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# **Organic and Inorganic Mercury in Neonatal Rat Brain Following Prenatal Exposure to Methylmercury and Mercury Vapor**

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**A list of abbreviations;**

ANOVA	Analysis of variance
CH <sub>3</sub> HgCl	Methylmercury chloride
CO <sub>2</sub>	Carbon dioxide
FAO/WHO	Food and Agriculture Organization/World Health Organization
GD	Gestational day
Hg	Mercury
Hr	Hours
IPCS	International Programme on Chemical Safety
IUPC	The International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LMM	Linear mixed effects models
LODs	Limits of detections
Log	Logarithmic
MeHg	Methylmercury
Na <sub>2</sub> CO <sub>3</sub>	Sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PND	Postnatal day
PTWI	Provisional tolerance weekly intake
U.S. EPA	United States Environmental Protection Agency

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## ABSTRACT

**Background:** Many populations are exposed to multiple species of mercury, predominantly organic mercury as methylmercury from fish, and inorganic mercury as mercury vapor from dental amalgams. Most of our knowledge of the neurotoxicity of mercury is based on research devoted to studying only one form at a time, mostly methylmercury.

**Objectives:** This research investigated prenatal exposure to methylmercury and mercury vapor on mercury concentrations in the brain of neonatal rats.

**Methods:** Female Long-Evans hooded rats were exposed to methylmercury (0, 3, 6 or 9 ppm as drinking solution), mercury vapor (0, 300 or 1000  $\mu\text{g}/\text{m}^3$  for 2 hr per day) or the combination of both, from 30 days prior to breeding thorough gestational day 18. On postnatal day 4, whole brains were taken from one male and one female from each of four litters in each group to assess organic and inorganic mercury in the brain by cold vapor atomic absorption spectrometry.

**Results:** Statistical analysis using linear mixed effects models showed that methylmercury dose was the primary determinant of both organic and inorganic brain mercury levels. For both outcomes, there were also significant interactions between methylmercury and mercury vapor exposure. These interactions were driven by the fact that among animals not exposed to methylmercury, animals exposed to mercury vapor had significantly greater organic and inorganic brain mercury levels than unexposed animals.

**Conclusion:** This interaction, heretofore not reported, suggests that coexposure to methylmercury and mercury vapor at levels relevant to human exposure might elevate neurotoxic risks.

## INTRODUCTION

Mercury toxicity is the focus of substantial public concern. Pronouncements about the safety of fish in the diet and about adverse health effects attributable to dental amalgams are the primary bases underlying this concern. Mercury occurs in different forms in the environment. Mercury species are classified as elemental mercury ( $\text{Hg}^0$ ), inorganic mercury ( $\text{Hg}^{2+}$ ,  $\text{Hg}^+$ ), and organic mercury such as methylmercury. However, many populations are exposed to more than one form. Almost all of our knowledge is based on research devoted to only one mercury species. How different species of mercury act in combination remains unclear, which leads to significant gaps in our understanding of mercury toxicity.

Methylmercury, whose main source of human exposure is through the consumption of contaminated fish, shellfish and sea mammals (U.S. EPA 1997), is a potent neurotoxicant both to the mature and developing central nervous system. Several studies have shown the potential adverse effects on child development of prenatal exposure to methylmercury (for a review, see Clarkson and Magos 2006). Observations on human populations demonstrate that methylmercury readily crosses the placental barrier, as indicated by cord blood levels higher than those seen in maternal blood (Vahter et al 2000). Methylmercury can penetrate into the fetal brain (Aschner and Aschner 1990; Choi et al. 1978; Davis et al. 1994; Day et al. 2005; Newland et al. 2006; Newland and Reile 1999, Stern et al. 2001), which allows for accumulation in the central nervous system. Because methylmercury is slowly converted to inorganic mercury in brain tissue, and as such resides there for many years (Charleston et al. 1995; Davis et al. 1994), the relative contributions of the intact organomercurial versus the inorganic metabolite to neuronal damage remains an open question (Magos 1986) with significant implications for risk assessment.

Another major source of mercury exposure is inorganic mercury in the form of mercury vapor. Exposure of the general population to mercury vapor occurs primarily through inhalation of mercury vapor released from dental amalgams (Brownawell et al. 2005; IPCS 1991). Although the levels from such exposures are considered "low" with respect to some known health effects, we know little about how those exposures might interact with methylmercury toxicity. Approximately 80 % of inhaled mercury vapor is retained and absorbed in blood as it passes through the pulmonary circulation (Hursh et al. 1976). Although mercury vapor introduced into the body is trapped and oxidized to divalent inorganic mercury in erythrocytes, part of the mercury vapor remains in the bloodstream long enough for it to be distributed to other tissues and reach the blood-brain barrier (Yoshida 2002). Non-ionized mercury readily penetrates the placental barrier (Clarkson 1972; Khayat and Dencker 1982) and is taken up by fetal tissues including brain. The ability of inhaled mercury vapor to accumulate in the fetal brain has also been shown in human and animal studies (Drasch et al. 1994; Morgan et al. 2002, 2006). Inhaled mercury vapor has been known to damage the adult central nervous system (Echeverria et al. 1998; Goldwater 1972). On the other hand, only a limited number of studies have shown behavioral changes in animals prenatally exposed to mercury vapor (Danielsson et al. 1993; Fredriksson et al. 1996). However, the study designs of these experiments were not relevant to human exposure because of rather high concentrations of mercury vapor and short durations of exposure.

