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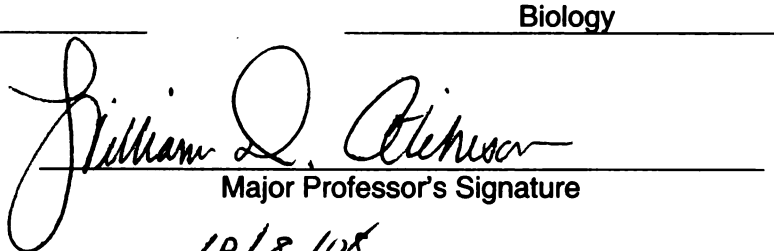
THE NEMATODE *CAENORHABDITIS ELEGANS:* A MODEL ORGANISM FOR STUDY OF METHYL MERCURY TOXICITY

presented by

Jason C. Tew

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Biochemistry and Molecular Biology



Major Professor's Signature

10/8/08

Date

THE NEMATODE *CAENORHABDITIS ELEGANS:* A MODEL ORGANISM FOR STUDY OF METHYL MERCURY TOXICITY

By

Jason C. Tew

A THESIS

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for the degree of

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ABSTRACT

THE NEMATODE *CAENORHABDITIS ELEGANS*: A MODEL ORGANISM FOR STUDY OF METHYL MERCURY TOXICITY

By

Jason C. Tew

Methyl mercury (MeHg), is toxic to the liver and kidneys, but its main target is the central nervous system (CNS), where it causes extensive damage to the cerebellum, especially in young developing nervous systems. The damage to the CNS causes a number of effects including the loss of motor control and reduced cognitive skills. Low dose MeHg can cause similar effects (such as decreased visual perception) that may not appear until later in life. The nematode *Caenorhabditis elegans* (*C. elegans*) is a valuable model organism that has been used extensively in neurobiology and may provide new insights in studying early developmental and chronic effects of MeHg. The effects of MeHg on lethality and growth in *C. elegans* were examined. Various concentrations (5 - 300 μM) were used to assess the whole concentration response range, and exposure was continuous after MeHg addition to the liquid worm culture. MeHg has an approximate LC_{50} of 220 μM at 6 h of exposure and 100 μM at 12, 24 and 48 h (approximately 1 generation time) exposures. Viability was assessed by a 10-sec observation of worm movement. Significant differences in body length, an effective measure of growth, were seen after 6 h of exposure to MeHg ≥ 100 μM, and at 48 h with exposure to MeHg ≥ 5 μM. Verapamil an L-type voltage-gated calcium channel antagonist (Ca_v 1.2) has been shown to alleviate MeHg effects *in vivo* and *in vitro* using mammalian models, but did not modify the effects of MeHg on *C. elegans in vivo*. Overall, it appears that *C. elegans* may provide an excellent model for MeHg research.

ACKNOWLEDGMENTS

I would like to thank Dr. William D. Atchison for the opportunity to pursue this research and for the learning experience involved. A special thanks to OVPRGS (Office of the Vice President for Research and Graduate Studies) and CVM (College of Veterinarian Medicine) of Michigan State University for funding this exploratory research to develop a new model organism for MeHg toxicity. I would also like to thank Dr. Ravindra K. Hajela for his insight and guidance, as well as his encouragement throughout this process.

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LIST OF ABBREVIATIONS

MeHg	Methyl mercury
[MeHg]	Methyl mercury concentration
CGC	*Caenorhabditis* Genetics Center
µl	microliters
ml	milliliters
µM	micromolar
mM	millimolar
M	molar
mg	milligram
g	gram
mm	millimeters
VGCC	Voltage-gated calcium channel(s)
LC_{50}	Lethal concentration that causes 50 % of a population to die
LD_{50}	Lethal dose that causes 50 % of a population to die
$[Ca^{2+}]_i$	Intercellular concentration of calcium ions
Ca^{2+}	Calcium ion
IP_3	Inositol 1,4,5-trisphosphate
GFP	Green fluorescence protein
LB	Luria-Bertani medium
DI	Deionized
L1	*C. elegans* larval stage 1

L2	*C. elegans* larval stage 2
L3	*C. elegans* larval stage 3
L4	*C. elegans* larval stage 4
ANOVA	Analysis of variance
w/v	weight per volume
rpm	revolutions per minute
g	force fo gravity
h	hour(s)
C. elegans	*Caenorhabditis elegans*
CNS	Central nervous system

INTRODUCTION

Inorganic and organic mercury are both potent environmental neurotoxicants. Inorganic mercury (Hg) has historically been linked to "mad hatters syndrome". Mad hatters syndrom is associated with behavioral changes (nervousness, timidity, embarrassed easily and moodiness) accompanied by lassitude, ataxia and mental impairments (Lishman, 1987; O'Carroll et al., 1995). Organic mercury is more neurotoxic than inorganic mercury. The present work has focused on methyl mercury (MeHg), one form of organic mercury, created in nature by bioconversion (methylation) of inorganic mercury by microbes. MeHg readily bioaccumulates in the food chain, primarily in fish. It easily crosses the blood-brain and placental barriers (Kerper et al., 1992; Ask et al., 2002). The ability of MeHg to cross these barriers has led to guidelines for limited fish consumption throughout much of the world, especially for pregnant and nursing women. MeHg exposure is still a concern, particularly for populations for which seafood is a large part of the diet (Renzoni et al., 1998). MeHg exposures are typically chronic low dose exposures associated with diet, although there have been two incidents of acute, widespread MeHg poisoning (Takeuchi et al., 1962; Bakir et al., 1973). Whether exposure is chronic or acute, the mechanisms of MeHg toxicity are not yet adequately understood, and are actively investigated by many laboratories.

Recently, a majority of MeHg studies have focused on low-concentration MeHg exposures for the following reasons: to identify any particularly sensitive targets; to model real world conditions of MeHg exposure; and because there are still questions

regarding the lowest concentration that induces neurotoxicity.

MeHg primarily affects the CNS causing neurotoxicity in adults, children and fetuses (Aschner and Aschner, 1990). MeHg exposure is more damaging to the CNS of children and particularly fetuses than to adults; in fact children exposed *in utero* may have MeHg induced impairments while the mother displays no toxic effect (Takeuchi, 1982). In the fetus, MeHg exposure can cause impairment of neurodevelopement (Choi, 1978), cognitive disabilities (including failure to reach developmental milestones) and/or loss of fine motor function (Grandjean et al., 1999).

The pattern of neuronal damage varies depending on the age of the individual at the time of exposure, and the duration and concentration of exposure. One common symptom is the atrophy of cells in the cerebellar cortex, particularly in the granule cell layer to the point that the granule cell layer virtually disappears (Choi, 1989). Within the granule cell layer MeHg accumulates in the Purkinje and Golgi cells and to a lesser extent in the granule and basket cells (Møller-Madsen, 1990, 1991; Leyshon-Sorland et al., 1994). Even though granule cells accumulate less MeHg, they may experience cell death while the neighboring Purkinje cells accumulate more MeHg without noticeable effects (Hunter and Russell, 1954; Eto et al., 1999; Edwards et al., 2005). Several hypotheses for this selective vulnerability have been postulated, but none have become the dominant paradigm in the field.

The mechanisms of selective neuronal cell death caused by MeHg are not well

understood. One complicating factor is the high chemical reactivity of MeHg, which has a strong affinity for thiol groups, meaning any protein with a cysteine or a methionine has potential MeHg binding sites (Harris et al., 2003; Krupp et al., 2008). The large number of potential targets suggests that MeHg may act through several cellular pathways. Indeed, multiple pathways of MeHg neurotoxicity have been reviewed, including; necrotic and apoptotic cell death, damage to cytoskeleton, changes in neurotranmitter systems, increase in reactive oxygen species, inhibition of protein synthesis and disruption of calcium homeostasis (Atchison and Hare; 1994; Castoldi et al., 2001; Limke et al., 2004).

One of the mechanisms of MeHg toxicity, that has been extensively studied in our laboratory, is disruption of calcium homeostasis of the cell. MeHg caused increased intercellular calcium concentration($[Ca^{2+}]_i$) has been demonstrated *in vitro* (Marty and Atchison, 1997) and *in vivo* (Mori et al., 2000). The mechanisms of increased intracellular calcium concentrations produced by MeHg are complex and probably involve a variety of ion channels including IP_3 (Tan et al., 1993), ryanodine (Bearrs, et al., 2001), acetylcholine (Bearrs, et al., 2001) and voltage gated calcium channels (VGCCs) (Marty and Atchison, 1997). In addition, it has been established that blocking some types of VGCCs delays the time to onset of MeHg-induced increases in $[Ca^{2+}]_i$, and has a protective effect on cell death (Hare and Atchison, 1995; Marty and Atchison, 1997). VGCC antagonists also have protective effects on rats exposed to MeHg *in vivo* (Sakamoto et al., 1996).

Methyl mercury causes cell death through apoptosis at lower concentrations and necrosis at higher concentrations (Castoldi et al., 2001). Both methods of cell death involve increased $[Ca^{2+}]_i$ levels (Kruman and Mattson, 1999). In granule cells in culture, 0.5 μM MeHg causes a notable increase in $[Ca^{2+}]_i$ after 15 min of exposure (Edwards et al., 2005). 100% of granule cells underwent apoptosis following 18 h of 1 μM MeHg exposure, or necrosis within 1 h of higher [MeHg] (5-10 μM) exposure (Castoldi et al., 2000). *In vivo*, rats dosed at 10 mg/kg per day for 10 days had neuronal necrosis in the cerebellar cortex and the brainstem, while rats given 4 mg/kg every other day for 20 days had damage to cerebellar granule cells consistent with apoptosis (Nagashima et al 1996; Nagashima, 1997). It is clear that one of the effects of MeHg is a profound change in calcium homeostasis. In fact, it has been proposed that one reason Purkinje cells are more resistant than granule cells to MeHg is because they have a greater Ca^{2+} buffering capacity due to expression of Ca^{2+} binding proteins such as calbindin D28K that are not evident in granule cells (Edwards et al., 2005).

The majority of studies exploring the mechanisms of MeHg toxicity have been conducted in cell culture, with some corresponding use of animal model studies, typically rat. Almost all these *in vitro* experimentations and a vast majority of the *in vivo* studies would have to be considered acute exposure paradigms. There have been few *in vivo* MeHg studies done that truly model chronic exposure such as those encountered in current environmental conditions. The nematode *Caenorhabditis elegans* (*C. elegans*) is an alternative animal model increasingly used throughout biological sciences. The simplicity of this organism may allow for a better correlation between *in vivo* and *in vitro*

acute MeHg toxicity findings, as well as, a relatively quick and inexpensive model for chronic exposure.

C. elegans is a microscopic free living soil nematode. It has become an important model organism over the last 30 yrs, since Sydney Brenner (1974) recognized it as an ideal model for multicellular eukaryotic organisms. Homologues to 60 % or more of human genes have been identified in the worm (Kaletta and Hengartner, 2006). The worms are mostly hermaphrodites, with generation times of 2.5 days (at 25°C) and a life span of 2-3 weeks. Each hermaphrodite on average produces 300+ eggs in its life, allowing for the culture of large numbers quickly (Hope, 1999). The cell linage of all 959 somatic cells (in the hermaphrodite) in the approximately 1 mm long adult worm have been mapped (Sulston et al., 1983) and individual cells can be viewed using standard bright field microscopy in these transparent animals. The complete DNA sequence is known (Ainscough et al., 1998). Additionally, gene manipulation (many mutant lines are stored at the *Caenorhabditis* Genetics Center (CGC)), cell isolation and gender manipulation can all be done relatively easily. The *in vivo* use of *C. elegans* as a model organism allows for the study of multicellular functional units (Kaletta and Hengartner, 2006), as well as examination of whole organism end points (such as feeding, reproduction and locomotion). In addition to organism-level examination, the worm allows for the study of individual cells or proteins. These features of the worm help to bridge the gap between current *in vivo* and *in vitro* models in many scientific fields.

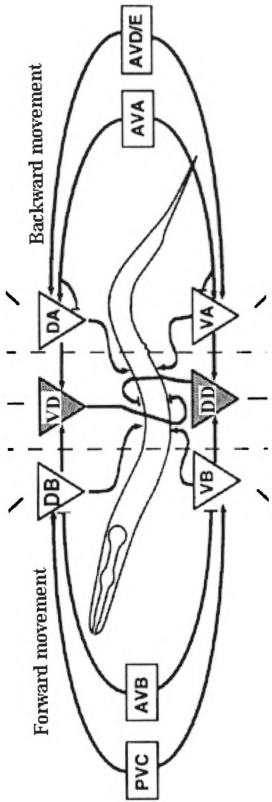
The use of *C. elegans* as a neuronal model is aided by a well characterized nervous system. The entire adult neurocircuitry of 302 neurons has been extensively mapped by electron microscopy, including all synapses and neuromuscular junctions (Chalfie and White, 1988). The nervous system includes 118 characterized neuronal subtypes (Hobert, 2005), with 6393 chemical synapses, 890 electrical junctions and 1410 neuromuscular junctions (Chen et al., 2006). The development of the *C. elegans* nervous system is invariant and well described (Sulston et al., 1983). Neuronal receptors, transmitters and transmitter release pathways are highly conserved between *C. elegans* and mammals, and the main excitatory neurotransmitter at motor nerve terminals is acetylcholine (ACh), the same as in vertebrates (Rand and Nonet, 1997; Bergmann, 1998). Overall, the nervous system of *C. elegans* may be the best characterized of any organism, and studies using the worm have led to further understanding of the mammalian system (Hatten, 2002; Hobert, 2005; Jin, 2005)

There has been a good deal of work done using *C. elegans* as a model organism for toxicity, with lethality, reproduction, life-span and protein expression as end points. When the worms are used to study neurotoxicity the endpoints have typically been behavioral changes (including locomotion and feeding), reporter expression and neuronal morphology. Acute lethality of several heavy metals (Zn, Be, Hg, Cd, Cu, Pb, Al and Sr) (Williams and Dusenbury, 1988) and organo-phosphate pesticides (Cole et al., 2004) are significantly correlated between LC_{50} values in *C. elegans* and mammalian LD_{50} values (rats and mice) using either regression analysis or Spearman's rank correlation coefficient respectively.

