



*Nicholas Ralston
Principal Investigator*

EFFECTS OF Hg TOXICITY ON SE-DEPENDENT MOLECULAR PATHWAYS

Key Personnel: Nicholas Ralston (EERC), Carla Ralston (EERC), Laura Raymond (EERC), Blaise Mibeck (EERC)

Project Description

In order to clearly understand the true toxicity risks of mercury exposure, selenium's involvement in mercury exposure risks needs to be addressed. Mercury–selenium interactions have been shown to occur throughout the mercury biogeochemical cycle, influencing mercury flux and release at every stage of its transition. Physiological interactions between mercury and selenium appear to greatly diminish methylmercury (MeHg) accumulation in fish and significantly reduce the risk associated with their consumption.

Elemental mercury (Hg^0) that becomes oxidized (Hg^{2+}) can eventually become methylated to form the readily bioaccumulated MeHg form that increases in tissue abundance in direct proportion with increasing age and position in the aquatic food chain. Inorganic forms of selenium such as selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are naturally present in varying concentrations in soils and waters, but are poorly reactive with mercury. However, these forms are converted to selenides (H_2Se , HSe^x) under reducing conditions in anaerobic environments within living cells, and these forms of Se have uniquely high-affinity ligands for binding with various forms of Hg. In anaerobic environments, both MeHg and Hg^{2+} will bind to selenides forming the insoluble compounds, mercury selenide (HgSe) and methylmercury selenide (MeHgSe). These insoluble compounds are poorly absorbed and consequently deposited in the sediments. Since the HgSe that is gradually formed in tissues of prey animals exposed to MeHg is very poorly absorbed in their digestive systems, the HgSe becomes deposited in the silt, thus retiring Hg that would otherwise bioaccumulate in aquatic food chains. Therefore, as a result of selenium's extremely high binding affinity for mercury, the formation of HgSe may not only be directly involved in the molecular mechanism of preventing Hg toxicity but may also be important in limiting Hg participation in biologic cycling.

This project evaluates the roles of Se in Hg toxicity, bioaccumulation, and retirement. Task 1 uses cell culture pretreated with varying amounts of Se to examine molecular effects of Se status on MeHg exposure and protection against cell death. Task 2 involves assessing selenium's role in Hg bioaccumulation in natural and artificial aquatic ecosystems.

Goal

The goal of this project is to assess chemical interactions between mercury and selenium in determining their physiological and environmental effects. The same chemical reaction that appears to be responsible for mercury toxicity and selenium-dependent protection against mercury toxicity is also likely to be involved in mercury retirement from aquatic ecosystems. The specific objectives of the proposed work will augment prior research investigating selenium's role in environmental and physiological effects of mercury exposure by performing the following tasks:

- Task 1 investigates biochemical consequences of exposure to toxic amounts of Hg on Se-dependent enzyme processes. Cell and tissue culture studies are being performed to evaluate selenium's effects on Hg toxicity by assessing cell proliferation, necrosis, and enzymatic processes.
- Task 2 assesses the influence of Se on Hg bioaccumulation in freshwater fish. Subtask 2.1 of the study examined Se concentrations in fish collected from a series of lake systems that vary in Se content. Subtask 2.2 of this study examines the effects of Se on Hg bioaccumulation in an artificial food web study. This involves aquarium fish fed controlled diets comprising invertebrates that were fed diets containing low levels of MeHg and varying amounts of selenium.

Rationale

Measuring the amount of mercury present in the environment or food sources provides an incomplete and inaccurate indication of mercury-associated risks if the presence and effects of selenium are not also considered. Because of the high affinity toward each other, mercury selectively binds with selenium and forms HgSe. Mercury selenide precipitates have extremely low solubility in water, ranging from 10^{-58} to 10^{-65} (1); thus, in this form, they are metabolically inert (2). This important Hg–Se interaction is apparent throughout the mercury cycle, influencing its transport, biogeochemical exposure, bioaccumulation, bioavailability, potential toxicological consequences, and remediation.

Physiologically, the interaction that forms HgSe is considered the basis of selenium's "protective" effect against mercury toxicity. Selenium is essential to support the synthesis of numerous types of selenoenzymes that are expressed in all tissues of all animals. Selenium has important roles in growth, development, reproduction, cardiovascular disease, and mood disorders. It has powerful antioxidant and anticancer properties, and it is essential for normal thyroid hormone homeostasis and immunity. Its involvement in medicine is becoming increasingly evident and has been specifically linked to many disease processes. Prior CATM-supported research suggests MeHg toxicity results from the interruption of normal processes of selenoenzyme synthesis because of the Hg sequestration of Se (3). This hypothesis implies that the protective effect of selenium against mercury toxicity is the result of supplemental selenium supporting normal selenoenzyme synthesis in otherwise compromised tissues.