Although studies of combined exposures are limited, we do know that both agents produce prenatal damage; both produce behavioral changes in animals at levels that do not produce clinically apparent toxicity; and both result in deposition in the brain of the same toxic species of mercury, namely, mercuric mercury. Yet, there are reasons to suggest that combined

exposure to methylmercury and inhaled mercury vapor might produce effects that differ from those seen from exposure to either agent alone: methylmercury is well known to affect developing central nervous system, while effects of inhaled mercury vapor on that system are not clear; metabolism of methylmercury differs from mercury vapor (Clarkson and Magos 2006); the brain pathology and the signs and symptoms of methylmercury poisoning differ from those of inorganic mercury (Clarkson and Magos 2006); although both methylmercury and mercury vapor result in deposition in the brain as mercuric mercury, divalent inorganic mercury,  $Hg^{2+}$ , is believed to be the proximate toxic agent in the case of poisoning from inhaled mercury vapor, whereas it does not appear to be the case for methylmercury (Clarkson and Magos 2006); delayed manifestation of the effects of methylmercury is seen in humans and animals (typical examples of latent toxicity in humans, including both acute and chronic methylmercury exposures), have been described (Weiss et al. 2005) and latent toxicities in animals have also been shown (Newland et al. 2004; Newland and Rasmussen 2000; Rice 1996; Yoshida et al. 2008), but is not known for inorganic vapor. Even so, despite the considerable public health implications of coexposure, there is only one report showing that exposure to methylmercury can further worsen adverse behavioral performance compared to inhaled mercury vapor alone (Fredriksson et al. 1996). In their experiment, rat dams were exposed to either mercury vapor ( $1800 \mu g Hg/m^3$  for 1.5 hr/day from gestational day (GD) 14 to 19), methylmercury ( $2 mg/kg/day$  from GD 6 – 9) or both.

The following experiments were designed, to broaden our understanding of the effects of joint exposure to mercury vapor and methylmercury, a situation that more realistically models human exposures which are both concurrent and chronic and that generally begin long before pregnancy.

## MATERIALS AND METHODS

### Subjects

Two experiments (Experiment 1 and 2) were conducted. Based on the preliminary analysis of Experiment 1, the doses of mercury for Experiment 2 were chosen. The basic experimental schemes were the same between the two experiments except for the mercury doses. The subjects were 99 (Experiment 1) and 96 (Experiment 2) female Long-Evans rats (Harlan, Indianapolis, IN) 8 – 9 weeks of age when they arrived at the University of Rochester Medical Center Vivarium (an AAALAC-certified facility). They were housed individually, in rooms maintained at  $23\pm 2^{\circ}\text{C}$  with a 12 hour light-dark cycle with light onset at 06:00, in polycarbonate breeder cages with wire covers and filter tops. The females were allowed free access to Teklad Global 2018 Rodent Diet (Harlan, Indianapolis, IN), which does not contain fish meal or animal protein, and drinking water solutions (described below) except when the rats were in the exposure chambers. We chose that diet to minimize uncontrolled mercury contamination (Weiss et al. 2005). The rats were randomly assigned to groups as shown in Table 1. Exposure chamber space and exposure durations limited the number of groups per experiment, so the group of 6 ppm methylmercury x  $300\ \mu\text{g}/\text{m}^3$  mercury vapor was excluded in Experiment 1. Long-Evans male rats (Harlan, Indianapolis, IN), 75 retired breeders in Experiment 1, and 80, 8-9 weeks of age, in Experiment 2, were received 3 weeks later, and housed under the same conditions and in the same rooms as the females.

All animals were treated humanely and with regard for alleviation of suffering. All were monitored daily by the research staff and personnel from the Division of Laboratory Animal

Medicine of the University of Rochester Medical Center. All experimental procedures were approved by the University Committee on Animal Research.

### **Exposure to mercury**

Different groups of rats were exposed to (1) methylmercury, as methylmercury chloride, via drinking water, (2) mercury vapor, (3) both methylmercury and mercury vapor, or (4) or not exposed (controls). Exposures occurred daily for 30 days prior to breeding to attain a stable mercury burden in the females, so as to simulate the human exposure pattern. Exposures continued until GD 18.

### **Mercury Vapor**

Before beginning the exposures, the females were first adapted for 1 week to the routine of transporting them from their home quarters to the inhalation facility, and then placing them in the exposure chambers. This was done to preclude excessive uptake of mercury during initial exposures, which would be expected in the absence of adaptation (Stern et al. 1996). The 2 hr exposure of female rats to mercury vapor was conducted in two adjacent hexagonal Rochester chambers (one control, one exposure) each having an internal capacity of 2 m<sup>3</sup> (Chen and Moss 1989). The chambers were supplied with filtered and conditioned outside air drawn into the chamber through an intake duct at the top and discharged into an exhaust manifold with a mercury-trapping filter at the bottom. Chamber temperatures were maintained at 22-24 °C. Metallic mercury was heated in a flask located adjacent to the exposure chamber. Vapor concentrations were controlled by adjusting the the flow of heated air passing over the mercury, which was then mixed with the main airstream flowing into the chamber. During exposure, the

mercury vapor concentration was monitored continuously with a UV mercury monitor. The calibration of the monitor was confirmed using a Jerome 431-X Mercury Vapor Analyzer (Arizona Instrument LLC, Chandler, AZ), which was certified by an independent testing laboratory. The output was connected to a desktop computer for both on-line monitoring and temporary storage of sampling data files. For exposures, the subjects were held individually in open-mesh metal cages. The 2 hr session was designated as beginning when the mercury concentration reached 66 % of the target concentration (~10 min; T90 about 1-2 min more). Once attained, the concentration was maintained at the target value ( $\pm 5\%$ ). At the end of the scheduled exposure, the mercury-vapor generator was turned off, and the airflow over the mercury was stopped. Chamber concentration declined rapidly, reaching less than  $30 \text{ ug/m}^3$  in just a few minutes. The females were then removed after another 30 minutes in order to completely exhaust the chambers. Exposure continued from 30 days before breeding through GD 18.

### **Methylmercury**

The females were dosed with a methylmercury chloride drinking solution 30 days prior to breeding through GD 18. For dosing, 100 ppm methylmercury stock solution was prepared weekly by dissolving crystalline methylmercury chloride ( $\text{CH}_3\text{HgCl}$ ; Alfa Aesar, Ward Hill, MA) in a 5 mM sodium carbonate ( $\text{Na}_2\text{CO}_3$ ; Mallinckrodt & Baker, Inc., Phillipsburg, NJ) buffer solution. The stock solution was diluted to produce requisite quantities of the dosing solutions. The sodium carbonate solution was also used for the 0 ppm control group. Solutions were provided in glass bottles with neoprene stoppers and stainless steel spouts.

Methylmercury (MeHg) concentration was confirmed by cold vapor atomic absorption spectrometry (described below) and found to be within 5 % of the target value.

### **Breeding and Litters**

After 30 days of exposure, individual females were randomly placed with the males at 16:00-17:00. The female's drinking solution was always kept on the same cage in which she was located, so that it was on the breeding cage only when she was physically present with the male. Vaginal smears were obtained at 06:00 and examined microscopically for the presence of sperm, and the female was then returned to her home cage. The day a sperm positive smear was observed was defined as GD 0. A male was paired with the same female(s) until a sperm-positive smear was observed, or after three successive nights, whichever came first. Some males were paired with a second female, which was always a member of a different exposure group.