Locomotion, feeding and other sub-lethal end points have been more extensively studied than lethality in *C. elegans* and have been correlated with neurotoxicity. Changes in locomotion would indicated an effect on the neuronal network composed of interneurons AVA, AVB, AVD and PVC that signal to A- and B-type motor neurons (control forward and backward movement) and D-type motor neurons (involved in coordinated movement (Figure 1)) (Driscoll and Kaplan, 1997). Computer tracking software allows for detailed analysis of worm movement including distance traveled, directional changes, shape of sinusoidal movement, body bends and head thrashes. Computer tracking studies have demonstrated a concentration-dependent decrease in locomotion when worms were exposed to organic pesticides, organic solvents and heavy metals for 4 h (Anderson et al., 2004). It was also observed that changes in locomotion were 15-50+ times more sensitive than the lethality response. In addition, locomotion was more sensitive to compounds known as neurotoxicants compared to chemicals that are not thought to be neurotoxicants (Anderson et al., 2004), but this difference between neurotoxicants and non-neurotoxicants was not seen when a 24 h exposure was used (Anderson et al., 2001). It has been suggested that 4 h exposure effects might demonstrate neurotoxicity, while the 24 h exposure was long enough for additional systems to be affected. Another possible explanation is that the worms reduce feeding within a few hours of being treated with a toxicant, and by 24 h starvation stress contributes to any functional impairment (Anderson et al., 2001). Indeed, feeding appears to be the most sensitive endpoint in *C. elegans* exposed to toxicants, although this varies depending on food availability and the individual toxicant (Anderson et al.,

Figure 1. *C. elegans* locomotory circuitry. (Inverted triangles) Representatives of the six major motor neuron classes; (rectangles) interneurons. Only one of each motor neuron class is shown, although each class has multiple members that are situated along the length of the ventral cord. The DA, DB, and DD motor neurons innervate dorsal muscles, and the VA, VB, and VD neurons innervate ventral muscles. The circuit comprising interneurons AVB and PVC and the B-type motor neurons directs forward locomotion; the circuit including interneurons AVA, AVD and AVE and the A-type motor neurons directs backward locomotion. The A- and B-type motor neurons are excitatory and use the neurotransmitter acetylcholine (white triangles); The D-class motor neurons are inhibitory and use the transmitter GABA (gray triangles). The D-class motor neurons receive synaptic input from other motor neuron classes rather than from interneurons - the D-class motor neurons synapse onto muscles situated opposite to those innervated by their presynaptic partners and function as reciprocal inhibitors that coordinate forward and back-ward movements. (Reproduced and modified from Driscoll and Kaplan, 1997)

FIGURE 1.


Backward movement
Forward movement
AVD/E
AVA
DA
VA
VD
DD
DB
VB
AVB
PVC

2001). Changes in feeding were evident within 2 h of exposure to 5 mg/l Cd (Anderson et al., 2001). Ultimately, it was found that feeding and movement were the most sensitive non-lethal endpoints at 4 h of exposure, but by 24 h of exposure movement, feeding, growth and reproduction (72 h) were all equally sensitive measures of toxicity (Anderson et al., 2001).

Other behavioral measures have been used to examine heavy metal neurotoxicity in *C. elegans.* Ye et al. (2008) have demonstrated that the heavy metals Al and Pb are able to reduce the worm's learning ability. This is in accordance with learning deficits seen in children exposed to Pb (concentrations of Pb measured in the blood ranged from 3-34 μg/dl. Canfield et al., 2003) and animals overexposed to Al (Yokel, 1985; Muller et al., 1990). Interestingly, the mechanism of this toxic effect was revealed through additional studies showing that vitamin E effectively reversed this learning loss in *C. elegans*, suggesting that oxidative stress was involved in Al and Pb neurotoxicity (Ye et al., 2008). In addition to heavy metal toxicity and neurotoxicity, *C. elegans* has been used extensively in studying neurotoxicity of pesticides and neurodegenerative diseases, although that is beyond the scope of this paper.

At the beginning of this project there were no previously published studies of the effects of MeHg on *C. elegans.* The purpose of this research was to find a working concentration range of MeHg exposure in *C. elegans* for future MeHg neurotoxicity studies. Using a protocol similar to that of Chu and Chow (2002), who examined lethality of several heavy metals in *C. elegans*, the concentration response of MeHg

induced toxicity was explored. To establish our methodology a cadmium concentration response was briefly conducted. Non-lethal endpoints have been extensively used when studying heavy metal toxicity and neurotoxicity in *C. elegans,* so body length was examined as a sub-lethal endpoint of MeHg toxicity. Lastly, the effect of verapamil, an L-type VGCC blocker, on MeHg effects in *C. elegans* was tested to examine if verapamil has a protective effect in *C. elegans* similar to what has been observed in cells and rats.

MATERIALS AND METHODS

Worm Lines

The worm lines used in this project were N2, NW1229 and KC136. N2 wild type and NW1229 worms (expressing Green Fluorescence Protein (GFP) that has been tagged to the dpy-20 gene) lines were both received from the CGC at the University of Minnesota. The dpy-20 (DumPY, shorter then wild type) gene encodes a protein with no known homologs outside of nematodes; it is required for normal body morphology and has a pan-neural expression (Hosono et al., 1982; Http://www.Wormbase.org, 2008). The KC136 worm line was received from Dr. King L. Chow, Professor of Biology at Hong Kong University of Science and Technology, with transgenic GFP attached to hsp16-2 (a heat shock protein encoding) gene (Chu and Chow, 2002).

Worm Culture

Worms were cultured following the procedure of Driscoll (1995) on nematode growth medium (NGM) agar with a bacterial lawn of *E. coli* OP50/1 obtained from the CGC.

The composition of NGM agar was:

3 g NaCl	(J.T.Baker A.C.S. reagent, Phillipsburg, NJ)
2.5 g peptone	(peptone from soybean (Fluka/Biochemika, Sigma-Aldrich Chemical Co., St. Louis, MO))
17 g agar	(Bacto Agar (Becton, Dickinson and Co, Franklin Lakes, NJ))
970 ml of distilled water	

These items were combined and autoclaved for 30 min and then cooled to 55-60°C. The following were then added:

1 ml fungizone (Gibco, Invitrogen, Carlsbad, CA)

1 ml of 5mg/ml cholesterol stock solutions

1 ml of 1 M $CaCl_2$ stock solution

1 ml of 1 M $MgSO_4$ stock solution

25 ml of 1 M KPO_4, pH 6 stock solution

10 ml of liquid agar were pipetted into 60 mm plates and 20 ml into 90 mm plates. The plates were cooled and dried at room temperature overnight (23-25°C), then stored upside down in closed bags at -20°C.

Stock solutions for NGM agar plates included:

5 mg/ml cholesterol w/v (Sigma-Aldrich Chemical Co., St. Louis, MO) dissolved in 100% ethanol (AAPER, Toronto, ON) and stored at -20°C.

1 M $CaCl_2$; autoclaved (J.T.Baker A.C.S. reagent, Phillipsburg, NJ)

1 M $MgSO_4$; autoclaved (J.T.Baker A.C.S. reagent, Phillipsburg, NJ)

1 M KPO_4: made by combining solutions of: 204.4 g KH_2PO_4 J.T.Baker A.C.S. reagent, Phillipsburg, NJ) in 1500 ml of water and another solution of 114.12g of K_2HPO_4 (J.T.Baker A.C.S. reagent, Phillipsburg, NJ) in 500 ml of water. The pH was adjusted to 6.0 then the combined solution was autoclaved.

The bacterial lawn was grown by pipetting 4 drops (approximately 30 µl/drop) of LB (Luria-Bertani) medium with saturated culture of *E. coli* on the 60 mm plates and 6 drops on the 90 mm plates and spreading the drops over the plates. The plates were then

wrapped with parafilm (Pechiney Plastic Packaging, Menasha, WI) to prevent drying and placed upside down in a 37 °C incubator overnight. The next day the parafilm wrap was replaced and the plates were then stored upside down at 4°C until used.

LB medium consists of (Sambrook and Russel, 2001):

950 ml deionized water	
10 g tryptone	(Bacto tryptone (Becton, Dickinson and Co, Franklin Lakes, NJ))
5 g yeast extract	(Becton, Dickinson and Co, Franklin Lakes, NJ)
10 g NaCl	(J.T.Baker A.C.S. reagent, Phillipsburg, NJ)

The pH was adjusted to 7.0 bringing then the total volume was brought up to 1 liter and then the medium was autoclaved. Usable amounts (50, 100, 200 ml) were then pipetted into flasks and autoclaved again. These autoclaved flasks were then stored on the bench top until used.

Saturated *E. coli* culture was grown by inoculating a flask from a previous *E. coli* suspension (that was stored at 4°C) using a heat sterilized inoculation loop. The newly inoculated flask's lid was taped in place and not closed tightly, then the flask was placed in an incubator shaker (New Brunswich Scientific, Edison, NJ) at 37°C and shaken at 225 rpm overnight (approximately 16 h).

Worms were transferred from plate to plate using a method similar to Driscoll's (1995). Using a heat sterilized scalpel to excise a chunk of agar from plates in which the food supply had run low, and placing that chunk on a new plate, the worm side of the

wrapped with parafilm (Pechiney Plastic Packaging, Menasha, WI) to prevent drying and placed upside down in a 37 °C incubator overnight. The next day the parafilm wrap was replaced and the plates were then stored upside down at 4°C until used.

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transferred agar chunk remained facing away from the new agar plate. The plates were then wrapped with parafilm and placed in an incubator upside down at the desired temperature, typically 20 °C.

Concentration response

Methyl mercuric chloride (Sigma-Aldrich Chemical Co., St. Louis, MO) and cadmium chloride (Sigma) were tested for lethality. Verapamil (Sigma) was tested for prevention of lethality at 50 μM and 150 μM MeHg . Assays were done in standard 12 well plates at room temperature (23-25 °C) on a Hot Shaker orbital shaker (Bellco Glass, Vineland, NJ) at 90 rpm.

Typically microscopic work was done using a Leitz Wetzler (Leica, Bannockburn, IL) light microscope model D65543 with a Nikon (Melville, NY) 4X objective, unless otherwise specified. Worms were synchronized by modifying a cleaning method described in Driscoll (1995) as follows:

> Adult gravid worms and eggs were washed off the plates by first adding 5 ml of K media to each 90 mm plate or 3 ml to each 60 mm plate and letting the solutions sit for a moment. The plate was washed by forcefully pipetting the solution up and down using a electric pipetter, followed by collecting the solution in a 15 ml centrifuge tube. Any remaining worms and eggs were carefully scraped off the plate using a cell scraper and added to the 15 ml centrifuge tube. This process is repeated for each plate, with the solutions from multiple plates combined into one 15 ml centrifuge tube. The centrifuge tubes were then spun at 1000 rpm (110 *g*) for 5 min.

K media as described in Chu and Chow included (2002):

50 mM NaCl	(J.T.Baker A.C.S. reagent, Phillipsburg, NJ)
30 mM KCl	(J.T.Baker A.C.S. reagent, Phillipsburg, NJ)
10 mM NaOAc	(J.T.Baker A.C.S. reagent, Phillipsburg, NJ)
bring pH to 5.5	

During this spin the cleaning solution was made by placing 550 µl of DI (deionized) water in a 1.5 µl centrifuge tube, then adding 300 µl of bleach (Clorox, Oakland, CA) and 150 µl of 5 M NaOH (Mallinckrodt, Hazelwood, MO) for a total volume of 1000 µl. One tube of cleaning solution was made for each 15 ml tube of worm solution.

The worm solution was aspirated off the worm/egg pellet in the 15 ml tubes at the end of the spin and then 800 µl of the cleaning solution was added to each tube. Approximately three to seven min. later when a majority of the worms had lysed or dissolved (this was monitored by placing a drop on a glass slide every minute after 3 min had passed and looking at it under the microscope), the cleaning solution was diluted to 15 ml using deionized water. The eggs were then pelleted by spinning the tubes at 3200-3600 rpm (1140-1440 *g*) for 5 min. The solution was aspirated off and then the eggs were resuspended in deionized water and spun again. Pelleting and resuspending were repeated twice, after which the solution was mostly removed and the eggs were suspended in the remaining solutions ~ 20-50 µl and plated onto an agar plate with an *E. coli* lawn. The plates sat face up on the bench top until the moisture was absorbed by the agar, then the plates were wrapped in parafilm and placed in a 20°C incubator upside down for16 h. As the eggs developed overnight

fresh confluent *E. coli* solution was grown (see procedure above).

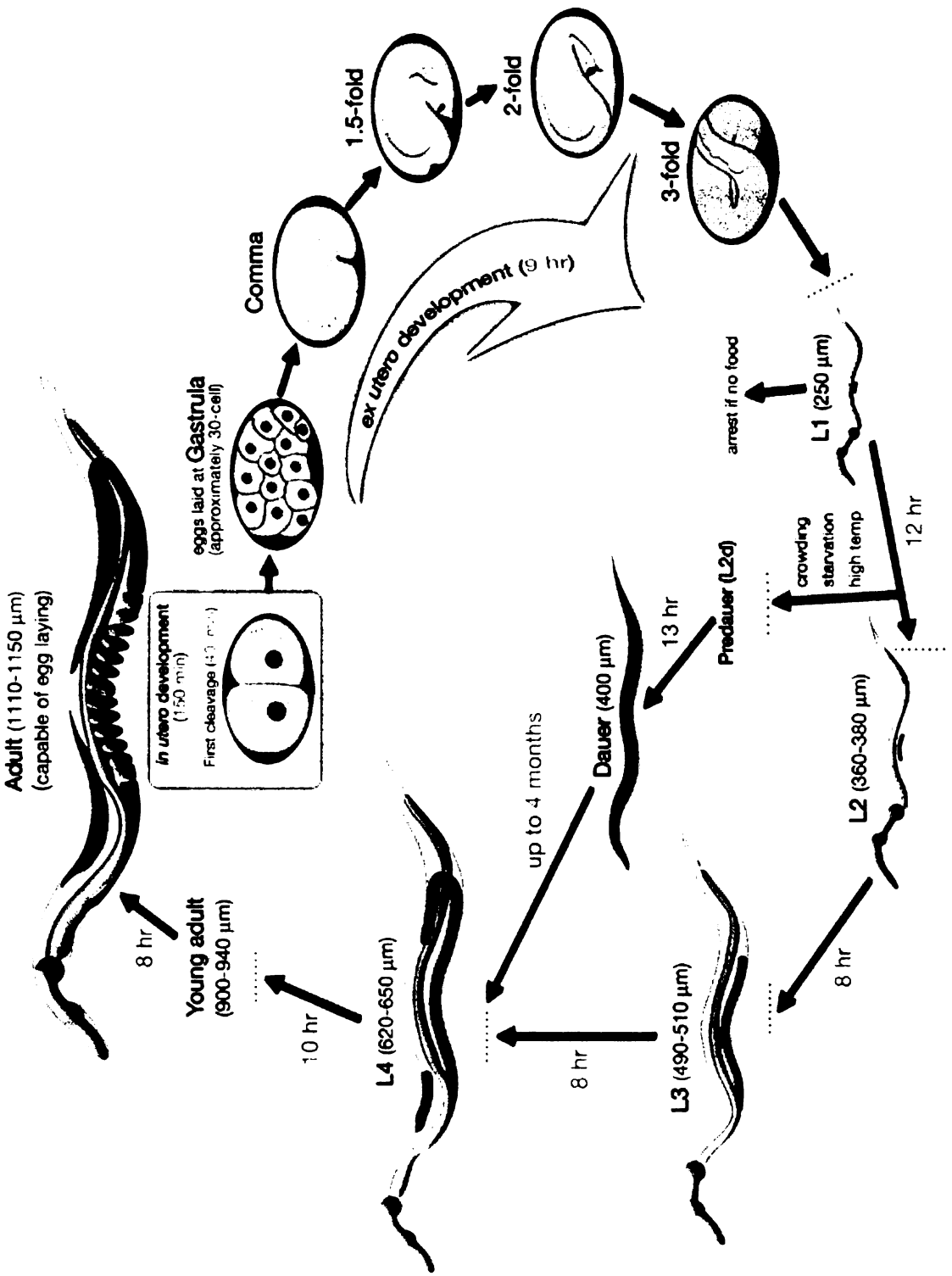
Fifty ml of fresh *E. coli* solution was concentrated by a factor of four in K medium (Chu and Chow). This was done by centrifuging the *E. coli* solution at 6000 rpm (4020 *g*) for 10 min then aspirating off the solution, followed by adding ~12.5 ml of K medium. Two to three mls of this new solution were aliquoted into 4 or 6 wells of a 12-well plate as follows: 3 ml of solutions in one well if there were 4 treatments and 2 ml if there were 6 treatments.

