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## EVALUATION OF SELENIUM'S ROLE IN HEAVY METAL BIOACCUMULATION AND TOXICITY

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### *Project Summary*

Mercury–selenium interactions appear to occur throughout the mercury biogeochemical cycle, influencing mercury flux and release at every stage of its transition, from initial release from geologic sources to its bioaccumulation and the potentially toxic effects of exposure, to final formation of stable complexes that temporarily or permanently retire mercury from further cycling in the environment. Physiological interactions between mercury and selenium appear to greatly diminish methylmercury accumulation in fish and significantly reduce the risk associated with their consumption. In order to clearly understand the true toxicity risks of mercury exposure, selenium's involvement in mercury exposure risks needs to be addressed, and bioaccumulation is a key factor influencing this pathway.

Inorganic forms of selenium such as selenate ( $\text{SeO}_4^{2-}$ ) and selenite ( $\text{SeO}_3^{2-}$ ) are naturally present in varying concentrations in soils and waters, but are poorly reactive with mercury. These forms are converted to selenides ( $\text{H}_2\text{Se}$ ,  $\text{HSe-X}$ ) under reducing conditions in anaerobic environments either exterior to (water/soil) or within living cells and become uniquely high-affinity ligands for binding with various forms of Hg. In anaerobic environments, both methylmercury (MeHg) and oxidized mercury ( $\text{Hg}^{2+}$ ) can combine with selenides to form the insoluble and, therefore, biologically unavailable compounds methylmercury selenide (MeHgSe) and mercury selenide (HgSe). Like other inorganic forms of mercury (oxides, sulfides, etc.) HgSe is poorly absorbed, HgSe is deposited in the sediments. Elemental mercury ( $\text{Hg}^0$ ) that becomes oxidized ( $\text{Hg}^{2+}$ ) 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. As a result of selenium's extremely high binding affinity for mercury, the formation of HgSe is directly involved in the molecular mechanism of Hg toxicity, but also may be important in limiting Hg participation in biologic cycling. The HgSe that is gradually formed in tissues of prey animals exposed to MeHg is very poorly absorbed by digestive systems of the predators that consume them. The poor bioavailability of Hg from HgSe retires Hg that would otherwise bioaccumulate in aquatic food chains.

This project evaluates the roles of Se in Hg toxicity, bioaccumulation, and retirement. Task 1 uses tissues pretreated with varying amounts of Se to examine molecular effects of Se status on  $\text{Hg}^{2+}$  and

MeHg exposure on Se-dependent protection against cell death. Task 2 involves assessing selenium's role in Hg bioaccumulation in natural and artificial aquatic ecosystems.

### *Goals and Objectives*

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

- Task 1 will investigate biochemical consequences of exposure to toxic amounts of Hg on Se-dependent enzyme processes. In collaboration with Dr. Othman Ghribi from the University of North Dakota's Neurology Department, molecular biology techniques will be used to assess Hg toxicity effects on apoptosis and Se-dependent effects on physiological processes involving  $\text{Hg}^{2+}$  and MeHg.
- Task 2 will determine the influence of Se on Hg bioaccumulation by freshwater fish. Subtask 2.1 of the study will examine Se concentrations in fish collected from a series of lake systems that vary in Se content. These fish have already been analyzed for Hg and/or MeHg contents. Subtask 2.2 of this study will examine the effects of Se on Hg bioaccumulation in an artificial food web study. This will involve a 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, 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.

Recently, investigations of a particular selenoprotein, thioredoxin reductases-1, offered a compelling new insight supporting this hypothesis (4). Thioredoxin reductases (TrxR) and their substrate thioredoxin (Trx) regulate a range of cellular systems. TrxR is critical in DNA production, gene

expression, cell survival, and embryogenesis. Thus TrxR enables basic processes and regulates multiple metabolic events. Since TrxR is considered essential for cell viability, it is an important target of many pharmaceutical drugs, such as anticancer and antirheumatic agents. However, recent investigations have shown that a truncated form of TrxR1 (GRIM-12, the gene associated with retinoid interferon-induced mortality) functions as a potent cell death-promoting factor. Remarkably, the only difference between the truncated and full-length protein is the presence of the two final selenocysteine amino acids in the full-length protein. This is the only protein known to have dual, opposing roles based on selenium availability. Considering Hg's ability to sequester selenium, this enzyme may be specifically important in the pathophysiology of Hg toxicity and Se deficiency.

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 (5). Elimination of Se-rich discharges of fly ash to an artificial lake caused a steady increase in Hg concentrations (6). Other studies have demonstrated that the environmental availability of Se is inversely related to Hg contents in fish (7, 8). 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 (9, 10), the influence of Se on Hg bioaccumulation in insects can be an important aspect influencing Hg bioaccumulation in fish.

