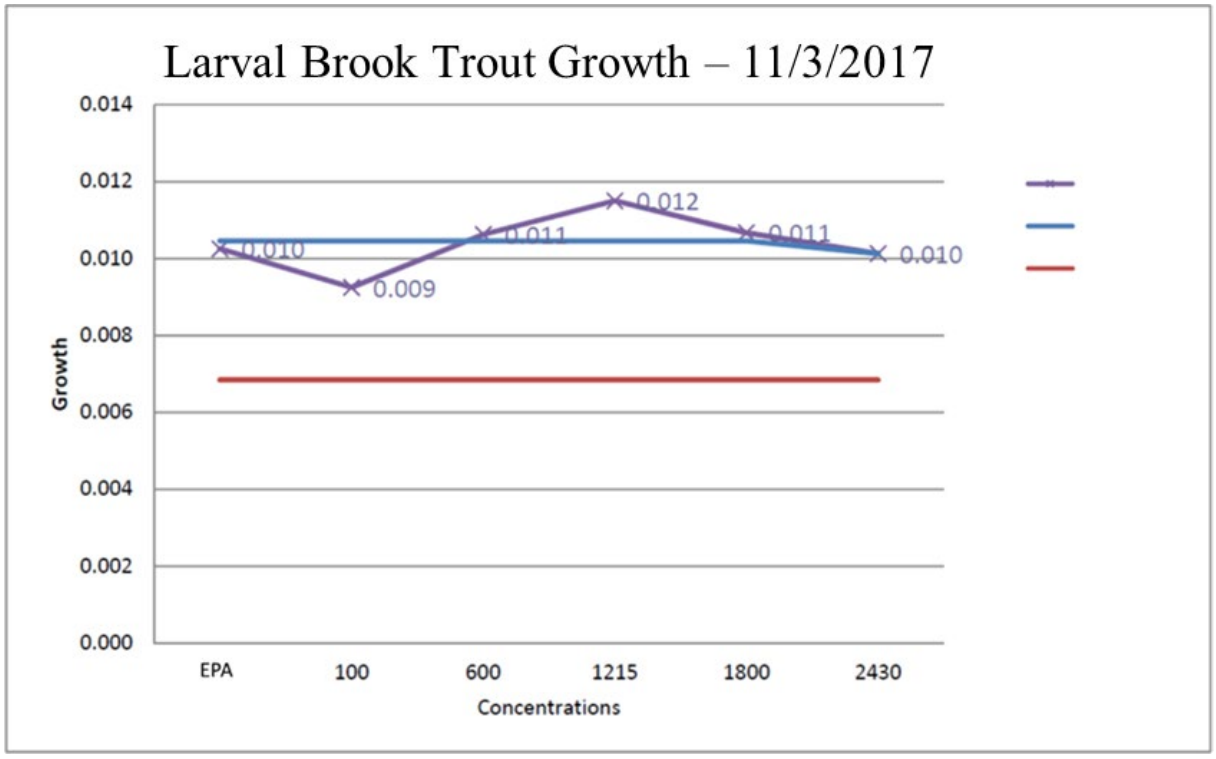

Figure 30. Larval Brook Trout growth showed no statistical difference between the control and effluent concentrations (LOEC >2,400 µS/cm).