The synchronized worms in the larval 1 (L1) and larval 2 (L2) stages of growth (Figure 2) were washed off the plate and collected (Chu and Chow, 2002), after hatching overnight, by spinning them at 2000-2500 rpm (440-690 *g*) for 5 min and removing the solution. Then the worm pellet was suspended in K medium at a minimum concentration of 3,000 worms/ml, determined by counting the worms in a 10 µl sample, in triplicate. 100 µl of worm solution were added to each ml of *E. coli* solution in the 4 or 6 wells of a 12-well plate and verapamil was added immediately if it was used. The background percent death was then calculated by counting the live and dead worms (moving versus non-moving, see below) in a 10 µl sample from the remaining worm suspension, done in triplicate.

The plates were then shaken for one hour, after which MeHg or cadmium was added. The plates were then shaken for 5 min before the cultures in each of the 4 or 6 wells were dispersed equally to 1 - 2 additional wells making use of all 12 wells and giving a

Figure 2. Life cycle of N2 wild type *C. elegans* at 22°C. 0 min is fertilization. The times associated with the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 min. postfertilization. Eggs are laid outside at about 150 min. postfertilization and during the gastrula stage. The length of the animal at each stage is marked next to the stage name in micrometers. (Figure reproduced and modified from Hall and Altun, 2008).

FIGURE 2.


Adult (1110-1150 µm)
(capable of egg laying)
8 hr
Young adult
(900-940 µm)
10 hr
L4 (620-650 µm)
8 hr
Dauer (400 µm)
up to 4 months
13 hr
Predauer (L2d)
crowding
starvation
high temp
12 hr
L1 (250 µm)
arrest if no food
L2 (360-380 µm)
8 hr
L3 (490-510 µm)
In utero development
(150 min)
First cleavage
eggs laid at Gastrula
(approximately 30-cell)
Comma
1.5-fold
2-fold
3-fold
ex utero development (9 hr)

replicate count of 2 - 3. Each twelve-well plate was counted as one replicate (n).

The cultures were concentrated by centrifugation (2000 rpm (300 *g*) for 2 min) at the appropriate time points, and 10 µl aliquots were placed on a glass slide under a coverslip for observation. The worms were scored based on whether they moved during a 10 sec observation; a minimum of 15 worms were scored per well. Length was measured using an ocular micrometer on one worm per well. The remainder of the worm suspensions was returned to the wells, discarded or kept for fluorescence microscopy as needed. This process was repeated for each time point.

Confocal microscopy

This work was done using a Leica TCS SL (Leica, Bannockburn, IL) confocal microscope with a 10X and a 60X oil emersion objective and the NW1229 worms. The concentrated worm suspension was used as in other experiments, and approximately 10 µl of 50 mM sodium azide was added to kill any living worms.

Fluorescence microscopy

This work was done on an Olympus IX70 inverted microscope (Olympus, Center Valley, PA) using a 20X objective. Images of the NW1229 and the KC136 worms were captured by taking 8 µl of concentrated worms and adding 2 µl of 50 mM sodium azide to kill any living worms. Then the procedure outlined in Driscoll (1995) was followed: a drop of liquid NGM agar was placed on a glass slide and immediately covered with another slide, that had two layers of labeling tape at either end, to flatten the agar droplet.

After the agar had solidified, the taped slide was carefully removed and the sample was placed on the agar pad and covered with a coverslip. Images were captured using an Olympus EVOLT E-330 digital SLR camera (Olympus, Center Valley, PA). All photo stitching was performed using Adobe Photoshop CS2 (Adobe, San Jose, CA).

Images of the N2 worms were also captured as described in the previous paragraph. The N2 worms were incubated for 15 min with 10 μM Sytox green nucleic acid stain (Invitrogen, Carlsbad, CA) before pictures were taken.

Data analysis

The percentage of dead animals was calculated by taking the average number of dead worms from the replicate wells on the same plate and subtracting the background percent of dead animals, then dividing this by the percentage of living animals at the start of the assay.

A minimum of six replicates (12-well plates) were used for each datum point, with each replicate consisting of 2-3 wells. Outliers (95 % confidence interval) for each datum point were removed from the statistical analysis (SigmaPlot10, Systat, San Jose, CA). Statistical analysis was done using a one-way ANOVA (analysis of variance) test with Dunnet's secondary test (SAS 9.1, SAS, Cary, NC). Data that failed the one-way ANOVA's Bennett's test for homogeneity of variance, were analyzed by nonparametric one-way ANOVA using the Kruskal-Wallis test, with each treatment individually tested for significance from control.

RESULTS

Initially, a concentration response was determined for *C. elegans* exposed to MeHg. MeHg effects on lethality in larval stage 1 and 2 (L1-L2) *C. elegans* were determined by calculating percent mortality then normalizing the percentage by subtracting the background mortality of the pre-assay worm culture. The concentration response to MeHg shows an LC_{50} between 200 and 300 μM (Figure 3) at 6 h of treatment, with 99 percent mortality at 300 μM. By 12 h of MeHg exposure, the LC_{50} had dropped to 100 μM and remained at 100 μM through 48 h of exposure. Older worms, mostly in the L4 life stage with some L3 animals, exposed to 200 and 300 μM MeHg had the same percent mortality as the younger worms (Figure 4). The effect of cadmium on percent mortality in *C. elegans* was briefly examined, because there were no previously published result examining MeHg effects on *C. elegans*. The observed cadmium LC_{50} was approximately 900 μM (Figure 5) after 48 h of exposure. Chu and Chow (2002), using a similar procedure, reported an LC_{50} value of 594 μM after 48 h of cadmium exposure. Williams and Dusenbery (1988) exposing *C. elegans* to cadmium on agar plates observed a 24 h LC_{50} of 3087 μM. The current results fall between these previously published fiugres.

The surviving worms at several MeHg concentrations failed to grow in size and develop to maturity (reproduction) (Figure 6). Body length was measured to quantify this effect on growth. By 6 h a significant reduction in body length was already evident between non-treated and treated worms, at MeHg concentrations ≥ 100 μM (Figure 7).

Figure 3. Mortality of *C. elegans* as a function of time and MeHg exposure.

Concentration response of organismal death in *C. elegans* exposed to MeHg in liquid culture of K medium with *E. coli* as the food source. All data points are significantly different than controls except for 12 h 5 and 10 μM and 24 h 5 μM (n of 6-61; see details in Table A.1). LC_{50} was between 200 - 300 μM at 6 h and is approximately 100 μM by 12 h and after. 300 μM MeHg caused close to 100 % death by 6 h.

FIGURE 3.

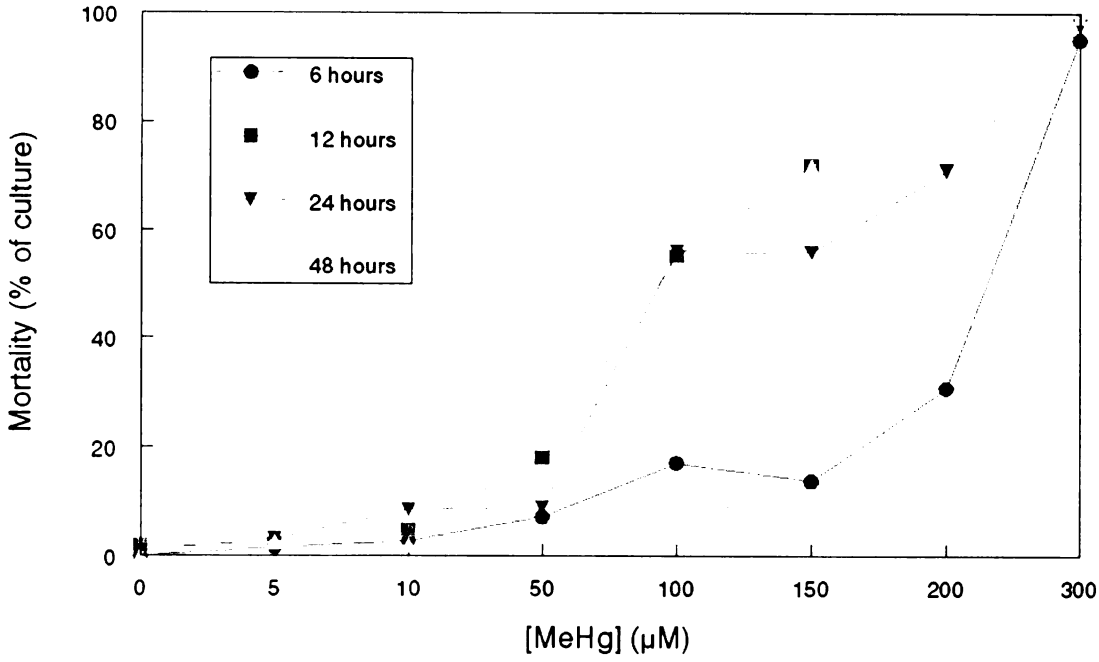

Mortality (% of culture)
100
80
60
40
20
0
6 hours
12 hours
24 hours
48 hours
0
5
10
50
100
150
200
300
[MeHg] (μM)

Figure 4. Comparative mortality of *C. elegans* when exposed to MeHg at different life stages for 24 h. Response of organismal death in L3/L4 life stage *C. elegans* exposed to MeHg for 24 h (n = 6). The percent mortality was the same as L1/L2 worms shown in Figure 3, and reproduced in this figure.

FIGURE 4.

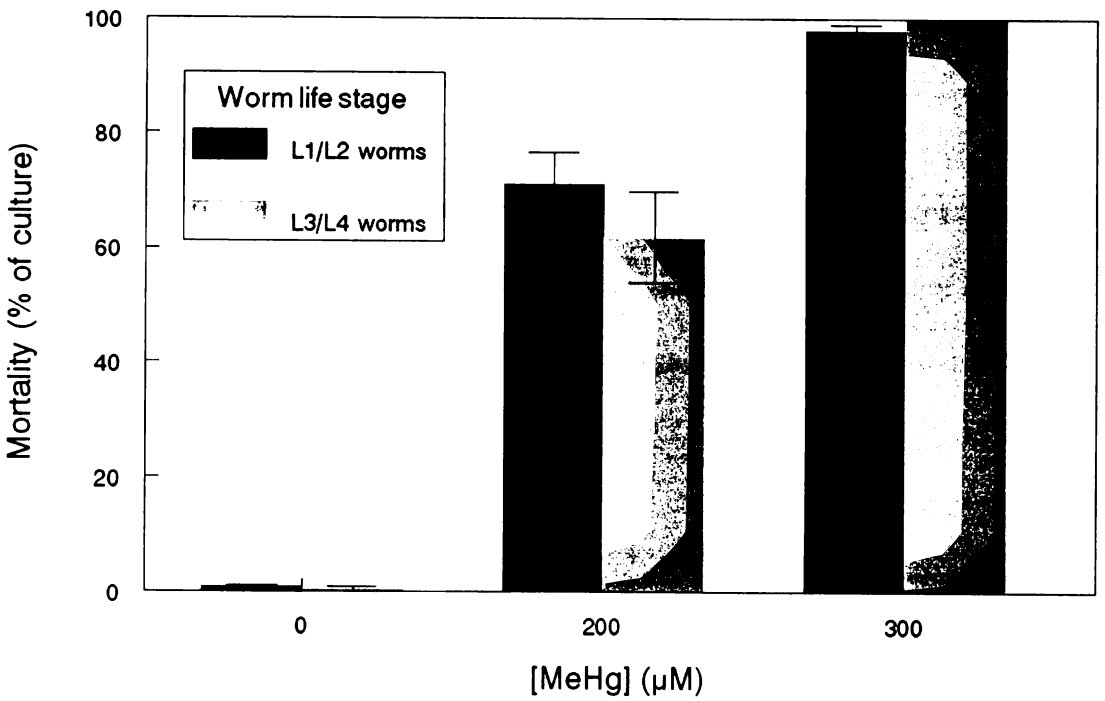

Mortality (% of culture)
100
80
60
40
20
0
Worm life stage
L1/L2 worms
L3/L4 worms
0
200
300
[MeHg] (μM)

Figure 5. Mortality of *C. elegans* as a function of cadmium exposure. Concentration response of organismal death in *C. elegans* exposed to cadmium in liquid culture of K medium with *E. coli* as the food source. LC_{50} was not seen at 24 h and was between 900 - 1000 μM at 48 h (n = 1).

FIGURE 5.