Immediately after the mercury vapor exposure on GD 18, blood was drawn from the tail of 4 females in each group into heparinized calibrated micropipets (Drummond Scientific Company, Broomall, PA). Collected blood samples were kept in 0.9 % NaCl (Sigma-Aldrich, St. Louis, MO) at 4 °C until assayed.

The day (up to 1300 hours) on which a litter was discovered was designated as postnatal day (PND) 1. Litter size, sex, body weight and overall health status of each pup were checked and recorded. On PND 4, one male and one female littermate from each of four litters in each group was sacrificed following CO<sub>2</sub> anesthesia. Whole brain was removed and weighed. Some brains were taken from smaller pups. The brains were kept at -12 °C until assayed.

On PND 4, litters were culled via random sampling to 3 pups of each sex per litter in Experiment 1, and 4 in Experiment 2. For culling, pups were separated by sex, then separated,

held individually in a fixed order, and weighed. A pup was culled immediately if its weight fell below 75% of the mean for that sex in that litter, unless needed for brain sampling. Then, following a list of random numbers equal to the number of remaining pups for that litter/sex, pups were kept or culled.

### **Mercury assays**

Mercury levels in brain and blood were determined by cold vapor atomic absorption spectrophotometry using flameless atomic absorption monitor (Laboratory Data Control Model 1235). All details of analysis and sample preparation were previously described (Magos 1971; Magos and Clarkson 1972). The method is based on the rapid conversions of mercury compounds into atomic mercury. Cadmium chloride-stannous chloride reagent is used to reduce total mercury (organic plus inorganic), whereas stannous chloride selectively reduces inorganic mercury. Organic mercury is the difference between total and inorganic mercury. Mercury in brain was determined after digestion with 40 % NaOH, and in blood after sample dilution with saline. For standards preparation, Mercury Reference Standard Solution (SM 114-100, Fisher Scientific, Fairmont, NJ) was used. Detection limits (LODs) and quantification limits (LOQs) were calculated from blank measurements following the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) (Currie 1999). The LODs (three times SD for blanks) were 19.5 ng/g for total mercury and 9.75 ng/g for inorganic mercury for brain and 11.5 µg/l for both total and inorganic mercury for blood, whereas limits of quantification were 65.0 and 32.5 ng/g, 38.3 µg/l, respectively. The values of some samples fell under the LODs. Half the LOD was applied to these samples when they were statistically analyzed. The method imprecision, calculated as the coefficient of variation for duplicate preparations measurements,

was 4 %. The analytical accuracy of mercury determination was evaluated using reference material (certified human blood samples from Centre de Toxicologie du Quebec, International Comparison Program, Canada). The results obtained were  $76.65 \pm 1.41$  ng /g (Lot M-08-14) vs. certified 79 ng/g (range 58-100 ng/g). Participation in external quality control programs also rendered highly satisfactory data.

## **Statistical analysis**

### ***Breeding outcomes and mercury in dam's blood***

The unit for analysis for breeding outcomes was the litter and for dam's blood was the dam. Outcomes (of the averaged parameters if needed for the breeding outcomes) were evaluated with a one-way ANOVA to determine differences among groups (defined by mercury vapor and methylmercury concentrations), and two-way ANOVA to determine the effects of methylmercury, mercury vapor and their interaction. Values of  $p < 0.05$  were considered statistically significant. We used a logarithmic (base e) transformation of many outcomes in order to satisfy model assumptions.

### ***Models for pup's brain mercury***

Linear mixed effects models (LMM) (McCulloch and Searle 2001) were applied using R software (Faraway 2006; see also [www.r-project.org](http://www.r-project.org)) to examine the relation between methylmercury dose, mercury vapor dose, sex, and experiment and two outcomes: organic and inorganic mercury brain levels. The two outcomes were examined in separate models. Data from experiments 1 and 2 were combined for both outcomes. The inclusion of the experiment term in the model allows the two experiments to have different intercepts. The mixed model includes a

random litter effect, which models the correlation between pups within a litter and allows the pup to be the unit of analysis. This general approach is also taken by Gray et al. (2009).

We started by treating both methylmercury dose and mercury vapor dose as continuous variables, and when the linearity assumption held, treated them as continuous variables in the final model. For both outcomes we started by considering a full model which also included all 2-way and 3-way interactions between mercury vapor dose, methylmercury dose, and sex. When the 3-way interaction was not significant, we fit a model without this term. We then considered models without 2-way interactions and without main and/or covariate effects, when these terms were not significant.

To satisfy model assumptions, it was necessary to use a logarithmic (base  $e$ ) transformation of each mercury brain level outcome. For both outcomes there was no difference in response between low and high mercury vapor dose, so this variable could be collapsed into two categories: no mercury vapor exposure, and any mercury vapor exposure. Neither sex of the pup nor experiment were significant predictors for either outcome, indicating in part that data from both experiments could be combined into a single model. For organic mercury brain levels, there was a linear relationship between the logarithm of organic mercury brain levels and the logarithm of methylmercury dose (after adding 0.1 to avoid taking the logarithm of 0), and our final model included three terms:  $\log(\text{methylmercury dose} + 0.1)$ , any mercury vapor exposure, and their interaction. The interaction allows the slope relating methylmercury dose to organic brain mercury outcome to differ for mercury vapor exposed compared to non-mercury vapor exposed pups.

For inorganic mercury brain levels, the relationship to methylmercury dose was not quite linear. Our final model for this outcome included three terms or groups of terms: three indicator

variables for methylmercury dose, any mercury vapor exposure, and their interactions. Sex and experiment were not significant predictors,

## **RESULTS**

### **Breeding outcomes**

Number of litters and number of pups per litter in each group on PND 1 and 4 are shown in Table 2 and 3. On PND 1, neither litter size (the averaged value with a group varied from 8.7 to 11.9 in Experiment 1 and 8.1 to 11.5 in Experiment 2) nor sex ratio within a litter (1.07 - 1.58 and 0.97 - 2.08 in Experiment 1 and 2, respectively) differed among the groups in both Experiment 1 and 2. Body weights on PND 1 did not differ. Number of pups in the 6- and 9-ppm methylmercury dose groups decreased between PND 1 and 4 in Experiment 2. Two-way ANOVA showed that methylmercury had a main effect on body weight of both male ( $p=0.0203$ ) and female ( $p=0.0055$ ) pups on PND 4 in Experiment 2 while such an effect was not seen in Experiment 1. Effects of mercury vapor and interaction of methylmercury and mercury vapor were not found.