Additionally, the availability of selenium for uptake by plant life in the aquatic environment may also have an important effect on bioaccumulation of Hg in the food chain. Likewise, the formation of HgSe complexes within the plants may be involved in phytoremediation—the use of plants to remove mercury from contaminated soil or water. 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. Therefore, in ecosystems where the geological and biological availability of Se supports increased Se physiology, increased HgSe formations should contribute to increased Hg retirement. In ecosystems where Se is either 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.

## *Approach*

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

Cerebral cortex and cerebellum from long rats will be dissected, trimmed of excess white matter, sliced, plated, and cultured in a humidified incubator atmosphere under conditions optimized for sustained culture. Organotypic slices are prepared as follows. Hippocampus, cerebral cortex, and cerebellum from adult animals were dissected, trimmed of excess white matter, and placed into chilled dissection media composed of Hibernate A (BrainBits) containing 2% B27 supplement and 2 mM L-glutamine (Invitrogen). Isolated tissue is placed on a wetted filter paper on the Teflon stage of a MacIlwain chopper for coronal sectioning (300  $\mu$ m thick). Sections are placed in new dissection media

and allowed to rest 5 minutes on ice before separating and plating five to ten slices on each membrane insert (Millipore). Inserts are placed in 35-mm culture dishes containing 1.1 mL growth media (Neurobasal A with 20% horse serum, 2 mM L-glutamine, 100 U/mL penicillin, and 0.05  $\mu$ M/mL streptomycin), and warmed 30 minutes prior to plating to ensure complete equilibration. Slices are exposed to a humidified incubator atmosphere (4.5% CO<sub>2</sub> and 35°C). Media are changed at DIV1 and every third day thereafter. At DIV4, slices are switched to a defined medium consisting of Neurobasal A, 2% B27 supplement, and 2 mM L-glutamine. On Day 10 postharvest, slices will be switched to cell culture media supplemented with varied concentrations of Selenium and incrementally increasing concentrations of MeHg and Hg<sup>2+</sup> (HgCl<sub>2</sub>) through five log order increases in concentration running from 1 nM to 10  $\mu$ M. Toxic effects, such as apoptotic responses, at these Hg and MeHg exposure levels will be assessed at regular intervals during posttreatment using western and northern blot techniques to assess time- and treatment-dependent effects on apoptosis initiation.

## **Task 2 – Selenium Analysis in Mercury-Polluted Lake Ecosystems**

### *Subtask 2.1 – Selenium Analysis in Freshwater Fish*

Approximately 100 archived lake fish samples from Minnesota and Florida state agencies and/or researchers will be acquired and analyzed for selenium. These fish have already been analyzed for Hg and MeHg contents by the respective state agencies of origin. The Hg, MeHg, and Se data sets will be compared and evaluated for statistically significant relationships.

### *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 = ~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  $\mu$ mol Se/kg). Small amounts of mercury (0.5  $\mu$ mol Hg/kg) were observed in the basal laboratory diets before addition of 0.5  $\mu$ mol MeHg/kg. Therefore, these three MeHg-supplemented diets contained 1.0  $\mu$ mol Hg/kg. The control group was fed a diet containing adequate selenium without any added MeHg. In Phase 1, groups of crickets (n = ~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 = ~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 will be fed to fish. These crickets have been crushed to a powder and will be fed as the only source of MeHg and Se in the fish diets. These diets will be fed to zebra fish in a controlled aquarium study consisting of 10 fish per treatment group. The fish will be analyzed for Hg, Se, and Hg–Se to establish Se-dependent effects on Hg accumulation and retention.

## ***Progress/Status***

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

#### *Organotypic Cell Cultures*

We have found organotypic slice systems have several distinct advantages over other in vitro systems since local connectivity between neurons, interneurons, and glia is maintained. This system also has several advantages over in vivo systems including simplified dose and time administration of

experimental agents. The methods for growing organotypic slices from adult rabbits have been optimized for sustained culture of hippocampus, cerebral cortex, and cerebellum. In the first series of experiments for this project, we attempted to grow organotypic cultures from rat brain slices but have been unsuccessful. Therefore, we have changed scope from using rat brains to using rabbit brains. These studies are ongoing.

## **Task 2 – Selenium Analysis in Mercury-Polluted Lake Ecosystems (SAMPLE)**

### *Subtask 2.1 – Selenium Analysis in Freshwater 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 in the annual report chapter describing our Year 14 studies.

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

Fish samples collected from lakes throughout Minnesota have been analyzed for their mercury contents. Lakes where both northern pike and yellow perch have been collected have been selected for this study. The lakes these fish originate from include the northern areas of the state that are known to have lower in-soil Se levels as well as lakes from central and southern regions that are generally richer in Se. A preliminary comparison of mercury levels in fish from the northern vs. south-central regions has been performed as described below.