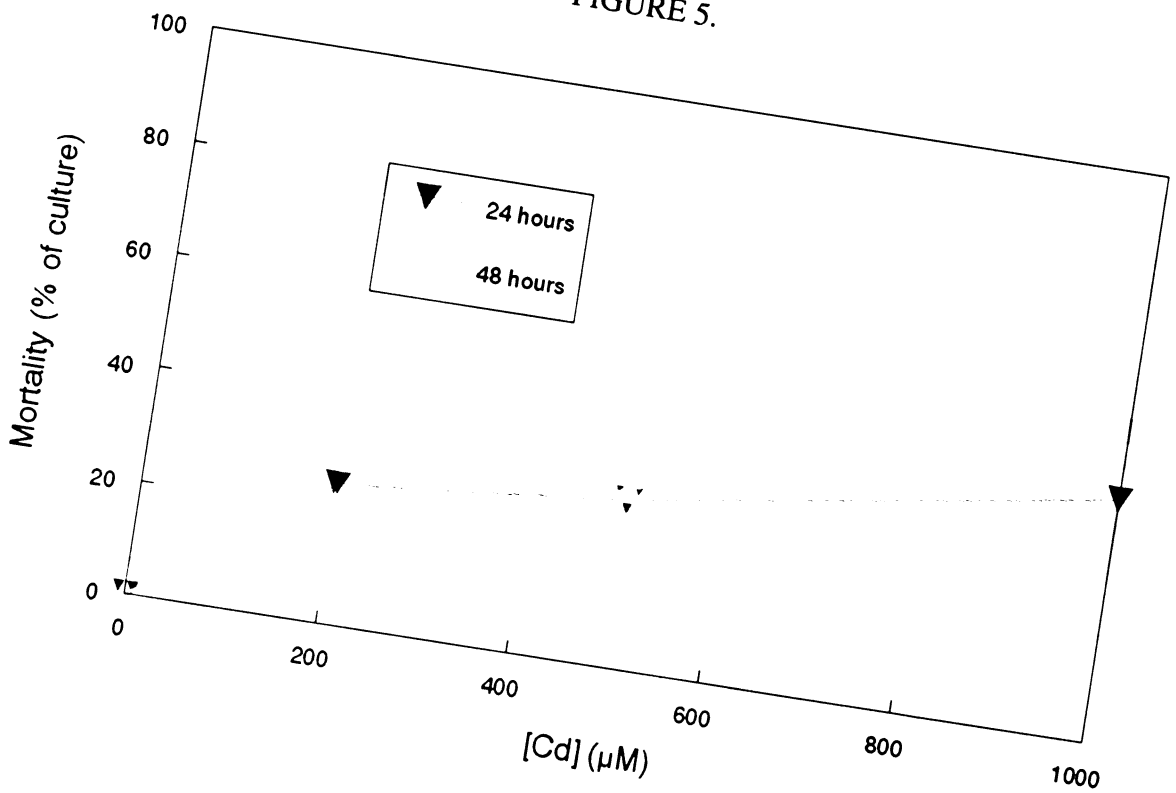

100
80
60
40
20
0
Mortality (% of culture)
24 hours
48 hours
0
200
400
600
800
1000
[Cd] (μM)

Figure 6. Comparative effects of several MeHg concentrations on *C. elegans* through 120 h exposure. Images correspond to MeHg concentrations seen in Figure 3 as follows: A is 0 μM, B is 5 μM, C is 10 μM, D is 50 μM, E is 100 μM and F is 150 μM. These images illustrate MeHg's effects on body length and reproduction. Smaller worms seen in frames A, B, C and D are second generation, and some third generation in A and B.

FIGURE 6

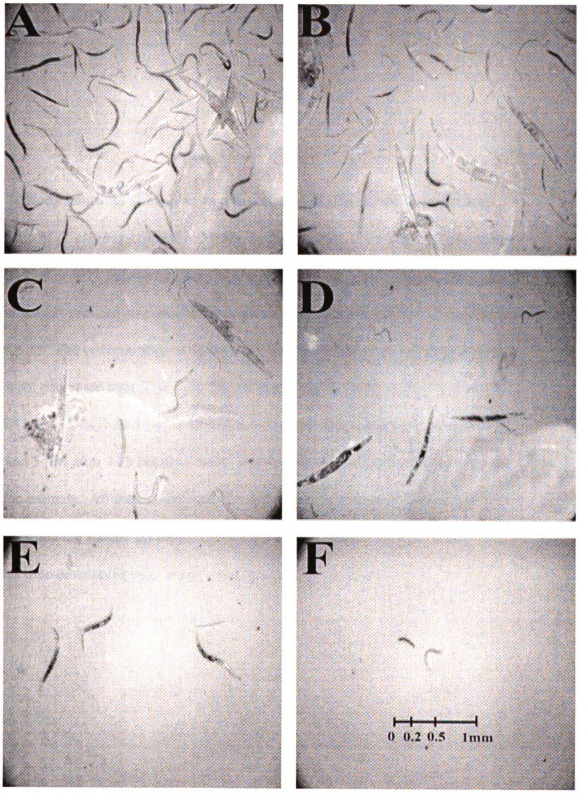


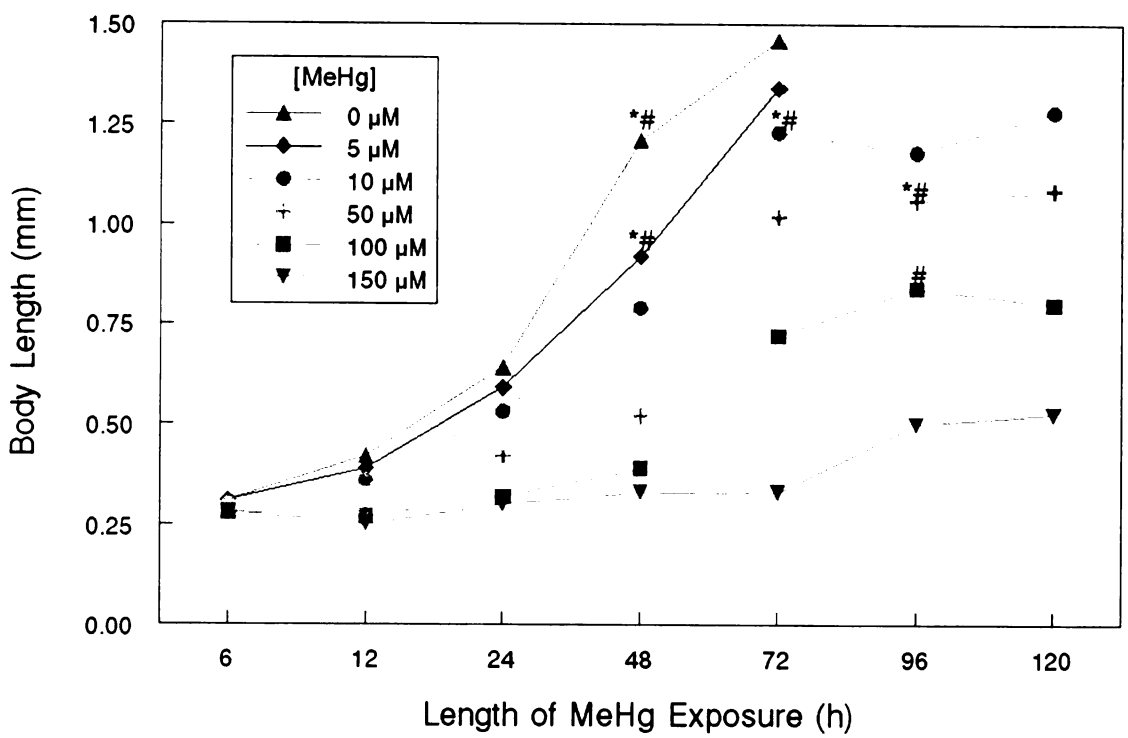
Figure 7. Body length of *C. elegans* as a function of MeHg exposure over time.

Body length of *C. elegans* exposed to MeHg, excluding data at 200 and 300 μM MeHg (shown in Figure A.1), because no growth was seen at these concentrations. Body length was significantly reduced at concentrations above 100 μM by 6 h (n = 7-21; see details in Table A.2). The concentration at which body lengths became statistically different from control decreased over time, with 50 μM being significant by 12 h (n = 5-6), 10 μM by 24 h (n = 5-24) and 5 μM at 48 h (n = 6-26). No data points were taken for the control and 5 μM after 72 h because is was not possible to distinguish first and second generation animals. All data points from 72 - 120 h had a replicate of 2 (n = 2).

* Denotes when the second generation of worms was first seen.

# Denotes time developing eggs inside adult worms were observed.

FIGURE 7.


[MeHg]
0 µM
5 µM
10 µM
50 µM
100 µM
150 µM
Body Length (mm)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
6
12
24
48
72
96
120
Length of MeHg Exposure (h)

At each subsequent time point, the lowest MeHg concentration that caused a significant difference in body length, compared to non-treated controls, decreased; with 50 μM becoming significant at 12 h, 10 μM at 24 h and 5 μM at 48 h. These observations suggest that body length is an excellent sub-lethal end point for analysis of MeHg effects in *C. elegans*. The body length studies were briefly carried out to 120 h to examine whether the worms would eventually reach the same length as non-treated controls. Generally, the worms treated with the lower concentrations of MeHg did reach maturity (reproduction) but never became quite as large as control worms (see Figure7). A second generation of worms was obvious and well established in non-treated control animals by 48 h. Worms treated with 5 μM MeHg appeared to have a delay/decrease in reproduction compared to non-treated worms, but the delay was less than 24 h, based on fewer second generation worms that appeared smaller than non-treated worms at the 48 h time point. There was approximately a 24 and 48 h delay in reproduction for worms treated with 10 μM and 50 μM MeHg respectively. Worms treated with 100 μM MeHg apparently reached maturity based on formation of eggs inside the worms at 96 h. The number of eggs that developed in the 100 μM MeHg-treated worms was reduced to 1-3 eggs per worm compared to 8 -12 eggs in non-treated control animals. Non-treated animals also accumulated 30 - 40 eggs over time; this is common in *C. elegans* grown in liquid culture (Stiernagle, 2006). In addition to reduced egg numbers at 100 μM MeHg, there was no observable second generation or egg laying. At 150 μM MeHg there was some growth, but no egg formation up to 120 h. The last two MeHg concentrations, 200 and 300 μM, stopped any increase in body length, and caused 76 and 99 percent mortality, respectively, by 48 h. They were subsequently omitted from the 120 h

experiment.

Verapamil was tested to see if it could reduce the effects of MeHg by blocking L-type VGCCs. Verapamil concentrations of 0, 5, 10, 25, 50 and 100 μM were tested on worms treated with 150 μM MeHg (Figures 8-15). A second verapamil experiment tested 0, 1, 5 and 10 μM verapamil at 0, 50 and 150 μM MeHg (Figures 16-21). There is a clear time dependent MeHg effect on percent mortality and body length in this study, however, verapamil offered no significant protection from the effects of MeHg on percent mortality or body length at 6, 12, 24 or 48 h. In fact, verapamil itself was toxic at the highest concentrations used, 50 and 100 μM, at 48 h (Figure 22).

Figure 8. Mortality of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 6 h. Concentration response of organismal death in *C. elegans* exposed to 150 μM MeHg and treated with L-type VGCC blocker verapamil for 6 h. There was no significant difference between control and any verapamil concentration tested (n = 10-11).

FIGURE 8.

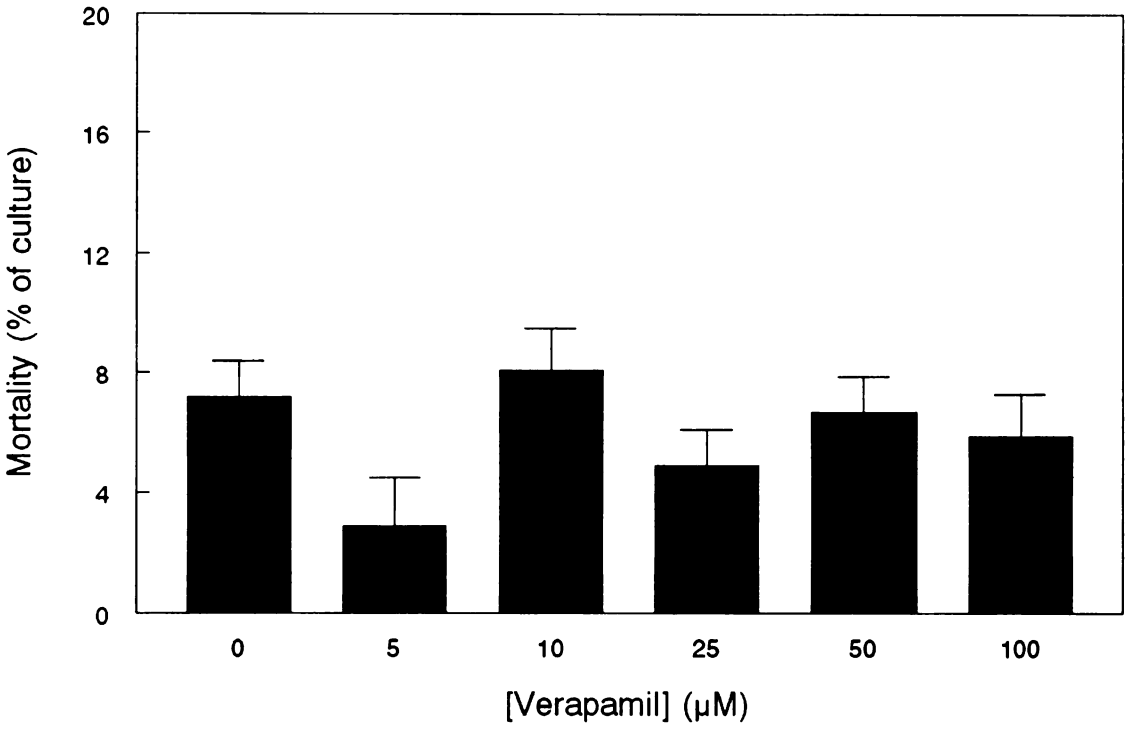


Figure 9. Mortality of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 12 h. Concentration response of organismal death in *C. elegans* exposed to 150 μM MeHg and treated with L-type VGCC blocker verapamil for 12 h. There was no significant difference between control and any verapamil concentration tested (n = 4).

FIGURE 9.

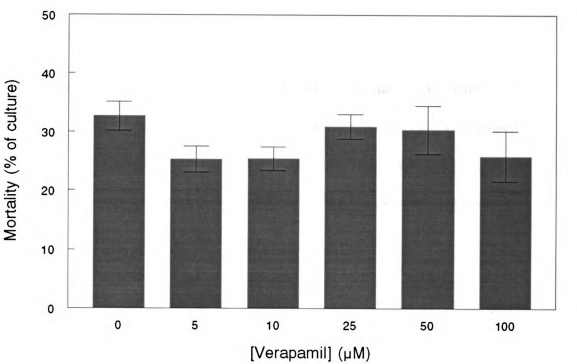

50
40
30
20
10
0
Mortality (% of culture)
0
5
10
25
50
100
[Verapamil] (μM)

Figure 10. Mortality of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 24 h. Concentration response of organismal death in *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 24 h. There was no significant difference between control and any verapamil concentration tested (n = 9-11).

FIGURE 10.

