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Effect of Selenium on Juvenile Bluegill Sunfish at Reduced Temperature

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Technical Report

Effect of Selenium on Juvenile Bluegill Sunfish at Reduced Temperature

by

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Reviews

The study design was reviewed by John Besser of USGS and Kevin Brix of EcoTox. The report was reviewed by an external panel of experts that included Jerry Diamond of TetraTech, David DeForest of Parametrix, David Janz of the University of Saskatchewan, and Stephen Klaine of Clemson University. Internal reviews were provided by Mick DeGraeve of GLEC and Charles Stephen, Dale Hoff and David Mount of EPA.

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Executive Summary

The final chronic value recommended in the 2004 draft update of the aquatic life ambient water quality criteria for selenium (7.91 µg/g dry weight) is based on a single study (Lemly 1993). This report presents results of toxicity assays designed to replicate Lemly's test, and to further explore how temperature affects the toxicity of selenium. Juvenile bluegill were exposed to three distinct combinations of selenium species and temperature. In exposure system one (ES1) and three (ES3), fish were exposed to six nominal concentrations of selenium in water (background, 1.25, 2.5, 5.0, 10, 20, 40 µg/L) and in diet (*Lumbriculus variegatus* with background, 1.25, 2.5, 5.0, 10, 20, or 40 µg/g dw) at two temperature regimes, 20°C decreasing to 4-5°C (ES1) and 20°C decreasing to 9°C (ES3). Exposure system two (ES2) had a temperature regime similar to ES1 (20°C → 4-5°C), but only one nominal concentration of selenium (5 µg/L in water and 5 µg/g dw in diet), incorporated in TetraMin as seleno-L-methionine. In ES1 and ES3 selenized yeast was fed to worms. ES2 duplicated Lemly's (1993) treatment with high fish mortality.

Average measured concentrations of total selenium in water were similar to target exposure concentrations. The proportion of selenate to selenite in each water tank remained close to the target ratio of 1:1. Average measured concentrations of selenium in worm tissues were within a factor of 1.5 of target concentrations for treatments aiming to reach 5.0, 10, 20, or 40 µg/g dw. The average measured tissue concentrations in the other two treatments (1.25, 2.5 µg/g dw) were between 2 and 3 times higher than target levels. Concentrations of selenium in fish tissues increased asymptotically with exposure period. Fishes exposed to lower concentrations of selenium in the water and in their food (worms) consistently displayed lower bioaccumulation rates and lower asymptotic concentrations of selenium in tissues. Rates of selenium accumulation in fish tissues were similar for corresponding ES1 and ES3 treatments up to day 112. Accumulation of selenium from that day until the end of the experiment (day 182) was higher in ES3 than in ES1 fish. Exposure of ES2 fish to seleno-L-methionine resulted in tissue concentrations of selenium approximately 2.5 times higher than in fish exposed to similar concentrations of selenium in worm tissues. At the end of the experiment, the average concentration of selenium in tissues of ES2 fish was 9.4 µg/g dw in one tank and 10.6 µg/g dw in another. The average concentration of selenium in tissues of ES1 fish exposed to a similar temperature regime and selenium concentration, was 4.0 µg/g dw.

This threshold was exceeded only in ES1 and ES3 treatments with a target concentration of selenium in the diet equal to 20 or 40 µg/g dw. Projection of the selenium concentration associated with the onset of mortality (>10%) in these treatments resulted in similar threshold values: 11.1, 11.6 µg/g dw for ES1 and 11.1, 13.8 for ES3. The projected EC_{20}values of 10.16 µg/g (9.81 – 10.52 µg/g, 95% CI), and EC_{10}, 9.56 µg/g (9.09 – 10.05 µg/g) for ES1 were lower than corresponding values for ES3, EC_{20} =14.02 µg/g (13.50 – 14.56 µg/g), EC_{10} = 13.29 µg/g (12.61 – 14.00 µg/g).

1.0 INTRODUCTION

The final chronic value of 7.91 µg/g dw recommended in the 2004 Draft Update of the Aquatic Life Ambient Water Quality Criteria for Selenium is based on one study (Lemly 1993), in which juvenile bluegill underwent "winter stress syndrome." Data from Lemly's study indicate that over-wintering fish may be more susceptible to the effects of waterborne and dietary selenium exposure due to increased sensitivity at low temperature. Lemly exposed juvenile bluegill sunfish in the laboratory to waterborne (1:1 selenite:selenate; nominal 5 µg Se/L) and food borne (seleno-L-methionine in TetraMin; nominal 5 µg Se/g dw food) selenium for 180 days with temperatures decreasing from 20 to 4°C. Given the importance of the data from the Lemly study in deriving the tissue-based final chronic value for selenium, the goal of this study is to determine tissue-based effect levels for selenium exposure over a simulated winter season at two temperature regimes, 20 to 4°C and 20 to 9°C. Besides the additional temperature regime, two prominent differences from the Lemly study include (1) a range of six selenium concentrations was included (aqueous and diet) to determine protective effect levels and (2) bluegill were fed the aquatic worm, *Lumbriculus variegatus*, which contained target levels of selenium accumulated by feeding the worms selenized-yeast. A separate system exposed juvenile bluegill to aqueous selenium and seleno-L-methionine in TetraMin under a 20 to 4°C temperature regime to mimic the Lemly study exposure design. The 182-day study began on April 30, 2007 and ended October 29, 2007.

1.1 SUMMARY OF LEMLY STUDY AND A COMPARISON OF TEST DESIGNS

Lemly exposed juvenile bluegill to aqueous and dietary selenium under intermittent flow-through conditions for 180 days. Tests were run at 4° and 20°C, with biological (histological, hematological, metabolic and survival) and selenium measurements made at 0, 60, 120 and 180 days. Fish were fed at a rate of 3% body weight per day. All treatments were initiated at 20°C, and then decreased at a rate of 2°C per week for 8 weeks to reach 4°C. The temperature was then maintained at that temperature for the remainder of the 180 days.

In the 20°C test, fish accumulated 6 µg/g dw selenium (whole-body) with no significant effect on survival (4.3% and 7.4% mortality in the control and treatment, respectively). In the 4°C test, fish exposed to selenium accumulated 7.9 µg/g dw (whole-body) selenium and significant mortality was observed after 120 (33.6%) and 180 days (40.4%) relative to the control (3.9%). Several hematological measurements were significantly different in both the warm and cold selenium exposures relative to controls. Both warm and cold selenium treatments also had greater O_2 consumption than controls. Fish lipid content in the cold selenium treatment decreased more than the cold control; lipid content did not decrease in either the warm control or the warm selenium treatment. The results suggest that significant mortality occurs in juvenile bluegill during winter months when tissue concentrations increase from 5.85 to 7.91 µg/g dw and lipid levels decrease to 6 percent.

Several design characteristics of Lemly's study were modified in the current study (Table 1.1). The notable changes, as stated above, were the addition of a more temperate temperature regime (20 to 9°C), and exposure to a range of six selenium aqueous and dietary concentrations and controls (Exposure Systems (ES) 1 and 3). The goal of the latter modification was to obtain a gradient in the response of the bluegill ranging from no observable effects in the low concentrations, to intermediate effects in the middle concentrations, to 100% affected in the high concentration. Such a range in response is needed for a reliable estimation of effect concentrations.

Another modification to the Lemly design was to feed the bluegill an aquatic worm, *Lumbriculus variegatus*, that had accumulated selenium to a gradient of levels through the consumption of selenized-yeast. Selenomethionine added to commercial fish food has been commonly used in exposure studies, but that may not be the predominate selenium species fish are exposed to in nature. Fan et al (2002) determined that selenomethionine was approximately 30% of the total selenium in biological tissues in several trophic levels. The use of a forage animal (*Lumbriculus*) that had accumulated selenium through the consumption of a trophic level 1 organism (selenized-yeast) was considered a more representative exposure to bluegill than the addition of selenomethionine to the diet. To have a direct comparison of the response of the bluegill in this study to the fish in Lemly's experiment, a repeat of Lemly's cold treatment (ES2) was run concurrent to ES 1 and 3. Due to space restrictions, only 2 replicates were used in ES2.

Table 1.1 Comparison in Selected Design Characteristics between Lemly and Current Studies

Design Characteristics	Lemly	Current Study Exposure System (ES)		
		ES1	ES2	ES3
Species	Juvenile bluegill sunfish	Juvenile bluegill sunfish		
Bluegill size at test initiation	50-70 mm total length	56-69 mm (mean = 60) total length[a]; 1.2-2.0 g (mean = 1.5) weight		
Aqueous exposure, nominal	1:1 ratio of selenite:selenate; 5 µg/L	1:1 ratio of selenite:selenate; 1.25, 2.5, 5, 10, 20, 40 µg/L	1:1 ratio of selenite:selenate; nominal 5 µg/L	1:1 ratio of selenite:selenate; 1.25, 2.5, 5, 10, 20, 40 µg/L
Dietary exposure, nominal	Seleno-L-methionine added to TetraMin; 5 µg/g	Se accumulated in *Lumbriculus* at six treatment conc'ns, 1.25, 2.5, 5, 10, 20, 40 µg/g dw	Seleno-L-methionine added to TetraMin; 5 µg/g	Se accumulated in *Lumbriculus* at six treatment conc'ns, 1.25, 2.5, 5, 10, 20, 40 µg/g dw
Feeding rate	3% body wt/day	4% body wt/day	3% body wt/day	4% body wt/day
Duration	180 days	182 days		
Temperature	Cold: 20 to 4°C;	20 to 4°C; after	20 to 4°C; after	20 to 9°C; after

Design Characteristics	Lemly	Current Study Exposure System (ES)		
		ES1	ES2	ES3
regime	decreased 2°C/week until 4°C reached Warm: 20°C constant	30 days at 20°C decreased 2°C/week until 4°C reached	30 days at 20°C decreased 2°C/week until 4°C reached	30 days at 20°C decreased 2°C/week until 9°C reached
Controls	No Se added to both cold and warm treatments	No Se added to water or worms	No Se added to water or TetraMin	No Se added to water or worms
Replication	3 reps/treatment	2 reps - controls only	2 reps/treatment	2 reps - controls only
Fish/replicate	70	100		

[a] Standard lengths of 44-54 mm (mean = 47) were converted to total length using conversion factor for bluegill of 1.278 (Beckman 1948).

2.0 METHODS

2.1 TEST ORGANISMS

2.1.1 Lepomis macrochirus

Juvenile bluegill (*Lepomis macrochirus)* used in the study were purchased from Osage Catfisheries in Osage Beach, Missouri. The juvenile bluegill, which were hatched in May 2006, were 38-51 mm in standard length, and arrived at Great Lakes Environmental Center's (GLEC) laboratory in Traverse City, Michigan on April 5, 2007. Upon arrival, the bluegill were physically inspected, and the initial weight and length of a subsample was recorded (average standard length: 47 mm; average weight: 1.0 gram). The bluegill were divided between two 400 liter flow-through tanks, each containing 350 L of dechlorinated water. The water temperature in the holding tanks at the time of stocking (12°C) was within 1°C of the shipping water temperature (11.8°C). Chilled dechlorinated water was supplied to each holding tank at the rate of 2 liters per minute. Both holding tanks were aerated continuously using large air stones supplied with compressed air from an oil-free air compressor.

Prior to test initiation, the bluegill were held for a period of 25 days, and during the first 14 days they were treated with salt and formalin to manage external parasites. Although no parasites were observed in the fish received on April 5, 2007, the fish were prophylactically treated for external parasites because *Dactylogyrus* or *Gyrodactylus* were observed on the bluegill in a previous shipment from the same source. Uniodized salt was added to the holding tanks on a daily basis to achieve an initial treatment concentration of 1 g/L, which was diluted over time as water flowed into the tanks. The treatment was performed for 19 consecutive days until one week prior to test initiation. The bluegill were also treated with formalin on two separate days, 5 days apart. On April 7, and 12,

2007, all the fish were moved to holding tank 1, where they were exposed to a nominal formalin concentration of 1 mg/liter in laboratory water for one hour. During the one hour formalin treatment, holding tank 2 was cleaned, rinsed thoroughly and filled with fresh dechlorinated water. After the fish were exposed to the formalin for one hour, they were then transferred to the fresh laboratory water in tank 2. Tank 1 was then disinfected, rinsed thoroughly, and filled with fresh dechlorinated water. The bluegill were once again divided between tanks 1 and 2 after the formalin treatment. None of the fish exhibited any overt signs of stress (i.e., surfacing or lethargy or death) during or after the salt or formalin treatments. No external parasites were observed on the bluegill during weekly monitoring prior to test initiation.

In the holding tanks the fish were fed frozen adult brine shrimp once daily until satiation. Each tank was siphoned every day after feeding to remove uneaten food and fecal material. Dissolved oxygen (D.O.), temperature, and pH were measured on a daily basis.

2.1.2 Lumbriculus variegatus

Selenium-dosed adult sized *Lumbriculus variegatus* (California blackworms) were used as the food source for the bluegill in the preliminary and definitive studies. Thirty-two pounds of *L. variegatus* were purchased from Bayou Aquatic and Reptile Supply in Ontario, California, arriving at GLEC on February 22, 2007, approximately 9 weeks prior to the initiation of the definitive study. Upon arrival, the *L. variegatus* were divided among 16 flow-through pans. The pans contained approximately 28 liters of water, and were aerated to maintain dissolved oxygen at an acceptable level (≥ 6 mg/L) to support the *L. variegatus.* Each pan of worms was fed daily, 3.2 grams of nutritional yeast suspended in 250 ml of dechlorinated water, until March 28, 2007. Beginning on March 28, 2007, each pan of worms was fed 3.2 grams of a mixture of nutritional yeast and selenized-yeast (SelenoSource™ AF 600[1]) to obtain a range of six concentrations. The yeast mixture was suspended in 250 ml of dechlorinated water, and fed to the worms on a daily basis until test initiation on April 30, 2007. Each tank was siphoned daily after feeding to remove uneaten food, fecal material, and detritus. D.O., temperature, and pH were measured daily (average measurements: dissolved oxygen, 7.9 mg/L; temperature, 15.0°C; and pH, 7.80).

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2.2 SELENIUM EXPOSURE

Three separate exposure systems were maintained concurrently in a trailer specifically designed for this study (Figure 2.1). In each system, fish were exposed for 182 days to selenium through water and diet; test initiation was April 30, 2007 and test termination was October 29, 2007. In Exposure Systems (ES) 1 and 3, juvenile bluegill were exposed to a series of six aqueous concentrations of selenium and fed selenium accumulated in *Lumbriculus variegatus*. The only difference between ES1 and ES3 was the water temperature regime: in ES1, temperature was maintained at 20°C for 30 days and then decreased 2°C/week until it reached 4°C, which was maintained until test termination. In ES3, the water temperature was maintained at 20°C for 30 days, and then decreased 2°C/week until it reached 9°C which, was maintained until test termination. In ES2, bluegill were exposed to one aqueous and one dietary selenium concentration. The temperature regime for ES2 was the same as for ES1. The nominal concentrations of selenium in the water and the target concentrations for selenium in the diet for each exposure system are given in Table 2.1. One hundred juvenile bluegill were added to each of the 20 test tanks at the start of the exposure period on April 30, 2007.

Table 2.1. Nominal exposure concentrations for Exposure Systems 1, 2 and 3.

Exposure System and temperature regime	Treatment Number (no. of replicates)	[Se] in Water, µg/L	Target [Se] in diet, µg/g dw	
			Lumbriculus	TetraMin
ES1 20 to 4°C	Control (2)	No added Se	Background	
	1 (1)	1.25	1.5	
	2 (1)	2.5	2.5	
	3 (1)	5	5	
	4 (1)	10	10	
	5 (1)	20	20	
	6 (1)	40	40	
ES2 20 to 4°C	Control (2)	No added Se	N.A.	Background
	5 (2)	5	N.A.	5
ES3 20 to 9°C	Control (2)	No added Se	Background	
	1 (1)	1.25	1.5	
	2 (1)	2.5	2.5	
	3 (1)	5	5	
	4 (1)	10	10	
	5 (1)	20	20	
	6 (1)	40	40	

The goals for selecting the target exposure conditions were to (1) attain a range of selenium concentrations in the juvenile bluegill that result in no response in the low exposures, intermediate response in the middle treatments and meaningful mortality in the high exposure conditions; and (2) achieve water and worm concentrations that are representative of field conditions. An assumption was made that the transfer of selenium from worm to bluegill was 1:1. This assumption was confirmed in selected exposure conditions in preliminary experiments.