Because of distinctions in the selenium contents of their soils, the lakes of northern Minnesota tend to have poorer selenium availability than lakes from the central and southern section of the state. It is very important to note that the lakes from these regions will differ in many important respects other than selenium availability. Therefore, although we are examining the hypothesis that “fish accumulation of mercury will be inversely related to environmental selenium availability,” the limitations of this study will not allow exclusive assignment of any observed differences to selenium alone. The findings of this study will either tend to confirm or tend to refute the hypothesis, but will need to be fully considered in the context of other potential differences that may cooperatively or antagonistically interact with selenium-dependent effects. Our expectation is that increased selenium bioavailability in lakes from southern and central Minnesota will cause fish collected from these lakes to have lower mercury levels than fish of comparable size collected from lakes in northern Minnesota.

This study will directly examine the selenium levels in fish from these lakes to establish the selenium bioavailability in their food webs, but this preliminary examination will simply compare mercury levels in fish from these regions to establish whether fish from northern Minnesota contain more mercury than fish from southern or central Minnesota.

Yellow perch (*Perca flavescens*) and northern pike (*Esox lucius*) that have previously been collected from eight northern and eight southern lakes of Minnesota and analyzed for their mercury contents have been selected for this preliminary study. The number of fish samples tentatively selected for inclusion from these lakes are approximately equal: 22–23 fish of each type from both regions. The observed amounts of mercury in these fish are shown in Figure 1.

It is clear from Figure 1 that northern pike accumulate much higher ( $p < 0.001$ ) total mercury concentrations than yellow perch. This difference is consistent with findings of previous studies and is

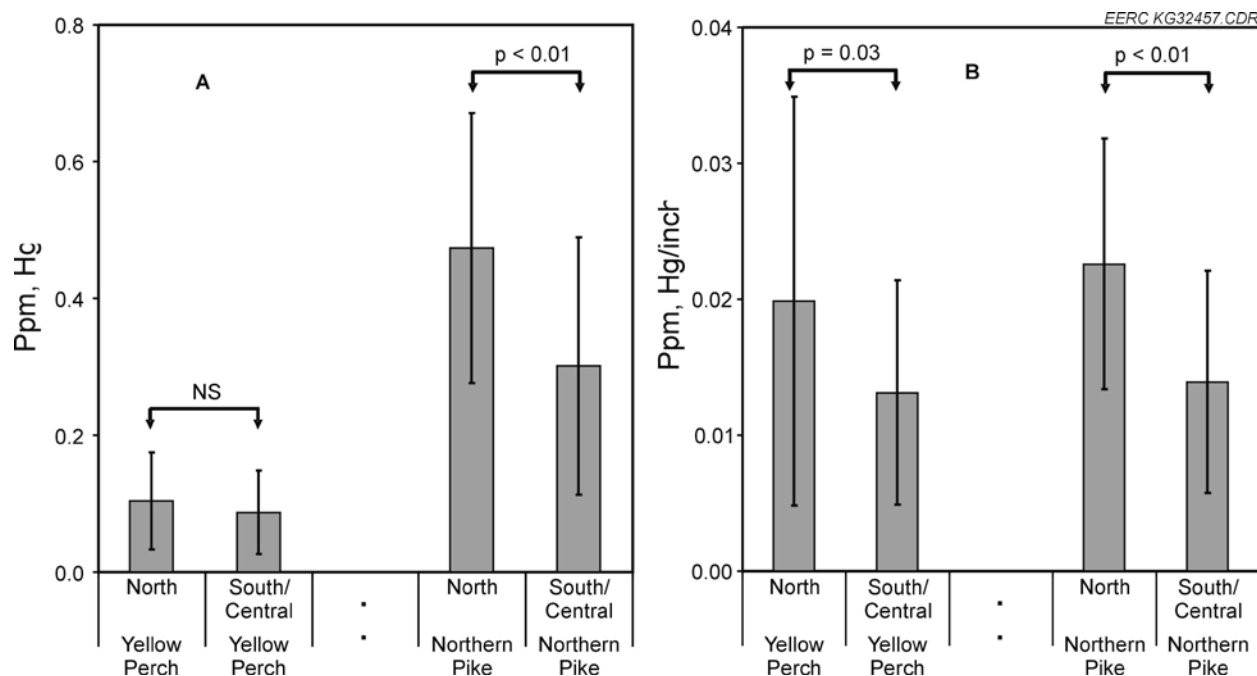


Figure 1. Panel A depicts the observed concentrations of mercury in yellow perch and northern pike from northern vs. south/central Minnesota lakes. Panel B shows the concentrations of mercury normalized to fish size.