### **Mercury in dam blood**

We compared the logarithm of mercury (total, inorganic and organic) levels in blood on GD 18 across groups, separately by experiment, as shown in Figure 1. There were significant differences among the groups ( $p < 0.0001$  for total, inorganic, and organic) in both experiments. Two-way ANOVA with interactions showed that in Experiment 1, the interactions between methylmercury and mercury vapor were significant for all 3 outcomes ( $p=0.0006$  for total,

$p=0.002$  for inorganic, and  $p=0.0002$  for organic, each for a 3 df test). In Experiment 2, the interactions were significant for inorganic ( $p<0.0001$ ), but not for total or organic mercury. Methylmercury was a very strong predictor of total, inorganic and organic mercury in both experiments ( $p<0.001$ ). With the exception of inorganic mercury in Experiment 2, mercury vapor did not affect mercury levels.

### **Mercury in pup brain**

Total, inorganic and organic mercury (mean  $\pm$  SD) in pup brains of each group are summarized in Table 4 and 5. Complete data are shown in the figures. Figures 2A and 2B show the observed (points) and fitted values (lines) of organic mercury in pup's brain, where both the brain levels and the methylmercury dose are on the logarithmic scale. The vapor and no vapor points are offset slightly in Figure 2A for clarity. There was an interaction of methylmercury and mercury vapor on organic brain mercury ( $p<0.0001$ , Figure 2A). Among animals that were exposed to mercury vapor, the predicted levels of brain organic mercury were larger among animals unexposed to methylmercury, but rose at a less steep rate as methylmercury exposure increased compared to animals with no mercury vapor exposure. Figure 2B shows the relationship between organic brain mercury and mercury vapor, separately by methylmercury concentration. As Figures 2A and 2B illustrate, organic brain mercury was very strongly predicted by methylmercury dose.

Methylmercury was also a very strong predictor of inorganic brain levels ( $p<0.001$  for each methylmercury dose) (Figure 3A). The observed (points) and fitted values (lines) of inorganic mercury in pup brains are shown in Figures 3A and 3B. As mentioned in the Methods section, for inorganic mercury brain levels, methylmercury dose was coded with three indicator

variables to distinguish between the 4 methylmercury dose levels. This analysis showed interactions between mercury vapor and the methylmercury dose indicator variables ( $p=0.02$  for the 3 df test).

In summary, the dose of methylmercury drove levels of both organic and inorganic mercury in pup brains. Exposure to mercury vapor lowered pup brain mercury levels at high methylmercury doses and increased them at low methylmercury doses as compared to animals not exposed to mercury vapor. Separate analysis showed that among the 40 animals with no methylmercury exposure, exposure to any mercury vapor ( $n=24$  animals) was associated with a higher brain mercury level than no exposure to mercury vapor ( $n=16$  animals) ( $p=0.02$  for both organic and inorganic mercury) (Figure 4).

## DISCUSSION

Prenatal exposure to the combination of methylmercury and mercury vapor had interactive effects on the levels of organic and inorganic mercury in rat neonatal brain. Surprisingly, mercury levels were increased by mercury vapor at low methylmercury doses, a finding relevant to human exposures, which typically occur at low concentrations.

Total mercury concentration in brains of pups exposed to 6 ppm methylmercury with no mercury vapor is comparable to that on PND 1 in previous studies, in which methylmercury dose and duration of exposure are similar to ours (Day et al. 2005; Newland et al. 2006; Newland and Reile 1999). In these studies, rat dams were exposed daily to methylmercury for 30 days or longer prior to breeding, simulating the predominant human exposure pattern of a stable diet. Although we did not determine whether steady state levels had been attained in the pregnant

females, Newland and Reile (1999) found no differences in brain mercury levels of PND 0 offspring of females that had been exposed for 28 or 45 days prior to breeding. Baseline mercury concentrations achieved by these studies, because they are consistent over time, are preferable to short-term exposures as a basis for risk assessments.

Pup brain mercury (total, inorganic and organic) concentrations increased with the dose of methylmercury, but this increase was not linear across the exposure groups. That is, total brain and organic mercury concentrations in pups exposed to 6 or 9 ppm methylmercury were more than two or three times those of pups exposed to the lowest (3 ppm) methylmercury dose. Newland and Reile (1999) also found that total mercury in pup brain at birth increased nonlinearly with the concentration of methylmercury (0.5 or 6.4 ppm in drinking water) when rat dams were exposed to methylmercury beginning 28 or 49 days prior to breeding and through gestation, similar to the present study design. Such nonlinearities have also been seen in nonhuman primates (Lushei et al. 1977). The present data suggest that extrapolation from high concentration exposures may distort estimates of brain mercury levels at lower methylmercury doses. The present data also suggests that with a logarithmic transformation of both pup brain mercury and methylmercury dose, a linear dose-response relationship may be reasonable. The increase in inorganic mercury associated with increased methylmercury doses was not similar to that measured by organic mercury levels. The increase seen in total and organic mercury as methylmercury dose increased was higher between 6 and 9 ppm methylmercury than between 0 and 3 ppm or 3 and 6 ppm, whereas that of inorganic mercury was lower. This complex pattern indicates that attempts to estimate brain levels of inorganic mercury on the basis of dose requires that experiments must rely on the lower methylmercury exposure levels that are relevant to human exposure levels.