While selenium's protective effect has been established in all investigated species of mammals, birds, and fish, additional studies suggest an important environmental role of Se in Hg bioaccumulation. Fish tissue containing Hg is inversely related to the abundance of Se present in the ecosystem. Se supplementation of lake waters in Sweden resulted in a 75%–85% reduction in Hg levels of fish over a 3-year period (4). Elimination of Se-rich discharges of fly ash to an artificial lake caused a steady increase in Hg concentrations (5). Other studies have demonstrated that the environmental availability of Se is inversely related to Hg contents in fish (6, 7). Studies such as these confirm the importance of Se-dependent Hg retirement from aquatic ecosystems, yet the mechanism involved remains undefined.

Although the forms of Hg and Se present in the water column are not readily interactive, the low solubility and poor bioavailability of Hg from HgSe formed in invertebrates of the food web may explain why fish MeHg seems to be inversely related to the abundance of environmental Se. Once the HgSe is formed within the cell, it becomes biologically unavailable. Therefore, the HgSe formed by the prey will pass through the digestive system of its predator and deposit in the silt of the ecosystem. Since invertebrates comprise up to 90% of the food consumed by fish (8, 9), the influence of Se on Hg bioaccumulation in insects can be an important aspect influencing Hg bioaccumulation in fish.

Approach

Task 1 – Effects of Hg Toxicity on Se-Dependent Molecular Pathways

HL60 cells grown in IMDM culture media supplemented with 20% bovine serum albumin have been tested to establish their sensitivities to methylmercury chloride and selenium using log and half-log concentration exposures. Six well plates were loaded with three mL of HL60 cells at 0.5×10^6 /mL. Cells were grown at 37°C in a fully humidified 5% CO₂ atmosphere for 3 days. During the growth period, cell viabilities, proliferation rates, and total cell counts were assessed at regular intervals.

Task 2 – Selenium Analysis in Mercury-Polluted Lake Ecosystems

Subtask 2.1 – Selenium in Freshwater Fish

Selenium analyses were performed on 468 fish of 40 different species from 137 sites (some with multiple fish samples) across 12 western U.S. states. Fish included all of the piscivores (n = 206) analyzed previously for Hg by Peterson et al. (10) and a random sampling of the remaining nonpiscivores (n = 262) from the same authors' original sampling of 2707 large fish. All Se analyses were done on freeze-dried fish homogenate samples by standard comparator instrumental neutron activation analysis (INAA) at the University of Missouri Research Reactor. The freeze-dried fish homogenate samples were irradiated for 5 seconds in a thermal flux of approximately 8×10^{13} n/cm²/s, and the samples were real-time-counted for 30 seconds following a 15-second decay. The mass of Se in a sample was quantified using the 162-keV peak from the decay of ⁷⁷mSe ($t^{1/2} = 17.45$ s). The area of the 162-keV peak was corrected for the direct spectral interference of hafnium by also monitoring the 214-keV peak from 179 mHf. Gravimetric comparator standards were prepared by pipetting approximately 0.5 µg of Se from a 1000 ± 30 ppm certified standard solution on paper pulp in a 0.25-dram high-density polyethylene (HDPE) vial. Six comparator standards were included with every analytical run: two standards at the beginning, two standards in the middle, and two standards at the end of the sample sequence. Samples were analyzed in duplicate and were reanalyzed if the relative standard deviation (RSD) exceeded $\pm 8\%$. The result for each sample was reported as the mean dry weight Se concentration. The dry weight result was corrected back to the as-received wet weight of each fish based on the difference between the original wet weight and the freeze dried weight of each fish tissue sample.

The fish were evaluated relative to a Hg threshold for wildlife (0.1 µg Hg/g) and the current tissue-based water quality Hg criterion for the protection of humans (0.3 µg Hg/g). Since Se appears to provide protection against Hg toxicity when Se:Hg molar ratios are >1:1, fish contents were also evaluated in relation to this threshold.