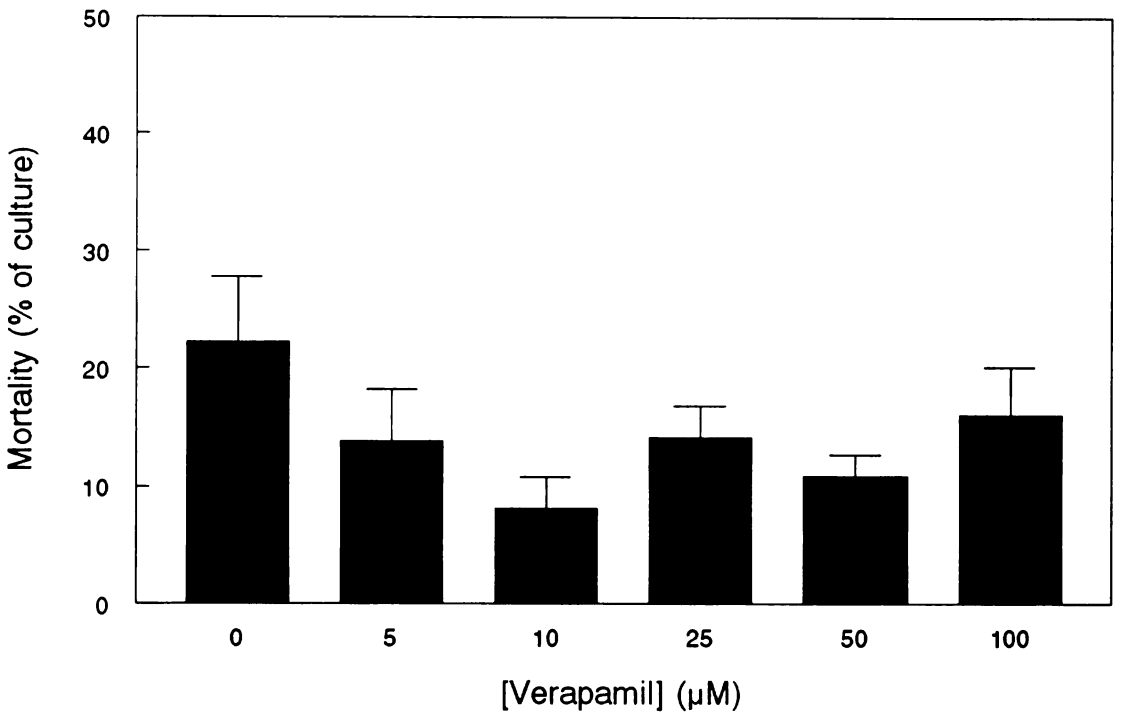

50
40
30
20
10
0
Mortality (% of culture)
0
5
10
25
50
100
[Verapamil] (μM)

Figure 11. Mortality of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 48 h. Concentration response of organismal death in *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 48 h. There was no significant difference between control and any verapamil concentration tested (n = 24-49).

FIGURE 11.

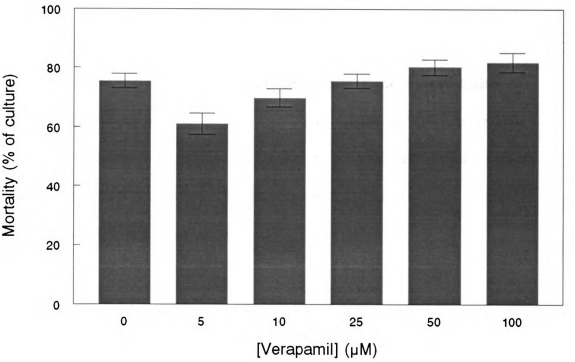

100
80
60
40
20
0
Mortality (% of culture)
0
5
10
25
50
100
[Verapamil] (μM)

Figure 12. Body length of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 6 h. Body length of *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 6 h. Body length was significantly different between control and worms treated with 100 μM verapamil (n = 10-11).

* Denotes significant difference than control.

FIGURE 12.

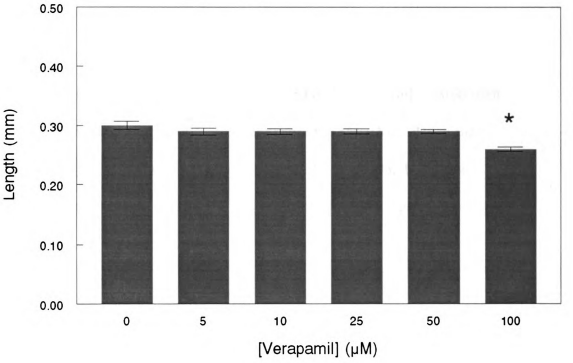


Figure 13. Body length of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 12 h. Body length of *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 12 h. Body length was not significantly different between control and any verapamil concentration tested (n = 4).

FIGURE 13.

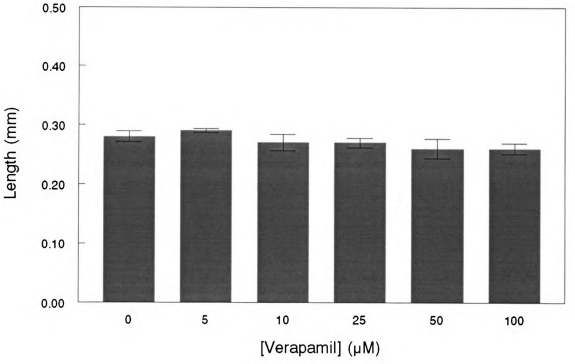


Figure 14. Body length of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 24 h. Body length of *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 24 h. Body length was not significantly different between control and any verapamil concentration tested (n = 10-11).

FIGURE 14.

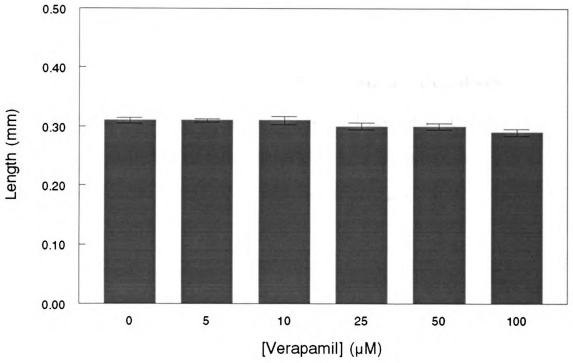


Figure 15. Body length of *C. elegans* exposed to 150 μM MeHg and treated with verapamil for 48 h. Body length of *C. elegans* exposed to 150 μM MeHg in the presence of the L-type VGCC blocker verapamil for 48 h. Body length was not significantly different between control and any verapamil concentration tested (n = 16-25).

FIGURE 15.

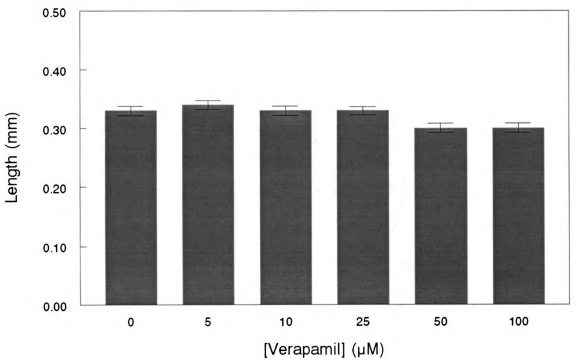


Figure 16. Mortality of *C. elegans* exposed to MeHg and treated with verapamil for 12 h. Concentration response of organismal death in *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 12 h. There was no significant difference between control and any verapamil concentration tested at any MeHg concentration (n = 5).

FIGURE 16.

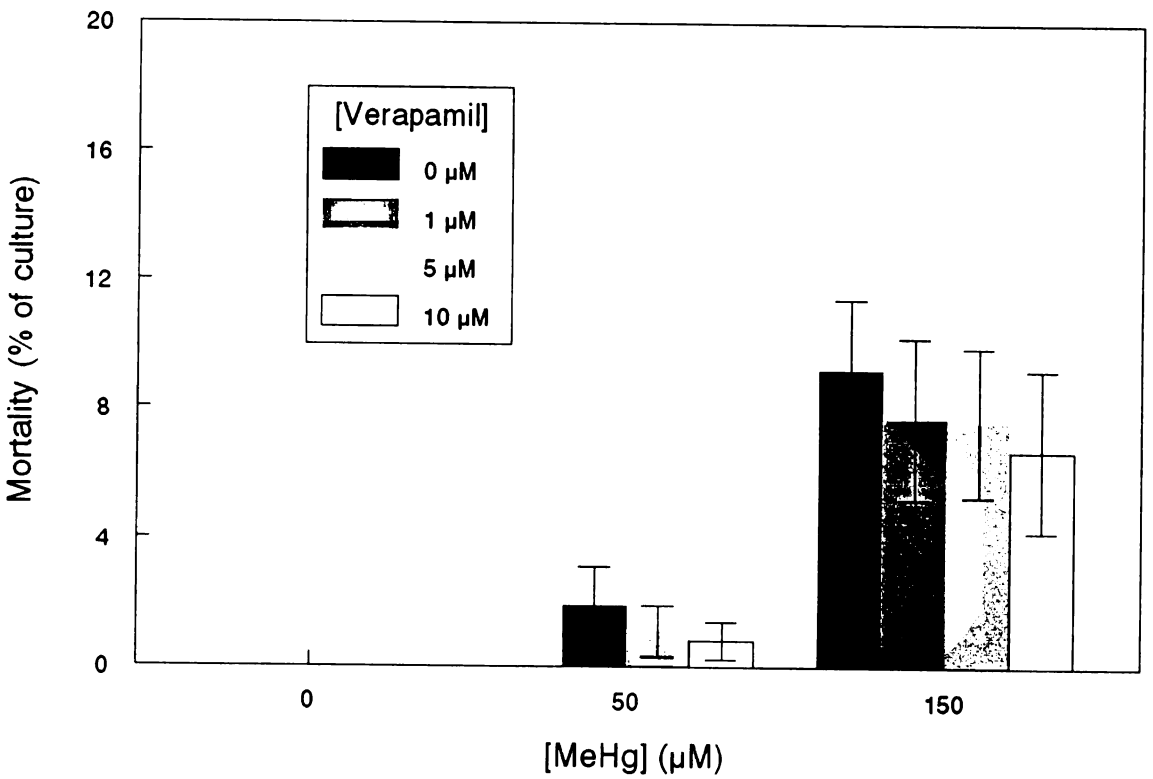

20
16
12
8
4
0
Mortality (% of culture)
[Verapamil]
0 μM
1 μM
5 μM
10 μM
0
50
150
[MeHg] (μM)

Figure 17. Mortality of *C. elegans* exposed to MeHg and treated with verapamil for 24 h. Concentration response of organismal death in *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 24 h. There was no significant difference between control and any verapamil concentration tested at any MeHg concentration (n = 5) .

FIGURE 17.

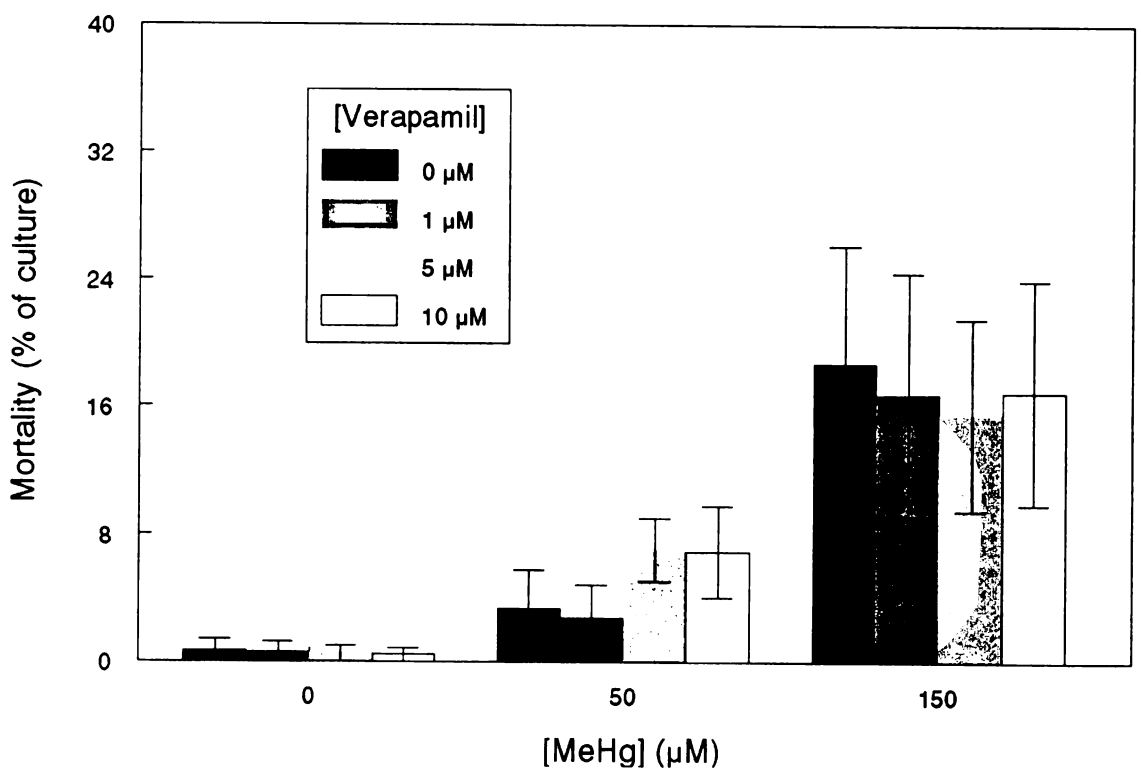

40
32
24
16
8
0
Mortality (% of culture)
[Verapamil]
0 µM
1 µM
5 µM
10 µM
0
50
150
[MeHg] (µM)

Figure 18. Mortality of *C. elegans* exposed to MeHg and treated with verapamil for 48 h. Concentration response of organismal death in *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 48 h. There was no significant difference between control and any verapamil concentration tested at any MeHg concentration (n = 5).

FIGURE 18.