In these experiments, inorganic mercury in pup brain increased with increasing methylmercury dose. This result is ascribed to the process by which methylmercury is converted to the inorganic form (Clarkson 1997; Clarkson and Magos 2006). Moreover, elevated levels of inorganic mercury have been found in the brains of humans and monkeys exposed to methylmercury (Davis et al. 1994; Vahter et al. 1995). One site for the process would be phagocytic cells present in many mammalian tissues, including the brain, that are capable of breaking the carbon-mercury bond (Suda and Takahashi 1990). Thus, inorganic mercury in brain tissue may arise from *in situ* metabolism of methylmercury. However, there is still a possibility that some of the inorganic mercury may not necessarily represent the *in situ* conversion of methylmercury. Instead, it may be derived from some distant source via the vapor pathway. Intestinal microflora are also capable of cleaving the carbon mercury bond (Rowland et al. 1987). As the vapor produced by reduction of inorganic mercury in the intestine or phagocytic cells in the liver readily crosses the blood-brain barrier to be oxidized in brain tissue (Clarkson 1997), some portion of the inorganic mercury in pup brain may arise from this process. However, it is not clear whether it occurs in the fetus or in the mother. As methylmercury readily crosses the placental barrier (Vahter et al. 2000), methylmercury transferred from mother to fetus may be converted to inorganic mercury in the fetus. Or, inorganic mercury converted from methylmercury in the mother may be transferred to the fetus via placenta and reach fetal brain as nonionized mercury, which also crosses the placental and blood-brain barrier (Clarkson 1972; Khayat and Dencker 1982; Yoshida 2002); either could occur. Contributions to pup brain inorganic mercury may differ between fetus and mother.

Mercury vapor exposure increased brain mercury (total, inorganic and organic) levels in the pups not exposed to methylmercury. The increase in brain mercury was not related to the

concentration of mercury vapor, which may suggest that the mercury level in the pup brains reached steady state before the time of brain sampling. However, Morgan et al. (2002, 2006) showed that, in the brains of neonates perinatally exposed to mercury vapor (1, 2, 4 or 8 mg/m<sup>3</sup> for 2 h/day during GD 6-15), total mercury concentrations increased with increasing exposure dose. The exposure concentrations they employed were higher, and the duration shorter, than those of the current study, which might contribute to the differing results. Although data on the elimination of inorganic mercury in the fetus and/or neonate after gestational exposure are sparse, factors of retention and/or elimination should be considered to evaluate the effect of prenatal exposure to mercury vapor on the brain mercury levels because of the time lag (6-7 days) between the last exposure and the brain sampling. There is unlikely to be any loss of inorganic mercury due to this process, because the methylation of inorganic mercury does not appear to take place to any significant extent in either human or animal tissues (Clarkson and Magos 2006).

So far, only one study has addressed prenatal coexposure to methylmercury and mercury vapor (Fredriksson et al. 1996). That study showed that brains of rat offspring (on PND 3) perinatally exposed to both methylmercury and mercury vapor contained more total mercury than those exposed to either form alone. Statistical analyses of the joint and single contributions of methylmercury and mercury vapor to total mercury seem not to have been performed, although coexposure resulted in slightly higher mercury levels in the brain (12 ng/g) than what would have been expected, considering the concentrations obtained after exposure to either methylmercury (4 ng/g) or mercury vapor (5 ng/g) alone. The present data suggest that coexposure to mercury vapor slightly lowered brain mercury levels at high methylmercury doses and increased them at low methylmercury doses. In the study by Fredriksson et al. (1996), the methylmercury dose was 2 mg/kg/day, which is higher than our highest dose, which would be approximately 700-750

$\mu\text{g}/\text{kg}/\text{day}$  if we extrapolate from Newland and Reile (1999). Similarly, their dose of mercury vapor was  $1.8 \text{ mg}/\text{m}^3$  for 1.5 h/day, which is also higher than our highest dose ( $1.0 \text{ mg}/\text{m}^3$  for 2 h/day). In addition, methylmercury exposure occurred only during GD 6 - 9 and that of mercury vapor occurred during GD 14-19, which means that the two exposures did not occur simultaneously. Different doses and duration of exposures might explain the different outcomes.

There may be several metabolic processes to account for the observation that exposure to mercury vapor lowered brain mercury levels at high methylmercury doses. Inorganic mercury, but not methylmercury, can induce the metal-binding protein metallothionein. Binding to this protein is generally regarded as a detoxication process (Clarkson 1997; Clarkson and Magos 2006) and has been proven to play an important role in the retention of mercury in tissue. Metallothionein induced by mercury vapor in the mother and/or in the fetus might prevent methylmercury and perhaps inorganic mercury from reaching the fetal brain. The conversion of methylmercury to inorganic mercury may also need to be considered (Clarkson 1996; Clarkson and Magos 2006). Inorganic mercury in the form of oxidized mercury has a limited capacity to cross the blood-brain and placental barriers (Clarkson 1997; Clarkson and Magos 2006). The presence of large amounts of mercury vapor might promote the oxidation of methylmercury in the mother and/or in the fetus, resulting in less mercury reaching the brain. However, there are no tests of this hypothesis.

The main source of human exposure to methylmercury is the diet, especially fish and seafood (U.S. EPA 1997). Dietary intake of methylmercury is estimated as 0.1 - 2.0  $\mu\text{g}/\text{kg}$  body weight per week for numerous national diets (IPCS 2004). EPA's current reference dose for methylmercury is 0.1  $\mu\text{g}/\text{kg}$  body weight/day (U.S. EPA 2001, 2009). The Fifty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established provisional

tolerance weekly intake (PTWI) of 200  $\mu\text{g}$  of methylmercury (3.3  $\mu\text{g}/\text{kg}$  body weight) for the general population, but noted that fetuses and infants might be more sensitive than adults to its toxic effects (IPCS 2000). . Since there has been no definitive separation of prenatal and postnatal exposure that would permit dose-response modeling, there are currently no data that would support the derivation of a child (vs. general population) reference dose (U.S. EPA 2001). Those values are relatively low compared to doses used in the present rat study. Rat blood has approximately ten times as much hemoglobin as mouse, monkey, or human blood (Magos 1987) and binds mercury. That means that rat blood has a higher capacity to bind mercury, so that a higher intake is required to compare neonatal brain mercury, a better biomarker, across species (Burbacher et al. 1990). However, the present study showed that, at lower methylmercury doses, exposure to mercury vapor increased both brain organic and inorganic mercury levels. In addition, the increase in brain mercury did not depend on the mercury vapor dose. That means that brain mercury levels might be higher than expected even if methylmercury intake is lower than the PTWI or EPA reference dose when fetuses are simultaneously exposed to mercury vapor even at levels as low as those attributable to dental amalgams. This might be one mechanism by which coexposure to dietary methylmercury and mercury vapor at levels relevant to human exposure elevates neurotoxic risks and may need to be taken into account for risk assessment calculations. Additional research is required to directly evaluate such outcomes.

