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MERCURY'S INTERACTION WITH SELENIUM

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Project Description

Environmental mercury can either bioaccumulate within an aquatic food chain as the readily absorbed and highly toxic form methylmercury (MeHg) or biologically retire as insoluble and inert forms such as mercury sulfide (HgS) and mercury selenide (HgSe). Based on ongoing CATM research, it appears that the extraordinarily high-affinity binding between Se and Hg is involved in many important aspects of MeHg bioaccumulation and pathophysiology. This project examines these interactions from multiple perspectives in order to assess the environmental benefits of Se binding with Hg and the physiological harms of Hg binding with Se.

Goal and Objectives

In order to better understand how Se limits Hg accumulation and how Hg harms Se physiology, it is important to study their interactions from multiple perspectives in an integrated research program. Research in previous years has used animal models to study Se-dependent protection against Hg toxicity and invertebrates to assess Se-dependent Hg retirement from aquatic ecosystems. To continue with an integrated analysis approach, the goals of this project are threefold: 1) to assess environmental factors of Hg and Se accumulation by analyzing plants and invertebrates and the potential ways of remotely detecting Hg and Se accumulation in plants; 2) to investigate the influence of Se status on MeHg toxicity at the cellular level in time-dose-dependent studies; and 3) to investigate effects of Hg accumulation in humans through the examination of Hg concentrations in normal heart tissues and in hearts removed during transplantation procedures performed on patients with ischemic heart disease or dilated cardiomyopathy.

Rationale

The importance of the Hg-Se interaction has many environmental and physiological implications that can influence MeHg exposure risks. Selenium, an important nutrient present in many foods including fish, is known to counteract Hg toxicity. It had previously been thought that selenium's potent protective

effect against Hg toxicity was due to its exceptional binding affinity for Hg. However, a different perspective on this interaction suggests MeHg toxicity is actually the result of this binding affinity. This mechanism involves mercury sequestering intracellular selenium, thereby inhibiting formation of vital selenoenzymes in the central nervous system. If this is the case, the greatest risk from MeHg exposure will occur in Se-poor populations whose diets originate from locally grown foods and freshwater fish. The Se-rich fields and pastures of the midsection of North America produce Se-rich foods that are broadly distributed through the centralized food distribution system. However, populations and individuals living in Se-poor regions may be at risk of having lower Se status if they primarily consume locally grown foods. Selenium-poor regions of the world include much of northern Europe, most of Africa, and many parts of Asia. Because of the particularly low soil Se levels in Finland and New Zealand, risks associated with Hg exposure would be far more pronounced than elsewhere. Populations in these countries and other Se-poor regions need to be cautious of consuming diets exclusively comprising foods grown in their local areas, since a low Se status will offer less Se-dependent protection against MeHg exposure. Fish originating from lakes in regions with low Se availability will be particularly risky to consume since these lakes will tend to display increased MeHg bioaccumulation in fish, and there will be far less Se available to counteract the toxicity of the MeHg that is consumed.

Selenium availability for uptake by plant life in the aquatic environment is likely to have an important effect on Hg bioaccumulation in the food chain. Formation of HgSe is minimal in the water column but greatly potentiated by the molecular species of Hg and Se that occur within living cells. Plant life, especially in the cases of Hg hyperaccumulator plant species, is likely to contribute to Hg retirement through HgSe formation as a result of biochemical processes that parallel those occurring in animal cells. Invertebrates that form the base of the aquatic food chain will contribute to Hg retirement also by facilitating HgSe formation. Likewise, selenium availability and uptake by plant and animal life in the aquatic environment will diminish the bioaccumulation of Hg in the food chain. Since HgSe is insoluble and extremely resistant to dissociation, Hg occurring in this form in the tissues of prey animals is poorly absorbed by predators that consume them. Therefore, if MeHg accumulation is reduced in the insects that make up 90% of the food consumed by fish, it will diminish the amount of MeHg accumulation in fish.

Currently, there are no techniques available that allow a rapid, precise, and inexpensive means of identifying areas that have high concentrations of heavy metals. Extensive sampling is currently needed to map soil Se or Hg concentrations. However, spectroscopic analysis of vegetation may provide a means of detection that can be used to identify either isolated or regional distributions of these elements. Remote sensing has shown that Se-rich soybean showed a spectral reflectance shift to shorter wavelengths in the long-wavelength edge of the chlorophyll absorption band centered at 680 nm and higher reflectance in the 550–650-nm region. Therefore, selenium incorporation in plants reportedly causes a specific shift in reflectance of light at 660 nm. This spectral shift may enable rapid and remote detection of Se distribution, thereby establishing a means of identifying areas of high- vs. low-Se soil availability. This approach is currently being assessed in a related CATM project. If it proves to be sufficiently sensitive, sensing through satellite imaging may eventually become possible.

Studies performed on men in eastern Finland indicate Hg exposure from consistently eating their local lake fish is associated with increased cardiovascular risk. Since eastern Finland has very poor Se availability, low Se and high MeHg contents in fish from that region may have caused impairments in selenoenzyme functions that may have contributed to cardiovascular impairments as a result of diminished free radical detoxification. The influence of Se on Hg methylation/demethylation rates and Hg-dependent losses of available Se for sustaining selenoenzyme activities is important metabolic interactions that need to be assessed for understanding these pathologies. However, these molecular effects are difficult to study in human or animal models. Therefore, for a thorough investigation of mechanisms involved in any biological issue, cell culture must be included. The specific advantage of cell culture methods is that they reduce the physiological and biochemical constraints imposed by in vivo

studies. Cell culture offers a completely controlled environment, thereby providing an opportunity to investigate very specific research questions and biological mechanisms.