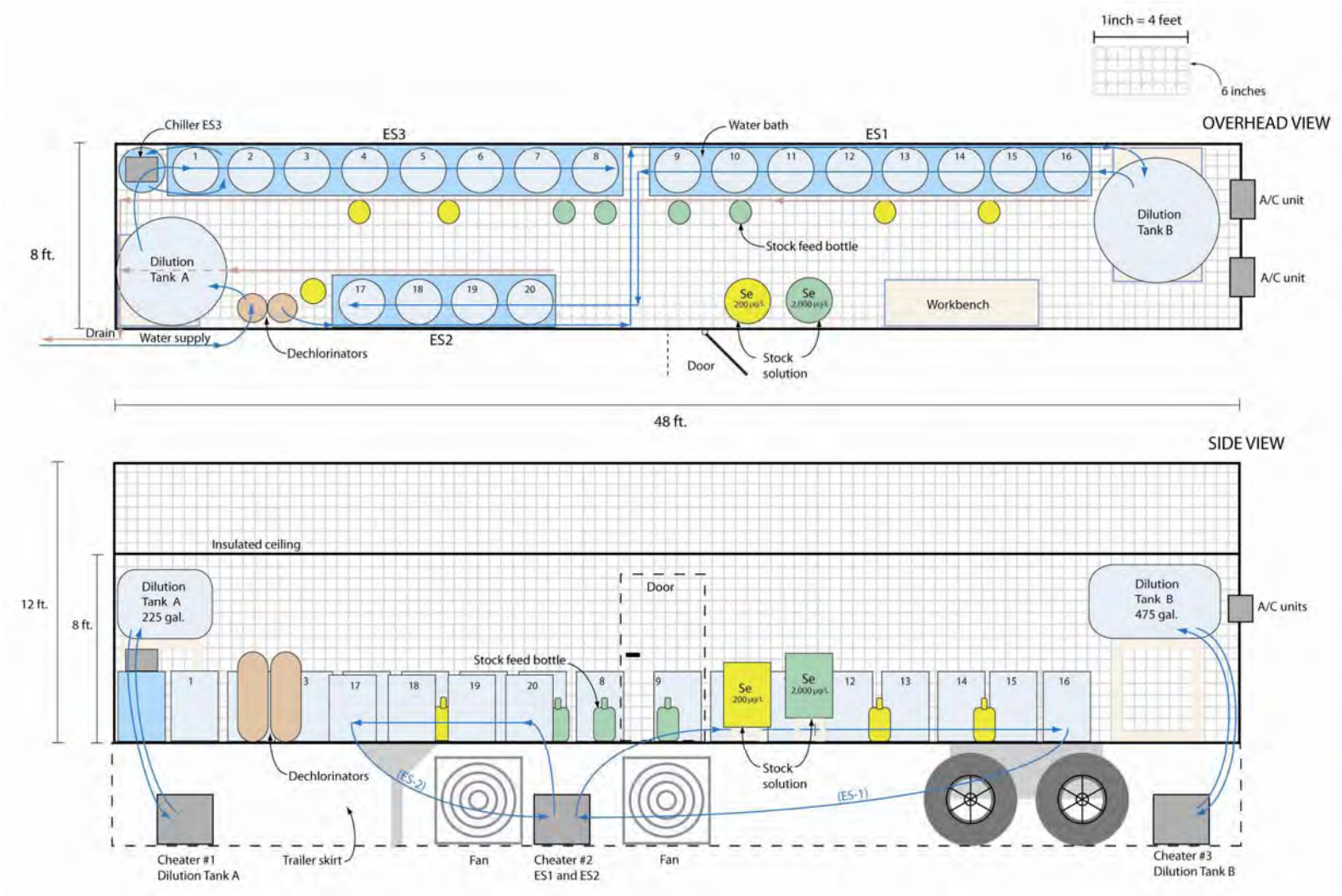


Figure 2.1. Floor plan of systems used in juvenile bluegill selenium study.

2.2.1 Aqueous Exposure

A 1:1 molar ratio (as selenium) of selenite and selenate was produced using sodium selenite (Na_2SeO_3; 99% Sigma-Aldrich), and sodium selenate decahydrate-(Na_2SeO_4 $10H_20$; 99%, Sigma-Aldrich). Concentrated stock solutions of selenate (4.67 g of sodium selenate/1 L of deionized water) and selenite (2.18 g of sodium selenite /L of deionized water) were prepared, and were combined to make two working stock solutions (200 and 2000 ug/L total selenium) that were used in the definitive study to achieve the target selenium concentrations. The 200 ug/L and 2000 ug/L stock solutions were prepared in 200 L calibrated carboys using dechlorinated tap water as the medium for the toxicant.

The combined selenite and selenate stock solutions were used to dose the bluegill exposure tanks for all three exposure systems. To achieve the target test concentrations, FMI (Fluid Metering, Inc) pumps delivered the stock solutions at a predetermined flow rate, while the dilution water (dechlorinated tap water) was delivered to the exposure tanks from a chilled head tank at a rate of 500 ml/minute (Figures 2.2 and 2.3). To ensure adequate mixing of dilution water and selenium prior to delivery to the exposure chambers, the stock solutions and dilution water flowed into a mixing vessel, which then drained into the designated 200 liter bluegill exposure tanks. The flow rates of the stock solutions and dilution water were measured approximately every 12 hours, and were adjusted as needed to be within $\pm$ 0.2 ml/minute for the stock solutions, and $\pm$ 5 ml/minute for the dilution water. Expected dilution water and stock solution flow rates (and designated stock solutions) for each of the six target concentrations for all three exposure systems are presented below.

	200 ug/L stock solution (1:1 selenite/selenate)			2000 ug/L stock solution (1:1 selenite/selenate)		
Target aqueous total [Se] in the test chambers	1.25 ug/L	2.5 ug/L	5.0 ug/L	10 ug/L	20 ug/L	40 ug/L
Stock solution flow rate to the test chambers (dilution water flow rate 500 ml/min)	3.13 ml/min	6.25 ml/min	12.5 ml/min	2.5 ml/min	5.0 ml/min	10 ml/min

To confirm the aqueous selenium exposure concentrations, 40 ml of test solution were collected from each exposure tank on a weekly basis. Samples were preserved with 1% HCL (instra-analyzed HCl 36.5-38%, J.T. Baker, Phillipsburg, NJ) in clean glass vials, and refrigerated until shipped to the analytical laboratory for analysis.

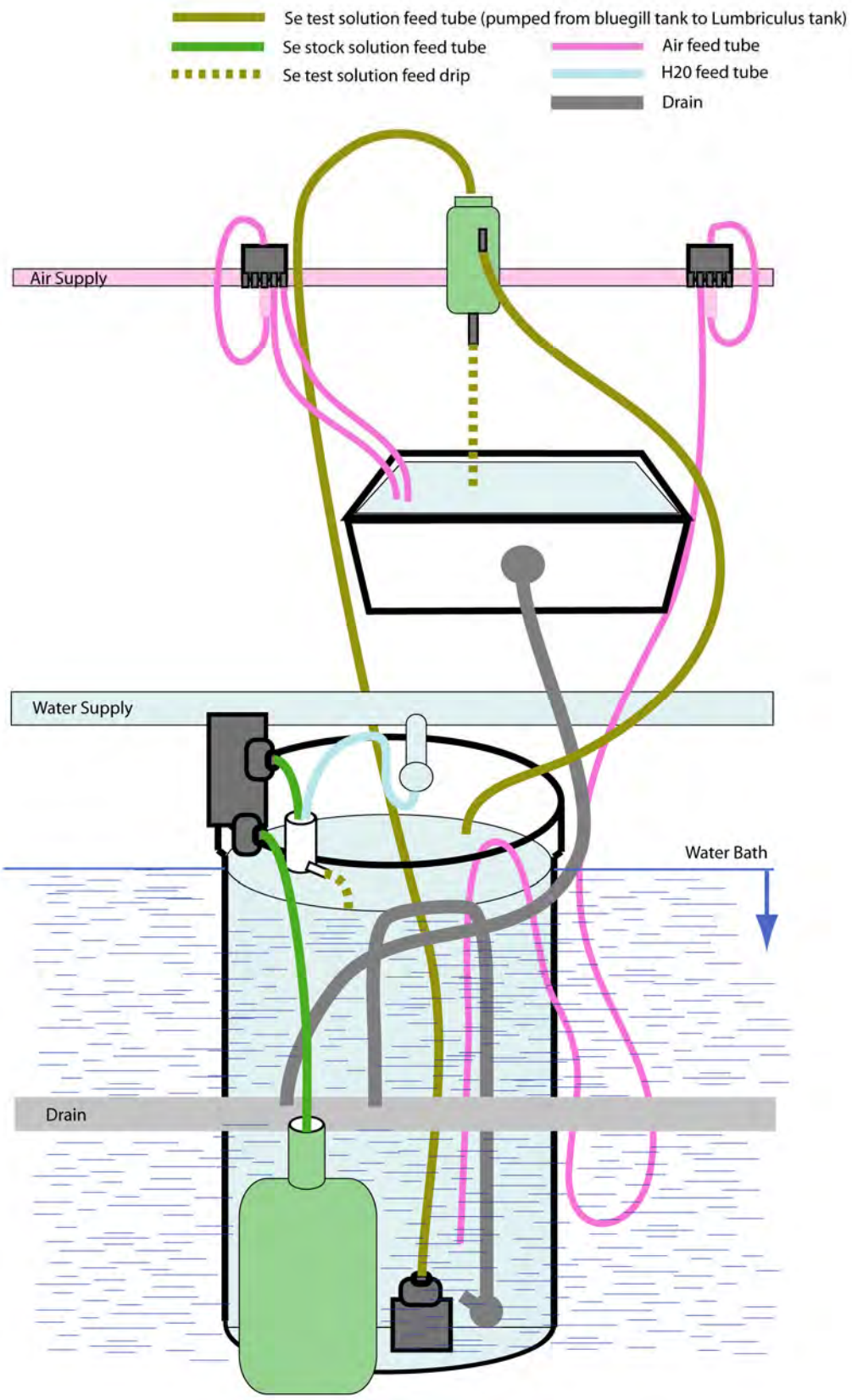


Figure 2.2. Tank system diagram for bluegill and *Lumbriculus* ES1 and ES3.

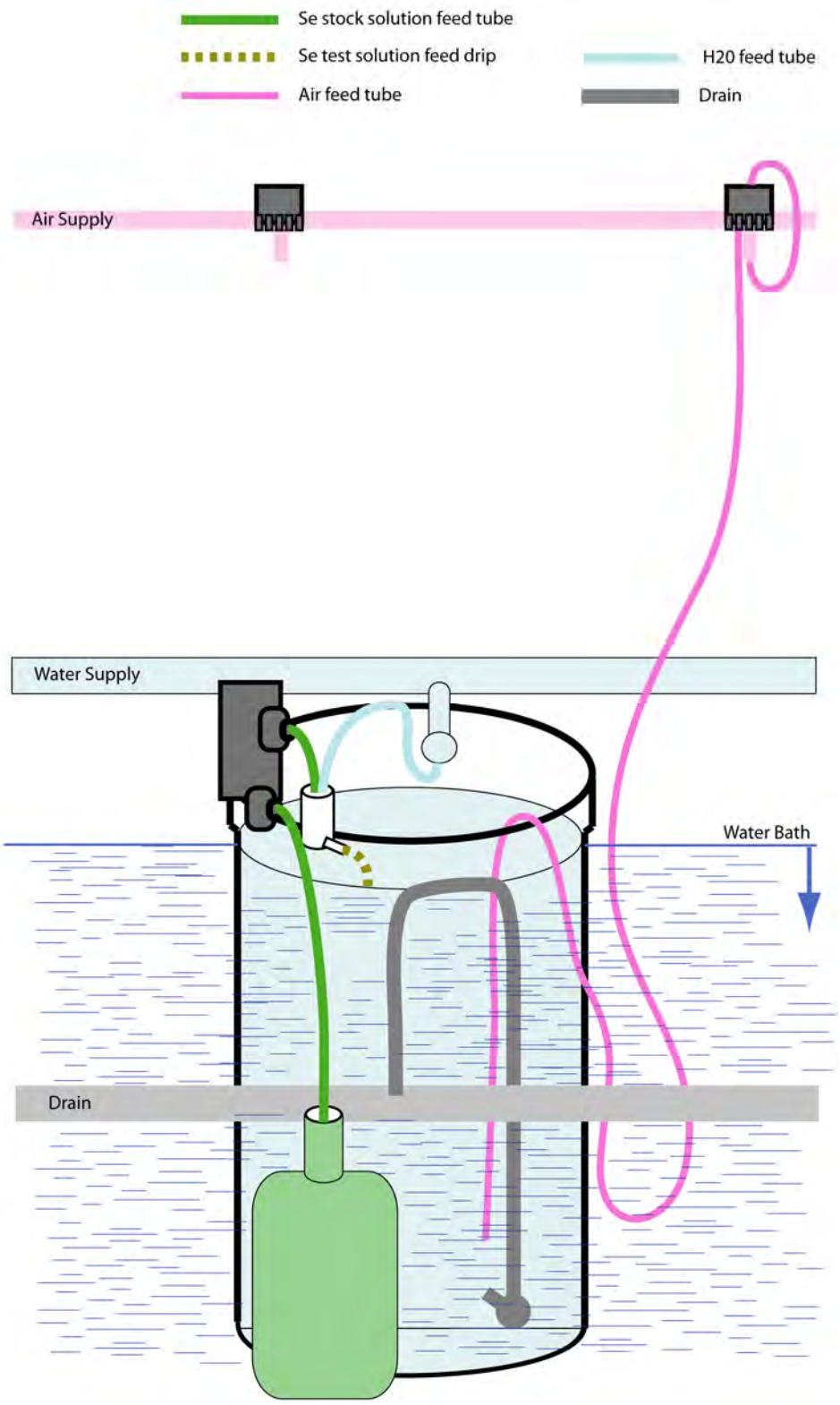


Figure 2.3. Tank system diagram for bluegill ES2.

2.2.2 Dietary Exposure

The fish in ES2 were fed a commercial fish flake food fortified with seleno-L-methionine, with the goal of achieving a nominal selenium concentration of 5 µg/g (dry weight), while the fish in ES1 and ES3 were fed *L. variegatus* which had accumulated selenium in their tissues. Selenium-spiked Tetramin fish flakes were prepared by adding 500 g of crushed fish flakes to 125 ml of a seleno-L-methionine (≥98%, Sigma-Aldrich, Lot# 016K1335) stock solution. The seleno-L-methionine stock solution was prepared by dissolving 62.06 mg of seleno-L-methionine in 1 liter of deionized water. The Tetramin flakes were finely ground using a glass mortar and pestal, and the seleno-L-methionine aqueous stock solution was added to achieve a moisture content of 25% (4.0 ug Se/g ww or 5 ug Se/g dw). The control food was prepared following the same procedures, except that deionized water without seleno-L-methionine was used to supply the 25% moisture. The crushed flakes and aqueous components were thoroughly mixed together to produce a paste. The dietary mixture was then weighed into aluminum pans in 5 gram aliquots, and compressed to form a cake. The TetraMin cakes were held in the freezer until they were needed. After preparation, three 5 g samples of the selenium-dosed and control TetraMin cakes were analyzed for total selenium. Four separate batches of selenium-dosed TetraMin cakes were prepared and used during the 182 day exposure. The dates and average measured total selenium (N = 3 or 4) for each batch were as follows: April 30 - May 17, 2007 (4.11 µg/g dw); May 18 – July 10, 2007 (5.77 µg/g dw); July 11 – September 6, 2007 (6.27 µg/g dw); and September 7 – October 29, 2007 (6.67 µg/g dw).

For ES2, the selenium-dosed TetraMin cakes were fed to the bluegills at a rate of 3% of their body weight (wet weight) per day, based on survival and the average weight measurements taken on days 0, 7, 30, 60, and 110. Control and selenium-dosed TetraMin cakes were held frozen throughout the study. As with ES1 and ES3, fish behavior was observed while eating, and the weight of food provided to the fish was recorded on the data sheets.

L. variegatus were exposed to selenium to create the dietary source for the bluegill in exposure systems 1 and 3. The *L. variegatus* in the two control and six treatment exposure chambers were fed 3.2 g of yeast suspended in 250 ml of dechlorinated water, once a day. The yeast was suspended in the dechlorinated tap water by placing the 3.2 g in a 500 ml Erlenmeyer flask and adding dechlorinated tap water to a 250 ml volume. The contents in the flask were then vigorously swirled in the flask throughout the feeding process.

Control worms were fed non-selenized nutritional yeast (Red Star™) and treatment worms were fed a mixture of selenized yeast (measured to be 826 µg/g) with non-selenized nutritional yeast to achieve the desired dietary selenium exposure and dietary requirements. The nominal concentrations of total selenium in the 6 selenized yeast preparations were 1.7, 3.3, 6.7, 13.3, 26.7, and 53.5 µg/g dw. These target concentrations were based on similar *Lumbriculus* exposures with selenized yeast (Besser et al. 2006) and confirmed in preliminary studies. The two yeast components were weighed on

calibrated scales, and combined in four liter HDPE Nalgene containers. The yeast combinations were initially mixed three separate times, at half-hour intervals, on a mechanical roller. Prior to test initiation, one 15 gram yeast sample from each dietary concentration was shipped to the analytical laboratory and analyzed for total selenium. To ensure consistency in the mixtures over time, each week of the study the yeast preparations were again mixed for a half hour on the mechanical roller.

The fish were fed *L. variegatus* at a rate of 4% of their body weight (wet weight) per day, based on survival and the average weight measurements made on days 0, 30, 60, and 112. A mass of worms was isolated, minus any overlying water, and held in a 100 glass mL beaker. Foreign material (yeast, algae, waste) was removed, excess water decanted, and the worms were weighed. Observations of the feeding activity of the bluegills while eating were made, and the weight of worms fed was recorded. Fish tanks were siphoned in the late afternoon to remove excess food and fecal matter.

Prior to initiating the study, the actual concentrations of selenium in the dietary samples (yeast, worms, and TetraMin cakes) were measured. During the study, both the worms and fish were fed after monitoring the morning flow rates and measuring the water quality characteristics in the overlying water. The average concentrations of total selenium measured in the worms sampled on days 0, 30, 60, 112 and 182 are given in Table 3.5 in the Results and Discussion section.

2.3 WATER QUALITY MEASUREMENTS AND OBSERVATIONS

During the 182-day study, observations on water quality, stock and dilution water flow rates, and test organism behavior and mortality were recorded on a daily basis. Samples for analysis of total selenium concentrations in the water were collected on a weekly basis, and once a month the samples were analyzed to determine selenium speciation. Test days 0, 30, 60, 112, and 182 were designated to sample worm tissue, and on test days 0, 7, 30, 60, 112, and 182 fish were sampled. Duplicate 5 g samples of *Lumbriculus* were collected from each of the 12 worm treatment tanks and from one of the two control tanks in each system. Triplicate fish samples, with each sample consisting of a three fish composite, were collected from each of the 16 fish treatment tanks (i.e., a total of 9 fish per tank), and from one of the two control tanks in each system.

Fish and worms were homogenized prior to shipping for selenium analysis. Tissue samples (e.g., a 3-fish composite) were homogenized with 10 ml deionized water in pre-cleaned 250 ml nalgene bottles using a pre-cleaned stainless steel tissue homogenizer. The samples were blended until completely homogenized (appearance smooth with no visible masses). The blended samples were transferred to the pre-labled 40 ml glass sample vial and 15 ml of deionized water was used to rinse out the 250 ml nalgene bottle. All equipment was cleaned using one percent HCl and rinsed with deionized water in between homogenization of different tissue samples. The samples were processed in order from lowest to highest nominal selenium concentration.

