Effects of dietary Selenomethionine on larval rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Increased selenium (Se) concentrations in water (>10 µg/L) have been measured in San Diego Creek, a tributary of the Upper Newport Bay in Orange County, California. The objective of this study was to develop tissue and dietary thresholds for Se in resident fish species in San Diego Creek. A 90-day dietary experiment was conducted to determine the effects of seleno-L-methionine (SeMe) on growth, survival, and whole-body accumulation in larval (24-day-old) rainbow trout. Decreased and oxidized glutathione (GSH-to-GSSG ratio) and thiobarbituric acid–reactive substances (TBARS) were also measured in livers of exposed animals to assess oxidative damage caused by Se. Fish food was spiked with SeMe to contain 4.6, 12, and 18 µg/g (dry weight) of Se. Fish exposed to SeMe for 90 days exhibited a significant decrease in body weight and fork length in the 4.6 and 12 µg/g Se treatments compared with controls. Whole body total Se concentrations increased significantly in fish fed 12 and 18 µg/g SeMe after 90 days compared with controls. Lipid peroxidation (TBARS) and GSH to GSSG ratios were not changed by SeMe treatment. Based on decreased growth after 90 days, a dietary Se lowest observed-effect concentration (LOEC) value of 4.6 µg/g and a Se body burden LOEC of 1.20 µg/g (wet weight) were estimated.

Introduction

Selenium (Se) is an element that occurs naturally in many soils and sediments and is an essential micronutrient for fish, birds, humans, and many microorganisms. Despite being a required micronutrient, Se is toxic at concentrations only slightly greater than nutritional requirements (Hilton *et al.* 1980). Although Se exists in many natural soil and water environments around the world, anthropogenic activities such as agricultural irrigation on Se-laden soils have led to significant site-specific areas of contamination. Inorganic Se can leach out of the soil and enter aquatic habitats by way of agricultural drainages and ground water discharges. Once in the waterways, microbial organisms tend to alter speciation into organic compounds such as seleno–amino acids that are passed up the food chain (Weiss *et al.* 1965). An example of a seleno–amino acid form that biomagnifies in food webs is selenomethionine (SeMe; Fan *et al.* 2002).

Increased water concentrations of Se (>10 µg/L) have been found in San Diego Creek, which is a tributary of the Upper Newport Bay in Orange County, California (CA). Selenium concentrations exceeded the 5 µg/L criteria for fresh water systems established by the United States Environmental Protection Agency. Concentrations of Se in San Diego Creek sediments ranged between 0.2 and 3 µg/g (California Regional Water Quality Control Board (CRWQCB) 2001). Whole body concentrations of Se in fish from San Diego Creek varied between 2 and 7 µg/g and did not exceed the values established for the protection of human health (20 µg/g wet weight). However, concentrations of Se in the fish did exceed levels of concern (>4 µg/g) on wildlife (CRWQCB 2001).

Recent studies in rainbow trout embryos reported that SeMe treatment resulted in the formation of superoxide and the subsequent consumption of reduced glutathione (GSH; Palace *et al.* 2004). GSH is a low molecular weight molecule form by three amino acids (tripeptide), which has an important role in the maintenance of cell membrane integrity, chemical metabolism, and protection from oxidative stress (Rogers and Hunter 2001). Since GSH is an important component of these fish systems it can be used as a sublethal endpoint to monitor fish health. Therefore, the measurement of the ratio of reduced GSH to oxidized GSH (GSSG) and the relative amount of lipid peroxidation are practical sublethal end points that can be used to provide an indication of oxidative stress within organisms (Gallagher *et al.* 1992, Jentzsch *et al.* 1996).

The objective of this study was to determine the body-burden and dietary thresholds for Se in a model fish species that would potentially assist regulators in

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evaluating Se effects on fish populations in San Diego Creek. The present study addressed this objective by exposing larval rainbow trout (*Oncorhynchus mykiss*) to dietary SeMe for 90 days and determining the effects of SeMe on survival, growth, and hepatic oxidative damage.

Methods

Fish exposure

Rainbow trout (*O. mykiss*) were selected as the model fish species because historic and recent records indicate the occurrence of steelhead trout populations in several streams of southern California (California Department of Fish and Game, personal communication, October, 2000). Larval rainbow trout (24 days old) were obtained from the Mojave Hatchery (California Department of Fish and Game, Victorville, CA). Selenium (Se) was mixed with dry fish food (Skretting, Vancouver, Canada) with appropriate amounts of SeMe (Aldrich Chemical, Milwaukee, WI) in a methanol solution to produce Se measured concentrations of 4.6, 12, and 18 µg/g (dry weight). The control treatment (fish food with methanol and no additional SeMe) contained 0.23 µg/g of Se. Each treatment had 5 replicates and each replicate contained 12 to 16 larval trout. The total number of fish used in the experiment was 300. Each fish was fed an average of 10 mg of fish food per day, for 30 days. Between 30 and 60 days of exposure they were fed 25 mg/fish and fed 40 mg/fish thereafter. The analysis of the fish diet showed that other elements in the food were present at typical concentrations (Table 1).