## CONCLUSIONS

There are interactive effects of joint exposure to methylmercury and mercury vapor during the prenatal period on pup brain organic and inorganic mercury. Mercury vapor increased

both forms of mercury in pup brain at lower methylmercury concentrations an outcome relevant to human exposure. Human fetuses exposed to both methylmercury and mercury vapor may have increased risks of neurodevelopmental toxicity in contrast to either species alone.

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**Table 1.** Group Assignment in two experiments

Mercury vapor ( $\mu\text{g}/\text{m}^3$ )	Methylmercury (ppm)			
	0	3	6	9
Experiment 1				
0	15 *	12 *	12 *	
300	12	12		
1000	12 *	12 *	12 *	
Experiment 2				
0	12 *	12 *	12 *	12
1000	12 *	12 *	12 *	12

Numerals show the number of females assigned to each group at the beginning of the experiments. Asterisks (\*) show the common groups between the two experiments. In Experiment 1, the group of 6 ppm methylmercury x 300  $\mu\text{g}/\text{m}^3$  mercury vapor was precluded because exposure chamber space and exposure durations limited the number of groups per experiment.

**Table 2.** Number of litters, number of pups per litter and body weight (mean  $\pm$  SD) on PND 1 and 4 in Experiment 1

Group (MeHg x Hg Vapor)		0 x 0	3 x 0	6 x 0	0 x 300	3 x 300	0 x 1000
Number of Litters	PND 1	14	10	10	12	10	10
	PND 4	14	10	9	12	10	10
Number of Pups per Litter	PND 1	10.8	11.7	8.4	11.8	11.9	10.7
	PND 4	10.8	11.6	9.2	11.8	11.5	9.3
Body Weight (g)							
Males	PND 1	6.40 $\pm$ 0.07	6.34 $\pm$ 0.11	6.61 $\pm$ 0.14	6.21 $\pm$ 0.16	6.10 $\pm$	
	0.16	6.47 $\pm$ 0.14	6.32 $\pm$ 0.11	6.48 $\pm$ 0.21			
0.85	PND 4	9.44 $\pm$ 0.80	9.11 $\pm$ 0.96	9.85 $\pm$ 1.24	9.07 $\pm$ 1.27	8.92 $\pm$	
		8.94 $\pm$ 1.17	9.09 $\pm$ 1.19	9.55 $\pm$ 1.72			
Females	PND 1	6.11 $\pm$ 0.10	6.06 $\pm$ 0.09	6.10 $\pm$ 0.11	5.92 $\pm$ 0.13	5.82 $\pm$	
	0.17	6.11 $\pm$ 0.11	6.01 $\pm$ 0.08	6.19 $\pm$ 0.16			
1.04	PND 4	9.02 $\pm$ 0.67	8.66 $\pm$ 1.03	9.36 $\pm$ 1.16	8.65 $\pm$ 1.11	8.52 $\pm$	
		8.38 $\pm$ 1.61	8.45 $\pm$ 1.32	9.10 $\pm$ 1.41			

MeHg; Methylmercury, Hg Vapor; Mercury vapor

Group mean is mean of mean within a litter. Body weight on PND 1 did not differ among groups.

**Table 3.** Number of litters, number of pups per litter and body weight (mean  $\pm$  SD) on PND 1 and 4 in Experiment 2

Group (MeHg x Hg Vapor)		0 x 0	3 x 0	6 x 0	9 x 0	0 x 1000	3 x 1000
Number of Litters	PND 1	10	11	7	11	10	10
	PND 4	10	11	7	7	10	9
Number of Pups per Litter	PND 1	9.4	10.7	8.1	10.9	11.4	10.2
	PND 4	9.2	10.6	7.4	12.6	10.3	11.1
Body Weight (g)							
Males	PND 1	6.42 $\pm$ 0.56	6.28 $\pm$ 0.26	6.53 $\pm$ 0.52	5.89 $\pm$ 0.64	6.05 $\pm$	
	0.42	6.22 $\pm$ 0.78	5.96 $\pm$ 0.49	6.07 $\pm$ 0.65			
1.47	PND 4	9.03 $\pm$ 1.37	8.87 $\pm$ 0.54	9.15 $\pm$ 0.85	7.33 $\pm$ 0.58	8.58 $\pm$	
		9.18 $\pm$ 0.80	8.20 $\pm$ 1.97	8.20 $\pm$ 1.58			
Females	PND 1	6.05 $\pm$ 0.46	5.94 $\pm$ 0.25	6.10 $\pm$ 0.62	5.59 $\pm$ 0.62	5.73 $\pm$	
	0.46	6.07 $\pm$ 0.42	5.69 $\pm$ 0.78	5.71 $\pm$ 0.55			
1.32	PND 4	8.73 $\pm$ 1.17	8.40 $\pm$ 0.60	8.45 $\pm$ 1.20	7.17 $\pm$ 0.92	8.12 $\pm$	
		8.47 $\pm$ 0.93	7.47 $\pm$ 2.60	7.06 $\pm$ 1.53			

MeHg; Methylmercury, Hg Vapor; Mercury vapor

Group mean is mean of mean within a litter. More pups were lost in the 9 ppm methylmercury groups with or without mercury vapor exposure between PND 1 and 4. Body weight on PND 1 did not differ. There was a main effect of methylmercury on body weight on PND 4 ( $p=0.0203$  for male and  $p=0.0055$  for female).