directly associated with their status as top predators in their lake ecosystems. Northern pike collected from northern and south/central Minnesota lakes were equivalent in size:  $20.9 \pm 0.6$  and  $21.3 \pm 1.0$  inches, respectively. However, yellow perch from south/central lakes ( $6.53 \pm 1.5$  in.) were larger ( $p = 0.1$ ) than perch collected from northern lakes ( $5.5 \pm 1.3$  in.). The greater size of perch collected from south/central lakes is likely to be due to a combination of greater abundance and more varied composition of the food webs in their lakes of origin and may involve other factors such as temperature-dependent effects on their growth. Since mercury accumulation is known to be directly associated with bioaccumulation from food, this size difference made it necessary to normalize the data. Therefore, mercury concentrations in all the fish samples were normalized in relation to size by dividing their observed mercury concentrations by the length of the fish. The normalized values (ppm Hg/in.) that result from this manipulation indicate the mercury levels in yellow perch and northern pike are nearly equivalent ( $p > 0.05$ ) on this basis. However, distinctions in mercury concentrations of fish samples from northern and south/central lakes are more readily evident.

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 of northern Minnesota, and lower mercury concentrations will be present in fish from regions of the state with richer selenium status. Until we obtain data regarding the selenium concentrations in these fish, it is not appropriate to assign differences in their mercury content to the bioavailability of selenium from these lake ecosystems. However, it is interesting to note that there are clear differences in the mercury contents of these fish that tend to coincide with expectations based on the selenium-retirement hypothesis.

### ***Quality Objectives Measurement/Data Acquisition***

The experiments in Task 1 are being conducted with carefully controlled treatment regimes so that it will be possible to clearly distinguish differences in brain tissue survival that are attributable to distinctions in mercury and/or selenium treatments.

Group treatments in Task 2.1 are carefully matched to ensure the fish grown in different aquaria are equivalent other than the diets they are being fed. Fish samples from this study and from Task 2.2 will be processed and analyzed using identical treatments to ensure elemental concentrations in the samples are precisely and accurately determined, other than the amounts of trace elements supplied in diets and growth medium in order to provide quantitative comparisons. Trace elements will be 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.

### ***References***

1. Dyrssen, D.; Wedborg, M. The Sulfur–Mercury (II) System in Natural Waters. *Water, Air, Soil Pollut.* **1991**, *56*, 507–519.
2. Nuttall, K.L. A Model for Metal Selenide Formation under Biological Conditions. *Med. Hypoth.* **1987**, *24*, 217–221.
3. Center for Air Toxic Metals. *Center for Air Toxic Metals 2005 Annual Report*; Annual Report of the Center for Air Toxic Metals, organized and administered through the University of North Dakota Energy & Environmental Research Center, for U.S. Environmental Protection Agency Contract No. R 827649-01; 2006..
4. Anestal, K.; Arner, Elias S.J. Rapid Induction of Cell Death by Selenium-Compromised Thioredoxin Reductase 1 but Not by the Fully Active Enzyme Containing Selenocysteine. *J. Biol. Chem.* **2003**, *278* (18), 15966–15972.
5. Paulsson, K.; Lindbergh, K. The Selenium Method for Treatment of Lakes for Elevated Levels of Mercury in Fish. *Sci. Total Environ.* **1989**, *87–88*, 495–507.
6. Southworth, G.R.; Peterson, M.J.; Turner, R.R. Changes in Concentrations of Selenium and Mercury in Largemouth Bass Following Elimination of Fly Ash Discharge to a Quarry. *Chemosph.* **1994**, *29* (1), 71–79.
7. Chen, Y.-W.; Belzile, N.; Gunn, J.M. Antagonistic Effect of Selenium on Mercury Assimilation by Fish Populations near Sudbury Metal Smelters? *Limnol. Oceanogr.* **2001**, *46* (7), 1814–1818.

8. Belzile, N.; Chen, Y.-W.; Gunn, J.M.; Tong, J.; Alarie, Y.; Delonchamp, T.; Yang, C.-Y. The Effect of Selenium on Mercury Assimilation by Freshwater Organisms. *Can. J. Fish. Aquat. Sci.* **2006**, *63* (1), 1–10.
9. Hall, B.D.; Bodaly, R.A.; Fudge, R.J.P.; Rudd, J. W. M.; Rosenberg, D.M.. Food as the Dominant Pathway of Methylmercury Uptake by Fish. *Water, Air, Soil Pollut.* **1997**, *100*, 13–24.
10. Boudou, A.; Ribeyre, F. Mercury in the Food Web: Accumulation and Transfer Mechanisms. In *Metal Ions in Biological Systems: Mercury and Its Effects on Environment and Biology*; Sigel, A.; Sigel, B., Eds.; Marcel Dekker Inc.: New York, **1997**.