Exposure was carried out in aerated, replicate 10-gallon glass aquaria that were partially submerged in a refrigerated water bath (at 10 ± 2°C). Each replicate received >1 L 12 ± 2°C replacement water every minute. Se concentrations in the water column were below levels of detection (0.05 to 0.1 µg/L) throughout the study. Water quality (pH, dissolved oxygen, conductivity, ammonia, and temperature) was measured weekly. Ammonia values ranged from 0 to 1.0 mg/L, pH values ranged from 7.0 to 8.4, and conductivity values ranged from 0.01 to 1.11 µS/cm. Temperature remained constant (10 ± 1°C).

The glass aquaria had glass lids and were exposed to partially shaded, natural light (regime of 12 hours dark to 12 hours light). Fish were acclimated for 3 days before exposure. Wet weight and fork length of a sample of 3 to 5 fish from each aquarium were measured as controls before the exposure began. Ten fish from each treatment (2 fish/aquarium) were sampled after 30, 60, and 90 days of exposure. Fork length, body weight, tissue Se concentrations, and hepatic GSH and TBARS were measured. All of the fish were killed with MS-222 (3-amino-benzoic acid ethyl ether) (Sigma Chemical, St. Louis, MO). Daily observations of mortality were made.

Se analysis

The diet for control fish was analyzed by Calscience Environmental Laboratories (Garden Grove, CA) for 18 elements including Se following USEPA 6010B and 7471A methods. Fish tissue analyses were conducted at the University of California, Riverside, CA. Samples of trout tissue (0.5 to 3.5 g) were digested in nitric acid, hydrogen peroxide, and laboratory-distilled water at 170°C and approximately 200 psi in a MARS 5 CEM microwave digester (Matthews, NC) for 30 minutes. To change the oxidative state of Se to Se (VI), the samples were heated at 90°C for 20 minutes in 12 mL of chloridic acid (HCl, Sigma Chemical) and 0.2 mL of 0.2 M K2S2O8 (Sigma Chemical) to a final HCl concentration of 6 M. The samples were analyzed using a Perkin-Elmer atomic absorption spectrophotometer (Norwalk, CT).

Table 1. Trace metal analysis of the unspiked fish food used in the experiment (ND = Not detected).

Metal	Result (µg/g)	Reporting Limit
Antimony	1.9	0.75
Arsenic	ND	0.75
Barium	5.8	0.5
Beryllium	ND	0.25
Cadmium	ND	0.5
Chromium	1.13	0.25
Cobalt	0.42	0.25
Copper	21.1	0.5
Lead	ND	0.5
Mercury	ND	0.08
Molybdenum	1.76	0.25
Nickel	1.57	0.25
Selenium	0.23	0.75
Silver	ND	0.25
Thallium	ND	0.75
Vanadium	1.23	0.25
Zinc	85	1

For quality assurance and control, a dried tomato leaf reference material (National Institute of Standards and Technology, Gaithersburg, MD) and matrix spikes were analyzed with the samples. The percent recovery for the tomato leaves ranged from 68% to 85%. The percent recovery for the spiked samples was >79%. The detection limit for the Se analysis ranged from 0.003 to 0.017 µg/g. All metal concentrations from fish were reported on a wet-weight basis, whereas food was measured as dry weight.

Hepatic lipid peroxidation and hepatic GSH-to-GSSG ratios

Total GSH and GSSG were measured using the method of Baker (1990). Trout liver tissue samples were homogenized in 1:9 v/v 5% 5-sulfosalicyclic acid (Aldrich Chemical) on ice. The homogenate was centrifuged, and triplicate 10-lL samples of supernatant (approximately 1.0 mg tissue) or standard was added to a 96-well microplate.

Two hundred ìL reaction buffer were then added to the wells for measurement of GSSG. Reaction buffer consisted of 5.40 mL NaH2-PO4/EDTA buffer (Aldrich Chemical), 2.80 mL 1 mM DTNB (0.15 mM final), 3.75 mL 1 mM NADPH (0.2 mM final), and 0.050 mL 12U GSSG reductase (1 U/mL final). The microplate was read at 10-second intervals during 2 minutes at 405 nm at room temperature (25°C). The GSH assay was similar to that previously described, except that the reaction buffer did not contain NADPH or GSSG reductase.