The toxic effects of MeHg and inorganic Hg in cultured cells vary somewhat with cell type and occur at slightly different concentrations (1), but differences in the molecular mechanisms associated with their respective pathologies have not been adequately studied. The entry of inorganic mercury into the brain is largely prevented by the blood–brain barrier, whereas organic mercury can pass the barrier more easily (2). However, it is interesting to note that a significant fraction of the total Hg bound in animal and human brains (3–5) is inorganic, a portion of which appears to originate from MeHg demethylation (6). In the brain, glial cells appear to be responsible for this demethylation activity (7). Methylmercury at a concentration of 0.1 μM has shown to be cytotoxic to certain cell lines (8) but no one has measured the direct effects of MeHg exposure on Se-dependent enzyme activities in cultured cells.

Unusually high concentrations of Hg and antimony (Sb) accumulation have been reported in heart tissues of human patients suffering from dilated cardiomyopathy (DCM). These levels have not been confirmed and may be due to analytical errors resulting from the small sample sizes required since the sampling used biopsy methods. However, if validated, this phenomenon could lead to further direction in understanding MeHg-associated cardiovascular disease. Since almost half of all heart transplants are performed on DCM patients, significant amounts of heart tissue can be accessed from the explanted heart removed during transplantation. The larger tissue samples will provide an opportunity for the highest-quality analytical evaluations so that these levels can be reassessed and validated. Likewise, selenium levels will also be assessed, since selenium plays an important role in Hg toxicity, and Hg accumulation seldom occurs without formation of HgSe.

Approach

Hg–Se Interactions in the Food Chain

Interactive effects of Hg on Se and Se on Hg accumulation will be investigated in a model invertebrate species (*Achaeta domestica*; house crickets) and in plants known to hyperaccumulate Hg and Se. Selenium enters the food chain when inorganic Se taken up from soil becomes nonspecifically incorporated into selenomethionine. When plant materials are consumed by animals, the inorganic selenium in the selenomethionine is slowly released and is subsequently used in de novo selenocysteine synthesis cycles that are continuous in animal tissues (See Figure 1).

Once created, a selenocysteine molecule cannot be reused, but must be degraded once more to release inorganic Se for a new cycle of de novo selenocysteine synthesis. Therefore, although selenomethionine and selenocysteine are the primary food forms of Se, inorganic selenium must be released from all these food forms before the selenium content of the food can be used by the cell. Since selenium from sodium selenite enters the selenium cycle directly without requiring initial degradation, it is the form that is most commonly used in Se nutrition studies. Therefore, although it is not a normal form found in food, it directly reflects the effects of the forms of selenium naturally present in foods. Food forms are a mixture of organic forms including selenomethionine and selenocysteine. Therefore, these studies will compare the interactions between MeHg and Se (both inorganic and organic forms).

The crickets from the trophic Level 1 study will be fed torula yeast-based diets prepared with graduated concentrations (0.1, 1.0, or 3 $\mu\text{mol Se/kg}$) of inorganic sodium selenite. The Se absorbed and retained in the tissues of these crickets will almost exclusively be present as selenocysteine since animal

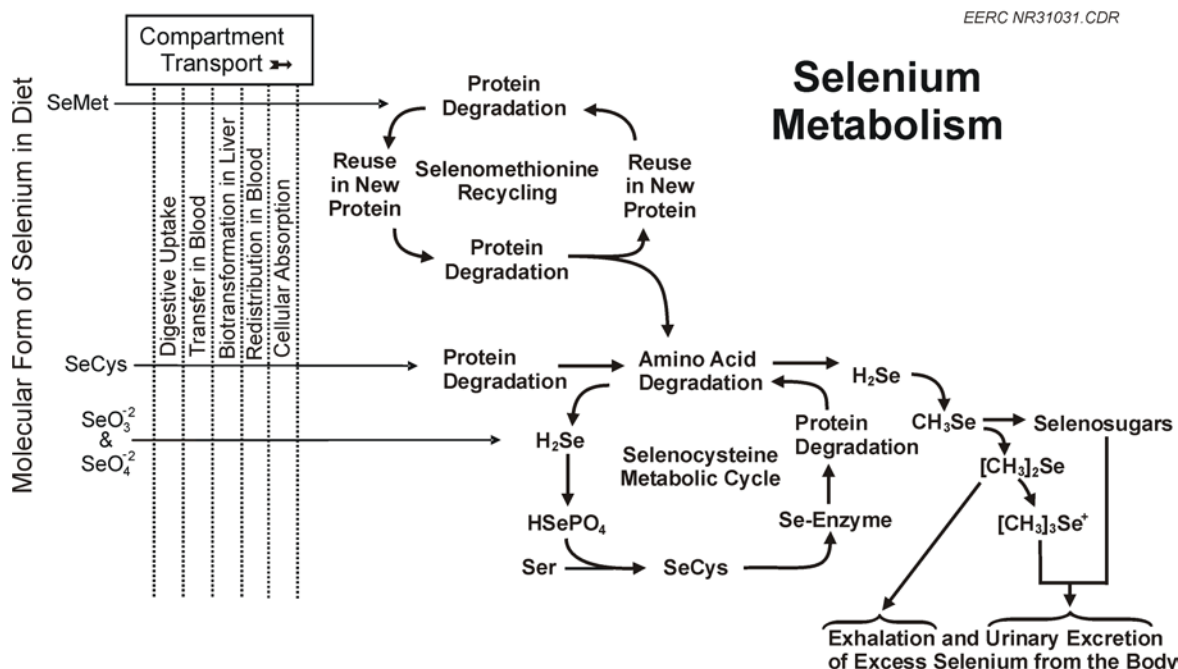


Figure 1. Selenium transport and form-dependent metabolism in animals.

tissues are unable to form selenomethionine. Absorption and retention of Se will be assessed in the presence and absence of 0.5 $\mu\text{mol MeHg/kg}$ added to the diets. At the end of the trophic Level 1 phase of the study, Se-dependent effects on Hg retention will be directly assessed in freeze-dried cricket tissues. Any MeHg-dependent effects on Se retention will be determined by comparing Se present in tissues of the 1- $\mu\text{mol Se}$, 1.0- $\mu\text{mol MeHg/kg}$ test group to the amount of Se present in tissues of the control group fed 1 $\mu\text{mol Se/kg}$ without any added MeHg.