Lipid content was measured in the bluegill on test day 0 and at test termination in each treatment. The method used was a standardized procedure developed by the EPA laboratory in Duluth, Minnesota. In summary, tissue samples were sequentially weighed, homogenized with an extraction solution of 3:2 hexane:isopropanol, centrifuged, and the supernatant solution decanted to a separatory funnel, where it is washed with a sodium sulfate solution. After the bottom aqueous phase was discarded, the organic phase was transferred to a 50 ml graduated cylinder fitted with a ground glass stopper, and the weight was measured and 5 ml duplicate aliquots of the lipid extract were pipetted to tared weighing pans. The pans were placed under a hood where the solvent was evaporated. The pans were transferred to a dessicator for removal of any remaining solvent and water. After 24 hours, the pans were weighed and the lipid content was calculated according to the following formula.

$$\%\,Lipid = \frac{(sample\ wt.)(sample\ vol./5ml)}{tissue\ wt.}$$

Oversight of the exposure systems included monitoring various overlying water quality characteristics (pH, dissolved oxygen, temperature, conductivity, and chlorine) on different days, and the flow rates of both the selenium stock solutions and dilution water twice a day.

Dissolved oxygen and temperature were measured daily in each *L. variegatus* and fish exposure tank. Dissolved oxygen was measured using a YSI 57 meter and an Orion probe. Temperature was measured two different ways; directly from each exposure tank using a digital hand-held thermometer with a stainless steel probe, and continuously at mid-depth in one tank in each exposure system using a submersible temperature data logger. An Orion 710 meter and probe was used to measure pH on Monday, Wednesday, and Friday in each *L. variegatus* and fish exposure tank. Conductivity was measured weekly in each bluegill exposure tank using a YSI 33 meter. The dechlorinated water head tank was monitored weekly for total chlorine, pH, dissolved oxygen, and conductivity. All meters were calibrated before each use, and the thermometer was calibrated at least every 2 weeks, or more frequently in the event of a planned temperature decrease.

Flow rates of the stock solutions and dilution water in the bluegill tanks were measured twice a day, approximately 12 hours apart. The treatment water from the bluegill exposure chambers was pumped to the worm exposure chambers to supply the aqueous selenium exposure for the worms. The worms therefore received the same aqueous exposure of selenium as the fish to which they were fed. The target flow rate from the bluegill tanks to the worm exposure chambers was 60 ml per minute. The target flow rate from the head tank and stock solution reservoir to the 200 L bluegill treatment tanks was 500 ml per minute, resulting in approximately 3.4 turnovers a day. Stock solutions were dispensed using a fluid metering pump (FMI), and the dilution water was delivered by gravity from the temperature controlled head tank.

2.4 DETERMINATION OF TOTAL SELENIUM IN WATERS AND TISSUES BY HYDRIDE GENERATION-ATOMIC FLUORESCENCE SPECTROMETRY (HG-AFS)

2.4.1 Fish and Worm Tissue Digestion

Dry weight determination: Vials containing suspensions of homogenized tissues in water were shaken vigorously and 5 ml aliquots pipetted into a pre-weighed aluminum trays. Samples were dried in an oven at 80°C for 24 hours, removed and placed in a desiccator for one hour (to cool down without drawing water from the atmosphere) prior to weighing on an analytical balance. Duplicate measurements were performed approximately every ten samples. Dry weight was calculated as the difference between the dry sample and the empty tray, and water content was calculated as the weight loss during drying.

Nitric acid digestion: Vials containing suspensions of homogenized tissue in water were shaken vigorously and 3.5 ml aliquots pipetted into new 40 ml I-Chem vials. 10 ml of concentrated nitric acid (Fisher, 16 M) was added, and samples were covered with a marble and digested on a hot plate set at 150°C for 1.5 hours. Digested samples were then allowed to cool and filled up to the mark on the vial (40 ml) with milliQ water. Each digestion batch also contained 3 blank samples, 3 spiked blank samples (1 each spiked with selenite (Se(IV)), selenate (Se(VI)) and selenomethionine (SeMet), and a certified reference material for each type of tissue present. One of every ten samples was prepared with a QC set: duplicate, matrix spike (spiked with SeMet) and a matrix spike duplicate.

2.4.2 Reagents

Reagent Blank (40% conc. HCl): 1 L of reagent grade concentrated HCl (Fisher) was added to 1.5 L milliQ water and inverted to mix. The resultant mixture is 4.8 M HCl.

Reductant (1% KBH_4 w/v in 0.4% NaOH): 16 g of 50% w/w NaOH (VWR) were added to approximately 1800 ml milliQ water and swirled to mix. 20 g of potassium borohydride (KBH_4, Aldrich) was added and swirled to dissolve powder completely, before the solution was filled to the 2 L mark with milliQ water.

Potassium persulfate (2% w/w): 0.6 g potassium persulfate ($K_2S_2O_3$, Fisher) was placed into a 40 ml I-Chem vial (same vial reused) and milliQ water was added to 30 g. The solution was shaken vigorously to dissolve the $K_2S_2O_8$.

Selenium standards: Working standards were prepared by diluting 1000 mg/L solutions of Se(IV), Se(VI) and SeMet to 1000 µg/L (50 µl to 49.95 ml milliQ), which were then further diluted to 100 µg/L (1 ml to 9 ml milliQ).

Certified reference materials: National Water Research Institute TM-DWS (30.6 µg/L Se) river water was used for calibration validation and as a water sample CRM. NIST

1566b Oyster Tissue (2.06 µg/g Se) was used as a CRM for worm tissues and NRC DORM-2 Dogfish Muscle Tissue (1.40 µg/g Se) was used as a CRM for fish tissues. NRC SELM-1 Selenium Enriched Yeast (2059 µg/g Se) was used as a CRM for yeast samples.

2.4.3 Sample Analysis

Sample preparation: All samples being analyzed were 40% HCl to match the calibration standards and not disrupt the function of the continuous flow HG-AFS. Sample preparation involved pipetting 12 ml of the sample into a conical flask (or, for diluted samples, less volume and the balance to 12 ml DI water); then, 8 ml of conc. HCl were added for a total volume of 20 ml. This results in a minimum dilution factor of 1.667x. For dilutions greater than 200x, serial dilutions were used, with intermediate steps prepared in Sarstedt tubes with milliQ water. All tissue samples were diluted at least 20x, in order to dilute the nitric acid introduced in the digestion step to the point where interferences with the HG procedure were eliminated.

Prereduction/oxidation step: Flasks containing properly diluted samples were weighed on a top-loading balance (to 0.01 g) and the mass recorded. 200 µl of potassium persulfate solution was added to the sample, which was then placed on a hot plate set at 200°C. A timer set for 15 minutes was started when the first sample on the hot plate began boiling, and samples were removed when the timer finished. This step was done in batches of approximately 10 samples at a time. Samples were allowed to cool prior to analysis. After HG-AFS analysis, this procedure yields total Se concentrations.

Selenium speciation analyses: For direct determination of Se(IV) in water samples, samples were measured without prereduction/oxidation. The concentration of Se(VI) was then calculated by difference between total Se and Se(IV), assuming that no other Se species besides Se(IV) and Se(VI) were present in the waters (which matches the way they were prepared).

Hydride Generation Atomic Fluorescence Spectroscopy (HG-AFS): A peristaltic pump was utilized to introduce reagent blank or sample into a gas-liquid separator at a rate of 10 ml min^{-1}, and combined with the reductant 5 ml min^{-1}. The mixing of highly acidic reagent/sample and reductant generates hydrogen gas and selenium hydride, SeH_2. This species is highly volatile and is swept into the AFS unit (Excalibur, P.S. Analytical) with argon as a carrier gas (300 ml min^{-1}, measured using a ball flow meter). 50 µl of *n*-octanol was added to the gas-liquid separator as a surfactant to smooth the HG process and reduce water droplet introduction to the AFS. The hydrogen gas was ignited to form a continuous flame for the duration of the analysis. Selenium passing through the flame was irradiated with a Photron hollow cathode lamp, and the intensity of fluorescence was then measured and recorded on a Hadley Tekscience printer. The lamp primary current was set to 20 mA and the boost to 25 mA. The intensity of fluorescence is proportional to the Se concentration in a sample, and peak heights could therefore be used to determine Se concentrations using a calibration curve.

Instrument Calibration: A calibration curve was made up using standards with concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 µg/L Se(IV) and no prereduction. An initial calibration validation was performed using a second Se(IV) standard and TM-DWS CRM again with no prereduction step. Coefficients of determination were always >0.995 and were >0.999 most of the time. A continuous calibration standard of approximately 1 µg/L Se(IV) was analyzed intermittently to track any sensitivity changes in the instrument due to lamp power, etc., and all analytical results were corrected for instrument drift.

Quality Assurance/Quality Control: For both water and tissue samples, prereduction/oxidation blank values were determined and subtracted from all samples. Blank spikes of Se(IV), Se(VI) and SeMet (1 µg/L) were analysed to determine recoveries of these species of Se, and were often several percent higher than non-prereduced samples. For tissue sample analyses, digestion blanks were quantified and subtracted proportionally to dilution from all samples. Digestion blank spikes were also analysed to assure quantitative recoveries following digestion. Certified reference materials suitable to the sample matrix were also analysed.

During analysis, quality assurance tests were conducted every 10-15 samples. For tissue digests, this included three extra vials of digested tissue: a duplicate sample aliquot and two more sample aliquots spiked with SeMet at levels 2-5x the expected value of Se to assess reproducibility and completeness of tissue digestion. Duplicate analysis of one of these vials was conducted as well as a SeMet spike added just prior to the prereduction step, performed in duplicate, to assess analytical reproducibility and accuracy. QA water samples were analysed in duplicate, as well as duplicate analyses of the same sample with 1 µg/L Se (as 50:50 Se(IV):Se(VI)) matrix spikes. Continuous calibration validation using Se(IV) without prereduction was performed every 5-10 samples in order to correct for changing sensitivity of the instrument.

3.0 RESULTS AND DISCUSSION

3.1 WATER QUALITY MEASUREMENTS

The water quality parameters measured in each tank (pH, dissolved oxygen, and conductivity) were within acceptable levels for toxicity tests, and remained consistent between treatments and throughout the 182 day exposure period (Table 3.1, Appendix A).

3.1.1 Temperature Measurements

The water temperatures measured in fish tanks generally followed the two temperature regimes targeted in the study design (Figures 3.1 – 3.3; Appendix A). Values from daily manual measurements in each tank were in agreement with records from the continuous logging probe. Average temperatures were within ± 0.5°C of the target temperatures in ES3, but 0.6 to 0.7°C higher than the targets in ES1 and ES2. The average and standard deviation of water temperatures, measured daily, for the target 4°C (day 80 through 182) ES1 tanks were 4.7°C and 0.25°C, and 4.6°C and 0.25°C in ES2 tanks.

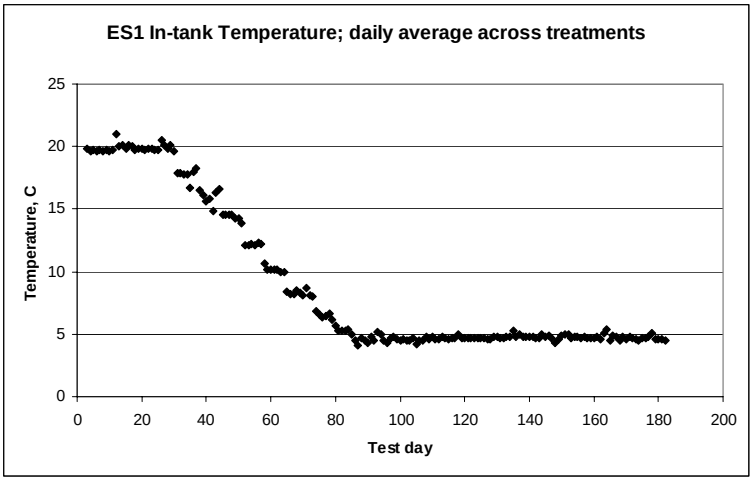


Figure 3.1. Daily average temperatures measured in each bluegill tank in Exposure System 1. Temperatures were averaged across treatments.

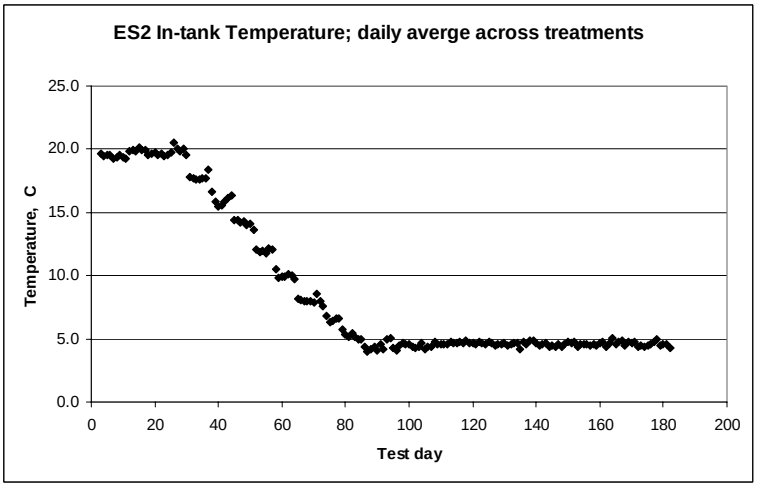


Figure 3.2. Daily average water temperatures measured in each bluegill tank in Exposure System 2. Temperatures were averaged across treatments.

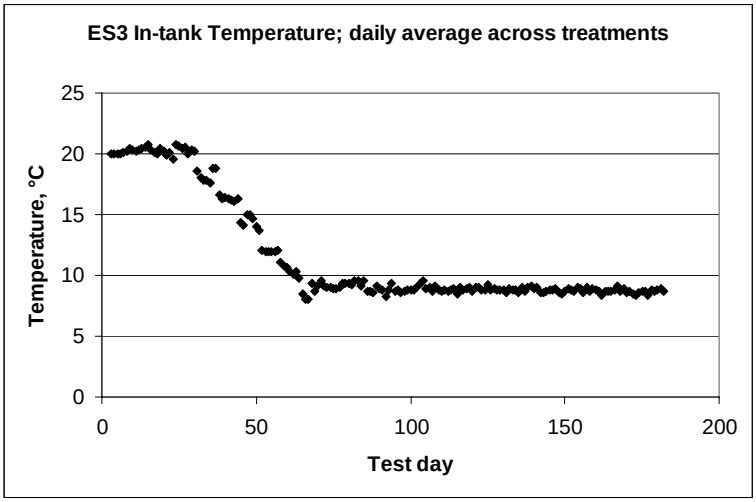


Figure 3.3. Daily average water temperatures measured in each bluegill tank in Exposure System 3. Temperatures were averaged across treatments.

Table 3.1. Average and range of pH, dissolved oxygen and conductivity in each tank. The pH and dissolved oxygen were measured daily. Conductivity was measured once a week in each tank during the 182 day bluegill study.

		pH, S.U.			Dissolved Oxygen, mg/L			Conductivity, µmhos/cm		
System	Treatment	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum
ES1	Control A	8.03	7.69	8.30	9.9	8.0	12.3	228	192	259
ES1	Control B	8.02	7.56	8.26	9.9	7.9	12.2	229	193	258
ES1	1	8.08	7.70	8.87	10.1	8.0	12.3	230	196	272
ES1	2	8.07	7.71	8.72	10.2	8.0	12.3	233	194	275
ES1	3	8.07	7.73	8.65	10.2	7.8	12.4	232	193	271
ES1	4	8.08	7.74	8.63	10.2	8.0	12.2	232	196	271
ES1	5	8.07	7.75	8.44	10.2	8.0	12.2	233	190	275
ES1	6	8.01	7.60	8.29	9.6	8.0	12.1	252	225	297
ES3	Control A	7.95	7.45	8.26	9.8	7.9	12.0	232	207	278
ES3	Control B	7.99	7.62	8.21	9.8	8.1	12.0	233	207	263
ES3	1	8.02	7.63	8.22	9.9	8.2	12.1	238	206	274
ES3	2	8.04	7.62	8.20	10.0	8.0	12.1	236	205	276
ES3	3	8.05	7.58	8.24	10.0	8.1	12.0	238	197	275
ES3	4	8.04	7.68	8.26	10.0	8.0	11.8	235	206	275
ES3	5	8.03	7.62	8.24	10.0	7.9	12.0	240	215	275
ES3	6	8.00	7.67	8.12	9.8	8.0	11.4	247	218	274
ES2	Control A	8.00	7.53	8.21	9.9	8.0	11.8	226	195	275
ES2	Control B	7.96	7.11	8.23	9.9	8.2	11.9	227	193	275
ES2	5A	8.00	7.46	8.20	10.1	8.1	12.2	228	197	274
ES2	5B	8.00	7.61	8.16	10.0	7.4	12.1	226	195	272

3.2 SELENIUM MEASUREMENTS

3.2.1 Selenium in Water

Total selenium concentrations in the bluegill tanks were similar to the target exposure concentrations (Table 3.2A; Appendix B). Measured concentrations were within 10% of the target concentrations with the exception of ES3 Treatments 1 and 2, and ES1 Treatment 1, which were within 12, 12 and 22% of the target concentrations, respectively. As a consequence of a technical error on day 154 of the study, the concentrations of selenium in the bluegill tanks for the last four weeks of the exposure period were negatively affected. On test day 154, the bottles containing the selenium stock solution were mislabeled with the incorrect stock solution concentration. The result of the mislabeling produced low selenium concentrations in all the bluegill tanks from day 154 through the end of the study, day 182. The average selenium concentrations in the water during this 4-week period was reduced across all treatments, from near target concentrations to less than 1 µg/L in ES2 and Treatments 1 through 3 in ES1 and ES3. The average selenium concentration during last 4 weeks in Treatment 4 (both ES1 and ES3) was less than 2 µg/L; in ES1 Treatment 5, 2.3 µg/L; and in ES3 Treatment 5, 7.9 µg/L.