**Table 4.** Total, inorganic and organic mercury concentrations (mean  $\pm$  SD) in pup brain on PND 4 in Experiment 1

Group (MeHg x Hg Vapor)	0 x 0 0 x 1000	3 x 0 3 x 1000	6 x 0 6 x 1000	0 x 300	3 x 300
<b>Total Mercury (ng/g)</b>					
Males	20 $\pm$ 7 50 $\pm$ 11	2773 $\pm$ 231 3244 $\pm$ 626	8997 $\pm$ 2949 8064 $\pm$ 1524	42 $\pm$ 13	2946 $\pm$ 208
Females	26 $\pm$ 15 51 $\pm$ 14	2723 $\pm$ 86 2685 $\pm$ 571	9262 $\pm$ 2810 7900 $\pm$ 2150	43 $\pm$ 7	2928 $\pm$ 383
<b>Inorganic mercury (ng/g)</b>					
Males	5 $\pm$ 0 18 $\pm$ 5	140 $\pm$ 23 172 $\pm$ 37	490 $\pm$ 209 350 $\pm$ 113	11 $\pm$ 5	150 $\pm$ 35
Females	7 $\pm$ 3 20 $\pm$ 3	140 $\pm$ 15 151 $\pm$ 32	505 $\pm$ 169 290 $\pm$ 149	13 $\pm$ 6	148 $\pm$ 30
<b>Organic mercury (ng/g)</b>					
Males	15 $\pm$ 7 33 $\pm$ 7	2632 $\pm$ 231 3072 $\pm$ 593	8507 $\pm$ 2749 7715 $\pm$ 1420	31 $\pm$ 13	2796 $\pm$ 180
Females	20 $\pm$ 12 32 $\pm$ 12	2583 $\pm$ 101 2534 $\pm$ 539	8757 $\pm$ 2658 7510 $\pm$ 2002	31 $\pm$ 4	2780 $\pm$ 359

MeHg; Methylmercury, Hg Vapor; Mercury vapor

**Table 5.** Total, inorganic and organic mercury concentrations (mean  $\pm$  SD) in pup brain on PND 4 in Experiment 2

Group (MeHg x Hg Vapor)	0 x 0 3 x 1000	3 x 0 6 x 1000	6 x 0 9 x 1000	9 x 0	0 x 1000
<b>Total Mercury (ng/g)</b>					
Males	26 $\pm$ 13 3286 $\pm$ 321	3469 $\pm$ 254 6796 $\pm$ 1684	8254 $\pm$ 1078 11795 $\pm$ 3156	16122 $\pm$ 4419	32 $\pm$ 8
Females	29 $\pm$ 16 3379 $\pm$ 287	3703 $\pm$ 72 6468 $\pm$ 1727	9703 $\pm$ 2216 13177 $\pm$ 5007	16545 $\pm$ 5941	33 $\pm$ 11
<b>Inorganic mercury (ng/g)</b>					
Males	8 $\pm$ 6 160 $\pm$ 48	167 $\pm$ 19 370 $\pm$ 75	452 $\pm$ 95 518 $\pm$ 107	612 $\pm$ 52	7 $\pm$ 3
Females	8 $\pm$ 7 156 $\pm$ 22	165 $\pm$ 18 366 $\pm$ 58	475 $\pm$ 83 668 $\pm$ 203	678 $\pm$ 151	5 $\pm$ 0
<b>Organic mercury (ng/g)</b>					
Males	18 $\pm$ 11 3127 $\pm$ 293	3302 $\pm$ 240 6426 $\pm$ 1622	7803 $\pm$ 1004 11277 $\pm$ 3052	15511 $\pm$ 4376	25 $\pm$ 7
Females	21 $\pm$ 15 3223 $\pm$ 276	3538 $\pm$ 62 6102 $\pm$ 1670	9228 $\pm$ 2150 12509 $\pm$ 4812	15867 $\pm$ 5811	28 $\pm$ 11

MeHg; Methylmercury, Hg Vapor; Mercury vapor

## Figure legends

**Figure 1.** Organic and inorganic mercury concentration in dam blood on GD 18

Concentrations are shown as mean  $\pm$  SD. Analyses using log transformed values show significant differences among the groups ( $p < 0.0001$ ) in both experiments. Two-way ANOVA with interactions showed that in Experiment 1, the interactions between methylmercury and mercury vapor were significant for all 3 outcomes ( $p=0.0006$  for total,  $p=0.002$  for inorganic, and  $p=0.0002$  for organic, each for a 3 df test). In Experiment 2, the interactions were significant for inorganic ( $p<0.0001$ ), but not for total or organic mercury. Methylmercury was a strong predictor of total, inorganic and organic mercury in both experiments ( $p < 0.001$ ). With the exception of inorganic mercury in Experiment 2, mercury vapor did not affect mercury levels

**Figure 2.** Organic mercury in pup brain by log scale of methylmercury (A) and absolute value of mercury vapor (B) on PND 4.

Data points show individual pups, with fitted values included in 2A.

The fitted values exhibited a clear linear relationship between the logarithm of organic mercury brain levels and the logarithm of methylmercury dose. The interaction allows the slope relating methylmercury dose to organic brain mercury outcome to differ for mercury vapor exposed compared to non-exposed pups. There was an interaction of methylmercury and mercury vapor on organic mercury values ( $p<0.0001$ ). Organic brain mercury is strongly predicted by methylmercury dose.

**Figure 3.** Inorganic mercury in pup brain by log scale of methylmercury (A) and absolute value of mercury vapor (B) on PND 4.

Data points show individual pups, with fitted values included in 3A. There is an interaction between mercury vapor and the methylmercury dose indicator variables ( $p=0.02$  for the 3 df test). Methylmercury was a very strong predictor of inorganic brain levels ( $p<0.001$  for each methylmercury dose).

**Figure 4.** Boxplots of log scale of organic and inorganic mercury brain concentrations on PND 4 for pups exposed to no mercury vs pups exposed to any mercury vapor.

The solid line within the box shows the median. The top and bottom of the boxes are the 75th and 25th percentile respectively. The broken lines extend to the largest or smallest observation that is within 1.5 times the length of the box. Among animals with no methylmercury exposure, exposure to any mercury vapor was associated with a higher mercury level ( $p=0.02$  for both organic and inorganic mercury).