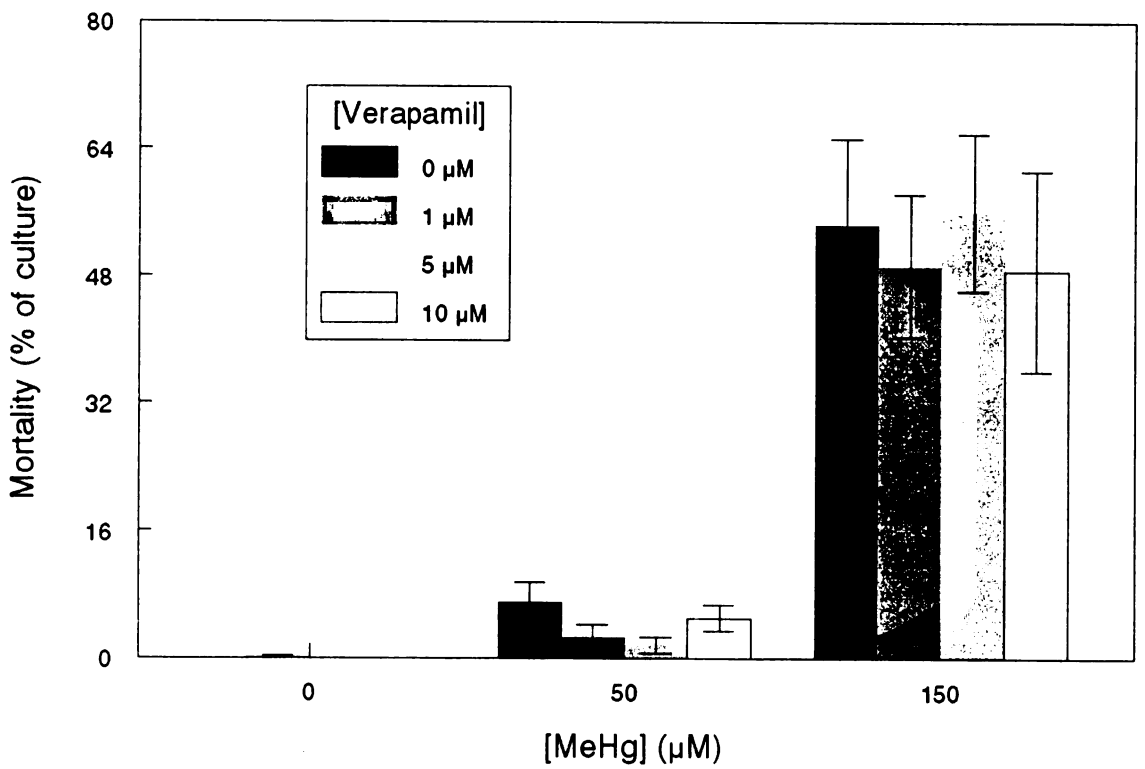

80
64
48
32
16
0
Mortality (% of culture)
[Verapamil]
0 µM
1 µM
5 µM
10 µM
0
50
150
[MeHg] (µM)

Figure 19. Body length of *C. elegans* exposed to MeHg and treated with verapamil for 12 h. Body length of *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 12 h. Body length was not significantly different between control and any verapamil concentration at any MeHg concentration tested (n = 5).

FIGURE 19.

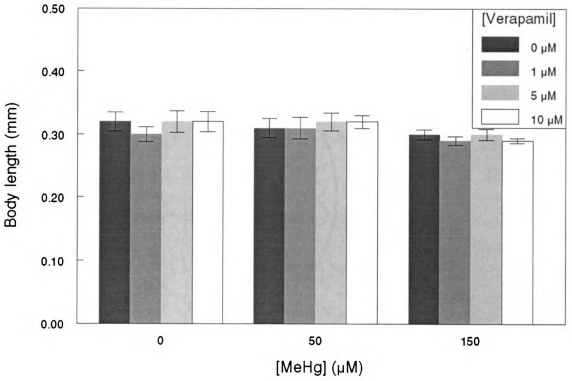

0.50
0.40
0.30
0.20
0.10
0.00
Body length (mm)
[Verapamil]
0 μM
1 μM
5 μM
10 μM
0
50
150
[MeHg] (μM)

Figure 20. Body length of *C. elegans* exposed to MeHg and treated with verapamil for 24 h. Body length of *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 24 h. Body length was not significantly different between control and any verapamil concentration at any MeHg concentration tested (n = 5).

FIGURE 20.

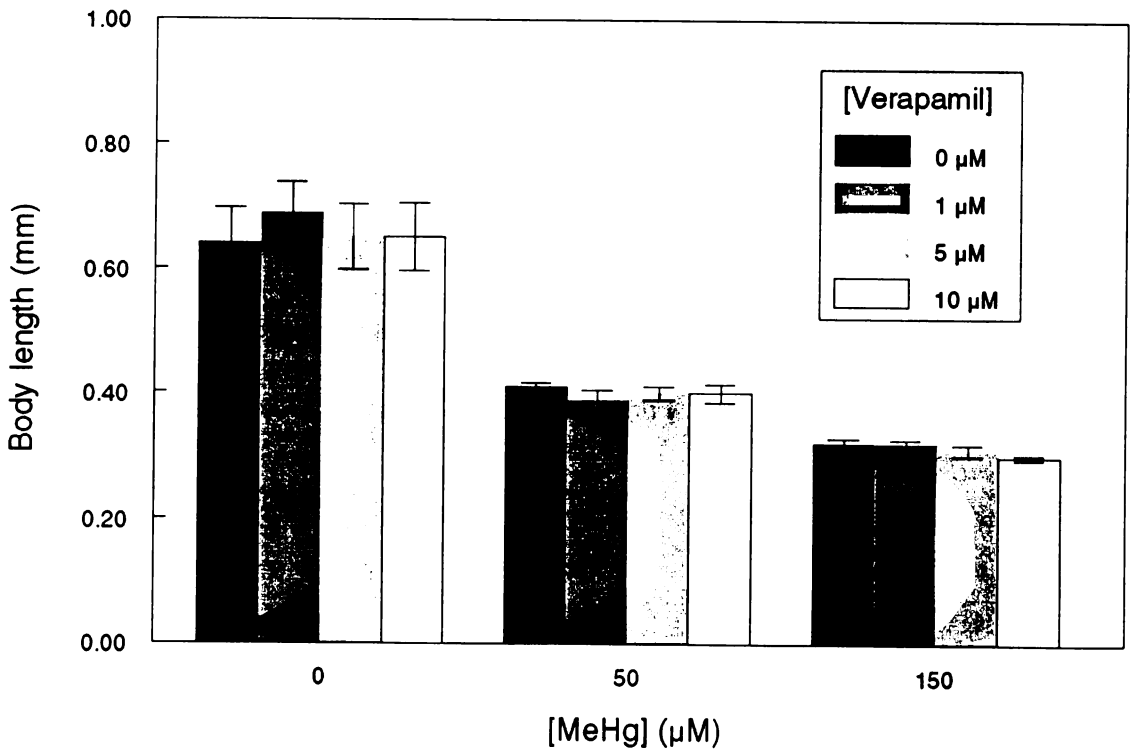

1.00
0.80
0.60
0.40
0.20
0.00
Body length (mm)
[Verapamil]
0 µM
1 µM
5 µM
10 µM
0
50
150
[MeHg] (µM)

Figure 21. Body length of *C. elegans* exposed to MeHg and treated with verapamil for 48 h. Body length of *C. elegans* exposed to 0, 50 or 150 μM MeHg in the presence of 0, 1, 5 or 10 μM of the L-type VGCC blocker verapamil for 48 h. Body length was not significantly different between control and any verapamil concentration at any MeHg concentration tested (n = 5).

FIGURE 21.

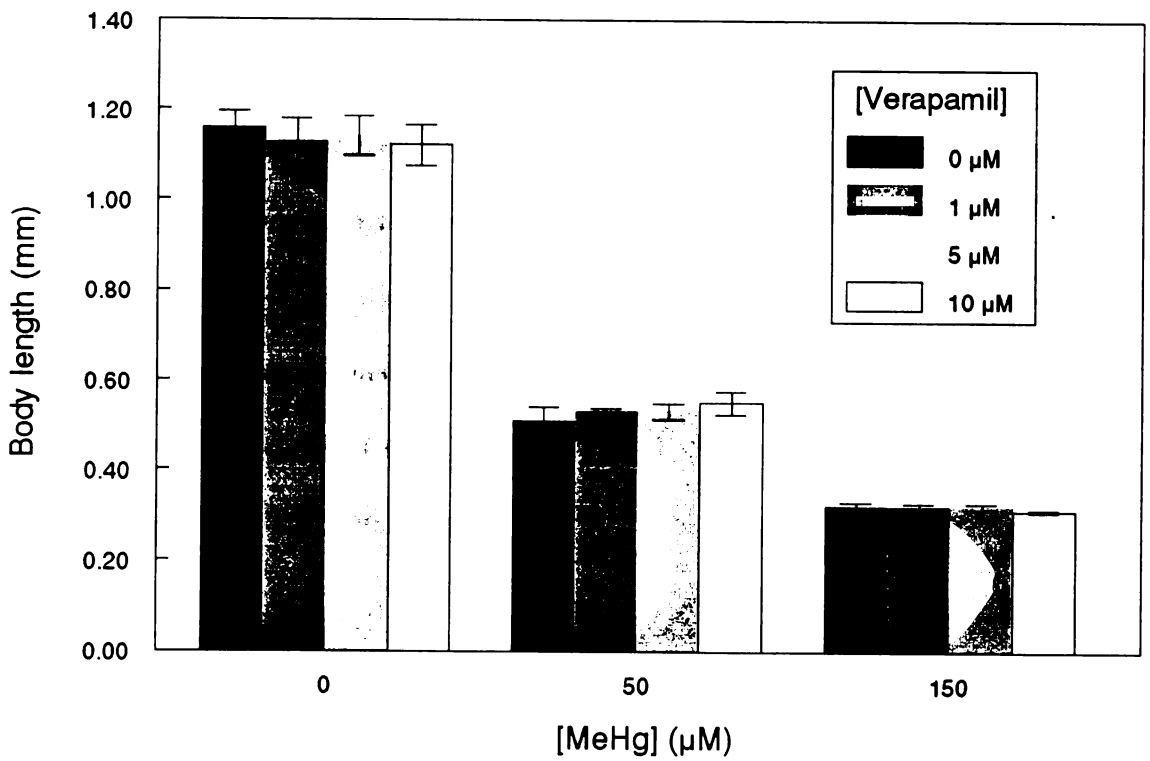
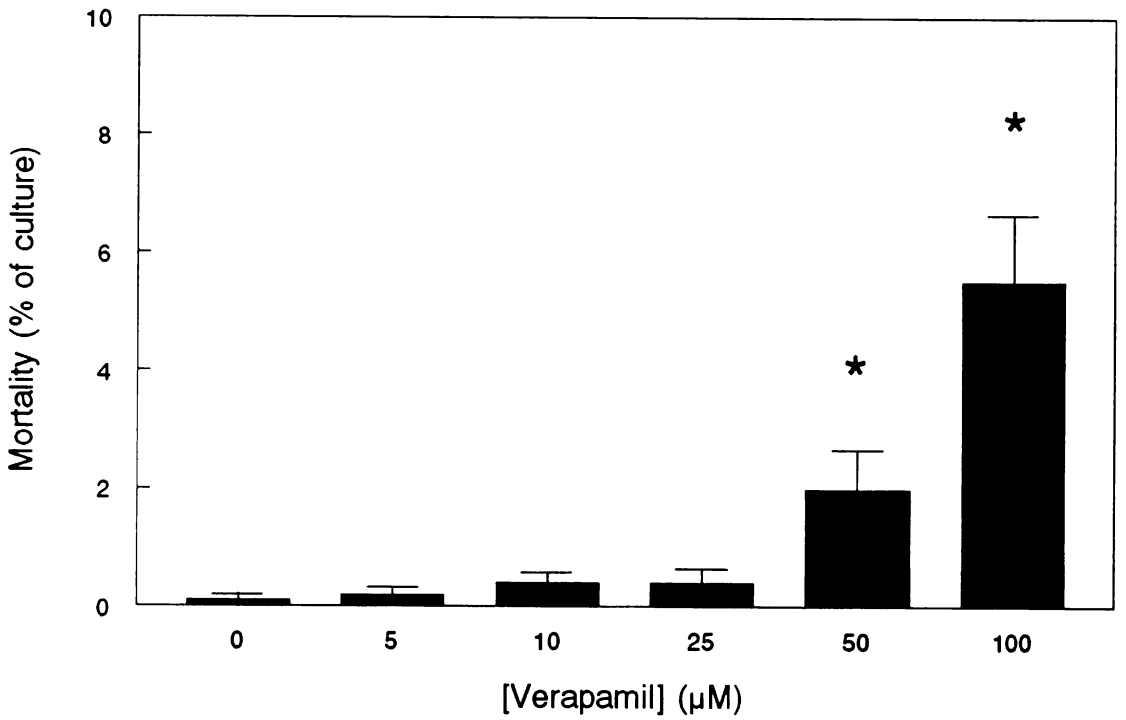

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Body length (mm)
[Verapamil]
0 µM
1 µM
5 µM
10 µM
0
50
150
[MeHg] (µM)

Figure 22. Mortality of *C. elegans* as a function of 48 h verapamil exposure.

Concentration response of organismal death in *C. elegans* exposed the L-type VGCC blocker verapamil for 48 h. There was a significant difference between control and the highest two verapamil concentration of 50 μM and 100 μM (n = 14-16) at 48 h, but not at the earlier time points (see appendix A).

* Denotes significant difference than control.

FIGURE 22.



DISCUSSION

At the time that this project was started, there had not been any studies on the effects of MeHg on *C. Elegans.* However, there were a few studies on the effects of cadmium on *C. elegans.* Chu and Chow (2002) reported an LC_{50} value of 595 μM after 48 h of cadmium exposure. Williams and Dusenbery (1988) reported a 24 h LC_{50} of 3087 μM when they exposed *C. elegans* to cadmium on agar plates in the presence of food and a LC_{50} of 1500 μM without food present. To examine if the current methodology was adequate, the effect of cadmium on *C. elegans* was tested for comparison to published results. Using a method similar to Chu and Chow (2002), a cadmium LC_{50} of 900 μM after a 48 h exposure was determined. This result suggests that my methodology is not dissimilar to other researchers, and could be applied to examination of MeHg effects on *C. elegans.*

Helmcke et al. (2008) recently reported LC_{50} values of 430 μM and 340 μM for L4 stage *C. elegans* exposed to MeHg for 6 h and 15 h respectively . Additionally, Helmcke et al. (2008) observed that older worms in L4 life stages (LC_{50} of 2.3 mM) are less sensitive to MeHg than younger L1 worms (LC_{50} of 1.3 mM) after a 30 min exposure. Similar results describing decreased sensitivity to cadmium in older worms have been reported with an LC_{50} of 5.52 mM in L4 worm compared to 595 μM in L1 worms with a 48 h exposure (Chu and Chow, 2002). The LC_{50} values of the current study for L1-L2 worms were 220 μM at 6 h and 100 μM at 12 h. We briefly examined the sensitivity of older worms to MeHg; these worms were treated with MeHg 24 h later than normal.

These later-treated worms were mostly in the L4 life stage with some in L3. Unlike what others have reported, the effects on these L4-L3 late treated worms were essentially the same as the L1-L2 worms (Figure 4). This difference is likely caused by variations in experimental conditions, such as food availability. Overall the lethality data indicate that relative toxicity, at least with respect to dose-sensitivity, in *C. elegans* is an adequate model of acute MeHg toxicity in mammals, when compared to cadmium.