Subtask 2.2 – Influence of Hg and Se on MeHg Accumulation in Crickets and Fish

In earlier CATM projects, nutritionally complete diets prepared with graduated Se and Hg contents were fed to large groups ($n = \sim 1000$) of crickets to emulate those arising from food webs with low, adequate, and moderate Se and MeHg levels (0.1, 1.0, and 3.0 $\mu\text{mol Se/kg}$). Small amounts of mercury (0.5 $\mu\text{mol Hg/kg}$) were observed in the basal laboratory diets before addition of 0.5 $\mu\text{mol MeHg/kg}$. Therefore, these three MeHg-supplemented diets contained 1.0 $\mu\text{mol Hg/kg}$. The control group was fed a diet containing adequate selenium without any added MeHg. In Phase 1, groups of crickets ($n = \sim 1000$) were grown to maturity while being fed these diets (~ 6 weeks), then terminated by freezing and freeze drying. The crickets from each group were powdered and analyzed for their mercury and selenium contents. In Phase 2, groups of crickets ($n = \sim 1000$) were fed diets composed of control diets supplemented with powdered crickets (1:2).

In the present study, the crickets grown through a two-stage food chain were fed to fish. These crickets had been crushed to a powder and were fed as the only source of MeHg and Se in the fish diets. These diets were fed to zebra fish in a controlled aquarium study consisting of ten fish per treatment group. Four 10-gallon aquariums, each equipped with under-gravel filters and fluorescent lamp and hood, were used in this study. Aquarium lighting (single 15-watt fluorescent lamp) was connected to a timer to provide a day/night cycle of 10 hr on, 14 hr off. Air pumps were used to power all under-gravel filters using fine bubble plastic diffusers. Aquaria were cycled for 38 days to stabilize pH and nitrate levels prior to adding fish. Fish were added to each tank and maintained for 27 days to ensure tanks were stable before starting the study. During this time, fish were fed 3 days a week using ~ 140 mg of Tetra Color Tropical Crisps. A Freshwater Test Kit was used to measure pH, ammonia, nitrite, and nitrates.

The fish used in this study are wild strain zebra danio (cyprinidae *danio rerio*). These were obtained from Florida-based breeder Aquatica Tropicals, Inc. On the day the feeding study was initiated, eight fish were randomly selected from the tanks to comprise the pretreatment group. These fish were used to establish initial body weight and mercury and selenium contents before the feeding study began. The fish were anaesthetized in club soda, patted dry, weighed, placed in individual ziplock bags, and stored frozen until analysis.

The remaining 40 fish were split into four treatment groups of ten each and placed in four 10-gallon tanks. On alternating days, three times a week, each group was fed ~ 70 mg of their assigned diets as detailed below. The diets consist of crickets from Level 2 of the food chain study that were freeze-dried and crushed into a fine powder for use as fish food. The diet assignments are shown below:

- Group 1 – 1.0 $\mu\text{mol Se}$, 0.0 $\mu\text{mol MeHg}$
- Group 2 – 0.1 $\mu\text{mol Se}$, 0.5 $\mu\text{mol MeHg}$
- Group 3 – 1.0 $\mu\text{mol Se}$, 0.5 $\mu\text{mol MeHg}$
- Group 4 – 3.0 $\mu\text{mol Se}$, 0.5 $\mu\text{mol MeHg}$

Progress/Status

Task 1 – Effects of Hg Toxicity on Se-Dependent Molecular Pathways

HL60 cells that were grown in the presence of graduated amounts of sodium selenite added to media were maintained with these selenium contents at the start of each passage. After two passages at these selenite addition levels, cells were exposed to the indicated MeHg concentrations. The proliferation rates supported by selenium are shown in Figure 1.