Figure 1

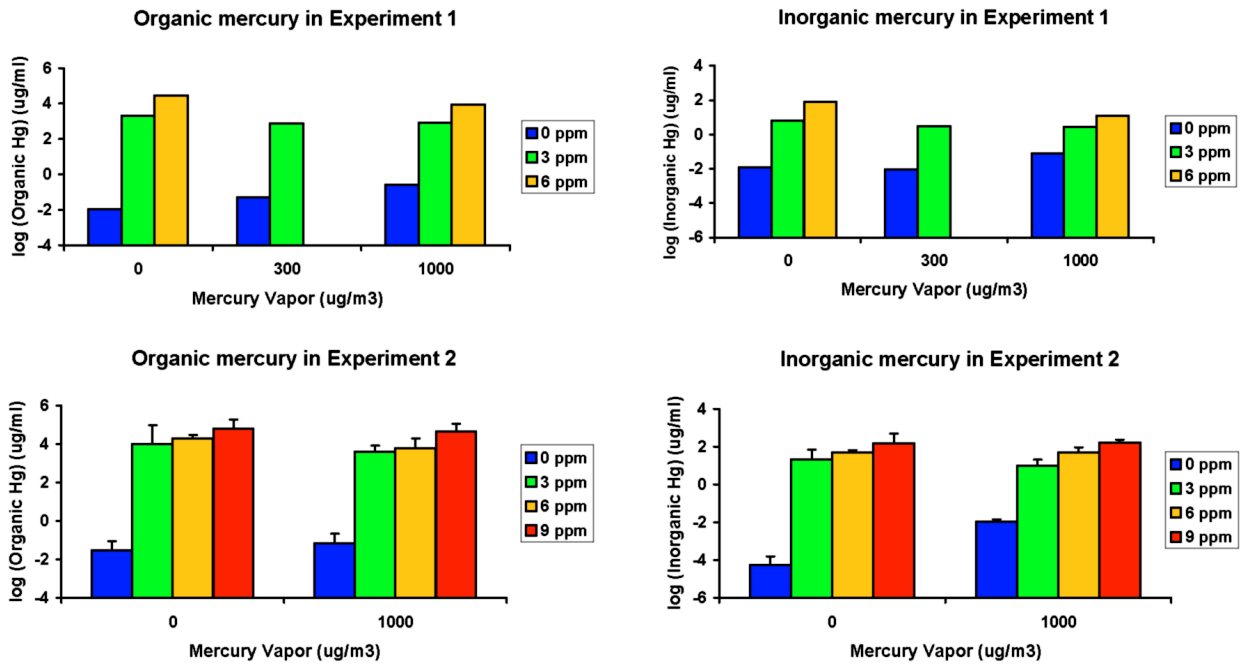


Figure 2

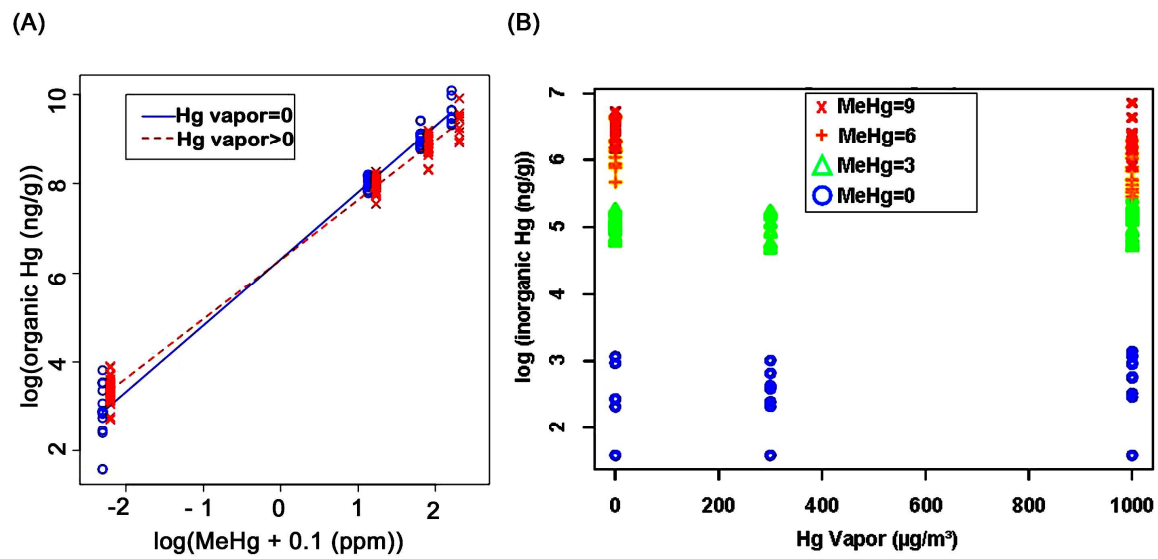
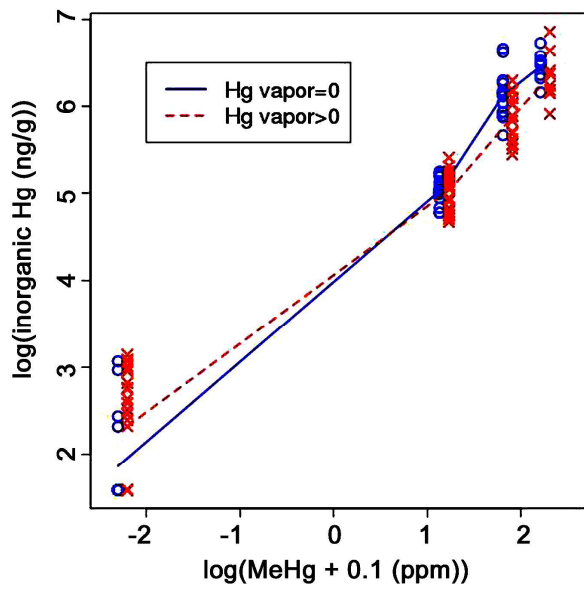


Figure 3

(A)



(B)

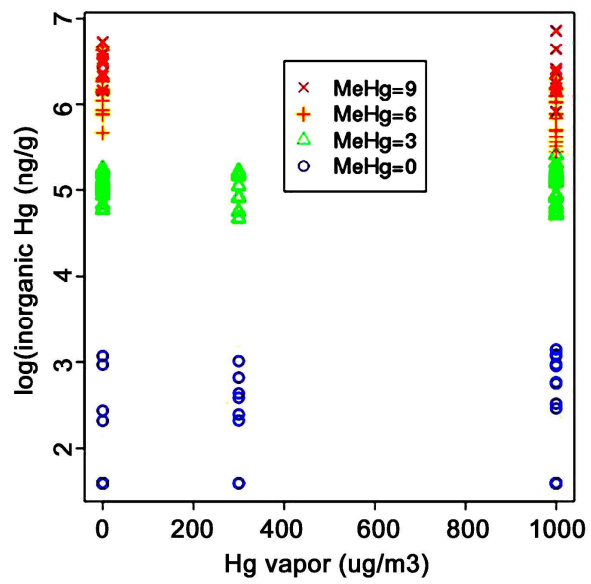


Figure 4