A summary of the average measured total selenium concentrations for the entire182 day exposure shows the aqueous exposure concentrations remained similar to target levels (Table 3.2B). The effect of this mistake is not considered meaningful for three main reasons: (1) the drop in selenium's aqueous exposure was limited to the last four weeks (15%) of the 26 week exposure; (2) the primary route of selenium exposure to the bluegill is through the diet for which target levels of selenium were maintained throughout the exposure period; and (3) the effects concentrations of most interest are expressed in terms of fish tissue concentrations, not as water concentrations.

Table 3.2A. Nominal and measured total selenium concentrations for all treatments. Average concentrations are based on weekly samples collected up to test day 154 of the exposure period.

		[Se] in water, µg/L through Day 154		
			Measured	
System	Treatment	Nominal	Average	Std. dev.
ES1	Control B	No added Se	0.21	0.11
ES1	1	1.25	1.52	0.32
ES1	2	2.5	2.61	0.73
ES1	3	5	5.44	0.50
ES1	4	10	9.66	1.22
ES1	5	20	20.3	2.6
ES1	6	40	41.4	6.0

		[Se] in water, µg/L through Day 154		
			Measured	
System	Treatment	Nominal	Average	Std. dev.
ES2	Control B	No added Se	0.21	0.12
ES2	5A	5	5.58	0.66
ES2	5B	5	5.61	1.35
ES3	Control B	No added Se	0.18	0.07
ES3	1	1.25	1.47	0.59
ES3	2	2.5	2.83	0.54
ES3	3	5	5.24	0.61
ES3	4	10	9.15	0.91
ES3	5	20	19.7	2.0
ES3	6	40	41.1	5.3

Table 3.2B. Nominal and measured total selenium concentrations for all treatments. Average concentrations are based on weekly samples collected throughout the 182 day exposure period.

		[Se] in water, µg/L		
			Measured	
System	Treatment	Nominal	Average	Std. dev.
ES1	Control	No added Se	0.19	0.12
ES1	1	1.25	1.32	0.55
ES1	2	2.5	2.26	1.08
ES1	3	5	4.70	1.82
ES1	4	10	8.47	3.14
ES1	5	20	17.6	6.9
ES1	6	40	41.4	6.0
ES2	Control	No added Se	0.23	0.19
ES2	5A	5	4.83	1.92
ES2	5B	5	4.85	2.23
ES3	Control	No added Se	0.17	0.07
ES3	1	1.25	1.28	0.71
ES3	2	2.5	2.45	1.05
ES3	3	5	4.70	1.63
ES3	4	10	7.95	3.07
ES3	5	20	18.0	5.2
ES3	6	40	41.1	5.3

The proportion of selenate to selenite in each bluegill tank remained similar to the target ratio of 1:1. Selenium speciation analysis of monthly water samples collected from each treatment resulted in an average ratio of 1.14:1 with a standard deviation of 0.31 (N = 84; 14 treatments and 14 monthly samples). The above speciation analysis was made by directly measuring selenite and then calculating the concentration of selenate by the difference between selenite and total selenium. This method was confirmed using direct measurement by Ion Chromatography Inductively Coupled Plasma (IC ICP) MS of both selenate and selenite in the test day 154 samples. The direct measurement of both species resulted in a ratio of 1.29:1 with a standard deviation of 0.29 (N = 9).

3.2.2 Selenium in Lumbriculus variegatus

The concentration of selenium in the worms in each of the treatments used to feed the bluegill in ES1 and ES3 varied somewhat over time (Tables 3.3 and 3.4, respectively; Appendix C). The average concentrations of the upper treatment levels (3 through 6) were within a factor of 1.5 of the target concentrations in the worms (Table 3.5). The average of measured selenium concentrations in the lowest two concentrations were between 2 and 3 times higher than the target levels.

To maintain a continuous supply of worms for feeding the bluegill, the population of *Lumbriculus* in ES1 and ES3 required supplementing three times during the 182 day exposure. A back-up culture of *Lumbriculus* was maintained in GLEC's main laboratory (i.e., not in the trailer) for the purpose of adding worms to the exposure system. The worms in the back-up culture were exposed to the same aqueous and dietary selenium treatments as in ES1 and ES3.

Table 3.3. Measured total selenium concentrations (µg/g dw) in *Lumbriculus variegatus* for all treatments in Exposure System 1.

Test	Treatment						
Day	Control	1	2	3	4	5	6
0	2.7	5.2	4.5	6.8	8.5	20.5	25.0
30	2.7	5.6	6.9	8.9	19.7	35.6	59.1
39*	2.4	2.6	3.3	3.8	5.8	10.0	16.9
60	2.5	4.2	5.9	8.5	12.3	35.7	44.6
85*	A	3.9	3.9	5.7	17.8	11.8	29.0
112	2.5	5.2	7.6	12.0	25.9	38.1	B
162*	1.7	4.5	5.9	7.5	12.2	33.2	B
182	1.9		4.4	6.6	11.1	20.6	B
Avg	2.3	4.5	5.3	7.5	14.2	25.7	34.9
SD	0.4	1.0	1.5	2.4	6.6	11.3	16.9

*Measurements made in back-up worms added to ES1 worm tanks.
A No sample collected.
B Treatment 6 discontinued due to complete mortality in bluegill tank.

Table 3.4. Measured total selenium concentrations (µg/g dw) in *Lumbriculus variegatus* for all treatments in Exposure System 3.

Test Day	Treatment						
	Control	1	2	3	4	5	6
0	2.0	3.9	4.8	7.0	11.3	16.4	38.4
30	2.4	5.7	5.0	8.2	13.7	35.1	63.5
39*	2.4	2.6	3.3	3.8	5.8	10.0	16.9
60	2.6	4.5	6.2	7.3	11.4	30.6	51.2
85*	A	3.9	3.9	5.7	17.8	11.8	29.0
112	2.7	5.5	6.9	8.7	29.0	36.3	81.3
162*	1.5	3.8	5.3	9.6	20.6	37.5	B
182	2.0	3.8	4.8	A	12.1	25.9	B
Avg	2.2	4.2	5.0	7.2	15.2	25.4	46.7
SD	0.4	1.0	1.2	1.9	7.1	11.3	23.5

*Measurements made in back-up worms added to ES3 worm tanks.
A No sample collected.
B Treatment 6 discontinued due to complete mortality in bluegill tank.

The worms from the supplementary culture were added to the *Lumbriculus* tanks in ES1 and ES3 on test days 39, 85 and 162. The concentration of selenium in the supplementary worms was measured just prior to addition to the test systems (see corresponding footnotes in Tables 3.3 and 3.4). Once added to the tanks the new worms joined the aggregations of the test system worms within a day. The concentrations of selenium in the supplementary worms were usually lower than the worms being maintained in the test system, but since all worms quickly co-mingled, the actual selenium concentrations being fed to the bluegill were assumed to be somewhere between the measured concentrations in the supplementary worms and the worms in the test system.

Table 3.5. Target and average measured total selenium concentrations in *Lumbriculus variegatus* for all treatments in Exposure Systems 1 and 3.

Treatment	Total Selenium in *Lumbriculus*, µg/g dw		
	Target	ES1 avg	ES3 avg
Control	Bkg	2.34	2.21
1	1.5	4.45	4.20
2	2.5	5.30	5.02
3	5	7.47	7.17
4	10	14.2	15.2
5	20	25.7	25.4
6	40	34.9	46.7

3.2.3 Concentrations of Selenium in Fish Tissues

Concentrations of selenium in fish tissues generally increased with exposure duration (Table 3.6; Appendix D). The asymptotic accumulation was modeled as

$$[Se]_{tissue} = a + (b (1 - \exp(-ct))) \qquad (I)$$

where t was the exposure time, expressed in days, and a, b, c were parameters estimated by nonlinear least squares regression (nls function in S-PLUS 6.2, Insightful Corporation). As the figures below illustrate, this model explained most of the variation in tissue concentrations of selenium over time.

Tissue concentrations of selenium in the ES1 control treatment averaged 2.11 µg/g, and varied little over time (Fig. 3.4). The average was 2.11 µg/g dw and the coefficient of variation (standard deviation/mean) was 0.083 (0.17/2.11). Changes in tissue concentrations of selenium over time in the control treatment of ES2 (Fig. 3.4) were larger than was the case for ES1. The average selenium concentration and coefficient of variation for the ES2 control were 1.85 µg/g and 0.23. Changes in tissue concentrations of selenium over time in the ES3 control treatment (average control tissue in ES3 was 2.20 µg Se/g) were also larger in ES3 (Fig. 3.4) than in ES1, yet the coefficient of variation (standard deviation/mean) for the ES3 control was only 0.17.

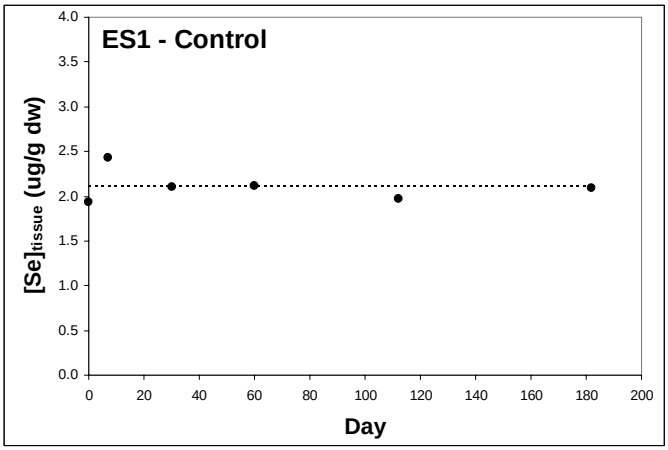
In ES1, measurements of tissue concentrations in fish exposed to the highest concentrations of selenium in the water (40 µg/L) and in diet (40 µg/g dw) were restricted to the first 60 days of exposure (Fig. 3.5) due to the high mortality of organisms in that treatment. Rates of selenium bioaccumulation in bluegill were higher in Treatment 6 than in Treatment 5 (Fig. 3.5). At day 7, tissue concentrations were 4.27 µg/g dw in Treatment 6 and 3.27 in Treatment 5. At day 60, concentrations of selenium in fish tissues increased to 8.62 µg/g dw in Treatment 5 and 12.66 µg/g dw in Treatment 6.

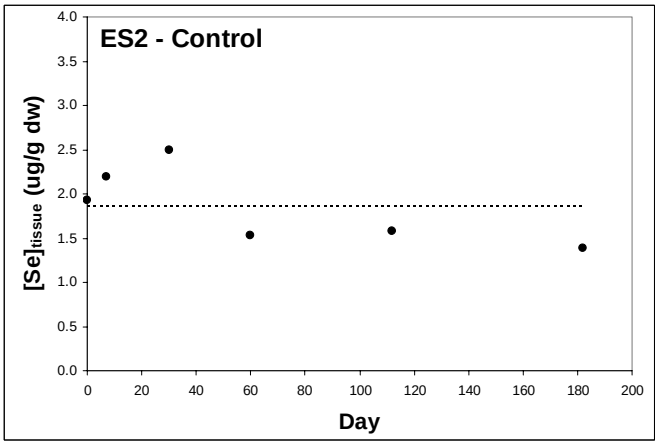
Bioaccumulation rates in fish exposed to lower concentrations of selenium in the water (≤10 µg/L) and in worms (≤10 µg/g dw) were consistently lower, as were the lower asymptotic concentrations of selenium in tissues (Fig. 3.5; Treatments 1 through 4). For instance, in Treatment 4 selenium concentrations reached 5.21 µg/g dw at day 60, 6.42 µg/g dw at day 112, and 6.72 µg/g dw at day 182. In Treatment 2 selenium concentrations reached 3.07 µg/g dw at day 60, 3.41 µg/g dw at day 112, and 3.15 µg/g dw at day 182 (Table 3.6).

In the two lowest ES1 treatments (1.5 and 2.6 µg/L in water, and 4.5 and 5.3 µg/g in worms), concentrations of selenium in bluegill tissues reached equilibrium at 2.8 µg/g dw in Treatment 1 and 3.2 µg/g dw in Treatment 2 after approximately 30 days of exposure (Fig. 3.5). In all other treatments, except Treatment 6 which was terminated early because of high mortality, tissue concentrations of selenium seemed to be approaching the asymptote at the end of the experiment.

Table 3.6. Measured total selenium concentrations in bluegill sunfish for all treatments in Exposure Systems 1, 2 and 3.

		Total Selenium in Whole Body Bluegill Tissue, µg/g dw						
ES1		Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
	Test Day	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)
	0	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)
	7	2.43 (0.31)	2.48 (0.11)	2.43 (0.18)	2.64 (0.06)	2.72 (0.07)	3.27 (0.27)	4.27 (0.44)
	30	2.10 (0.21)	2.85 (0.10)	3.10 (0.04)	2.94 (0.13)	4.24 (0.22)	6.62 (0.23)	10.21 (0.36)
	60	2.11 (0.02)	2.70 (0.20)	3.07 (0.05)	3.69 (0.25)	5.21 (0.30)	8.62 (0.45)	12.66 (0.45)
	112	1.98 (0.04)	3.16 (0.11)	3.41 (0.08)	3.99 (0.26)	6.42 (0.05)	11.60 (0.43)	
	182	2.08 (0.10)	2.56 (0.21)	3.15 (0.25)	4.02 (0.21)	6.72 (0.09)	10.71 (0.55)	
ES3		Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
	Test Day	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)	Average (SD)
	0	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)
	7	2.50 (0.10)	2.60 (0.29)	2.38 (0.10)	2.82 (0.20)	3.19 (0.33)	4.29 (0.20)	6.13 (0.62)
	30	2.24 (0.41)	2.44 (0.26)	2.70 (0.16)	3.13 (0.10)	3.95 (0.16)	6.06 (0.36)	11.07 (0.92)
	60	2.70 (0.22)	2.88 (0.08)	3.04 (0.39)	3.79 (0.24)	5.54 (0.21)	9.50 (0.91)	15.14 (0.96)
	112	2.16 (0.14)	2.49 (0.10)	3.10 (0.12)	3.64 (0.16)	6.54 (0.21)	11.50 (0.25)	17.24 (0.30)
	182	1.67 (0.21)	3.20 (0.27)	3.83 (0.47)	5.48 (0.24)	9.38 (0.63)	16.01 (0.30)	
ES2		Control	5A	5B				
	Test Day	Average (SD)	Average (SD)	Average (SD)				
	0	1.93 (0.21)	1.93 (0.21)	1.93 (0.21)				
	7	2.19 (0.19)	3.55 (0.25)	3.08 (0.50)				
	30	2.49 (0.15)	7.05 (0.76)	7.51 (1.18)				
	60	1.53 (0.03)	8.23 (1.55)	8.09 (0.67)				
	112	1.57 (0.01)	8.97 (1.28)	9.45 (1.73)				
	182	1.38 (0.06)	9.41 (1.63)	10.61 (0.38)				





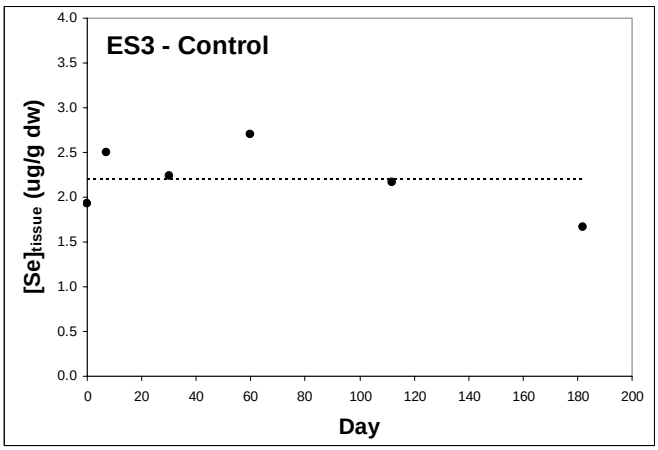


Figure 3.4. Concentrations of selenium in juvenile bluegill tissues over time of exposure in Controls for Exposure Systems (ES) 1, 2 and 3. The dotted line represents the average concentration.