Quantification of malondialdehyde (MDA) has been used in past decades as a marker of lipid peroxidation. One of the most widely used methods involves the use of 2-thiobarbituric acid (TBA or 4,6-dihydroxy-2-mercapto-pyrimidine). The adduct formed during the reaction can be measured by spectrophotometry and spectrofluorometry (Jentzsch *et al.* 1996). Biologic specimens contain a mixture of thiobarbituric acid–reactive substances (TBARS) including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. The concentration of TBARS returns to normal levels with time depending on the presence of antioxidants. In practice, TBARS concentration is expressed in terms of MDA equivalents (Kwon and Watts 1964). MDA, a byproduct of lipid peroxidation, was measured using the TBARS (Sigma Chemical) assay (Jentzsch *et al.* 1996).

Data analysis

After tests for homogeneity of variance were completed, temporal and dose-related end points were compared using two-way analysis of variance. Dunnet's test was performed if significance among different means was observed to determine which groups were significantly different from the control (p greater than or equal to 0.05). The lowest observed-effect concentration (LOEC) was determined as the lowest food or tissue concentration producing a statistically significant adverse effect.

Results

Fish survival and growth

Approximately 95% of the cumulative mortalities occurred within the first 17 days of exposure without any relationship to dose. Thirty percent mortality was observed in controls and the lowest Se treatment (4.6 µg/g), whereas 6% and 13% mortality, respectively, were observed at the two higher doses.

Fish growth was significantly impacted after 90 days for the 4.6 and 12 µg/g treatments compared with control fish ($p < 0.05$). Control fish at the end of the experiment had an average fork length of 7.70 cm, whereas the length for SeMe exposed fish ranged between 6.84 and 7.37 cm (Table 2). Fish fork length increased throughout the experiment, but the rate of increase slowed markedly after 60 days in all treatments. Trout body weight at the end of the experiment averaged 5.17 g for control fish and ranged between 3.45 and 3.82 g for the exposed fish. Fish in all treatments exhibited a similar rate of weight gain during the experiment up to 60 days, then a nearly two-fold increase was observed in controls from 60 to 90 days.

Se accumulation

A positive relation between Se dose and accumulation was observed at each sampling time (Table 2) with r^2 values of 0.8831, 0.8894, and 0.9886 for 30, 60 and 90 days, respectively ($n = 4$). Tissue concentrations of Se were variable and not significantly different from the control fish after 30 days, although mean Se concentrations were two to four times higher than the control ($p > 0.05$). Likewise, a trend toward a dose-dependent increase of tissue concentrations was observed after 60 days, but variability prevented the observation of statistically significant differences between the treatment doses ($p > 0.05$). However, significantly higher concentrations of Se

Table 2. Rainbow trout growth after four different selenomethionine exposures (SeMe as (µg/g). Results shown are average values at 0, 30, 60 and 90-days, standard deviation in parenthesis (*= Statistical significant difference from control (p <0.05), ND = Not determined).

Time	Treatment (µg/g dry weight)	Weight (g)	Fork Length (cm)	Tissue Concentration (µg/g wet weight)
0	Control	0.37 (0.30)	3.14 (0.41)	ND
30	Control	1.33 (0.92)	4.66 (0.41)	0.46 (0.20)
	4.6	1.25 (0.21)	4.84 (0.29)	1.05 (0.77)
	12	1.33 (0.30)	5.09 (0.46)	1.81 (1.04)
	18	1.31 (0.37)	4.97 (0.50)	1.60 (0.93)
60	Control	2.96 (0.92)	6.91 (0.56)	1.24 (0.54)
	4.6	2.33 (0.63)	6.69 (0.67)	1.70 (0.72)
	12	2.52 (0.38)	6.88 (0.35)	1.83 (0.94)
	18	2.59 (0.24)	6.92 (0.24)	2.62 (1.22)
90	Control	5.17 (1.09)	7.70 (0.33)	0.31 (0.20)
	4.6	3.45 (0.35)*	6.93 (0.19)*	0.58 (0.21)
	12	3.45 (0.35)*	6.84 (0.68)*	1.20 (0.21)*
	18	3.82 (0.62)	7.37 (0.62)	1.41 (0.27)*

were observed in fish exposed to the 12 and 18 µg/g treatments after 90 days ($p < 0.05$). The average concentration of Se in fish tissue decreased between 60 and 90 days in all treatment groups, including controls, with tissue concentrations returning to 30 day values.