Since crickets are opportunistic cannibals, crickets in the trophic Level 2 study will be fed selenocysteine in the form of ground crickets from trophic level 1. To ensure adequate nutritional status, the powdered cricket meal will be mixed with Se-free torula yeast-based diets containing adequate amounts of nutrients. At the end of trophic Level 2 phase of the study, diet-dependent effects on Se and Hg retention will be directly assessed in freeze-dried cricket tissues. Crickets from the trophic Level 2 study will be used as food in the trophic Level 3 study involving fish.

Hg–Se Interactions in Plants

To investigate the spectral reflectance shift of vegetation induced by Se, Hg, and Hg binding of Se), *Polypogon monspeliensis* (rabbitfoot grass) and *Eichornia crassipes* (water hyacinth) plants were grown under controlled conditions and provided basal nutrient solutions prepared with or without Hg and Se added at graduated concentrations. As the plants grew, Hg- and Se-dependent spectral reflectance of *P. monspeliensis* leaves was measured by spectrophotometer (as reported in a related CATM project). At the end of the project, Hg and Se concentrations in plant tissues were determined by elemental analysis.

MeHg–Se Interactions at the Cellular Level

To understand the environmental and epidemiological effects of mercury exposure, it is important to understand mercury mechanisms at the cellular level, particularly in the central nervous system. Our

approach for this study is to use cell cultures grown in the presence of media containing graduated concentrations of Se, and subjects to MeHg and inorganic Hg added at incremental log concentrations ranging from unexposed to toxic levels. Parallel studies of these Se-conditioned and Hg exposed cultures will be challenged for specified time periods to establish the time- and dose-dependent effects of MeHg toxicity and Se's protective effects against toxicity. The end points measured include cell proliferation rates, viability, mercury and selenium concentrations in the cells, Se-dependent enzyme activities, and rates of MeHg demethylation.

Hg's Effects on Cardiovascular Disease

Working with research pathologists and surgeons at the Mayo Clinic in Rochester, Minnesota, tissue samples have been collected from the explanted hearts removed from patients undergoing heart transplantation in response to ischemic heart disease (IHD) and DCM. The Hg and Se contents of these samples are being assessed in comparison to heart tissues without recognizable pathology collected at autopsy from patients dying of accidental causes. The elemental analysis data will be compiled and statistically compared in order to confirm or refute the reported observations of unusually high Hg levels in DCM hearts.

Progress/Status

Hg–Se Interactions in the Food Chain

Diets used in this study were based on the AIN-93G formula customized through use of low-Se torula yeast as the protein source (Teklad, Madison, Wisconsin). Table 1 contains composition details. Low-Se torula yeast basal diets measured by hydride generation–atomic fluorescence spectroscopy (HG-AFS; P S Analytical, Deerfield Beach, Florida) were found to contain $0.10 \pm 0.03 \mu\text{mol Se/kg}$ ($0.0 \pm 0.01 \text{ ppm Se}$). This is a low but apparently nutritionally adequate level of Se for animal life (9). These basal diets were augmented with Na_2SeO_4 to adjust Se concentrations to levels that reflect the nutritionally relevant range of dietary Se concentrations. Diets for this study were low-Se, adequate-Se, or rich-Se prepared to contain $\sim 0.1, 1.0,$ or $3.0 \mu\text{mol Se/kg}$, respectively.

Table 1. Modified Mineral Mix and Composition of AIN-93G Torula Yeast-Based Diets

Mineral Mix Composition	g/kg	Diet Ingredients	g/kg
Calcium Carbonate, anhydrous, 40.04% Ca	555.26	Torula yeast	300
Sodium Chloride, 39.34% Na	73.5	DL-methionine	6.7
Copper Carbonate, 57.47% Cu	0.143	Arginine	0.1
Potassium Iodate, 59.3% I	0.01	Tryptophan	0.3
Ammonium Paramolybdate, 4 Hydrate, 54.34% Mo	0.008	Soybean oil	70
Sodium Metasilicate, 9 Hydrate, 9.88% Si	1.45	Mineral mix	35
Chromium Potassium Sulfate, 12 Hydrate, 10.42% Cr	0.275	Cellulose	50
Lithium Chloride, 16.38% Li	0.017	Vitamin mix	10
Boric Acid, 17.5% B	0.082	Choline bitartrate	1
Sodium Fluoride, 45.24% F	0.064		
Nickel Carbonate, 45% Ni	0.032		
Ammonium Vanadate, 43.55% V	0.007		
Powdered Sucrose	369.15		
	Total 1000	Total 1000	

Note: Sodium selenite additions replaced sucrose in Se-supplemented diets. Mineral mix was added at 35 g/kg (3.5%) of total diet prior to mixing.