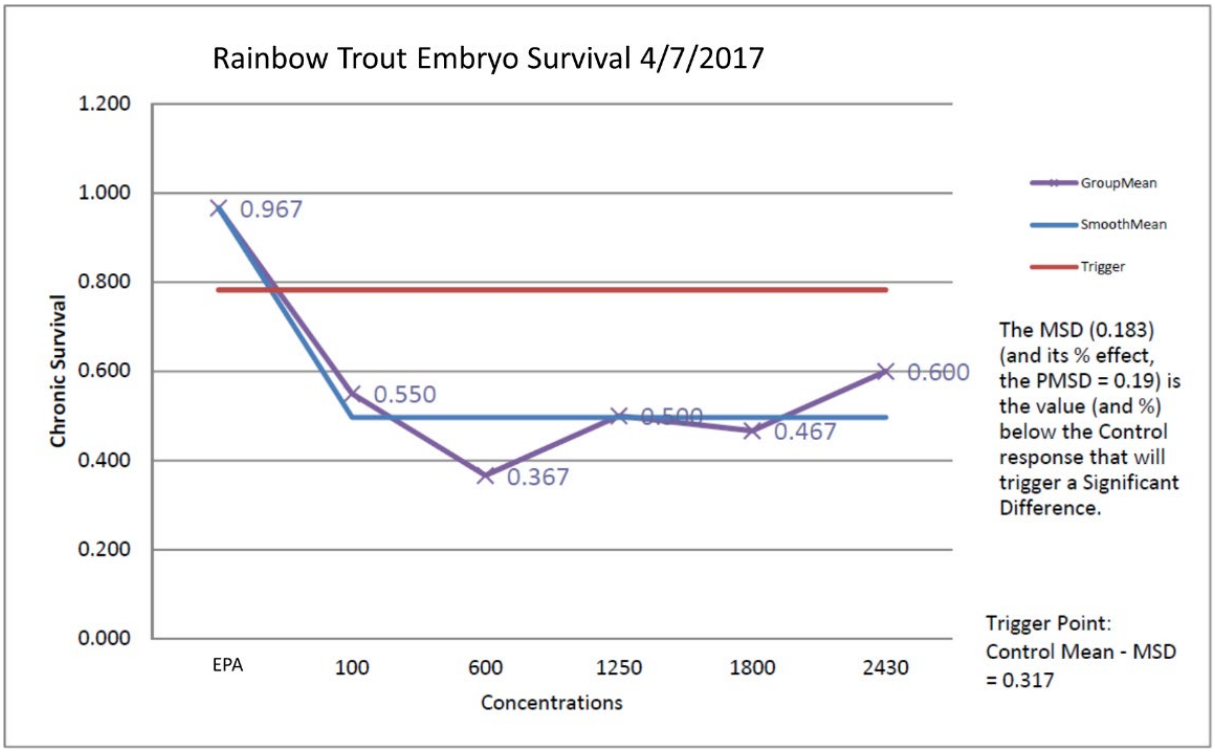

Figure 31. Rainbow Trout embryo survival had a NOEC of 600 µS/cm and a LOEC of 1,200 Ss/cm.

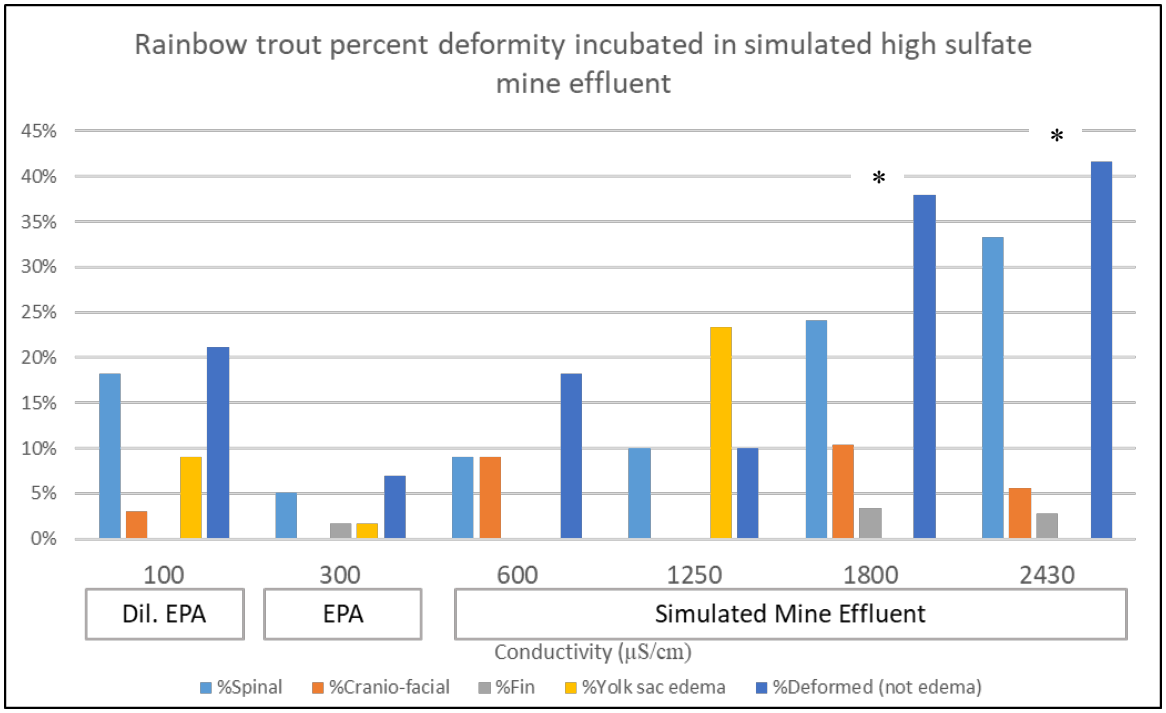

Figure 32. Rainbow trout larval deformities after incubation in simulated high sulfate mine effluent. Deformity rates significantly different from the EPA water control are indicated by *.