Hepatic lipid peroxidation and hepatic GSH-to-GSSG ratios

Lipid peroxidation (Figure 1) and GSH-to-GSSG ratios (Figure 2) were unchanged by SeMe treatment. Lipid peroxidation after 90 days of exposure ranged from 0.03 to 0.08 mol/mL MDA among the treatments. GSH-to-GSSG ratios ranged from 1.71 to 2.04 after 90 days of exposure. These differences were not statistically significant, and no trends were detected.

Discussion

This study demonstrated that both the duration and concentration of dietary SeMe exposure negatively impacted larval trout growth. After 90 days of dietary exposure to SeMe, growth of rainbow trout was significantly diminished at 4.6, 12, and 18 µg/g concentrations of Se, resulting in a dietary threshold (LOEC) of 4.6 µg/g. These results are consistent with other studies showing that dietary Se (as SeMe) was toxic to fish in the range of 3 to 5 µg/g (Hamilton *et al.* 1990). When rainbow trout were fed 13-µg/g selenite for 20 weeks, fish exhibited decreased growth and survival (Hilton *et al.* 1980). Another study with rainbow trout showed that fish fed 11 µg/g (dry weight) for 16 weeks had decreased body weight compared with those fed 7-µg/g selenite (Hilton and Hodson 1983). A value of 4 µg/g for invertebrates has been recommended as a threshold for a hazing program for birds by the California Department of Fish and Game (Hamilton 2002 and 2004).

Direct relationships between Se tissue accumulation and dietary dose were observed in all treatments. However, the relationship between Se accumulation and duration of exposure was not as clear. The fish contained lower concentrations of total Se after 90 days compared with tissue samples from the 60-day time points. A similar trend was also seen in the controls. The data trends indicated that a physiologic change may have occurred in the fish during the 60- to 90-day interval. Evidence of such a change is shown by increased weight gain during this period. Larval rainbow trout mature into juveniles between 2.5 and 13 cm (Westers 2001), the same size range of the fish at the 60- to 90-day transition (Table 2). The decreases in Se body burden observed between 60 and 90 days likely occurred because of the relative increases in total body mass, which would have decreased overall concentrations. Alternatively, changes in Se uptake or enhanced excretion produced by the fish becoming juveniles during that period of time may also explain the data. Perkins *et al.* (1997) observed that hepatic concen-

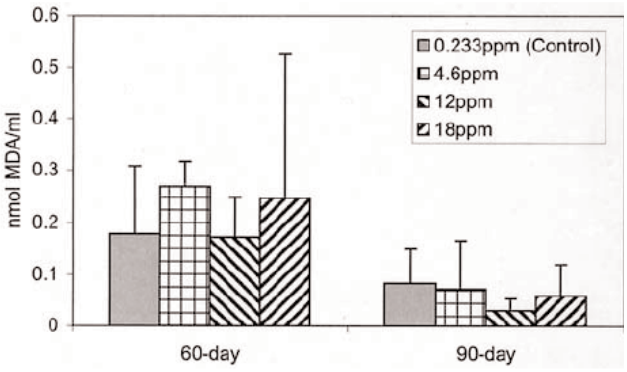

Figure 1. Effects of dietary SeMe treatment on hepatic lipid peroxidation in larval rainbow trout after 60 and 90 days of exposure.

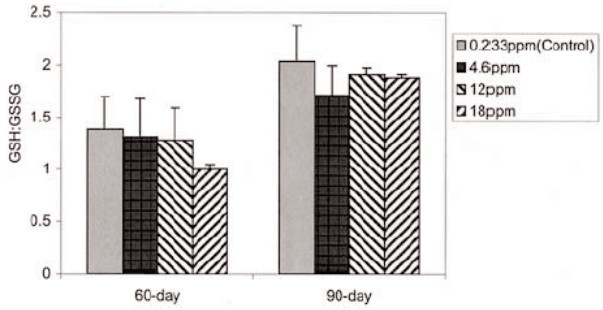

Figure 2. Effects of dietary SeMe treatment on hepatic GSH-to-GSSG ratios in larval rainbow trout after 60 and 90 days of exposure.

trations of the essential metal, copper, in channel catfish exposed to aqueous copper sulfate for 10 weeks reached a maximum concentration at 6 weeks and then decreased. Hamilton *et al.* (1990) observed a similar phenomenon with Chinook salmon and demonstrated a 20% decrease in total Se in control animals after 90 days. Given that Se is an essential nutrient, its rate of uptake, metabolism, or excretion may have been influenced by developmental changes in the fish.