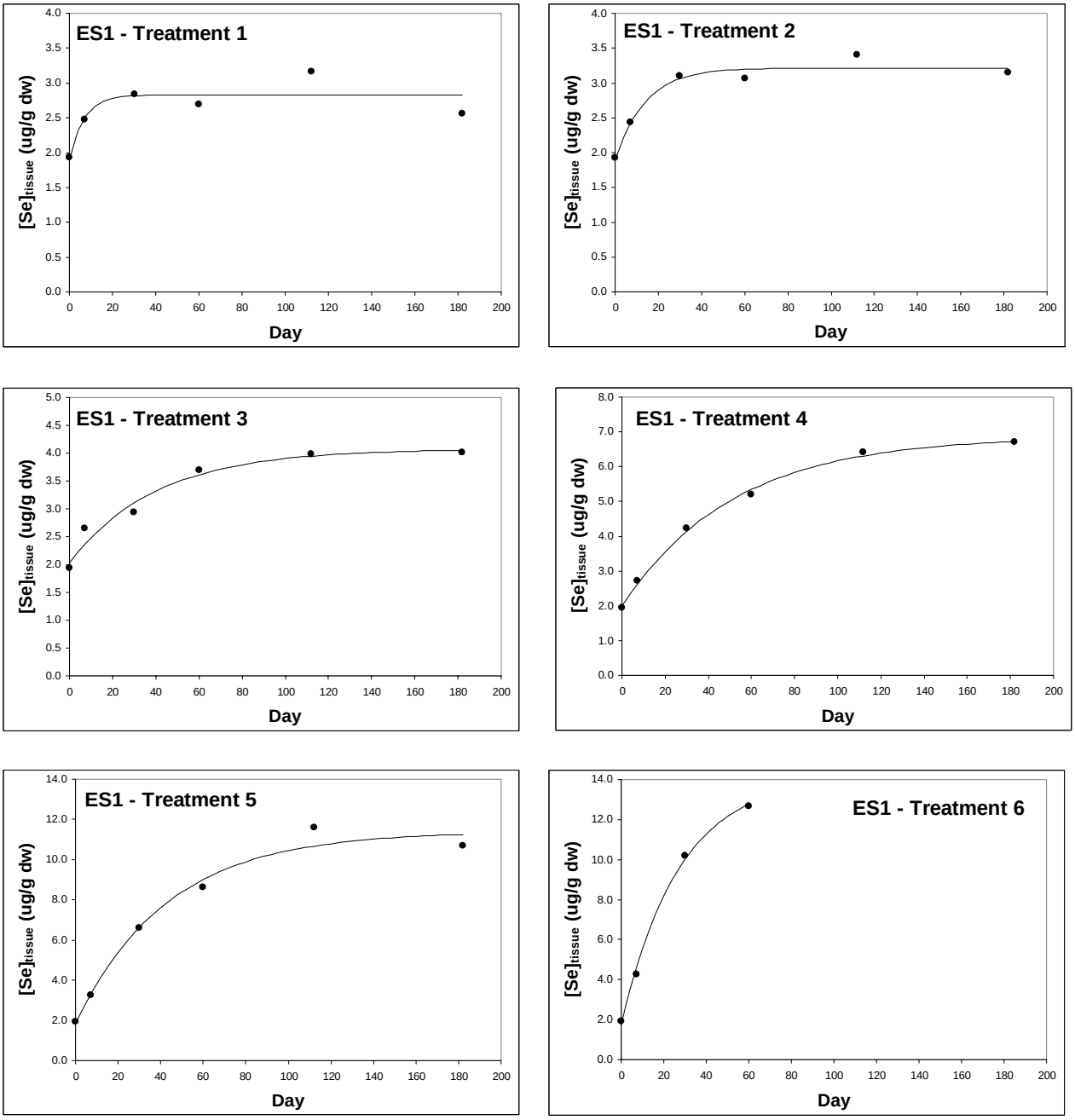


Figure 3.5. Concentrations of selenium in juvenile bluegill tissues over time of exposure in Exposure System 1 (ES1) Treatments 1 through 6. Dots represent measured values and the solid line represents projections from the fitted model (I):

Treatment 1: *a* = 1.9008, *b* = 0.9275, *c* = 0.1470
Treatment 2: *a* = 1.8944, *b* = 1.3218, *c* = 0.0715
Treatment 3: *a* = 2.0309, *b* = 2.0425, *c* = 0.0248
Treatment 4: *a* = 1.9839, *b* = 4.8816, *c* = 0.0194
Treatment 5: *a* = 1.8629, *b* = 9.5322, *c* = 0.0231
Treatment 6: *a* = 1.7580, *b* = 12.411, *c* = 0.0365

Up to day 112, concentrations of selenium in ES3 (20 → 9°C) fish were similar to tissue concentrations of selenium in corresponding treatments of ES1 (20 → 4°C). From day 112 to the end of the experiment on day 182, selenium accumulated in ES3 fish at faster rates (Table 3.6, Fig. 3.6). The difference in selenium accumulation between ES1 and ES3 fish during this period could be attributed to the decreased feeding observed in ES1 fish and the continued feeding by ES3 fish. On day 112, tissue concentrations of selenium in Treatment 1 were 3.16 µg/g dw for ES1 and 2.49 µg/g dw for ES3. On this day, tissue concentrations in Treatment 5 were 11.60 µg/g dw for ES1 and 11.50 µg/g dw for ES3. At the end of the experiment, tissue concentrations in Treatment 1 were 2.56 µg/g dw for ES1 and 3.20 µg/g dw for ES3. Concentrations of selenium in Treatment 5, were 10.71 µg/g dw for ES1 and 16.01 µg/g dw for ES3. At the end of the experiment, tissue concentrations in most ES1 treatments were increasing at slow rates. In contrast, tissue concentrations in most ES3 treatments were still increasing at fast rates at that time (day 182). In fact, for Treatments 5 and 4 (Fig. 3.6) it is not clear what would be an appropriate estimate for the asymptote. The observation that the bluegill are still accumulating selenium after 182 days of exposure in ES3 Treatments 1 through 5 cannot be explained by the variability in selenium concentrations in their diet (*Lumbriculus*). Although there was some variability in selenium levels in the worms, there was no apparent increase in concentration during the latter half of the exposure period (Tables 3.3 and 3.4).

Just as in ES1 (4°C), the bioaccumulation rates in fish in ES3 (9°C) exposed to lower concentrations of selenium in the water (≤10 µg/L) and in worms (≤10 µg/g dw) were consistently lower as were the asymptotic concentrations of selenium in tissues (e.g., Fig. 3.6). For instance, in Treatment 4 selenium concentrations reached 5.54 µg/g dw at day 60, 6.54 µg/g dw at day 112, and 9.38 µg/g dw at day 182. Selenium concentrations in Treatment 2 reached 3.04 µg/g dw at day 60, 3.10 µg/g dw at day 112, and 3.83 µg/g dw at day 182 (Table 3.6).

The accumulation of selenium approached steady-state in the bluegill exposed to ES2 Treatments 5A and 5B (Figs 3.7a and b). Although the solid line projection in Figures 3.7a and b indicate steady-state was reached, the point measurements on test days 120 and 180 show a gradual increase in the selenium tissue concentration, that is likely due to the progressively higher concentrations of selenium in the TetraMin fed to the bluegill. As described in the Methods section, four batches of the selenium-spiked TetraMin were fed to the bluegill in the 182-day study (test days 0-17, 4.11 µg/g; test days 18-71, 5.77 µg/g; test days 72-129, 6.27 µg/g; and test days 130-182, 6.67 µg/g). It is likely steady-state would have been reached if the dietary selenium concentration was constant.

Tissue concentrations of selenium in the ES2 Treatment (nominal 5 µg/L in water and 5 µg/g dw in the TetraMin) were far higher than tissue concentrations in comparable exposures in ES1 and ES3 Treatment 3 (nominal 5 µg/L in water and 5 µg/g dw in worms). At the end of the experiment, tissue concentrations in Treatment 3 of ES1 and ES3 reached 4.0 and 5.5 µg/g dw, respectively, and 9.4 and 10.6 µg/g in Treatment 5A and 5B of ES2, respectively.

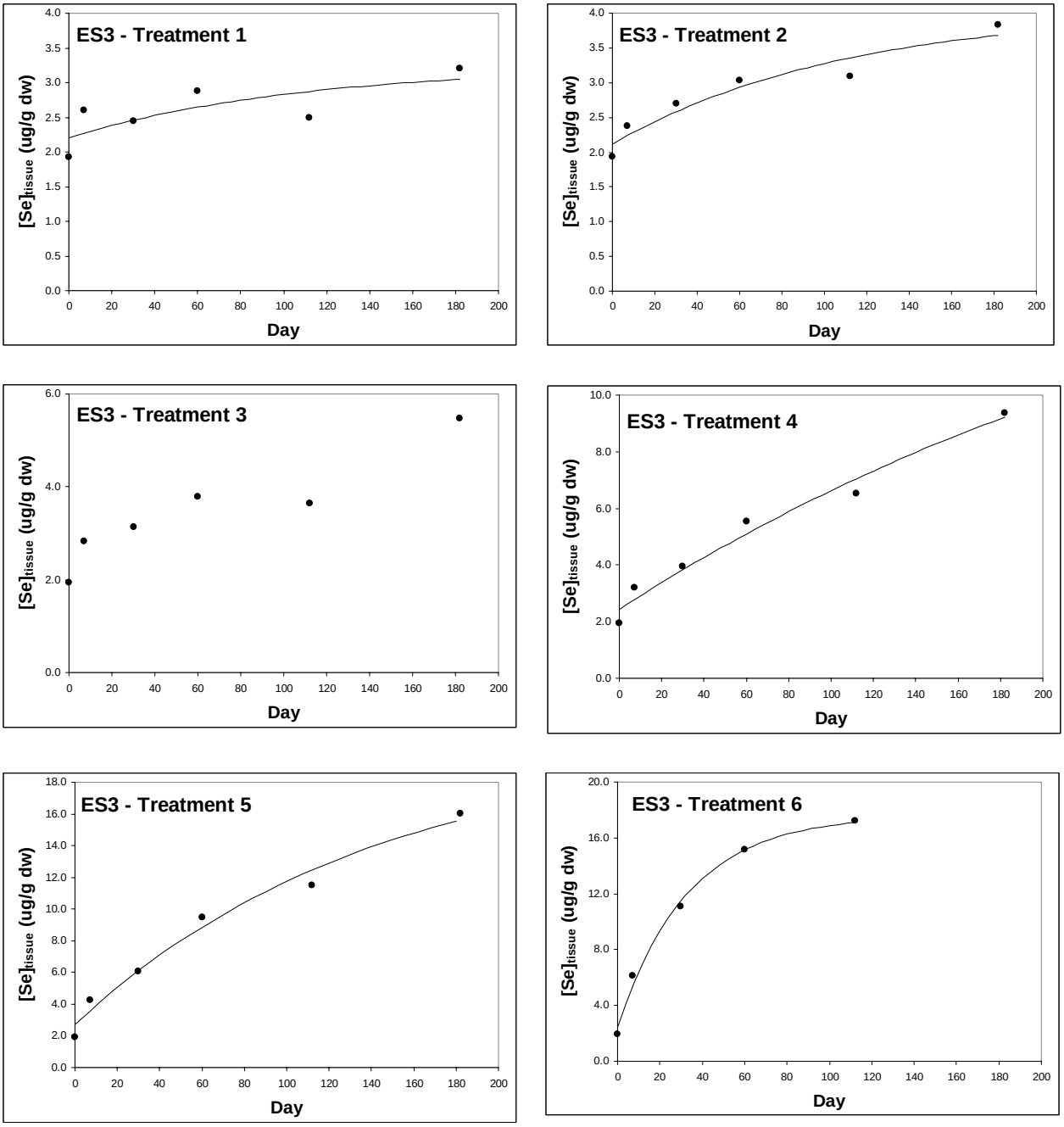


Figure 3.6. Concentrations of selenium in juvenile bluegill tissues over time of exposure in Exposure System 3 (ES3) Treatments 1 through 6. Dots represent measured values and the solid line represents projections from the fitted model (I):

Treatment 1: *a* = 2.2066, *b* = 1.0280, *c* = 0.0093
Treatment 2: *a* = 2.1157, *b* = 1.9311, *c* = 0.0092
Treatment 3: no model line fitted; no convergence in estimates of parameters
Treatment 4: *a* = 2.4325, *b* = 15.831, *c* = 0.0031
Treatment 5: *a* = 2.6954, *b* = 17.938, *c* = 0.0070
Treatment 6: *a* = 2.3920, *b* = 15.211, *c* = 0.0303

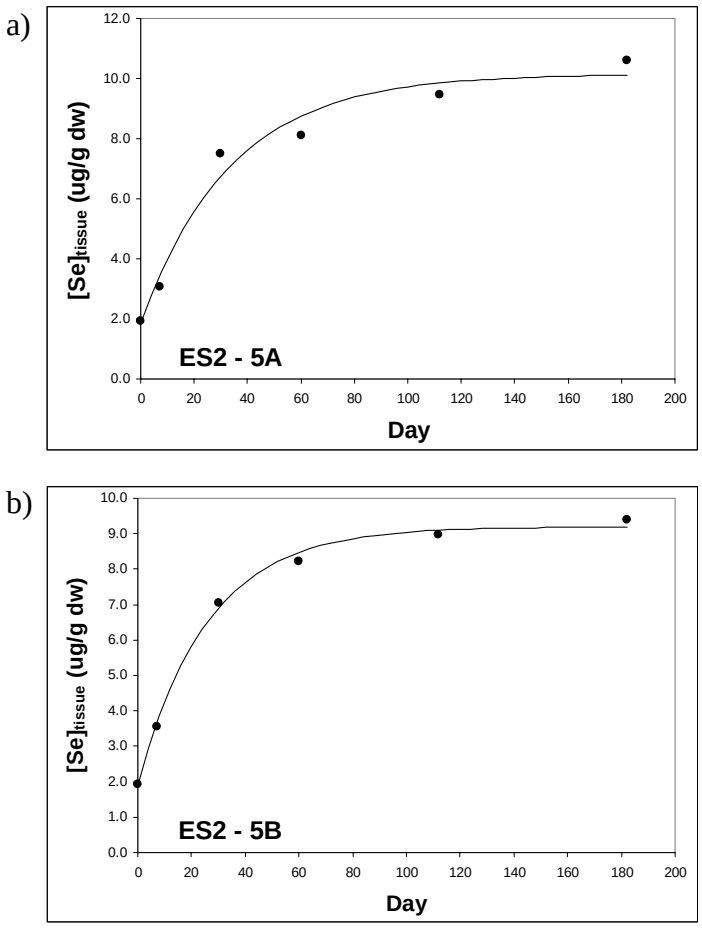


Figure 3.7. Concentrations of selenium in juvenile bluegill tissues over time of exposure. Dots represent measured values and the solid line represents projections from the fitted model (I): a) *a* = 1.8437, *b* = 8.3022, c = 0.02973; b) *a* = 1.9114, *b* = 7.2843, *c* = 0.03848.

The higher bioaccumulation in the ES2 exposure system was apparently due to the form of selenium to which the fish were exposed. The ES2 fish were fed a commercially prepared fish food, TetraMin, to which seleno-L-methionine was added. ES1 and ES3 fish were fed worms which accumulated selenium by ingesting selenized-yeast. Preliminary investigations of the specific forms of selenium in the worms fed selenized-yeast and TetraMin spiked with selenomethionine show selenomethionine was the dominant soluble species in both diets. The TetraMin also contained trace amounts of selenate and selenite, but selenomethionine was 76% of total selenium after mineralization with nitric acid. The soluble selenium in the worms consisted of 71% selenomethionine, 19% selenocystine and approximately 5% selenite and 5% selenate. The soluble fraction of selenium in the worms, however, was only 15% of the total selenium indicating a large part of the selenium in the worms was proteinaceous and less bioavailable. This large portion of insoluble selenium in the worms was likely the reason less selenium was accumulated in ES1 and ES3 Treatment 3 relative to ES2.

3.3 SURVIVAL ANALYSIS OF JUVENILE BLUEGILL SUNFISH

Estimates of juvenile bluegill survival take into account the removal of individuals from the test population during the experiment. Individuals were removed for sampling tissue concentrations, or because they suffered accidental deaths unrelated to selenium toxicity. Removal of fish from the test reduces the number of individuals at risk of mortality due to selenium toxicity. The time when fish are removed (e.g., the number of days after the experiment started) is informative, because it reveals the period over which the removed fish remained alive. Ignoring removed fish will result in inaccurate estimates of survival (*S*). For instance, consider a hypothetical example where 100 fish are exposed to selenium for 300 days; 50 die due to selenium toxicity and 50 are removed the day before the test ends. Disregarding the time when fish are removed would lead to $S = 0.0$, while proper acknowledgment of fish removal time would result in $S \approx 0.5$.

At each of four sampling dates (day 7, 30, 60, and 112), nine juvenile bluegills were removed for measuring tissue concentrations of selenium. Therefore, over the duration of the experiment, 36 fish were removed from each tank (from a total of 100). The total number of fish removed from each tank ranged from 36 to 37 in ES1 (9 and 29 in controls), to 36 to 39 in ES3 (18 in controls). A mechanical malfunction on day 159 caused 23 deaths unrelated to selenium toxicity in Treatment 5 of ES1 (total number of fish removed: 36 + 23 = 59). The 23 fish were lost through the opening to the outflow pipe located at the bottom of the ES1 Treatment 5 tank due to the dislodging of the screen covering the opening.

If $r(t_i)$ is the number of individuals at risk just before time t_i and d_i is the number of deaths in the interval, $I_i = [t_i, t_{i+1})$, then survival (*S*) at time *t* can be estimated as

$$\hat{S}(t) = \prod \frac{r(t_i) - d_i}{r(t_i)} \qquad \text{(II)}$$

The product (Π) is calculated for each period in which one or more deaths occur. Equation (II) is the Kaplan-Meier estimator (Venables and Ripley 2002). It was used to calculate the proportion of survival in treatments with ten or more deaths (10% mortality). Confidence intervals for survival estimates were based on Greenwood's formula,

$$\text{var}\left(\hat{S}(t)\right) = \hat{S}(t)^2 \sum \frac{d_j}{r(t_j)\left[r(t_j) - d_j\right]}$$

Computations were performed with the survfit function in the R software version 2.6.0 (R development core team 2007).

Substantial mortality (>50%) was observed in treatments where tissue concentrations of selenium exceeded 11 μg/g, which only occurred in ES1 and ES3 Treatments 5 and 6 (Table 3.7). The timetables of deaths in these treatments, as well as respective estimates

of survival are presented in Tables 3.8-3.11. The survival curves for these treatments are illustrated in Figures 3.8-3.11. Juvenile bluegill survival in ES1 Treatment 6 was similar to survival in the corresponding ES3 exposure throughout the experiment, despite the fact that concentrations of selenium in fish tissues in the latter treatment were consistently higher, 15.1 vs. 12.7 µg/g at day 60 (the last measurement in ES1 Treatment 6). Survival of fishes in Treatment 5, though, was lower in ES3 than in ES1. Concentrations of selenium in tissues of these fish were similar up to day 112, at which time the differences in survival between the exposure systems were already pronounced (0.93 in ES1 vs. 0.63 in ES3). Mortality in other ES1 and ES3 Treatments (1 through 4) was very low (Table 3.7; Appendix E); mortality did not exceed seven in any tank over the entire duration of the experiment (182 days).

Table 3.7. Total number of deaths attributed to background mortality and selenium toxicity in each treatment of ES1, ES2, and ES3 (initial *N*=100) over the experiment's duration (182 days). All three exposure systems (ES1, ES2, ES3) had two control tanks. The ES2 treatment with a target diet concentration of 5 µg Se/g dw also had two replicates.

Treatment	ES1	ES2	ES3
Control (#1, #2)	0, 7	0, 0	1, 1
1	5		0
2	1		1
3	0	0, 2	0
4	3		3
5	24		38
6	68		61

The estimate of survival for control B in ES1 (S = 0.924) did not raise concerns about excessive mortality, because there was zero in control A. No deaths occurred in ES2 controls. In the ES2 Treatment, two fish died in Treatment 5B, and none in Treatment 5A.