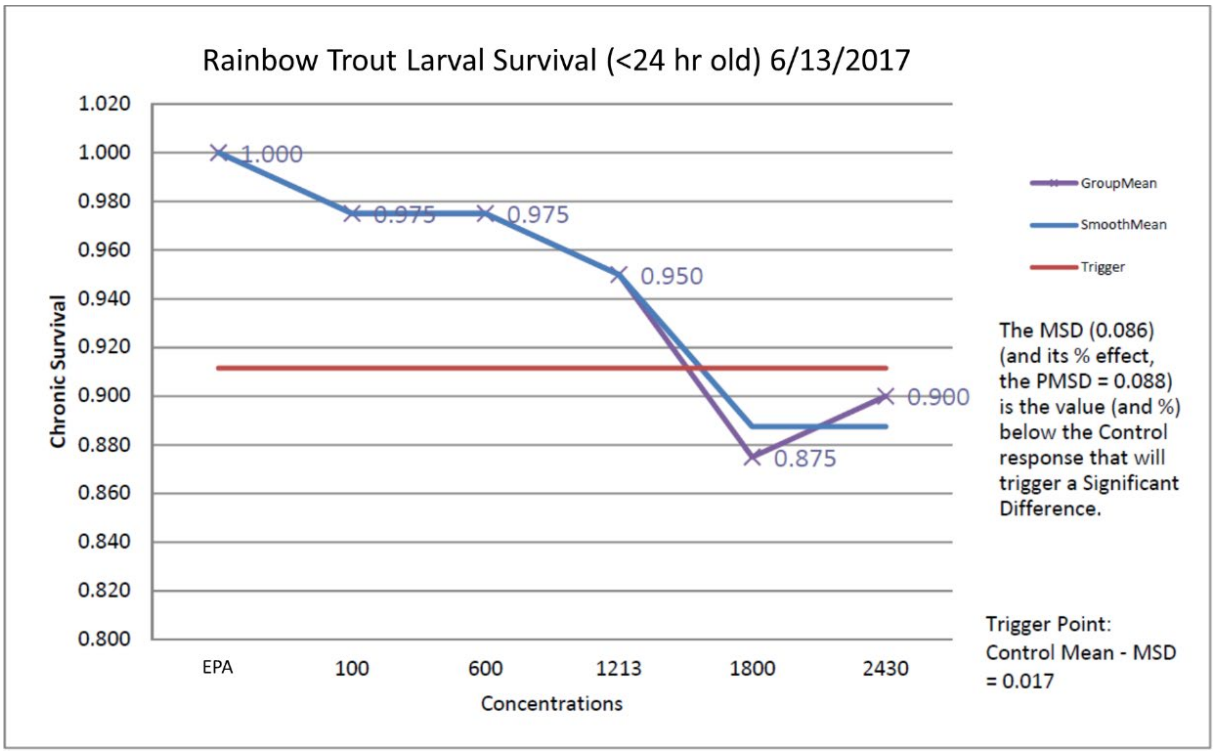

Figure 33. Rainbow trout larval (<24 hours old) survival with an NOEC of 1,200 µS/cm and a LOEC of 1,800 µS/cm.