Several studies using *C. elegans* as a toxicity model have focused on changes in growth, reproduction, movement patterns and feeding habits as sub-lethal endpoints of toxicity (Anderson et al., 2001 , Boyd et al., 2003, Helmcke et al., 2008). They have shown that these sub-lethal measures are excellent predictors of toxicity. In the present study, MeHg effects on growth were examined by measuring body length. Body length decreased as MeHg concentration increased, with concentrations as low as 5 μM (the lowest concentration tested) having a significant effect at 48 h of exposure, demonstrating the sensitivity of growth as a measure of toxicity in *C. elegans*. Examination of other behavioral end points may also prove useful.

To use *C. elegans* as a model for MeHg neurotoxicity it would be ideal to identify if there are any specific target neurons. In an attempt to identify potential neural targets Helmcke et al., (2008) used a systematic approach. They looked at individual neural groups tagged with GFP or its derivatives. However, as of their last report no target neural group had been identified. In our efforts to identify target neurons, a GFP tagged to a heat shock protein (KC136) was used but the results were inconclusive, because of

inconsistent GFP visualization. Additionally, we used another worm line (NW1229) displaying pan-neuronal GFP expression. It was thought that loss of fluorescence due to cell death may reveal target neurons that are most susceptible to MeHg. No neuronal targets were identified using pan- neuronal GFP worms due to long lasting fluorescence that persists long after cell death, in fixed slides. However, the use of confocal microscopy offers the ability to examine and identify small variations in GFP intensity, and may allow for identification of neuronal targets in the future.

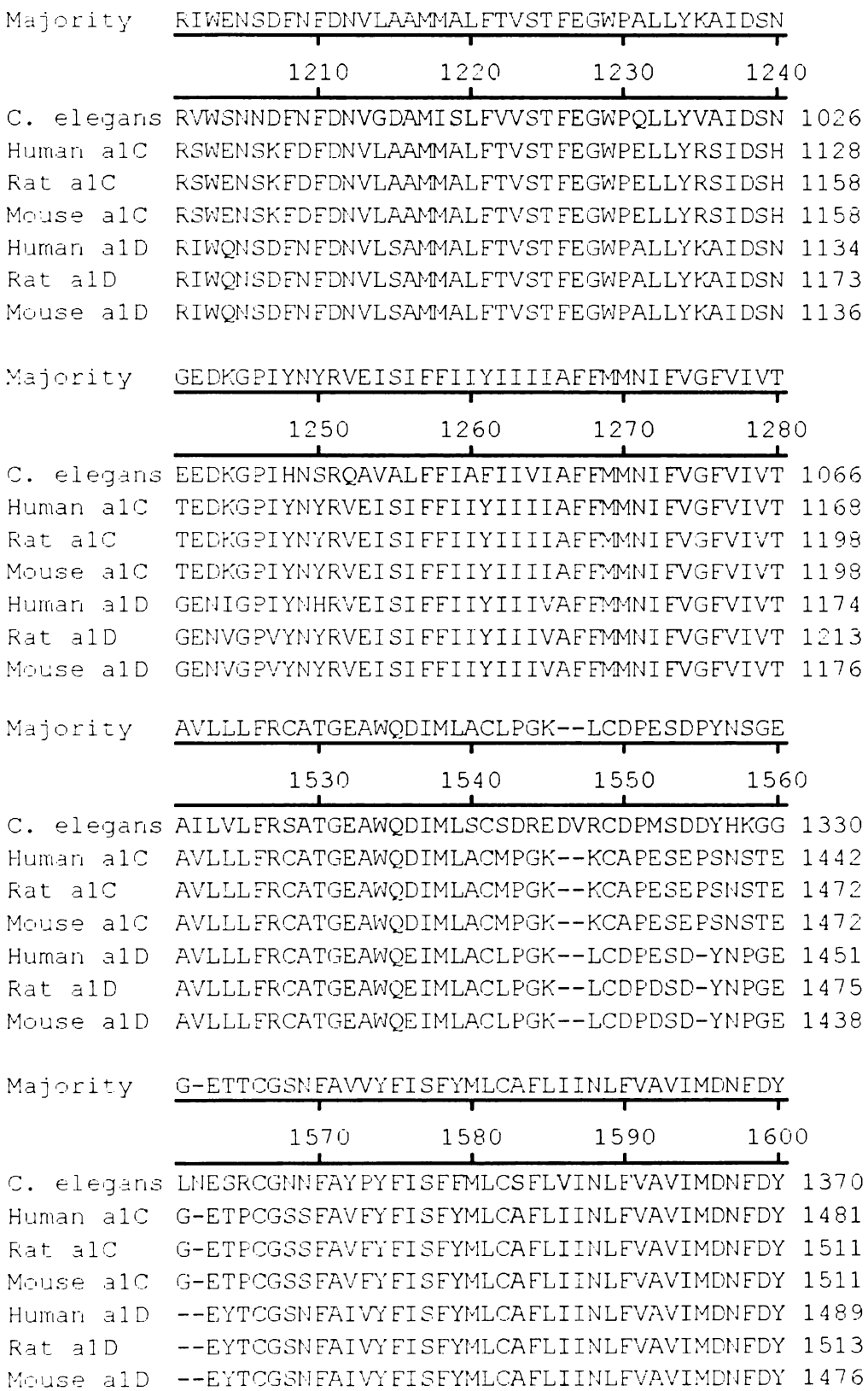
C. elegans has homologues to most major classes of ion channels in mammals (Bergmann, 1998). This protein homology suggests that MeHg's toxic effects could be similar in mammals and *C. elegans*. One known effect of MeHg is disruption of $[Ca^{2+}]_i$. It is also known that increased $[Ca^{2+}]_i$ is an important factor in cell death (Kruman and Mattson, 1999). Blocking L-type VGCC with a dihydropyridine (nifedipine for example), ω-conotoxin-MVIIC and verapamil can mitigate the effect of MeHg (Hare and Atchison, 1995; Wu, 1995; Sakamoto et al., 1996; Marty and Atchison, 1997). There is only one L-type VGCC expressed in *C. elegans* and it is homologous to the mammalian L-type channels, and is blocked by the same L-type Ca^{2+} channel antagonists including verapamil (Jospin et al., 2006; Franks et al., 2008). In the present study verapamil was chosen primarily because it is more chemically stable than other L-type channel blockers. In addition, in an assay similar to the current one, our lab has previously shown that verapamil at concentrations of 25, 50 and 100 μM can significantly mitigate the lethal effect of MeHg on *C. elegans* (Kolselke et al., 2007). Using the current protocol, *in vivo* use of verapamil caused no reduction on MeHg induced mortality or reduction in body

length. Interestingly, Kwok et al. (2006), found that verapamil had no effect on L-type VGCC *in vivo* during a screening of possible L-type channel blockers using *C. elegans*, though other compounds did have an effect on the worms. Sequence analysis suggests that verapamil should interact with the *C. elegans* channel, similar to the mammalian channels (Figure 23). The lack of any verapamil effect is puzzling because the L-type VGCC is heavily expressed in the pharynx of the worm (Lee et al. 1997), suggesting that verapamil and other L-type VGCC blocking compounds should easily reach these channels. It may be that *C. elegans* has some mechanism of excluding some xenobiotics from being ingested. Indeed, Kwok et al. (2006) observed that only L-type VGCC blockers that had an effect *in vivo* reached concentrations similar to that present in the medium. Another possibility is that verapamil might be quickly dealt with through protective pathways similar to those used on some other xenobiotics to which *C. elegans* is resistant (Vatamaniuk et al., 2001). Whatever the mechanism of verapamil detoxification, exposure to relatively high concentrations of 50 and 100 μM caused some statistically significant lethality. It should be noted that the observed verapamil lethality may be caused by interactions other than with L-type VGCC.

The concentrations of MeHg used in this work were generally high compared to concentrations typically used in cellular work (0.2 - 5 μM) (Marty and Atchison, 1997; Edwards et al., 2005). Concentrations as high as the observed LC_{50} of 100 μM would undoubtedly be lethal to most cells, suggesting that the lethality seen in this project is associated with cell death in the smooth muscle of the worms digestive track. Thus, the

Figure 23. Comparative sequence alignment of α_1 subunits of L-type VGCC from several species. Selected segments from a sequence alignment between α_1 subunits of *C. elegans* L-type VGCC and two (the α_{1C} and α_{1D}) mammalian L-type VGCCs from 3 species (human, rat and mouse). Several amino acids that have been shown to be critical for verapamil interactions are highlighted (shaded gray boxes). The glutamates at consensus sequence positions 1227 and 1532 are invariant across L-type and non-L-type VGCC and form part of what is known as the Ca^{2+} selectivity filter. The additional highlighted amino acids are part of the channel pore and are in α-helical structures. (reviewed in Hockerman et al., 1997; Catterall et al., 2005; Lipkind and Fozzard, 2008)).

FIGURE 23


Majority RIWENSDFNFDNVLAAMMALFTVSTFEGWPALLYKAIDSN
1210 1220 1230 1240
C. elegans RVWSNNDFNFDNVGDAMISLFVVSTFEGWPQLLYVAIDSN 1026
Human a1C RSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSH 1128
Rat a1C RSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSH 1158
Mouse a1C RSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSH 1158
Human a1D RIWQNSDFNFDNVLSAMMALFTVSTFEGWPALLYKAIDSN 1134
Rat a1D RIWQNSDFNFDNVLSAMMALFTVSTFEGWPALLYKAIDSN 1173
Mouse a1D RIWQNSDFNFDNVLSAMMALFTVSTFEGWPALLYKAIDSN 1136
Majority GEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVT
1250 1260 1270 1280
C. elegans EEDKGPIHNSRQAVALFFIAFIIVIAFFMMNIFVGFVIVT 1066
Human a1C TEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVT 1168
Rat a1C TEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVT 1198
Mouse a1C TEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVT 1198
Human a1D GENIGPIYNHRVEISIFFIIYIIIVAFFMMNIFVGFVIVT 1174
Rat a1D GENVGPVYNYRVEISIFFIIYIIIVAFFMMNIFVGFVIVT 1213
Mouse a1D GENVGPVYNYRVEISIFFIIYIIIVAFFMMNIFVGFVIVT 1176
Majority AVLLLFRCATGEAWQDIMLACLPGK--LCDPESDPYNSGE
1530 1540 1550 1560
C. elegans AILVLFRSATGEAWQDIMLSCSDREDVRCDPMSDDYHKGG 1330
Human a1C AVLLLFRCATGEAWQDIMLACMPGK--KCAPESEPSNSTE 1442
Rat a1C AVLLLFRCATGEAWQDIMLACMPGK--KCAPESEPSNSTE 1472
Mouse a1C AVLLLFRCATGEAWQDIMLACMPGK--KCAPESEPSNSTE 1472
Human a1D AVLLLFRCATGEAWQEIMLACLPGK--LCDPESD-YNPGE 1451
Rat a1D AVLLLFRCATGEAWQEIMLACLPGK--LCDPDSD-YNPGE 1475
Mouse a1D AVLLLFRCATGEAWQEIMLACLPGK--LCDPDSD-YNPGE 1438
Majority G-ETTCGSNFAVVYFISFYMLCAFLIINLFVAVIMDNFDY
1570 1580 1590 1600
C. elegans LNESRCGNNFAYPYFISFFMLCSFLVINLFVAVIMDNFDY 1370
Human a1C G-ETPCGSSFAVFYFISFYMLCAFLIINLFVAVIMDNFDY 1481
Rat a1C G-ETPCGSSFAVFYFISFYMLCAFLIINLFVAVIMDNFDY 1511
Mouse a1C G-ETPCGSSFAVFYFISFYMLCAFLIINLFVAVIMDNFDY 1511
Human a1D --EYTCGSNFAIVYFISFYMLCAFLIINLFVAVIMDNFDY 1489
Rat a1D --EYTCGSNFAIVYFISFYMLCAFLIINLFVAVIMDNFDY 1513
Mouse a1D --EYTCGSNFAIVYFISFYMLCAFLIINLFVAVIMDNFDY 1476

direct comparison between the two systems is difficult. Therefore it is suggested that future work use sterilized worms and increased assay length with MeHg concentration of 10 μM (we noticed significant decreases in growth and development at this concentration) or less, to avoid killing everything by massive poisoning. In addition, a complete evaluation of behavioral end points, such as feeding and movement would be ideal in longer experiments, for comparison to human clinical symptoms. Another reason for beginning a longer assay is: MeHg poisoning, whether *in vivo* or *in vitro* and whether acute or chronic, is associated with a silent latency period (Weiss et al., 2002). The current 48 hour assay is probably shorter than the latency period of low dose MeHg toxicity. In fact, the worms' two week life span may not be long enough to study low dose MeHg toxicity because of the latency period.

Another area of future focus should be the use of intact animals in elecrophysiology work. Much of our understanding about MeHg neurotoxicity has come from elecrophysiology studies and confirming these in a living organism would help bridge the gap between *in vitro* and *in vivo* findings. Electophysiology work would be helped by identification of cells highly susceptible to MeHg toxicity. It is possible that the most likely target neurons will be in the neural ring surrounding the pharynx. The neural ring functions like a CNS for the worm. The sensory neurons of the "nose" region are another likely target. The use of labeled MeHg along with DAPI staining of nuclei might be the most effective method of identifying any susceptible target cells. In additon, further exploration of MeHg effect on calcium homeostasis *in vivo* might be performed using calcium fluorophores. One promising calcium fluorophore used in *C. elegans* is the

genetically-tagged Cameleon.

Lastly, knock down, knock out, laser ablation, protein tagging and other methods of examining cells and individual proteins are relatively easy in *C. elegans*. These features of the worm open up additional research potential, including the possibility of studying MeHg effects on cytoarchitecture or reactive oxygen species *in vivo*. The exploration of non-worm transfected genes such as calbindin D28K is also possible. Cells from *C. elegans* embryos can be isolated and cultured providing a source of non-transformed cells for future work. In summary, *C. elegans* appears to have promise as a model organism for studying MeHg, however future work should focus on non-lethal end points or cellular/protein/gene function.

APPENDIX

APPENDIX A

ADDITIONAL DATA

Figures 1 & 4

Statistics for Figures 1 & 4, with a breakdown of replicates, average and standard errors for each datum point (Tables A.1 & A.2). In the main body of the paper it was stated that 200 and 300 μM MeHg arrested growth in the worms (Figure A.1).