Diets that had been prepared with no MeHg added were found to contain $0.51 \pm 0.35 \mu\text{mol Hg/kg}$ ($0.10 \pm 0.07 \text{ ppm Hg}$), apparently arising in conjunction with one or more of the component materials comprising the basal diet. This level of Hg contamination is similar to what has previously been observed in laboratory animal diets (10). Therefore, the actual Hg concentrations in the diet after the MeHg additions were ~ 0.5 and $1.0 \mu\text{mol MeHg/kg}$ (~ 0.2 and 10.0 ppm Hg). Each of the Se diets was prepared with either low or high amounts of MeHg added in the following manner. Diets provided by Teklad were prepared with only 60 g/kg of oil/kg, 1% less than the AIN-93 recommended levels. This purposeful omission allowed MeHg to be added in safflower oil to be added at 1% wt/wt basis to complete the 70 g oil/kg recommended for this diet. Required amounts of MeHgCl (Sigma-Aldrich, St. Louis, Missouri) were dissolved in safflower oil and mixed for 30 minutes to ensure homogeneous distribution. Diets were prepared in 1.5-kg batches using 15 g of oil distributed evenly over 1485 g and completely mixed together for 5 minutes to obtain an even distribution of MeHg in the diet. After mixing, representative fractions of these diets were set aside for total Hg analysis.

The crickets from the trophic Level 1 study were fed torula yeast-based diets prepared with 0.1, 1.0, or 3.0 $\mu\text{mol Se/kg}$ as Na_2SeO_4 . Dietary Se significantly influenced tissue Se ($p < 0.00001$), but the amount of Se absorbed and retained in the tissues indicates significant homeostatic regulation is occurring. Concentrations of Se in tissues of crickets fed a 0.1- $\mu\text{mol Se/kg}$ diet ($2.89 \pm 0.29 \mu\text{mol Se/kg}$) were not much lower than those in tissues of crickets fed 1.0- $\mu\text{mol Se/kg}$ diet ($3.41 \pm 0.08 \mu\text{mol Se/kg}$). Crickets fed diets containing 3.0 $\mu\text{mol Se/kg}$ contained more Se ($6.68 \pm 0.30 \mu\text{mol Se/kg}$) than the other crickets ($p > 0.05$). Dietary Se did not appear to influence accumulation of Hg in trophic Level 1 crickets and crickets that were fed 1.0 $\mu\text{mol Se/kg}$ diets supplemented with MeHg did not contain different ($p > 0.05$) amounts of Se than crickets fed diets containing 1.0 $\mu\text{mol Se/kg}$ without added MeHg. Since the amount of Hg accumulated in crickets fed the basal diets containing 0.5 $\mu\text{mol Hg/kg}$ was so low, the Hg in those diets appears to have been present as the less readily absorbed inorganic form.

Crickets in the trophic Level 2 study that were fed ground crickets from trophic Level 1 contained uniformly greater tissue Se concentrations. Homeostatic regulation was clearly evident as gains in concentrations of Se in tissues of crickets fed Group 2 crickets contained $5.92 \pm 0.77 \mu\text{mol Se/kg}$, while those eating Group 3 and 4 crickets contained $7.76 \pm 0.71 \mu\text{mol Se/kg}$ and $7.13 \pm 0.64 \mu\text{mol Se/kg}$, respectively. In Trophic Level 2, crickets fed crickets containing the greatest amount of Se did not have the highest tissue Se themselves and the statistically significant relationship between diet and tissue Se concentrations was no longer apparent. Crickets from the trophic Level 2 study are currently being used as food in the trophic Level 3 study involving fish.

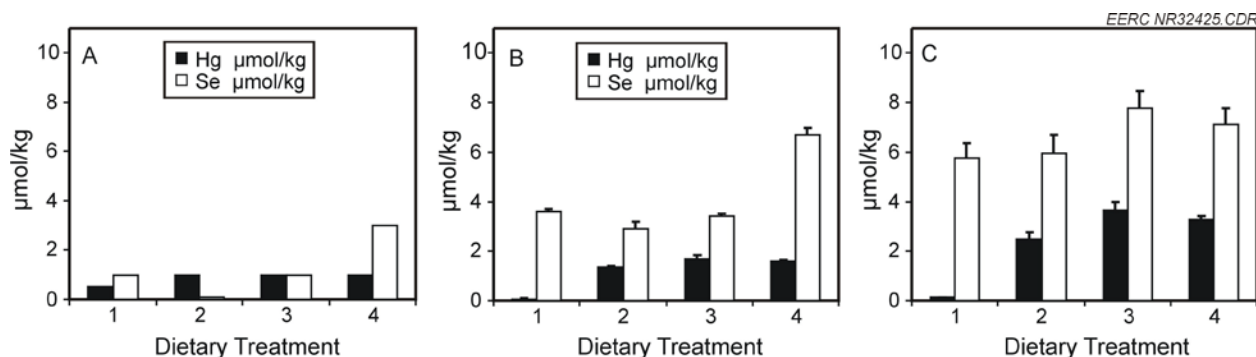


Figure 2. Hg and Se contents of diets and tissues of crickets in food chain study (dry weight basis).

Hg–Se Interactions in Plants

The best analytical precision in trace element analysis is attained using dry sampling methods, thus avoiding potential variance due to differences in water contents of individual samples. Previous work has established that the forms of Hg and Se present in plant materials were not lost during heat drying since the observed concentrations were equivalent regardless of whether analysis was performed on wet or freeze-dried homogenates. Leaves and stems of *P. monspeliensis* collected at the end of Task 1 were dried for 24 hours at 70°C in a laboratory oven prior to sampling. Because of their higher water contents and denser tissues, leaves, stems, and roots of *E. crassipes* collected at the end of Task 2 were oven-dried for 48 hours prior to sampling for elemental analysis.

Accumulation of Hg and Se in *P. monspeliensis*

Germination of *P. monspeliensis* seeds obtained from the National Plant Germplasm Resources Laboratory was less than 20%, so *P. monspeliensis* seeds were instead obtained from Sandeman Seeds (Lalougue, France). The germination rate of these seeds (sets of 100 seeds examined in triplicate) was $95 \pm 2\%$. Twenty milligrams of *P. monspeliensis* seeds was planted in potting soil in 12-inch-diameter pots. Plants were watered with 200 mL of DynaGro Plant Growth Solution daily throughout the study. Pots containing seeded soil were covered with transparent lids until germination was complete. Plants were grown indoors in ambient sunlight under temperature-controlled conditions (20°–25°C) during the entire study.