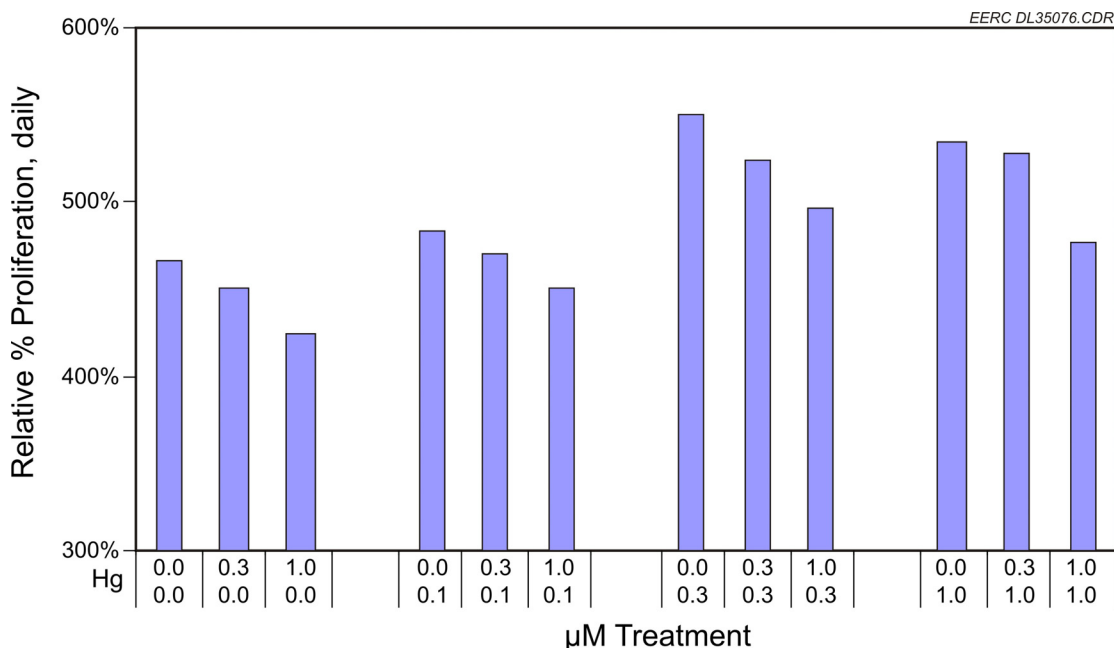


Figure 1. Effects of selenium pretreatment on proliferation in methylmercury-treated HL60 cells.

In cells that were not subjected to methylmercury challenge, there was a gradual increase in proliferation rates in HL60s as selenite concentrations increased to 0.3 μM and declined at 1 μM Se and higher concentrations. The negative effects of exposure to 0.3 and 1.0 μM MeHg were Hg concentration-dependent, but proliferation rates were also increased as Se pretreatment concentrations increased.

Task 2 – Selenium Analysis in Mercury-Polluted Lake Ecosystems

Subtask 2.1 – Selenium Analysis in Freshwater Fish

This study examines the hypothesis that mercury bioaccumulation in fish will be inversely related to selenium bioavailability in the food chain that supports the fish. Based on this hypothesis, it would be expected that higher mercury concentrations will be observed in fish from low-selenium lakes such as those of northern Minnesota, and lower mercury concentrations will be present in fish from lakes with richer selenium status. Although it was not possible to obtain data regarding the selenium concentrations from Minnesota fish, working together with the EPA research group in Corvallis, Oregon, and the Research Reactor Group at the University of Missouri, the influence of Se status on Hg in fish collected from rivers and streams throughout the western United States was assessed.

Whole body Se and Hg concentrations in 468 fish representing 40 species from 137 sites across 12 western U.S. states were analyzed. Fish included all of the piscivores (n = 206) analyzed previously for Hg by Peterson et al. (10) and a random sampling of the remaining nonpiscivores (n = 262) from original sampling of 2707 large fish. As expected, Hg concentrations in piscivores were greater (more than double) those of nonpiscivores. However, mean Se concentrations were greater for nonpiscivores than for piscivores. Mean Hg concentrations (μg/g) by fish group indicate piscivores pose a risk relative to the wildlife threshold of 0.1 μg Hg/g, but the nonpiscivores present a mixed picture. Individual pikeminnow, walleye, sauger, bass, and pike also exceeded the human health MeHg criterion (0.3 μg/g

for file) as it relates to whole fish Hg concentrations ($\geq 0.185 \mu\text{g/g}$) (10). If assessments were based on Hg content alone, many of these individual fish would go on the “Do Not Eat” list.

About 56% of the fish sampled had Hg concentrations that exceeded the wildlife Hg threshold, while 12% exceeded the human health consumption criterion. However, 97.5% of these fish contained more Se than Hg (molar ratio >1), leaving only 2.5% with Se:Hg ratios <1 . All but one of the fish with Se:Hg <1 , were of the genus *Ptychocheilus* (pikeminnow). The results of this study have been prepared as the manuscript that has been submitted to the journal, *Science of the Total Environment*, and is currently being reviewed.