The growth results for the 90-day experiment indicate a body-burden threshold (LOEC) of 1.20 µg/g (wet weight). This value is below tissue residue thresholds (approximately 4 µg/g dry weight) reported by others (Maier and Knight 1994, Lemly 1995, Hamilton 2002, 2004). These differences are likely related to measurement of body burdens at a unique physiologic stage of the animal or the use of wet-weight normalization for Se tissue residues. Other possibilities for the lower threshold include the use of younger fish, an ambient light-to-dark cycle, and (potentially) a lack of Se in the gut contents of fish during whole-body measurements at 90 days (although feeding behavior was never impaired throughout the study). These results demonstrate the difficulty of using body burden residues as toxicity thresholds for essential elements in species that are developmentally immature.

Although growth was impaired in the fish after 90 days, indicators of oxidative stress with the liver were unchanged. The GSH-to-GSSG ratio and the quantification of lipid peroxidation yielded no significant differences, which indicates that the Se concentrations used did not cause oxidative stress. The lack of response of these sublethal end points indicates that the observed growth impacts were caused by mechanisms other than changes in the GSH-to-GSSG ratio or lipid peroxidation. Even if prone to selenoxide formation and GSH oxidation, GSH did not appear to be depleted in the livers of any of the exposed animals, thus indicating either redox cycling did not occur or other cellular defenses against oxidative stress were available to prevent injury. These results contrast with studies in trout embryos and juvenile trout in which GSH-to-GSSG ratios have been shown to be significantly decreased by Se (Palace *et al.* 2004, Schlenk *et al.* 2003). It is well recognized that Se induces GSH peroxidase, which catabolizes lipid hydroperoxides to the corresponding lipid hydroxide (Burk 1997). Consequently, one may speculate that protection against lipid peroxidation may occur in Se-treated animals. However, the lack of GSH depletion in the current study is unclear and suggests the induction of other feedback loops enhancing either reducing equivalents (i.e., NADPH) or GSH synthesis. Clearly, more work is needed to understand the molecular mechanisms of Se toxicity in non-mammalian organisms.

Recent studies of fish tissue Se in San Diego Creek and other channels in southern California indicated that resident fish species might be impacted by Se (CRWQCB 2001). Tissue Se concentrations for red shiner (*Notropis lutrensis*), measured by the State of California's Toxic Substances Monitoring Program (TSMP), ranged from 0.4 to 1.6 µg/g (wet weight), which is higher than the tissue effects threshold obtained for larval trout (Figure 3). The TSMP data

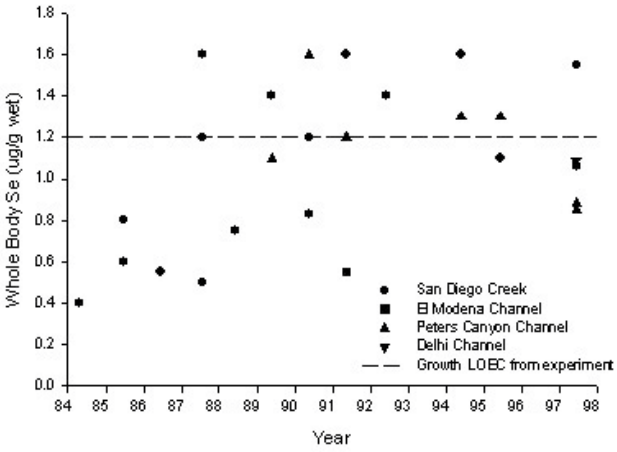

Figure 3. Whole-body concentrations of Se in red shiner (N. lutrensis) collected from San Diego Creek and other channels in the Newport Bay watershed (data from CRWQCB 2001). Dashed line represents growth LOEC determined in the current study (1.2 µg/g wet weight).

represent adult fish of a different species, which may be more tolerant than the larval trout used in this experiment. Species differences have been noted with regard to Se intoxication with carp (*Cyprinus carpio*) and mosquitofish (*Gambusia affinis*) being very resistant (Hamilton 2004). Interestingly, red shiner were one of three species, including fathead minnow and the central plains killifish (*Fundulus zebrinus*), that were noted to be reproductive in the highly seleniferous Sweizer Lake (Barnhart 1957). Given the differences in body-burden concentrations between 60- and 90-day fish, developmental differences between the species may also be a factor affecting sensitivity to Se toxicity among species. The relative sensitivities of red shiner of differing developmental stages to Se are not known. Thus, use of the trout threshold is likely a conservative estimate of risk to fish populations in San Diego Creek.

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