Table 3.8. Timetable of deaths and respective estimates of fraction survival for ES1 Treatment 6. All survival values projected by the Kaplan-Meier estimator.

time (day)[a]	no. at risk	no. deaths	fraction survival	std error	low 95%CI	up 95%CI
43	82	4	0.951	0.024	0.906	0.999
44	78	9	0.842	0.040	0.766	0.924
45	69	2	0.817	0.043	0.738	0.905
46	67	2	0.793	0.045	0.710	0.885
47	65	1	0.781	0.046	0.696	0.875
51	64	2	0.756	0.047	0.669	0.855
55	62	1	0.744	0.048	0.655	0.845
56	61	1	0.732	0.049	0.642	0.834
57	60	2	0.707	0.050	0.615	0.813
58	58	4	0.659	0.052	0.564	0.770
59	54	3	0.622	0.054	0.525	0.736
60	51	2	0.598	0.054	0.500	0.714
61	40	3	0.553	0.056	0.453	0.674
62	37	1	0.538	0.056	0.438	0.660
63	36	3	0.493	0.057	0.393	0.619
65	33	11	0.329	0.056	0.236	0.458
66	22	1	0.314	0.055	0.222	0.443
68	21	1	0.299	0.055	0.209	0.427
72	20	2	0.269	0.053	0.183	0.396
73	18	3	0.224	0.050	0.145	0.347
74	15	3	0.179	0.046	0.108	0.297
76	12	1	0.164	0.045	0.096	0.280
80	11	1	0.149	0.043	0.085	0.263
81	10	2	0.120	0.039	0.063	0.228
84	8	3	0.075	0.032	0.032	0.173

[a] Mortality counts were checked and recorded daily. The number at risk, number deaths and survival reflect the timing of the fish deaths. For example, on day 47, one fish was observed dead, no fish were observed dead on days 48 through 50 and 2 fish were found dead on day 51.

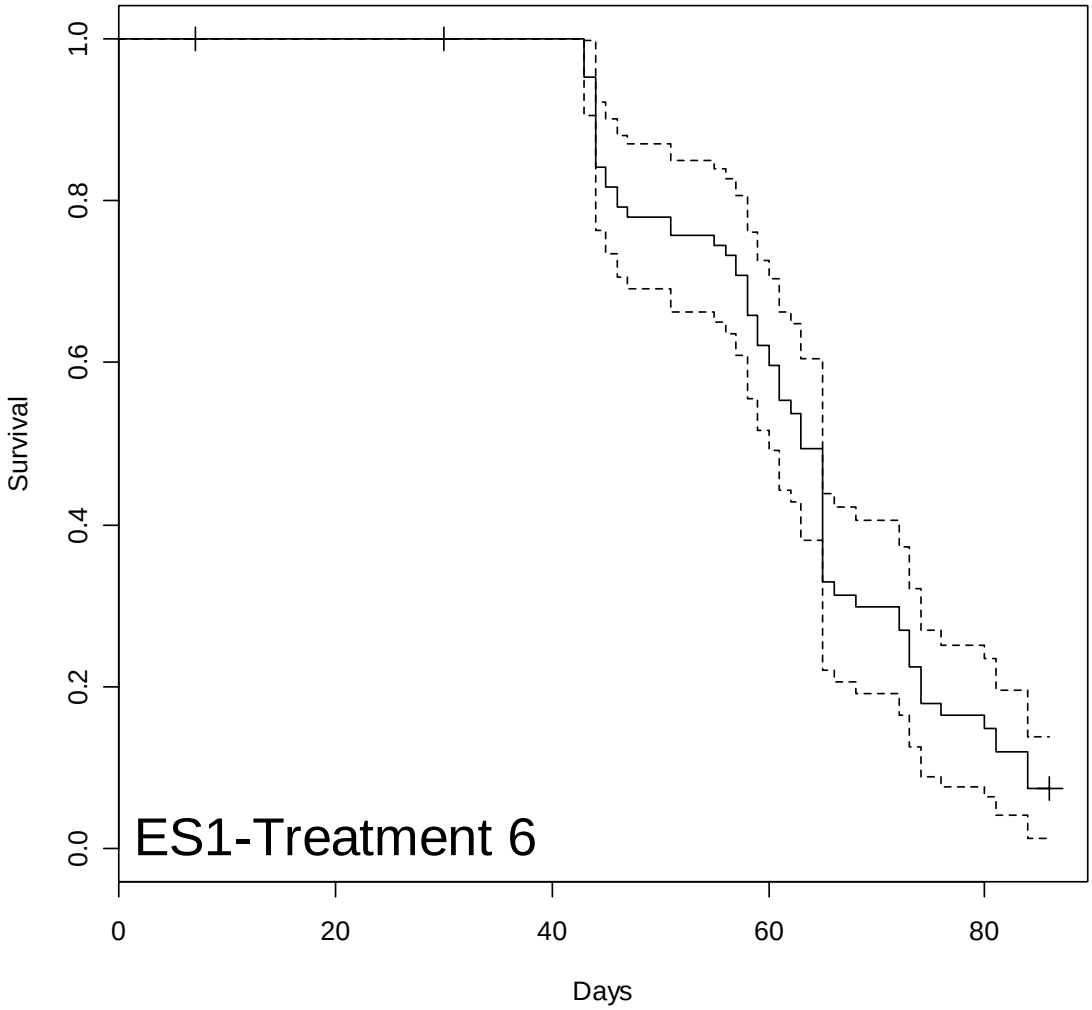


Figure 3.8. Survival curve of juvenile bluegill exposed to selenium. Dashed lines represent the 95% confidence interval for estimates of survival (solid line). The "+" sign indicates dates when data were censored.

Table 3.9. Timetable of deaths and respective estimates of fraction survival for ES1 Treatment 5. All survival values projected by the Kaplan-Meier estimator.

time (day)[a]	no. at risk	no. deaths	fraction survival	std error	low 95%CI	up 95%CI
90	73	1	0.986	0.014	0.960	1.000
93	72	1	0.973	0.019	0.936	1.000
94	71	2	0.945	0.027	0.894	0.999
95	69	1	0.932	0.030	0.875	0.991
130	59	1	0.916	0.033	0.853	0.983
150	58	1	0.900	0.036	0.832	0.973
153	57	1	0.884	0.039	0.811	0.963
155	56	1	0.868	0.041	0.791	0.953
157	55	3	0.821	0.047	0.734	0.919
158	52	2	0.789	0.050	0.697	0.894
160[b]	27	1	0.760	0.056	0.657	0.879
161	26	1	0.731	0.061	0.620	0.861
169	25	7	0.526	0.079	0.392	0.706
177	18	1	0.497	0.080	0.363	0.681

[a] Mortality counts were checked and recorded daily. The number at risk, number deaths and survival reflect the timing of the fish deaths. For example, on day 161, one fish was observed dead, no fish were observed dead on days 162 through 168 and 7 fish were found dead on day 169.

[b] The 23 fish accidentally lost from the tank due to the screen from the outflow tube being dislodged were accounted for using the Kaplan-Meier estimator (Equation II).

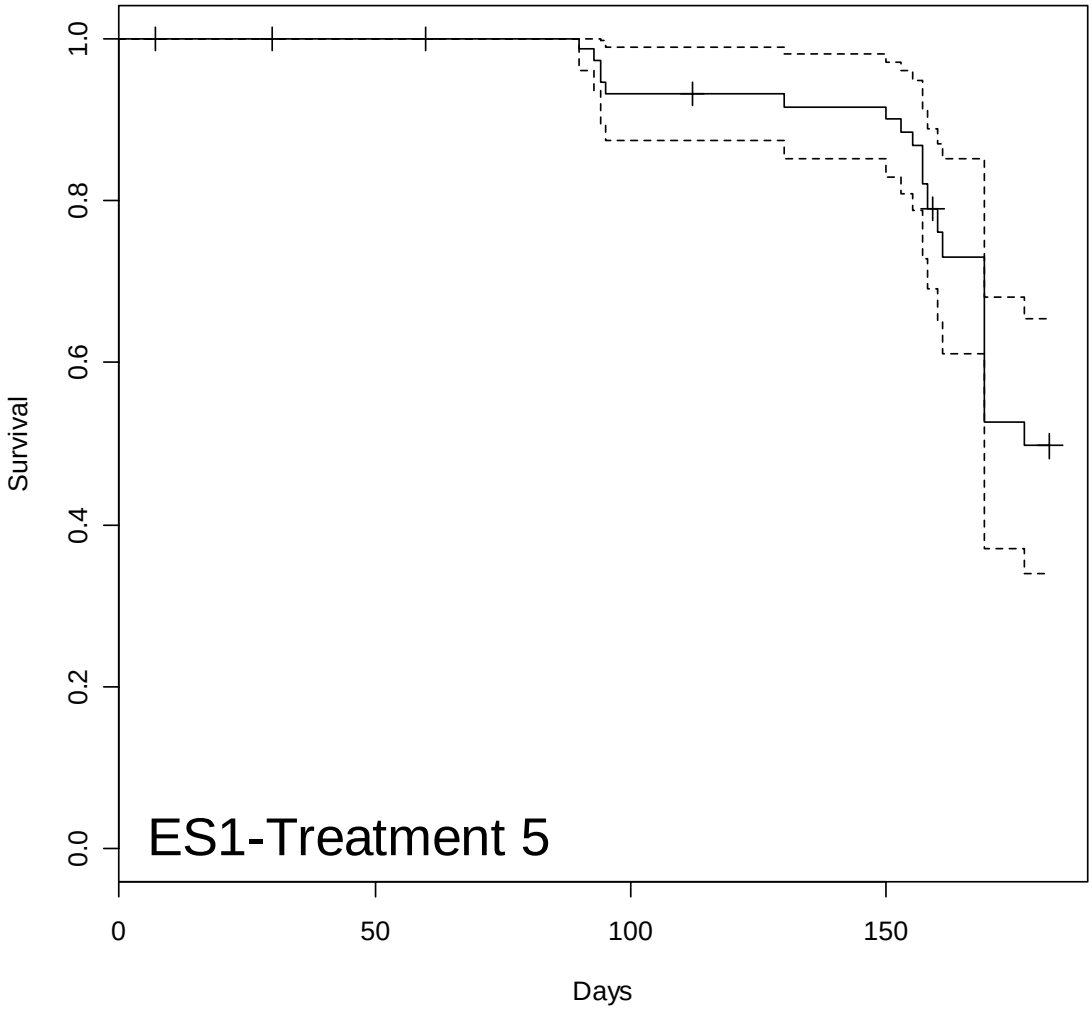


Figure 3.9. Survival curve of juvenile bluegill exposed to selenium. Dashed lines represent the 95% confidence interval for estimates of survival (solid line). The "+" sign indicates dates when data were censored.

Table 3.10. Timetable of deaths and respective estimates of fraction survival for ES3 Treatment 6. All survival values projected by the Kaplan-Meier estimator.

time (day)[a]	no. at risk	no. deaths	fraction survival	std error	low 95%CI	up 95%CI
36	82	1	0.988	0.012	0.964	1.000
43	81	3	0.951	0.024	0.906	0.999
45	78	1	0.939	0.026	0.889	0.992
46	77	2	0.915	0.031	0.856	0.977
47	75	3	0.878	0.036	0.810	0.952
48	72	1	0.866	0.038	0.795	0.943
49	71	1	0.854	0.039	0.780	0.934
61	61	2	0.826	0.043	0.746	0.913
65	59	2	0.798	0.045	0.713	0.892
66	57	2	0.770	0.048	0.681	0.870
67	55	1	0.756	0.049	0.665	0.858
68	54	4	0.700	0.053	0.603	0.811
70	50	2	0.672	0.054	0.573	0.787
71	48	2	0.644	0.056	0.544	0.762
72	46	5	0.574	0.058	0.471	0.699
73	41	4	0.518	0.058	0.415	0.646
74	37	3	0.476	0.059	0.374	0.605
75	34	1	0.462	0.058	0.360	0.592
76	33	3	0.420	0.058	0.320	0.550
77	30	1	0.406	0.058	0.307	0.536
78	29	3	0.364	0.057	0.268	0.493
80	26	1	0.350	0.056	0.256	0.479
81	25	1	0.336	0.056	0.243	0.464
84	24	1	0.322	0.055	0.230	0.450
86	23	2	0.294	0.054	0.205	0.420
88	21	1	0.280	0.053	0.193	0.405
90	20	1	0.266	0.052	0.181	0.390
91	19	3	0.224	0.049	0.146	0.344
93	16	3	0.182	0.046	0.111	0.297
95	13	1	0.168	0.044	0.100	0.281

[a] Mortality counts were checked and recorded daily. The number at risk, number deaths and survival reflect the timing of the fish deaths. For example, on day 49, one fish was observed dead, no fish were observed dead on days 50 through 60 and 2 fish were found dead on day 61.

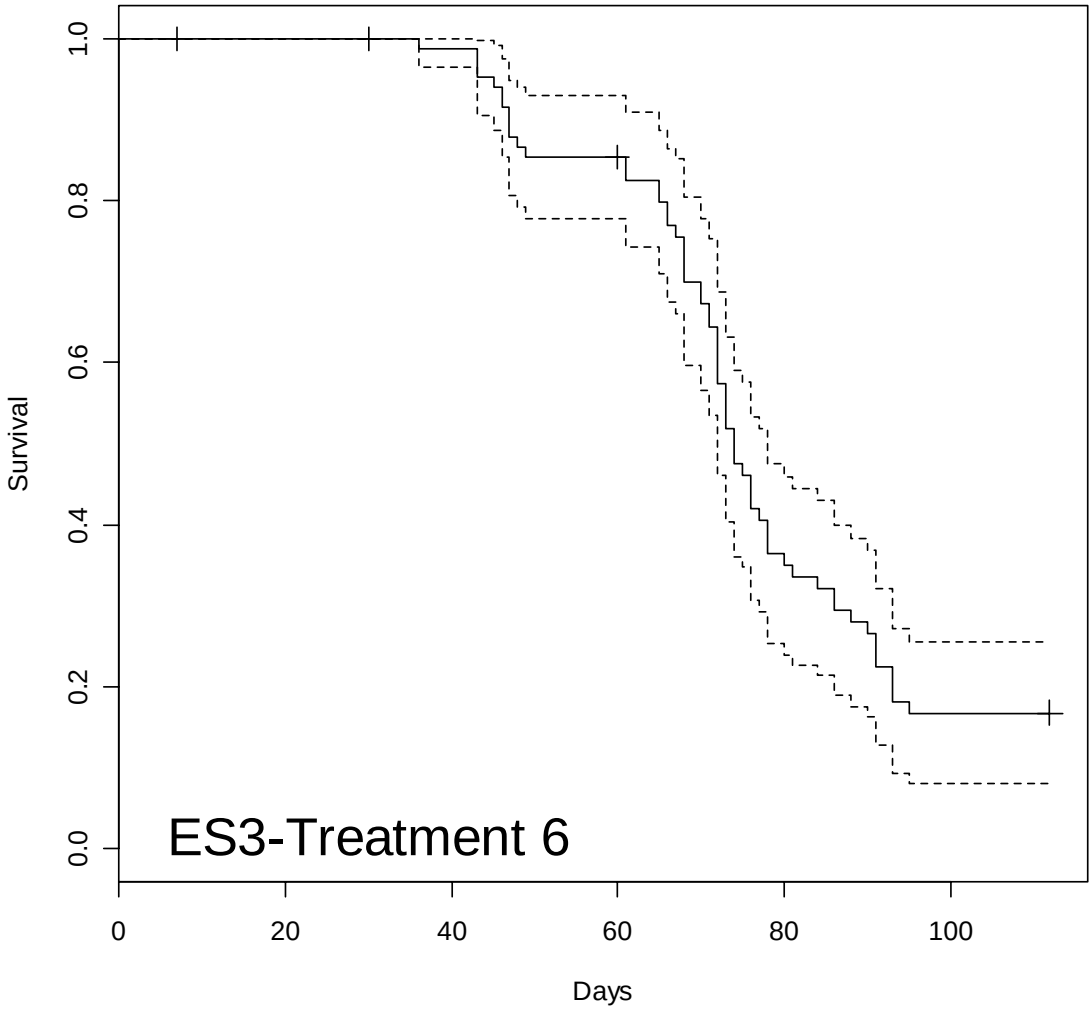


Figure 3.10. Survival curve of juvenile bluegill exposed to selenium. Dashed lines represent the 95% confidence interval for estimates of survival (solid line). The "+" sign indicates dates when data were censored.

Table 3.11. Timetable of deaths and respective estimates of fraction survival for ES3 Treatment 5. All survival values projected by the Kaplan-Meier estimator.

time (day)[a]	no. at risk	no. deaths	fraction survival	std error	low 95%CI	up 95%CI
78	73	2	0.973	0.019	0.936	1.000
88	71	3	0.932	0.030	0.875	0.991
90	68	2	0.904	0.035	0.839	0.974
91	66	5	0.836	0.043	0.755	0.925
95	60	1	0.822	0.045	0.738	0.914
101	59	9	0.696	0.054	0.598	0.811
106	50	3	0.655	0.056	0.554	0.774
107	47	1	0.641	0.057	0.539	0.761
109	46	1	0.627	0.057	0.525	0.749
115	36	1	0.609	0.058	0.506	0.734
122	35	1	0.592	0.059	0.487	0.719
123	34	1	0.574	0.060	0.469	0.704
130	33	2	0.540	0.061	0.433	0.673
143	31	1	0.522	0.061	0.415	0.658
150	30	1	0.505	0.062	0.397	0.642
157	29	1	0.487	0.062	0.380	0.625
160	28	1	0.470	0.062	0.363	0.609
169	27	1	0.453	0.062	0.346	0.593
171	26	1	0.435	0.062	0.329	0.576

[a] Mortality counts were checked and recorded daily. The number at risk, number deaths and survival reflect the timing of the fish deaths. For example, on day 95, one fish was observed dead, no fish were observed dead on days 96 through 100 and 9 fish were found dead on day 101.