When the plants were approximately 4 inches high, the supplementation phase of the study began, using a fully crossed 3×3 factorial (nine growth treatment groups) study design to examine dose effects of Se and Hg on one another's elemental uptake. During this phase, in addition to the 200 mL of diluted Dyna-Gro solutions, plants were supplemented with weekly additions of solutions prepared with 100 mL of graduated concentrations of mercury and selenium (50 mL of supplementation solution of each element). The solutions contained low, medium, or high levels of Se as sodium selenite (0.01, 100, or 300 mmol $\text{Na}_2\text{SeO}_4/\text{kg}$, respectively) and low, medium, or high levels of Hg as mercury chloride (0.01, 100, or 300 mmol HgCl_2/kg , respectively). Plants were supplemented weekly for ~8 weeks. At the end of the study, plant leaves and stems were harvested from each treatment group and stored in trace metal-free plastic bags at -85°C until ready for analysis.

The amount of Se in leaves of *P. monspeliensis* plants grown without appreciable amounts of added Se or Hg was $0.83 \pm 0.24 \mu\text{mol Se/kg}$ ($0.070 \pm 0.02 \mu\text{g Se/g}$). As seen in Panel A of Figure 3, addition of 100- or 300-mM Se solutions resulted in minor increases in Se incorporation, but when Hg was added, Se accumulation in leaves was greatly increased. The roots on these plants have not been analyzed yet, so it is not known whether the effect of Hg on Se levels in the plants represents an accentuated uptake from the soil or redistribution from the roots.

The amount of Hg in leaves of *P. monspeliensis* plants grown without appreciable amounts of added Se or Hg was $0.15 \pm 0.04 \mu\text{mol Se/kg}$ ($0.029 \pm 0.008 \mu\text{g Hg/g}$). Panel B of Figure 3 shows that addition of 100- or 300-mM Se solutions synergistically increased Hg contents of the leaves when Hg was also added. Supplementation with 100 or 300 mM Se resulted in 6- and 50-fold increases in Hg accumulation in leaves. Once again, until the roots are analyzed, it is not known whether these results reflect an increased uptake from the soil or an increased redistribution of Hg from the roots.

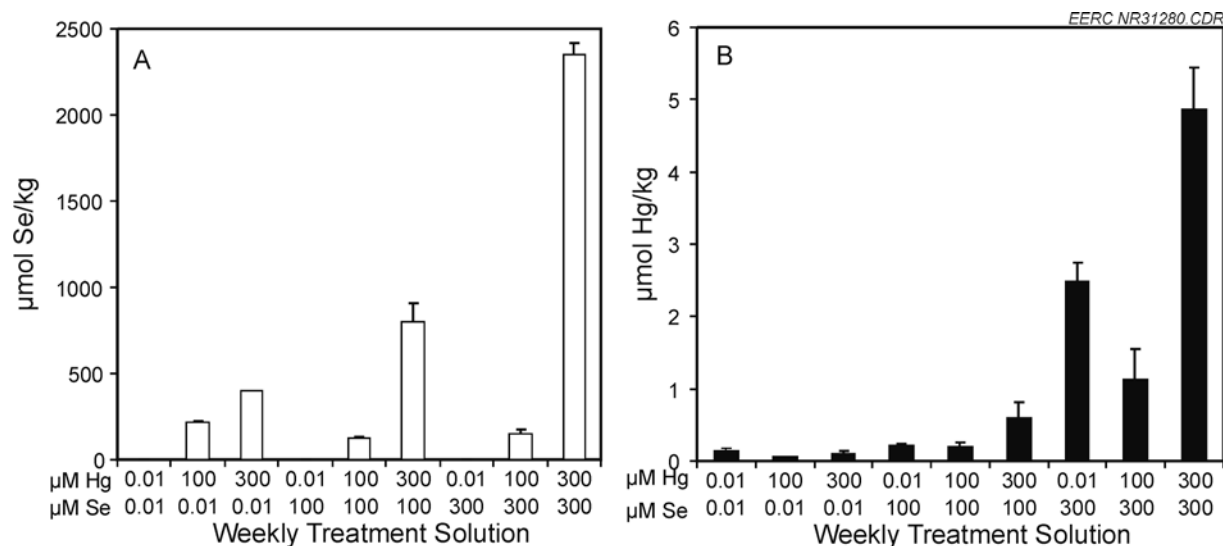


Figure 3. Se (A) and Hg (B) concentrations in leaves and stems of *P. monspeliensis* grown in soil treated weekly with solutions containing the indicated concentrations of Hg and Se.

Accumulation of Hg and Se in *E. crassipes*

Twenty-seven individual 4-inch-diameter *E. crassipes* plants were obtained from Pond Factory Plants (<http://pondfactory.com/home.php>). Individual plants were transferred to trace metal-free plastic containers prepared with 1500 mL of a 1:1500 solution of DynaGro (Dyna-Gro Company, San Pablo, California). The plants were weighed and randomly assigned to Hg and Se treatment groups (three plants/group; 27 total plants) in a fully crossed 3×3 factorial (nine growth treatment groups) study of time- and dose-dependent interactive effects of these elements. Plants were supplemented weekly with 20 mL of either low, medium, or high Na_2SeO_4 (0.01, 100, or 300 mmol Se/kg, respectively) and 20 mL of either low, medium, or high HgCl_2 (0.1, 100, or 300 mmol Hg/kg, respectively). Plant growth was monitored during the ~8-week treatment study. At the end of the study the entire plant was harvested and divided into leaves, stems, and roots that were stored frozen in trace metal-free plastic bags at -85°C until ready for analysis.