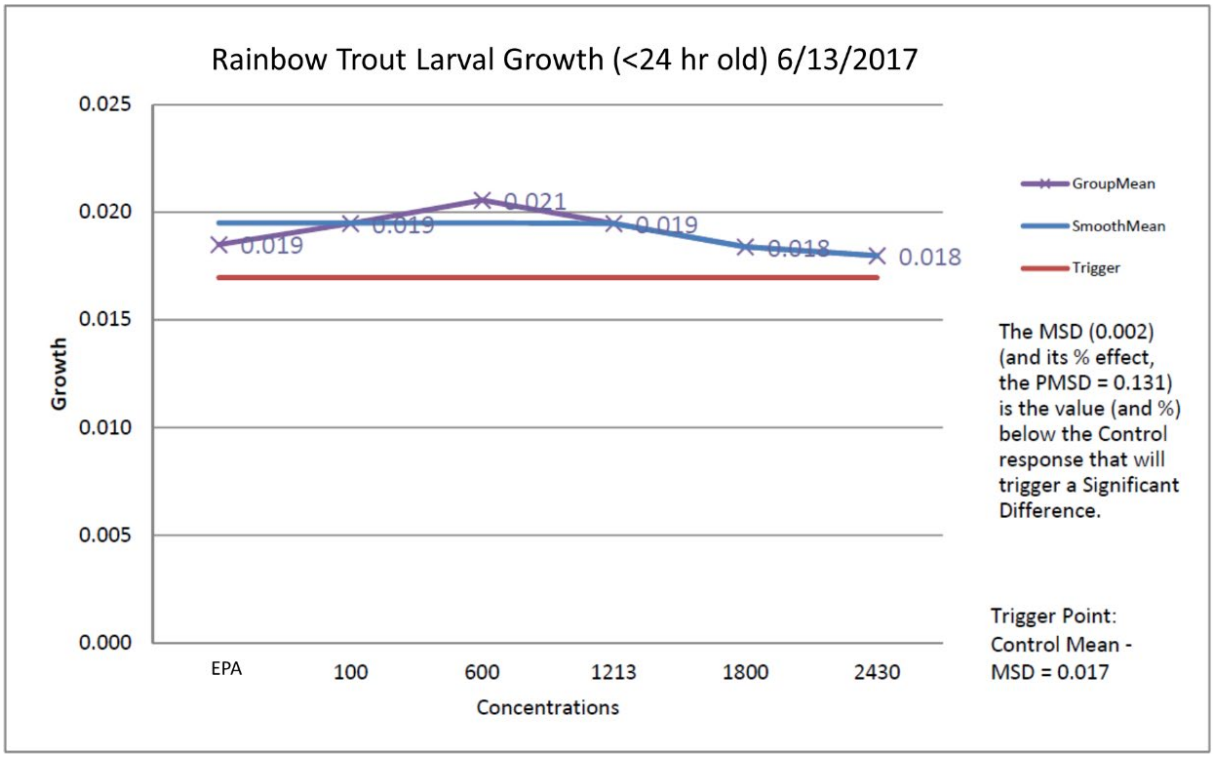

Figure 34. Growth of Rainbow Trout Larvae (<24 hours old).