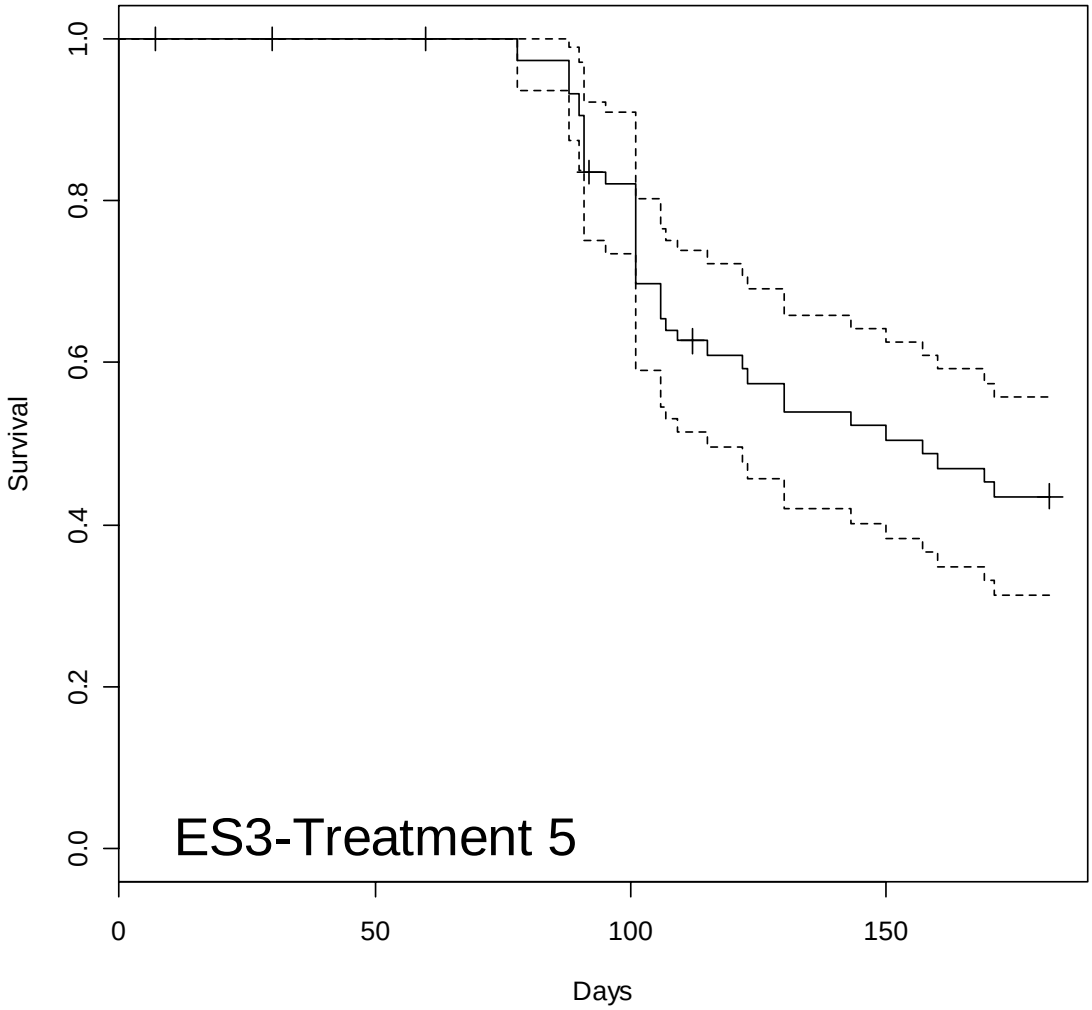


Figure 3.11. Survival curve of juvenile bluegill exposed to selenium. Dashed lines represent the 95% confidence interval for estimates of survival (solid line). The "+" sign indicates dates when data were censored.

3.3.1 Overlay of Survival and Bioaccumulation Plots

Plots of selenium bioaccumulation and fraction survival (*S*) of juvenile bluegill over time were overlaid for estimating the concentration of selenium associated with the onset of mortality ($S < 0.9$). Figures 3.12 – 3.15 display observed and projected concentrations of selenium in fish tissues, as well as Kaplan-Meier estimates of survival over the duration of the experiment (182 days), or until all of the fish had died.

ES1 – Treatment 6
Survival of juvenile bluegill was 0.95 at day 43 and 0.84 at day 44 (Fig. 3.12). At day 43, the concentration of selenium in fish tissues was estimated as 11.58 µg/g dw.

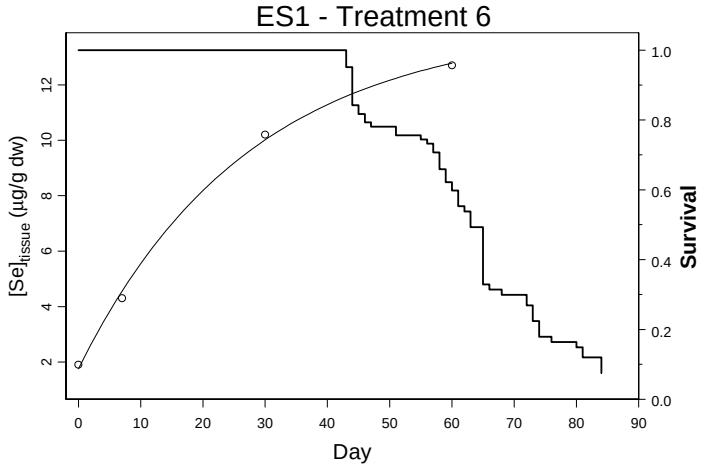


Figure 3.12. ES 1 Treatment 6 overlay of increasing selenium accumulation (measured points and fitted asymptotic curve), and decreasing fraction survival.

ES1 – Treatment 5
Survival of bluegill was 0.90 at day 150 and 0.88 at day 153 (Fig. 3.13). At day 151, the concentration of selenium in fish tissues was estimated as 11.10 µg/g dw.

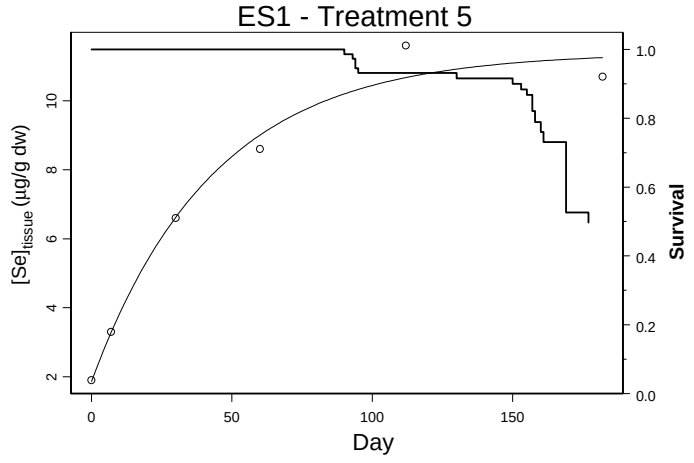


Figure **3.13**. ES1 Treatment 5 overlay of increasing selenium accumulation (measured points and fitted asymptotic curve), and decreasing fraction survival.

ES3 – Treatment 6

Survival of juvenile bluegill was 0.92 at day 46 and 0.88 at day 47 (Fig. 3.14). At day 46, the concentration of selenium in fish tissues was estimated as 13.83 µg/g dw.

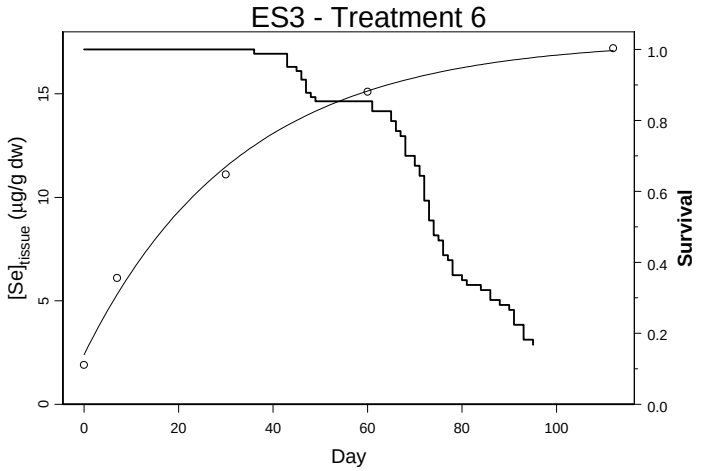


Figure **3.14**. ES3 Treatment 6 overlay of increasing selenium accumulation (measured points and fitted asymptotic curve), and decreasing fraction survival.

ES3 – Treatment 5

Survival of bluegill was 0.90 at day 90 and 0.84 at day 91 (Fig. 3.15). At day 90, the concentration of selenium in fish tissues was estimated as 11.09 µg/g dw.

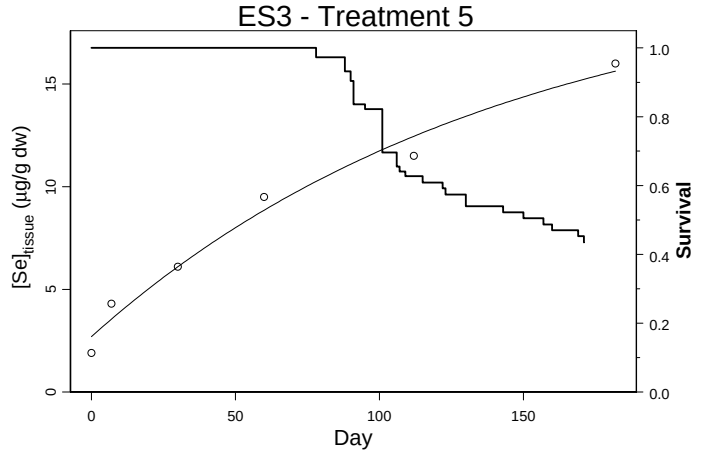


Figure 3.15. ES3 Treatment 5 overlay of increasing selenium accumulation (measured points and fitted asymptotic curve), and decreasing fraction survival.

3.3.2 Estimates of Effect Concentrations

Effect concentrations (EC) for selenium were projected by rearranging a logistic model that quantified the proportion of juvenile bluegill survival as a function of selenium concentration in fish tissues. The TRAP software (U.S. EPA 2002) was used to fit the logistic equation and Kaplan-Meier estimates of effect concentrations. Survival was our selected endpoint because we could estimate it while taking censored data into account; fishes removed from the experiment for reasons other than selenium toxicity. Analysis of the concentration effect was based on the concentrations of selenium in fish determined by several approaches. The first approach used selenium concentrations in fish measured at the last day of the experiment, or at the last day when tissue samples were collected before no fish were left in the tank (day 60 for ES1 Treatment 6 and day 112 for ES3 Treatment 6). If the final measurement of selenium concentrations precedes much of the mortality, as observed in Treatment 6 of ES1 (final measurement at day 60, Fig. 3-12), then estimates of effect concentrations are likely to be biased low. The second approach used the average of the last two measurements for ES1 Treatment 5 and a calculated concentration for ES1 Treatment 6. Averaging the last two Treatment 5 values was done because one appears high (day 112 value is above the modeled line) and one low (day 182 value is below the line) (Figure 3.13). The last measurement for ES1 Treatment 6 value is day 60 although fish survived through day 84; Equation I was used to estimate a selenium concentration for day 84. The third approach used the nonlinear regression Equation I to calculate all treatment values for ES1 and ES3. The following data were entered in the TRAP software:

	ES1				ES3		
Treatment	surv	$[Se]_{tissue}$, µg/g dw			surv	$[Se]_{tissue}$, µg/g dw	
		last meas.[a]	avg T5, calc T6[b]	calc all[c]		last meas.[a]	calc all[c]
Control	0.962	2.08	2.08	2.08	0.988	1.67	1.67
1	0.988	2.56	2.56	2.83	1	3.2	3.05
2	0.984	3.15	3.15	3.22	0.988	3.83	3.68
3	1	4.02	4.02	4.05	1	5.48	4.92
4	0.962	6.72	6.72	6.72	0.96	9.38	9.22
5	0.497	10.71	11.16	11.25	0.435	16.01	15.63
6	0.075	12.66	13.59	13.59	0.168	17.24	17.09
EC_{10} (95% CL)		9.27 (8.86-9.69)	9.40 (8.92-9.91)	9.56 (9.09-10.05)		14.00 (13.40-14.62)	13.29 (12.61-14.00)
EC_{20} (95% CL)		9.78 (9.49-10.09)	10.02 (9.67-10.39)	10.16 (9.81-10.52)		14.64 (14.19-15.11)	14.02 (13.50-14.56)

[a] Last measured selenium concentration

[b] The Treatment 5 value is the average of the last two measured values. Treatment 6 was calculated using equation (I) described in Section 3.2.2, $[Se]_{tissue} = a + (b (1 – \exp(-ct)))$

[c] All treatment values were calculated using equation (I) for the final day of the particular treatment. The final day of treatment was day 182 for all treatments except Treatment 6 which was day 85 for ES1 Treatment 6 and day 112 for ES3 Treatment 6.

A plot of the proportion of juvenile bluegill survival as a function of the logarithm of selenium concentration using the last measured concentration (Fig. 3.16) in ES3 reveals very low mortality up to 10 µg/g ($\log([Se]_{tissue}) = 1.0$) and a steep decline in survival at concentrations above it. Consequently, the EC_{20}, 14.64 µg/g (14.19 – 15.11 µg/g, 95% confidence interval) was similar to the EC_{10}, 14.00 µg/g (13.41 – 14.62 µg/g). Analysis of the calculated values yielded a similar relationship although because the estimated concentrations were slightly lower than the last measured values, the EC values were also slightly lower: EC_{20} = 14.02 µg/g (13.50-14.56 µg/g); EC_{10} = 13.29 µg/g (12.61-14.00 µg/g).

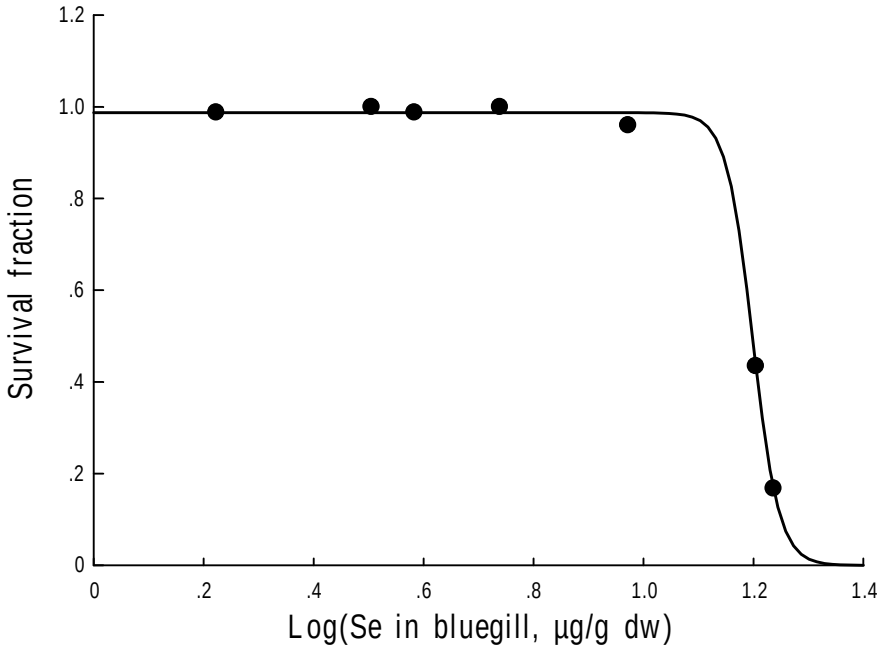


Figure 3.16. Survival of juvenile bluegill as a logistic function of the logarithm of the final selenium concentration in fish tissues. Concentration-survival curve for ES3.

Similar results were obtained for the analysis of fish survival as a function of selenium concentration in ES1 based on the first approach (last measured values) (Fig. 3.17) as well as approaches two and three. In ES1 though, the logistic model projects a steep decline in survival at a lower threshold concentration of selenium in fish tissues. Not surprisingly then, the projected EC_{20} and EC_{10} values for all three approaches (see table above) were lower than correspondent values for ES3.

EC10s (as well as EC20s) calculated using the different ways of estimating exposure differed by only a few percent. (see table for EC values). The EC values determined from the calculated values are considered the best estimates because they represent an integration of all the measured values during the exposure period.

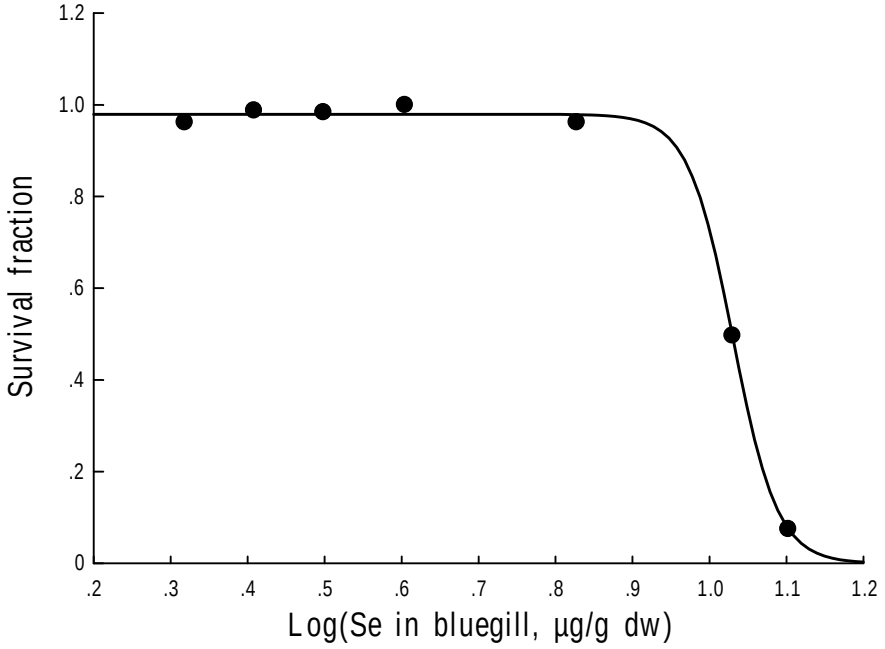


Figure 3.17. Survival of juvenile bluegill as a logistic function of the logarithm of the final selenium concentration in fish tissues. Concentration-survival curve for ES1.