Subtask 2.2 – Influence of Hg and Se on MeHg Accumulation in Insects and Fish

Sample groups of 10 zebra danio (*Danio rerio*) of equivalent size have been added to each of four identically prepared individual aquaria (total of 40 fish). These fish are currently being fed diets composed of powdered crickets that were grown as part of the insect food chain study described previously.

The fish contained a small amount of mercury upon arrival, but those that consumed crickets that had been fed $0.5 \mu\text{mol MeHg}$ accumulated more Hg in their tissues. As has been noted in rats fed dietary selenium, mercury bioaccumulation in tissues increases with increasing dietary selenium levels ($y = 0.1835x + 0.022$, $R^2 = 0.64$, $F = 22.8$ $p < 0.001$). The increased bioaccumulation of mercury appears to occur in association with selenium, potentially resulting in formation of mercury selenides that would be poorly bioavailable. Analysis of second and subsequent generations is needed to confirm this hypothesis. Provided the assumption regarding formation of mercury selenides is correct, it is expected that the second and all subsequent levels of the fish food chain will experience less mercury bioaccumulation since increased mercury retirement will be occurring.

Quality Assurance/Quality Control

An EERC quality management system (QMS), authorized and supported by EERC managers, is in effect and governs all programs within the organization. Additionally, the CATM Program has a quality assurance plan (QAP) in effect that addresses trace metal emissions research at the EERC. The CATM QAP has been reviewed and accepted by EPA. This project follows the quality manual in order to obtain statistically valid and physiologically meaningful results regarding the interactions of mercury and selenium.

Quality Objectives

The experiments in Task 1 were conducted with carefully controlled treatment regimes so that they are repeatable and it is possible to clearly distinguish differences in the assessed end points. In order to provide quantitative comparisons, group treatments in Task 2.1 are carefully matched to ensure the fish grown in different aquaria are equivalent other than the amounts of trace elements supplied in diets and growth medium.

Measurement/Data Acquisition

Cultures are grown under identical conditions other than their treatment media concentrations. Cell counts and enzymatic analyses are performed in duplicate according to their respective kit instructions,

and each study is repeated a minimum of three times. The same instruments, types of plating supplies, and batch media/serum are used to ensure accurate repeatability. Fish samples from this study and from Task 2.2 are processed and analyzed using identical treatments to ensure elemental concentrations in the samples are precisely and accurately determined. Trace elements are determined in batches that include certified quality control samples analyzed relative to calibration standards according to established protocol.

Assessment and Validation

Protocols being used in this project have been established and are maintained to ensure accurate and precise analytical results are obtained. All sampling, instrument calibrations, and quality control considerations are included in the protocols. Quality control samples including analytical blanks and certified reference materials are included in each batch to ensure validity of observed analysis values.

Status

The project is currently ongoing and near completion. Final enzymatic studies in cell cultures are being performed, and data sets are being analyzed for statistical significance. All freshwater and laboratory-controlled fish analysis are completed and the Hg, MeHg, and Se data sets have been compared and evaluated for statistically significant relationships. Final reporting and writing has begun, and material is being prepared for presentation at various conferences. Likewise, this information will be disseminated to support further related research studies.

Potential Applications and Benefits

Potential Users and Real-Life Applications

The findings of these studies will provide important information for EPA, state fish and gaming agencies, the U.S. Food and Drug Administration, and the World Health Organization. This information may assist these agencies in making regulatory policy decisions regarding mercury exposure. Recognizing the influence of mercury–selenium relationships on mercury bioaccumulation in fish and the physiological impacts of consumption will help these agencies in assessing the risks of human mercury exposure.

Environmental and/or Health Benefits

The availability of selenium for uptake by fish and plant life in the aquatic environment may have an important effect on bioaccumulation of Hg in the food chain. Likewise, the formation of HgSe complexes within plants may be involved in Hg phytoremediation—the use of plants to remove mercury from contaminated soil or water. In ecosystems where the geological and biological availability of Se supports increased Se physiology, increased HgSe formation may contribute to increased Hg retirement. In ecosystems where Se either is absent from soils or is poorly available because of low pH or other limiting factors, the paucity of intracellular Se will limit Hg retirement as HgSe formation. Certain plants are known to accumulate large amounts of Hg and Se, but the interactions of the two elements in these plants have been inadequately explored. Since Hg hyperaccumulator plants are also known to be Se accumulators, the potential for HgSe formation is reasonable to consider. These aspects may have implications for environmental treatment of high mercury remediation and bioaccumulation.

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