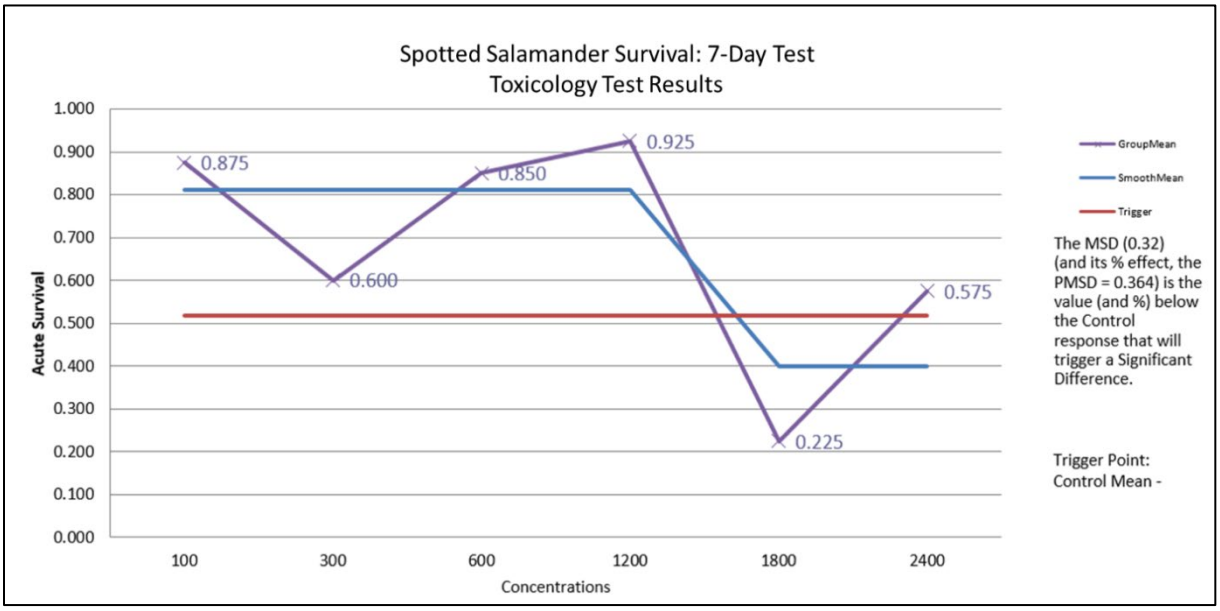

Figure 355. Survival of spotted salamanders was not significant with an LOEC > 2,400 µS/cm.

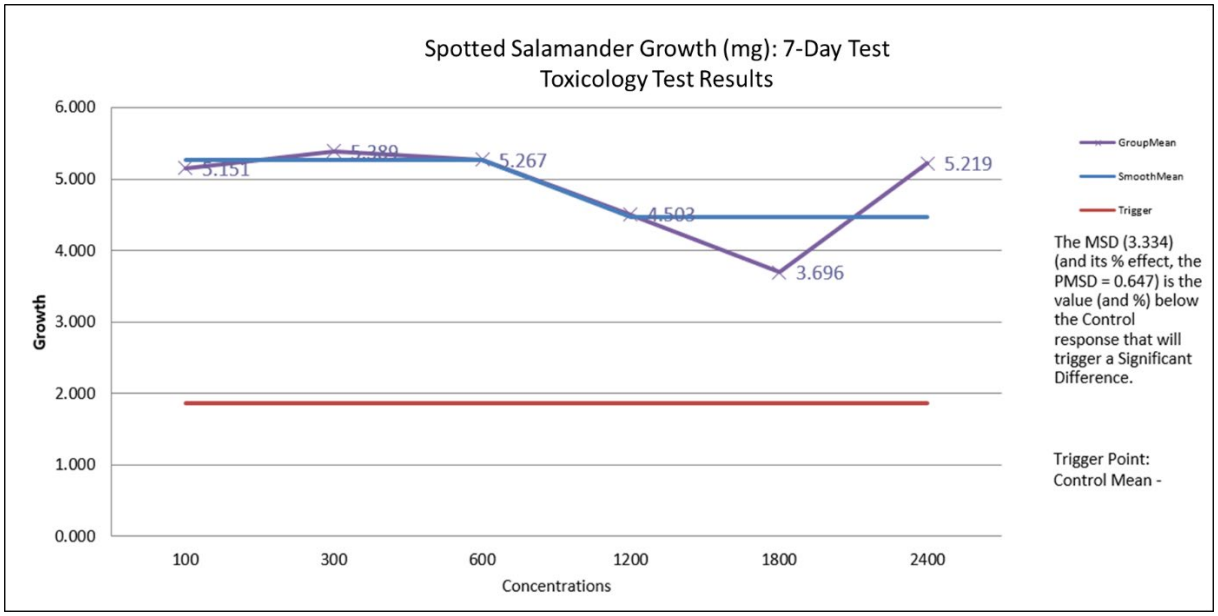

Figure 36. Mean growth (mg) for spotted salamander larvae during a 7-day conductivity toxicology test (LOEC > 2,400 µS/cm).