Samples of ~0.2 g were taken from each treatment group and weighed into single-use, sealable trace element-free 50-mL digestion tubes (Environmental Express, Mt. Pleasant, South Carolina) with every tenth sample being prepared in duplicate and with elemental spike recovery samples being prepared accompanying each batch. Each sample digestion batch included analysis blanks and reference materials (dogfish muscle certified reference material DORM-2, National Research Council of Canada, Ottawa, Ontario, Canada) with certified Hg and Se concentrations.

Samples were treated with 5 mL of HNO_3 (Fisher trace metal grade, Fisher Scientific, <http://www.fishersci.com>) and heated at 85°C in deep cell hot blocks (Environmental Express) for 24 hours in capped tubes to preserve samples from trace element contamination. Samples were cooled, 1.5 mL of 30% H_2O_2 (Fisher Certified A.C.S., Fisher Scientific) was added, and samples were recapped and returned to heating in the dry block at 85°C for 8 hours more. Samples were cooled and 15 mL of 12 N HCl (Fisher Trace Metal Grade, Fisher Scientific) was added. Samples were heated at 90°C for 90 minutes to reduce Se(VI) to Se(IV). Samples were cooled and diluted to 50 mL with double-distilled water. Samples were further diluted into instrumental calibration ranges and analyzed for Hg content by cold-vapor atomic absorption spectrophotometry using a CETAC M-6000A (CETAC Technologies,

Omaha, Nebraska) and Se was analyzed by hydride generation–atomic absorption spectroscopy (HG–AAS) using a PerkinElmer Dual Millennium Excelibur (PerkinElmer Analytical, Deerfield Beach, Florida). Before data from sample analysis runs were entered into the database, Hg and Se concentrations in sample digestion blanks and elemental recoveries in samples of certified reference materials were evaluated to qualify the analysis batch data for inclusion.

Total Hg and Se mass concentrations (parts per million) for each sample were converted to molar concentrations (micromole per kilogram). Means and standard deviations of molar concentrations of Hg and Se were calculated and graphed for each treatment group. Elemental concentrations of Hg and Se in tissues of different dietary treatment groups were analyzed using ANOVA to assess Hg–Se interactions and compared using t-tests (Microsoft Office Excel, <http://office.microsoft.com>).

The amount of Se incorporated into leaves of *E. crassipes* grown in water that was treated with low levels of Hg and Se was $0.36 \pm 0.06 \mu\text{mol Se/kg}$ ($0.030 \pm 0.004 \mu\text{g Se/g}$). As seen in Panel A of Figure 4, when solutions containing an additional 100 or 300 mM Se were added, the amount of Se in the leaves increased 10- and 20-fold, respectively. Increasing Hg exposure resulted in increased Se distribution into the leaves when Se was low, but leaves of plants supplemented with 100-mM Se solutions tended to have diminishing leaf Se in the presence of increasing Hg. Leaves of plants supplemented with 300 mM Se were observed to have more Se as increasing Hg was added. Further work will be needed to determine whether this indicates formation of HgSe complexes as hypothesized or whether these results reflect a shift in compartment partitioning that increased Se distribution into the leaves.

Leaves of plants grown in water without added Hg or Se contained $0.37 \pm 0.08 \mu\text{mol Hg/kg}$ ($0.070 \pm 0.02 \mu\text{g Hg/g}$). Plant incorporation of Hg was less sensitive to Se-dependent effects than Se incorporation was to Hg effects (Panel B of Figure 4). Amounts of Hg incorporated appeared to be maximal in all groups independent of added Se, so increases in its rate of uptake may not have been possible to observe using the conditions employed in this study. The roots and stems from the plants in these treatment groups will be analyzed to determine whether similar effects occurred throughout the plant or varied by compartment.

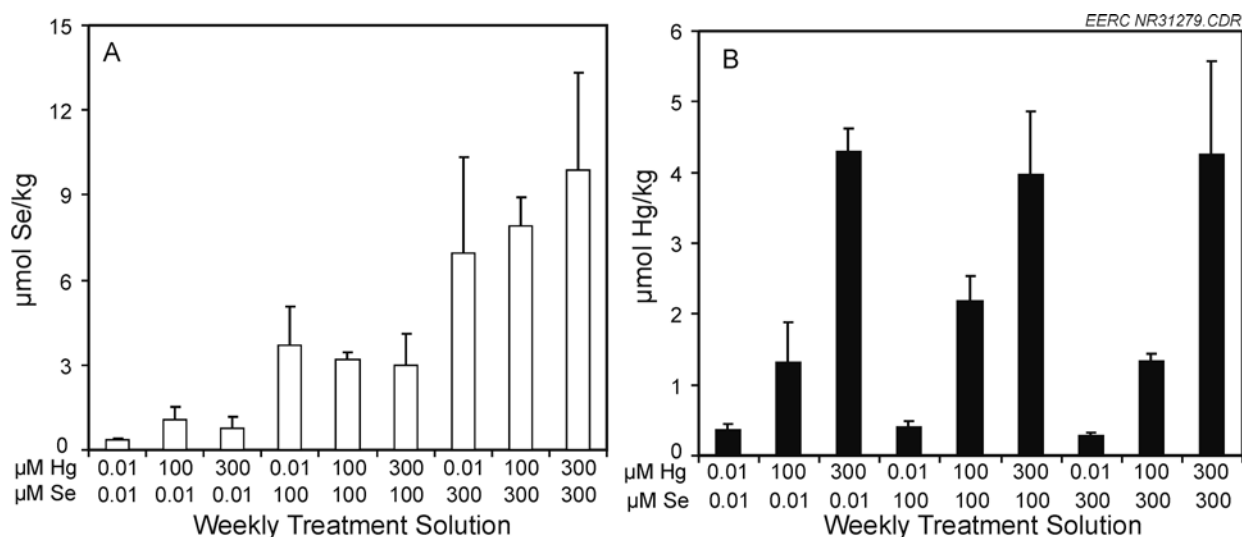


Figure 4. Se (A) and Hg (B) concentrations in leaves of *E. crassipes* grown in water treated weekly with solutions containing the indicated concentrations of Hg and Se.