The EC_{10}s determined by the conventional concentration-survival analysis using TRAP were supported by the concentrations observed to represent the onset of mortality when the survival data was overlain with the accumulation data. The onset of mortality concentrations at the 10% effect level were within 20% of the associated EC_{10} values for ES1 and ES3 (see table below).

Method		ES1	ES3
TRAP, concentration-survival	EC_{10}	9.56 µg/g	13.29 µg/g
Onset of mortality (10% effect)	Treatment 5	11.10 µg/g	11.09 µg/g
	Treatment 6	11.58 µg/g	13.83 µg/g

The concentration-survival analysis indicated selenium is 39% more toxic to the bluegill when the temperature approaches 4°C (EC_{10} = 9.56 µg/g) compared to 9°C (EC_{10} = 13.29 µg/g). This difference was less clear when looking at the concentrations determined by the onset of mortality. The ES3 Treatment 6 estimate of effect (13.83 µg/g) followed the same trend of decreased sensitivity at the higher temperature, but the ES3 Treatment 6 estimate (11.09 µg/g) was nearly the same as the two ES1 estimates (T5 = 11.10 µg/g; T6 = 11.58 µg/g). The distinction between the temperature regimes is more apparent in a comparison of tissue concentrations of selenium in the fish and their associated mortalities. There was zero to 4% mortality through Treatment 4 in both ES1 and ES3, however, selenium reached 9.38 µg/g dw in the warmer ES3, whereas it only reached

6.72 µg/g dw in ES1 (see table in Section 3.2.2). The number of mortalities increased markedly in Treatment 5 with comparable levels of 50% in ES1 and 56.5% in ES3. However, the last measured selenium concentrations in the fish were not comparable: 10.71 µg/g dw in ES1 and 16.01 µg/g dw in ES3. The same pattern of greater sensitivity to selenium in the colder exposure system was observed in Treatment 6 where a tissue concentration of 12.66 µg/g dw killed 92.5% of the fish in ES1 and the higher concentration 17.24 µg/g dw killed 82.5%.

The concentration-effect analysis was considered to be a better assessment of the effect of selenium coupled with temperature on the bluegill test population because it used a wider set of data to estimate effect. Also, the onset-of-mortality approach compares increasing accumulation with increasing mortality within each treatment during the course of the test; the conventional application of TRAP compares the end-of-test results between treatments.

Although both approaches assume no delayed mortality, the Onset of Mortality is more sensitive to the assumption than TRAP's use of the end-of-results. This is because the selenium concentrations were rising at a greater rate at the onset of 10% mortality, and the approach assumes that death is caused by the concentrations occurring at the time of death, not the concentrations occurring say 20 days earlier.

3.4 GROWTH, LIPID ANALYSIS, AND BEHAVIOR OF JUVENILE BLUEGILL SUNFISH

Growth of juvenile bluegill was not negatively affected by the selenium exposures used in the study. Within each system, the length and weight of the fish did not show a decreasing trend as the exposure concentrations increased (Tables 3.12 and 3.13; Appendix F). Growth was greater in ES2 and ES3 than in ES1. The greater growth in ES3 can be explained by continued active feeding by the bluegill throughout the 182 day exposure. The fish contained in ES1 fed minimally once the temperatures reached 5°C. Even though ES2 fish exhibited the same decrease in feeding activity, their length and weight were greater than fish in ES1, the other 4°C exposure.

The average body condition factor, K, showed a similar lack of response to selenium exposure concentration (Table 3.14). K values tended to increase during the exposure period.

The lipid content of the bluegill did not decrease during the 182 day exposure. The percent lipid values measured in the bluegill upon receipt at the laboratory and on test day 0 were 2.51% and 3.04%, respectively. These initial values were similar to the values measured in control and each treatment fish in ES1 after 182 days of exposure (Table 3.15; Appendix G). Lipid values for the fish treated in ES2, and for ES3 fish in Treatments 3, 4, and 5 appeared to slightly increase over the exposure period.

The fish in the two colder exposure systems, ES1 and ES2, displayed similar feeding behavior. Bluegill actively fed on Tetramin in the control and treatments in ES2 through test day 77 as the temperature approached 5°C, after which feeding was minimal. A

marked reduction in the consumption of *Lumbriculus* in ES1 was observed in fish in the control and Treatments 1 through 5 on test days 81 to 83. The bluegill in ES1 Treatment 6 reduced their feeding behavior earlier, on test day 53, presumably due to effects of the selenium exposure. Erratic swimming was also observed in ES1 Treatment 6 of a few fish on test day 54.

Since the temperature in ES3 did not reach 5°C, the temperature at which feeding was reduced in ES1 and ES2, the feeding behavior of fish in the controls and Treatments 1 through 4 in ES3 was active throughout the entire 182 day exposure. The feeding activity in ES3 Treatments 5 and 6 was noticeably reduced on test days 75 and 74 apparently due to selenium exposure.

3.5 COMPARISON OF RESULTS BETWEEN LEMLY AND CURRENT STUDIES

A comparison of Lemly's cold treatment plus selenium to EPA's ES2 results is the most direct evaluation of the two studies. Although both studies exposed juvenile bluegill to nominal selenium concentrations of 5 µg/L in the water and 5 µg/g in TetraMin, there were differences. Lemly began his temperature decline of 2°C per week at the start of the test whereas the current study maintained 20°C for 30 days prior to initiating the temperature decline. Lemly reached 4°C, and the current study reached 4.6°C. The current study used 2 replicates with 100 fish/replicate and Lemly used 3 replicates with 70 fish in each replicate tank. Lemly measured oxygen consumption on 15 fish randomly selected from each treatment and control on test days 60, 120 and 180; oxygen consumption was not measured in the current study. The oxygen consumption measurement required transferring the selected fish to a separate chamber and then reintroducing the fish back to the exposure tank. Lastly, EPA's test duration was 182 days and Lemly's was 180 days. Of these differences, we expect only the first to have any possible significant effect on the results.

A comparison of the accumulation of selenium in the bluegill in these treatments from these two studies suggests that at first the current study fish accumulated more during the respective exposure periods. Lemly's fish accumulated 5.85 µg/g after 60 days and 7.91 µg/g by the end of the 180 days. The bluegill in the current study accumulated approximately 8 µg/g of Se after 60 days and 10 µg/g after 182 days. A closer look at three exposure conditions may explain these differences. First, the background concentration of selenium in bluegill was 1 µg/g in Lemly's fish and approximately 2 µg/g in the current study fish. Second, the measured concentration of selenium in the TetraMin was 16% higher in ES2 at a time-weighted average concentration of 6.01 µg/g dw; Lemly's TetraMin was measured at 5.16 µg Se/g dw. The difference was even greater when considering the last two batches of TetraMin that were fed to the bluegill during test days 72-182 in the current study were progressively higher in selenium concentration than the first two. The time-weighted average selenium concentration in last two TetraMin batches was 6.46 µg/g dw, 25% higher than Lemly's diet. The ratio of selenium in fish to selenium in diet between the two studies is comparable: Lemly ratio = 1.53; current study ratio using the time-weighted average of the last two TetraMin batches = 1.55. The third consideration that may explain the difference in Se accumulation is the longer exposure period at 20°C in ES2. After considering the above,

the accumulation of selenium in the two studies was similar, but the fish in the current study contained more selenium at the beginning, middle and end of the test.

Although the accumulation of selenium was similar between the two studies, there was a difference in survival of fish. Lemly observed 40% mortality by the end of the 180 days whereas no meaningful mortality was observed in ES2. This difference is larger when considering the mortalities occurred in Lemly's fish when selenium concentrations in the bluegill increased from 5.85 to 7.91 µg/g compared to no effect on survival up to 10 µg/g in ES2. The latter observation of no mortality at 10 µg/g is consistent with the results from ES1 and ES3 which saw no meaningful effects on survival until the selenium concentrations in the bluegill approached 11 µg/g.

As stated above, Lemly removed 15 fish from each treatment for oxygen consumption measurement and then returned these fish to the exposure tanks. There is the possibility that the fish removed from the cold plus selenium treatment were sufficiently stressed by the exposure conditions that the additional handling stress contributed to the mortality observed in this treatment. Between test days 60 and 180, 56 fish died Lemly's cold plus selenium treatment. Even if stress due to handling affected all the fish used in the oxygen consumption measurements (up to 30 fish), it does not explain all the mortality that was observed and therefore does not explain the difference between the two studies.

Lemly found meaningful decreases in body condition factor, K, and lipid content in his cold plus selenium treatment. K decreased from 4 at the start of the test to 2.2 by the end with the most dramatic decrease occurring between days 60 and 120. In contrast, the average condition factor of the bluegill in ES2 was 3.2 at test initiation and 5.3 at test termination. A similar comparison was observed with the measurement of lipid content of the bluegill in the two studies. Note: Lemly determined lipid based on dry weight. In order to make a direct comparison, Lemly's lipid values were converted to wet weight assuming the fish were 75% moisture. The percent lipid at the start of Lemly's 180 day exposure was 3.25 and decreased to 1.5 by the end of the test. The lipid content in the fish in ES2 did not decrease as evidenced by 3% at test start and 3.5% at test end.

In summary, the direct comparison between the results of the current study's ES2 and Lemly's cold plus selenium treatment shows similarity in the accumulation of selenium in the bluegill, but a meaningful difference in the toxicity of selenium. Lemly's fish displayed toxicity to selenium at concentrations 2 to 4 µg/g dw lower than the current study. The difference in toxicity is apparently also reflected in the difference observed in the body condition factor, K, of the two test populations. K increased in the current study over the exposure period, whereas K decreased in Lemly's fish.

A comparison can be made between Lemly's cold plus selenium and the current study's Treatment 3 in ES1 and ES3. The exposure conditions in the latter tests were nominal 5 µg/L in the water and average measured selenium concentrations 7.47 and 7.17 µg/g in *Lumbriculus* in ES1 and ES3. Selenium in the bluegill in ES1 appeared to reach steady-state around 4 µg/g compared to around 8 µg/g in Lemly's study.

The fish in the warmer ES3 did not appear to reach steady-state; the whole body selenium concentration on day 182 was 5.5 µg/g. As discussed at the end of Section 3.2, the

apparent difference in selenium accumulation is due to the form of selenium in the diet. The TetraMin contained the more bioaccumulative seleno-L-methionine whereas *Lumbriculus* contained a mixture of selenium species with some not as bioaccumulative as seleno-L-methionine.

The toxicity of selenium to the bluegill in Lemly's cold plus selenium and ES1 can be compared by an examination of when meaningful toxicity occurred in the fish. Lemly's fish had a sharp increase in mortality after day 60 when mortality went from 5 to 10% over a couple of days. The concentration of selenium in Lemly's fish was approximately 6 µg/g during that period. As discussed in Section 3.3.1, survival of bluegill in ES1 Treatment 6 decreased from 95 to 84% over days 43 and 44 when the bluegill selenium concentration was 11.6 µg/g. A similar concentration in bluegill (11.1 µg/g) was observed in ES1 Treatment 5 at the point where survival dropped below 90%. The relative difference between these two threshold values, that is, the selenium concentration determined by the Onset of Mortality in the current study (average of ES1 and ES3 = 11.4 µg/g dw) divided by concentration of selenium in Lemly's fish when mortality increased from 5 to 10% (6 µg/g dw) is 1.9.

Similar to that discussed above in the direct comparison between ES2 and Lemly's cold plus selenium treatment, the body condition factor in ES1 and ES3 did not decrease over the exposure duration as did Lemly's fish (Table 3.14). There was less of an increase in K over the exposure period in ES1 and ES3 than there was with ES2 fish but it was still markedly different than the decrease from 4 to 2.2 in K observed in Lemly's fish. Since K is a reflection of the overall health of the bluegill, it directly relates to the differences observed in the toxicity of selenium in the two studies.

Table 3.12. Average standard lengths (mm) in bluegill based on samples taken for chemical analysis; N = 9 for each average value.

		exposure day					
system	treatment	0	7	30	60	112	182
ES1	control	47	49	48	49	49	53
ES1	1	47	48	47	50	53	51
ES1	2	47	49	50	49	50	51
ES1	3	47	49	49	51	50	51
ES1	4	47	49	50	50	51	51
ES1	5	47	48	51	50	50	52
ES1	6	47	50	49	52		
ES3	control	47	48	51	46	51	56
ES3	1	47	50	50	50	52	56
ES3	2	47	49	48	53	51	55
ES3	3	47	48	48	49	50	55
ES3	4	47	50	46	50	50	56
ES3	5	47	49	48	47	54	57
ES3	6	47	47	45	48	51	
ES2	control	47	51	53	59	60	56
ES2	5A	47	50	52	55	54	60
ES2	5B	47	48	54	55	57	57

Table 3.13. Average weights (g) in bluegill based on samples taken for chemical analysis; N = 9 for each average value.

		exposure day					
system	treatment	0	7	30	60	112	182
ES1	control	1.51	1.81	1.59	1.74	1.67	2.38
ES1	1	1.51	1.58	1.35	1.85	2.05	1.89
ES1	2	1.51	1.70	1.71	1.88	1.94	2.13
ES1	3	1.51	1.63	1.65	1.90	1.97	2.13
ES1	4	1.51	1.62	1.78	1.74	2.07	2.00
ES1	5	1.51	1.56	1.79	1.83	1.99	2.08
ES1	6	1.51	1.75	1.54	2.31		
ES3	control	1.51	1.69	1.72	1.24	1.95	2.37
ES3	1	1.51	1.73	1.54	1.74	2.08	2.86
ES3	2	1.51	1.67	1.48	2.20	1.86	2.29
ES3	3	1.51	1.57	1.48	1.75	1.96	2.25
ES3	4	1.51	1.71	1.25	1.67	2.09	2.75
ES3	5	1.51	1.66	1.50	1.39	2.73	3.64
ES3	6	1.51	1.51	1.32	1.68	2.38	
ES2	control	1.51	2.00	2.19	3.34	3.57	2.76
ES2	5A	1.51	1.79	2.26	2.93	2.81	3.32
ES2	5B	1.51	1.65	2.63	2.83	2.85	2.92

Table 3.14. Average body condition factor (K)* bluegill based on samples taken for chemical analysis; N = 9 for each average value.

		exposure day					
system	treatment	0	7	30	60	112	182
ES1	control	3.21	3.67	3.30	3.51	3.39	4.49
ES1	1	3.21	3.32	2.89	3.69	3.89	3.73
ES1	2	3.21	3.51	3.45	3.83	3.86	4.13
ES1	3	3.21	3.35	3.37	3.73	3.98	4.19
ES1	4	3.21	3.33	3.56	3.49	4.08	3.90
ES1	5	3.21	3.28	3.50	3.64	3.96	4.00
ES1	6	3.21	3.51	3.17	4.42		
ES3	control	3.21	3.50	3.35	2.72	3.85	4.27
ES3	1	3.21	3.45	3.09	3.52	3.98	5.08
ES3	2	3.21	3.39	3.08	4.17	3.66	4.17
ES3	3	3.21	3.24	3.11	3.56	3.92	4.11
ES3	4	3.21	3.44	2.70	3.32	4.16	4.92
ES3	5	3.21	3.41	3.13	2.97	5.03	6.34
ES3	6	3.21	3.21	2.92	3.50	4.70	
ES2	control	3.21	3.94	4.11	5.69	5.94	4.93
ES2	5A	3.21	3.60	4.38	5.32	5.18	5.53
ES2	5B	3.21	3.43	4.87	5.17	5.00	5.12

* K = (100 x weight (g))/standard length

Table 3.15. Lipid content (%) in juvenile bluegill at the start and end of the exposure period.

			lipid content	
system	treatment	test day	average	std. dev.
NA	Arrival of fish at lab	-25	2.51%	0.02%
NA	test day 0	0	3.04%	0.04%
ES1	control-1	182	2.35%	0.07%
ES1	control-2	182	2.67%	0.07%
ES1	1	182	2.69%	0.38%
ES1	2	182	2.05%	0.03%
ES1	3	182	2.49%	0.02%
ES1	4	182	2.67%	0.01%
ES1	5	182	2.26%	0.08%
ES2	control-1	182	5.79%	0.01%
ES2	control-2	182	4.42%	0.01%
ES2	5A	182	4.04%	0.02%
ES2	5B	182	3.06%	0.04%
ES3	control-1	182	2.88%	0.11%
ES3	control-2	182	1.96%	0.08%
ES3	1	182	2.74%	0.02%
ES3	2	182	2.82%	0.03%
ES3	3	182	4.24%	0.03%
ES3	4	182	3.92%	0.08%
ES3	5	182	3.74%	0.00%

4.0 SUMMARY

The goal of the 182-day exposure to juvenile bluegill sunfish was to determine tissue-based effect levels for selenium exposure over a simulated winter season at two temperature regimes, 20 to 4°C and 20 to 9°C. The following bullets summarize the findings:

- Juvenile bluegill sunfish appear to be more sensitive to selenium in waters reaching 4-5°C than 9°C. The EC_{20} and EC_{10} estimates for the exposure in which temperature decreased from 20 to near 4°C were 10.16 and 9.56 µg/g dw, respectively, while the EC_{20} and EC_{10} estimates for the exposure that began at 20°C and systematically lowered to 9°C were 14.02 and 13.29 µg/g dw, respectively.
- The accumulation of selenium in the juvenile bluegill was affected by the form of selenium in the diet of the fish. Under a similar temperature regime and exposure period, bluegill receiving an artificial diet spiked with seleno-L-methionine (ES2 treatments 5A and 5B) accumulated 2.5 times the selenium accumulated by bluegill receiving a natural diet of selenium accumulated in *L. variegatus* (ES1 Treatment 3).
- The accumulation of selenium in the juvenile bluegill was affected by temperature. Fish exposed to dietary selenium via *L. variegatus* accumulated up to 39% more selenium in the 20 to 9°C regime than in the 20 to 4°C regime.
- The accumulation characteristics of seleno-L-methionine in juvenile bluegill in the current study were similar to that observed in Lemly's study.
- The toxicity of selenium to juvenile bluegill was approximately 1.9 times less in the current study than that observed in Lemly's study.
- The juvenile bluegill in the current study did not decrease in body condition factor and lipid content as they did in the Lemly study.

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