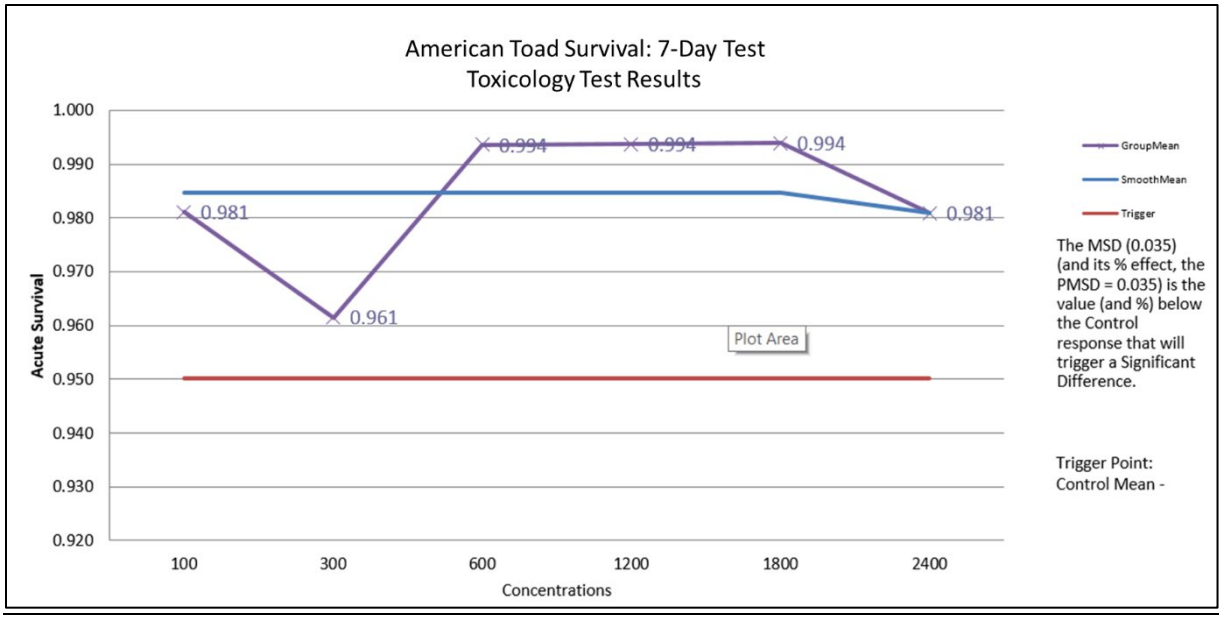

Figure 37. American toad larvae survival during a 7-day conductivity toxicity test (LOEC > 2,400 µS/cm).

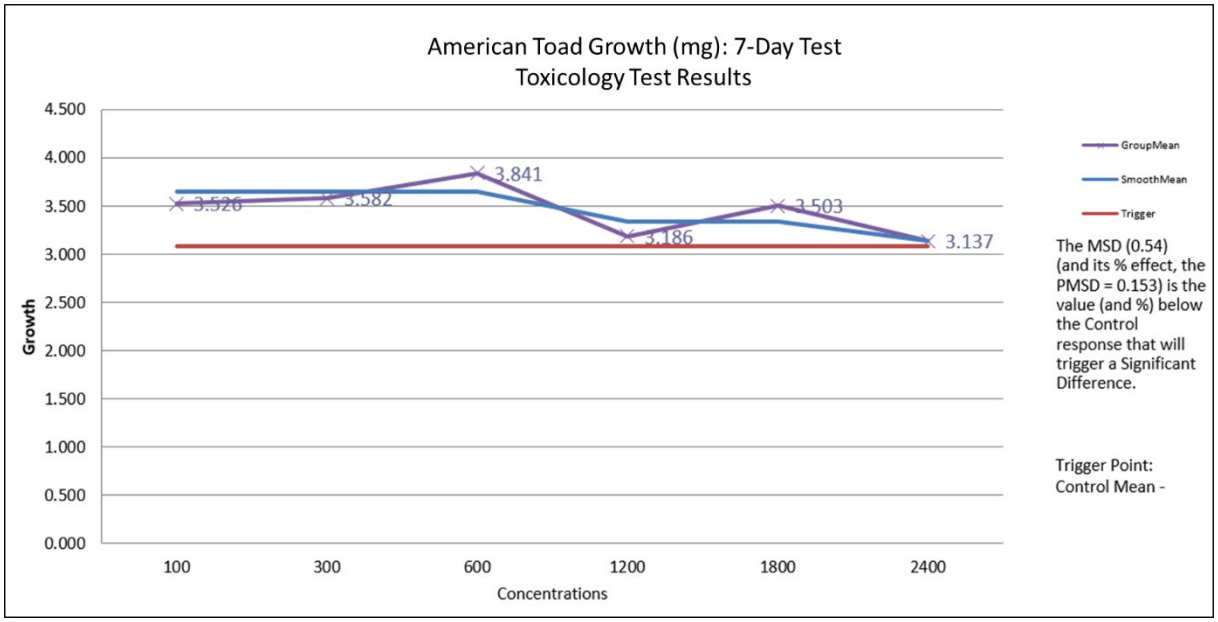

Figure 38. American toad larval growth a 7-day conductivity toxicity test (LOEC > 2,400 µS/cm).