MeHg–Se Interactions at the Cellular Level

The cell culture study has only recently begun because of issues beyond our control that prevented us from occupying the cell laboratory. Arrangements have been made for temporary use of Dr. Ghribi's cell lab in UND's neuroscience building. The approach for this study is to use cell cultures grown in the presence of media containing graduated concentrations of Se and subject them to MeHg and inorganic Hg added at incremental log concentrations ranging from unexposed to toxic levels. Parallel studies of these Se-conditioned and Hg-exposed cultures will be challenged for specified time periods to establish the time- and dose-dependent effects of MeHg toxicity and Se's protective effects against toxicity. The end points measured include cell proliferation rates, viability, mercury, and selenium concentrations in the cells, Se-dependent enzyme activities, and rates of MeHg demethylation.

The cell line growth curve of human neuroblastoma SH-SY5Y cells administered specified concentrations of methylmercury and methylmercury chloride has been established and is shown in Figure 5. SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium–nutrient mixture F12 ham (DMEM-F12) supplemented with 10% fetal bovine serum. When the cells had reached 80% confluence, the cells were incubated with methylmercury or mercury chloride for 24 hours. Methylmercury was administered at 0, 1, 5, 10 or 50 μM , and mercury chloride was administered at 0, 1, 5, and 10 mM. The viability of SH-SY5Y cells was determined by adding MTT to the SH-SY5Y cell cultures to reach a final concentration of 1 mg/mL according to the manufacturer's recommendations. Values obtained in the presence of methylmercury or mercury chloride were normalized against values obtained in the absence of treatments. Three repeats were carried out for each measurement.

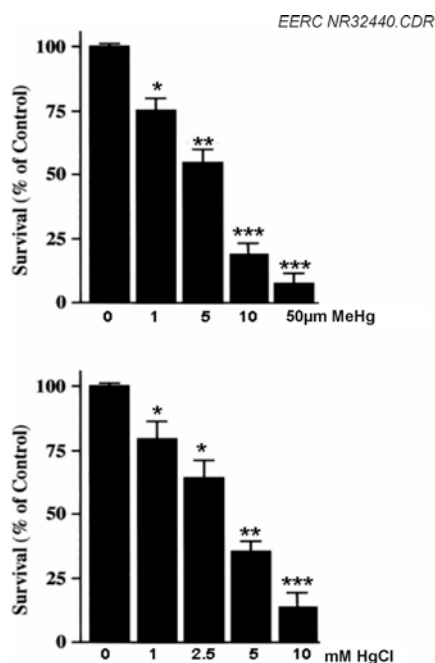


Figure 5. Growth rates of human neuroblastoma SH-SY5Y cells administered the indicated concentrations of methylmercury and methylmercury chloride. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student *t* test).

Selenium treatment effects are in progress. Our approach is to use unsupplemented media as our basal concentration and then supplement with various concentrations of Se to determine the protective effects. The range of sodium selenite to be used in the studies has been established using prepared stock solutions of sodium selenite in PBS: Flask 1 – 0 added; Flask 2 – 0.5 μM ; Flask 3 – 1 μM ; Flask 4 – 2 μM ; Flask 5 – 3 μM ; Flask 6 – 5 μM ; Flask 7 – 10 μM . Cell counts and viability were determined each day \times 7 days.

Mercury's Effects on Cardiovascular Disease

Sample aliquots of \sim 0.5 g weighed to 0.0001 g from each heart sample were transferred into single use, trace element-free 50 -mL digestion tubes (Environmental Express, Mt Pleasant, South Carolina), with every tenth sample being prepared in duplicate and with elemental spike recovery samples being performed accompanying each batch. Each digestion batch included blank and certified standard reference materials (dogfish muscle certified reference material DORM-2, National Research Council of Canada, Ottawa, Ontario Canada).

Samples were treated with 5 mL of 16 N nitric acid (Fisher trace metal grade, Fisher Scientific, www.fishersci.com) and heated at 85°C in deep cell hot blocks (Environmental Express, Mt. Pleasant, South Carolina) for 24 hours in capped tubes to preserve samples from trace element contamination. Samples were cooled, 1.5 mL of 30% hydrogen peroxide (Fisher Certified A.C.S., Fisher Scientific, www.fishersci.com) was added, and samples were recapped and returned to heating in the dry block at 85°C. Samples were cooled, and 15 mL of 12 N HCl (Fisher trace metal grade, Fisher Scientific) were added. Samples were heated at 90°C for 90 minutes in order to reduce Se(VI) to Se(IV). Samples were cooled and diluted to 50 mL with double distilled water. Samples were analyzed for Hg content by cold-vapor atomic absorption spectrophotometry using a CETAC M-6000A (CETAC Technologies Inc, Omaha, Nebraska), and Se was analyzed by hydride generation atomic fluorescence spectroscopy using a P S Analytical Dual Millennium Excalibur (PS Analytical, Deerfield Beach, Florida).

Mercury and selenium concentrations in normal hearts and hearts removed from patients with DCM or IHD are shown in Figure 4. Normal hearts contained $3.73 \pm 0.54 \mu\text{mol Se/kg}$ and $0.47 \pm 0.16 \mu\text{mol Hg/kg}$ ($0.295 \pm 0.43 \mu\text{g Se/g}$, $0.093 \pm 0.033 \mu\text{g Hg/g}$). No age- or sex-related differences in tissue trace element concentrations were noted in normal or diseased patient groups. Hearts of patients with DCM contained $3.65 \pm 0.59 \mu\text{mol Se/kg}$ and $0.39 \pm 0.29 \mu\text{mol Hg/kg}$ and hearts of patients with IHD contained $3.50 \pm 0.43 \mu\text{mol Se/kg}$ and $0.17 \pm 0.11 \mu\text{mol Hg/kg}$.

No significant differences in selenium contents were noted between any of the study groups (Figure 6). Compared to normal hearts, mercury contents of DCM hearts were not significantly different, but hearts of patients with IHD contained significantly less mercury ($p < 0.000001$). Mercury contents of hearts from IHD patients were also lower than hearts of patients with DCM ($p = 0.01$).

In contrast to the previous investigation that noted extraordinarily high mercury contents in heart tissues of patients with DCM, this study finds the concentrations of mercury in DCM and normal hearts are indistinguishable. However, heart tissues from the IHD population used as a control group were found to contain significantly less mercury than was present in normal or DCM hearts. Since tissue mercury levels are usually directly related to seafood consumption, the low mercury levels noted in hearts from IHD patients may indicate low seafood consumption by this population.

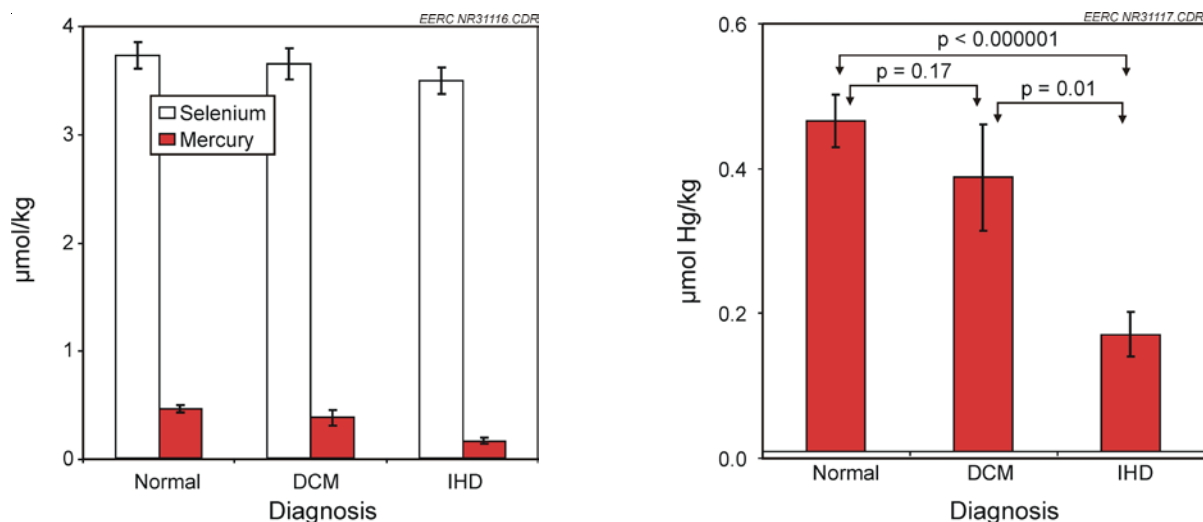


Figure 6. Mercury and selenium concentrations in normal hearts and hearts removed from patients with DCM or IHD.

It is unknown why the Frustaci investigation observed elemental concentrations that are so much higher than this study, but the small sample mass (~ 1 mg) of the biopsy would greatly increase the effect of any contamination artifacts encountered during sample processing. Since the reported mercury concentrations of the heart biopsy tissue samples increased as ejection fraction decreased, it is also possible a component of the radioopaque solution used for visually positioning biopsy-sampling instruments may have contained mercury that contaminated the biopsy sample. These solutions may have either contained or appeared to have contained mercury. This possibility is being investigated.

Quality Objectives Measurement/Data Acquisition

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.

In the dietary treatment study performed on crickets, being able to measure, contrast, and compare the weight gains and survival of crickets fed diets that varied in mercury and selenium contents provides a measure of their concentration-dependent effects.

To validate consistency and validity in cell culture studies, repeat studies will be performed to evaluate the effects of exposure to the forms and concentrations of Hg. At indicated times, the parameters are measured and the concentrations and time-dependent protective effects of selenium against Hg toxicity are evaluated and individually plotted. Multiple independent assessments are performed, and a minimum of three separate experimental collections of data points for each treatment group are included in determining the mean values and standard deviations for the survival and propagation effects. Selenoenzyme activity in cultured cells will be similarly measured in multiple independent assessments using established kit methods and their accompanying pools standards and controls, independent measurements of three or more sets of samples assessing total Hg, Se, and MeHg contents will be

performed using large-scale batches of cultured cells to permit parallel assessments of total Hg and organic Hg.

In the study of elemental distributions in human heart samples, the quality objective is to obtain analytically accurate and precise determinations of elemental contents. Trace elements were determined in batches that include certified quality control samples analyzed relative to calibration standards according to established protocol.

Assessment and Validation

This project was designed with a time line and milestones that facilitate determining whether or not the objectives and the goal of the project are achieved. The protocols 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 will be included in each batch to ensure validity of observed analysis values.

Potential Applications and Benefits

The findings of these studies will provide important information for EPA, the U.S. Department of Energy, the U.S. Food and Drug Administration, the U.S. Department of Agriculture, and the World Health Organization. This information may assist these agencies in making regulatory policy decisions regarding mercury exposure. Assessing selenium's regional distribution and environmental and physiological involvement in mercury toxicity issues will help these agencies assess the importance of selenium nutrition in estimating risks of human mercury exposure and identify populations at risk and potential methods for protection and remediation.

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