Some observations and data points suggested that larger worms (worms that were growing) died first in the 150, 200, 300 μM MeHg while the smaller worms persisted. This is indicated by the decreasing size of worms from one time point to the next at these three MeHg concentrations. Analysis of these data showed that the worms were not statistically smaller at latter time points, thus the idea that larger worms die earlier was not substantiated.

Mercury Chloride

Earlier work done in this lab by Koselke et al. (2007) had examined the lethality of inorganic mercury chloride as well as MeHg on *C. elegans* using a scaled down version of the current method with a different shaker. Using the current methods described in this thesis, inorganic mercury was briefly examined for comparison to the previous work. Mercury chloride concentrations of 5, 10 and 50 μM had minimal lethal effect on the worms (Figure A.2), whereas, Koselke et al. (2007) had found 18 and 50 % mortality at 10 and 50 μM respectively. The differences between these results are likely caused by changes in the procedure, based on the sensitive nature of this assay discovered by using

various shakers and assay volumes. The earlier work was done in smaller volumes with slower shaker speeds.

Verapamil Cytotoxicity

Verapamil cytotoxicity at 48 h in the worm at the 2 highest concentrations tested (50 and 100 μM) has already been described in the main body of the paper. Verapamil cytotoxicity was not evident at the earlier time points of 12 h and 24 h (Figure A.4-A.5). Furthermore, verapamil had no statistically significant effects on the length of the worm at 12, 24 or 48 h (Figures A.6- A.8).

Table A.1. Statistical data for Figure 3. The detailed list of datum points from Figure 3 with replicate numbers, averages and standard errors.

TABLE A.1.

Length of MeHg exposure	[MeHg] μM	n	Average Mortality %	S E (Standard Error)
6 h	0	19	0.0	0.0
6 h	5	13	1.6	0.6
6 h	10	12	2.8	0.8
6 h	50	21	10.7	2.0
6 h	100	21	24.4	3.3
6 h	150	21	15.5	2.4
6 h	200	8	30.8	5.2
6 h	300	7	95.1	1.9
12 h	0	6	2.0	1.4
12 h	5	6	2.2	0.9
12 h	10	6	4.8	1.8
12 h	50	6	18.1	5.4
12 h	100	6	55.4	6.9
12 h	150	6	72.1	9.9
24 h	0	18	0.3	0.1
24 h	5	12	3.3	1.2
24 h	10	13	8.5	1.9
24 h	50	22	10.8	2.2
24 h	100	24	56.1	5.9
24 h	150	23	61.5	5.8
24 h	200	10	71.1	5.2
24 h	300	7	99.8	0.2
48 h	0	61	0.0	0.0
48 h	5	6	2.6	1.3

Table A.1 cont.

48 h	**10**	**6**	**2.0**	**1.1**
48 h	**50**	**35**	**14.2**	**1.4**
48 h	**100**	**53**	**52.4**	**3.7**
48 h	**150**	**54**	**73.3**	**3.5**
48 h	**200**	**30**	**75.9**	**5.3**
48 h	**300**	**24**	**99.5**	**0.1**

Table A.2. Statistical data for Figure 7. The detailed list of datum points from Figure 7 with replicate numbers, averages and standard errors. Also includes data at 200 and 300 μM MeHg shown in Figure A.1.

TABLE A.2.

Length of MeHg exposure	[MeHg] μM	n	Average Body Length (mm)	S E (Standard Error)
6 h	0	20	0.31	0.01
6 h	5	13	0.31	0.01
6 h	10	13	0.30	0.01
6 h	50	21	0.30	0.01
6 h	100	21	0.28	0.01
6 h	150	21	0.28	0.01
6 h	200	8	0.28	0.01
6 h	300	7	0.28	0.01
12 h	0	6	0.42	0.03
12 h	5	6	0.39	0.02
12 h	10	6	0.36	0.01
12 h	50	6	0.31	0.01
12 h	100	6	0.27	0.01
12 h	150	5	0.25	0.01
24 h	0	24	0.65	0.02
24 h	5	12	0.60	0.01
24 h	10	13	0.53	0.02
24 h	50	24	0.42	0.01
24 h	100	21	0.30	0.01
24 h	150	21	0.30	0.01
24 h	200	10	0.30	0.01
24 h	300	5	0.29	0.02
48 h	0	26	1.23	0.02
48 h	5	6	0.92	0.06

Table A.2 cont.

48 h	**10**	**6**	**0.79**	**0.04**
48 h	**50**	**19**	**0.50**	**0.02**
48 h	**100**	**26**	**0.38**	**0.02**
48 h	**150**	**24**	**0.32**	**0.01**
48 h	**200**	**15**	**0.28**	**0.01**
48 h	**300**	**6**	**0.25**	**0.01**
72 h	**0**	**2**	**1.46**	**0.02**
72 h	**5**	**2**	**1.34**	**0.06**
72 h	**10**	**2**	**1.23**	**0.04**
72 h	**50**	**2**	**1.02**	**0.03**
72 h	**100**	**2**	**0.72**	**0.06**
72 h	**150**	**2**	**0.33**	**0.04**
96 h	**10**	**2**	**1.18**	**0.01**
96 h	**50**	**2**	**1.06**	**0.05**
96 h	**100**	**2**	**0.84**	**0.11**
96 h	**150**	**2**	**0.50**	**0.06**
120 h	**10**	**2**	**1.28**	**0.03**
120 h	**50**	**2**	**1.09**	**0.04**
120 h	**100**	**2**	**0.80**	**0.00**
120 h	**150**	**2**	**0.53**	**0.03**

Figure A.1. Body length of *C. elegans* as a function of time and 200 or 300 μM MeHg exposure. Body length of *C. elegans* exposed to MeHg at 200 and 300 μM MeHg. Body length was significantly different than control at all points measured (n = 7-61; see details in Table A.2). Control data are duplicated from Figure 7.

FIGURE A.1.

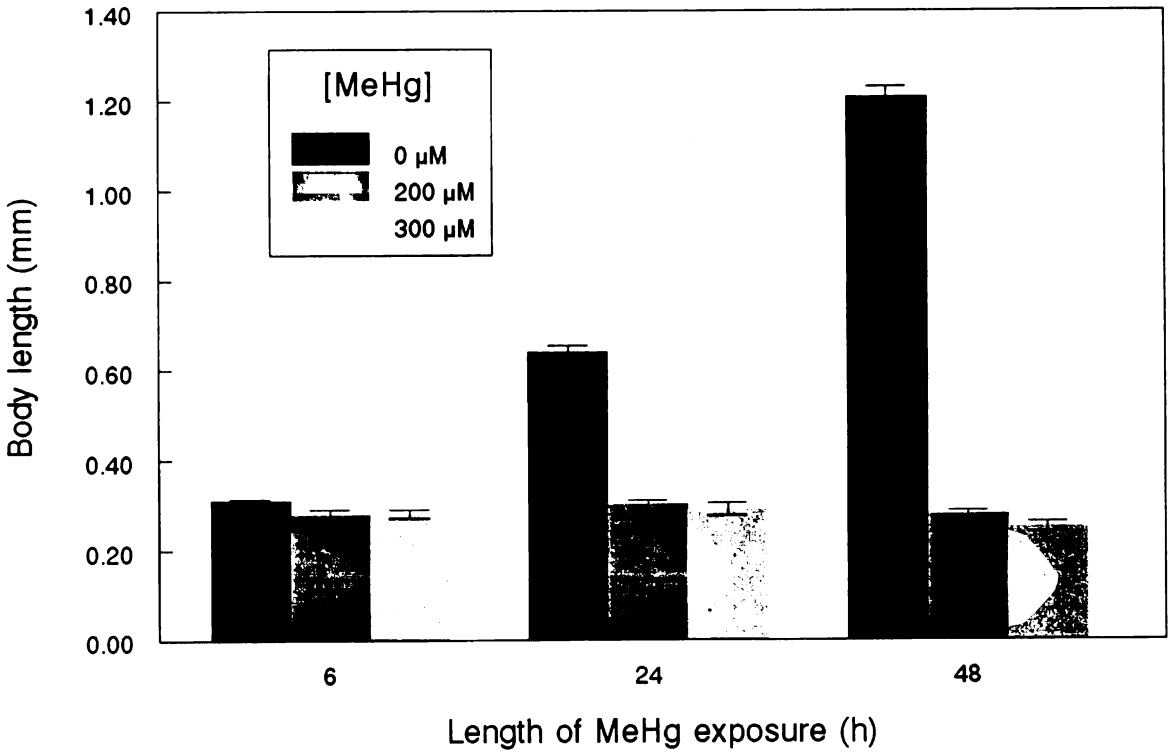

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Body length (mm)
[MeHg]
0 µM
200 µM
300 µM
6
24
48
Length of MeHg exposure (h)

Figure A.2. Mortality of *C. elegans* as a function of mercuric-chloride exposure for 24 h. Concentration response of organismal death in *C. elegans* exposed to low levels of mercury Hg^{2+} for 24 h (n = 1).

FIGURE A.2.

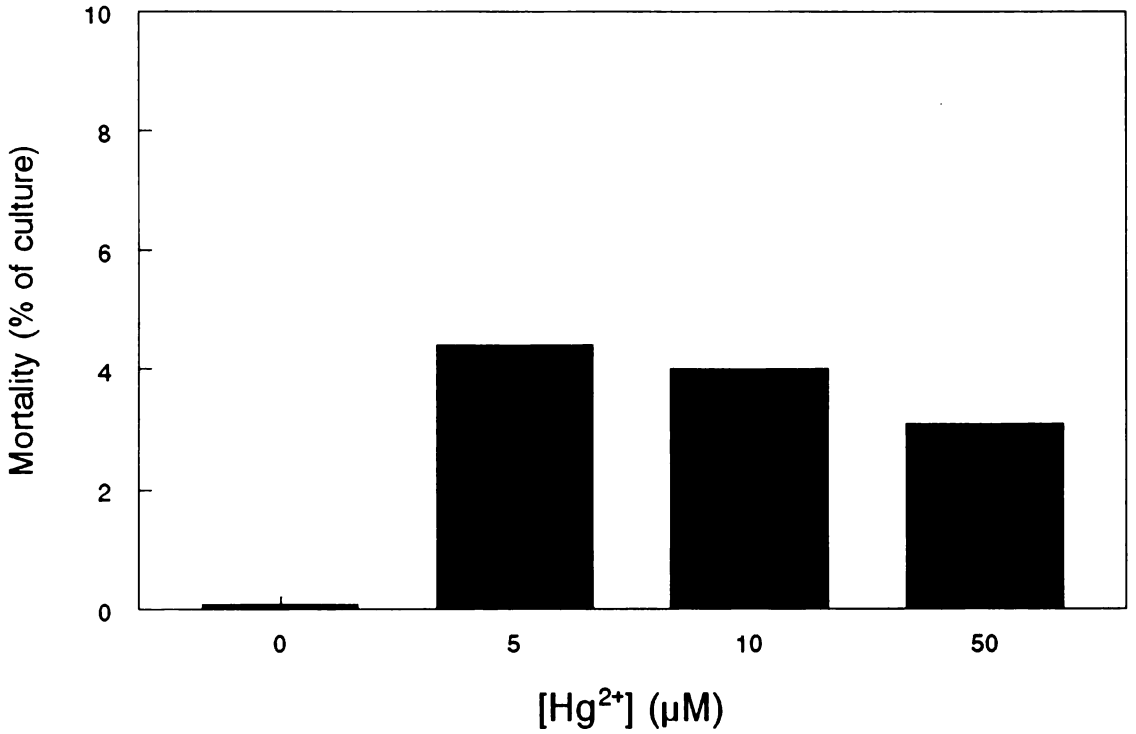


Figure A.3. Mortality of *C. elegans* as a function of mercuric-chloride exposure for 48 h. Concentration response of organismal death in *C. elegans* exposed to low levels of Hg^{2+} for 48 h (n = 1).

FIGURE A.3.

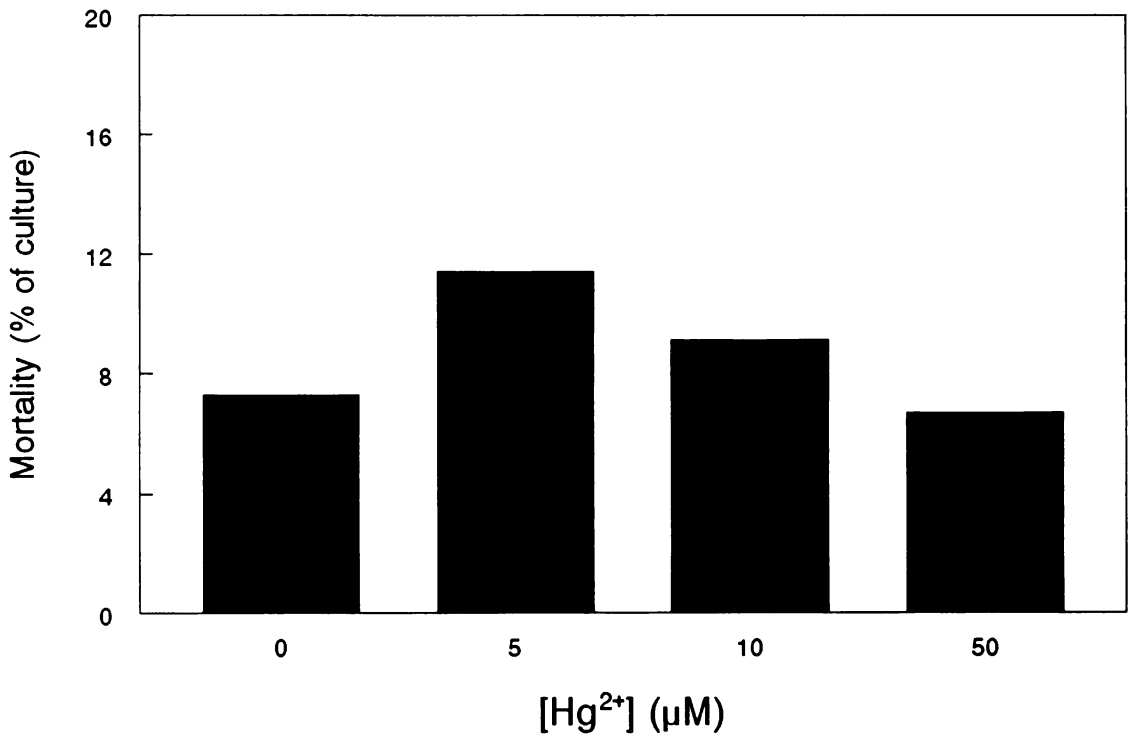


Figure A.4. Mortality of *C. elegans* as a function of 12 h verapamil exposure.

Concentration response of organismal death in *C. elegans* exposed to the L-type VGCC blocker verapamil for 12 h. There was no significant difference between control and any verapamil concentration tested (n = 8-9).

FIGURE A.4.

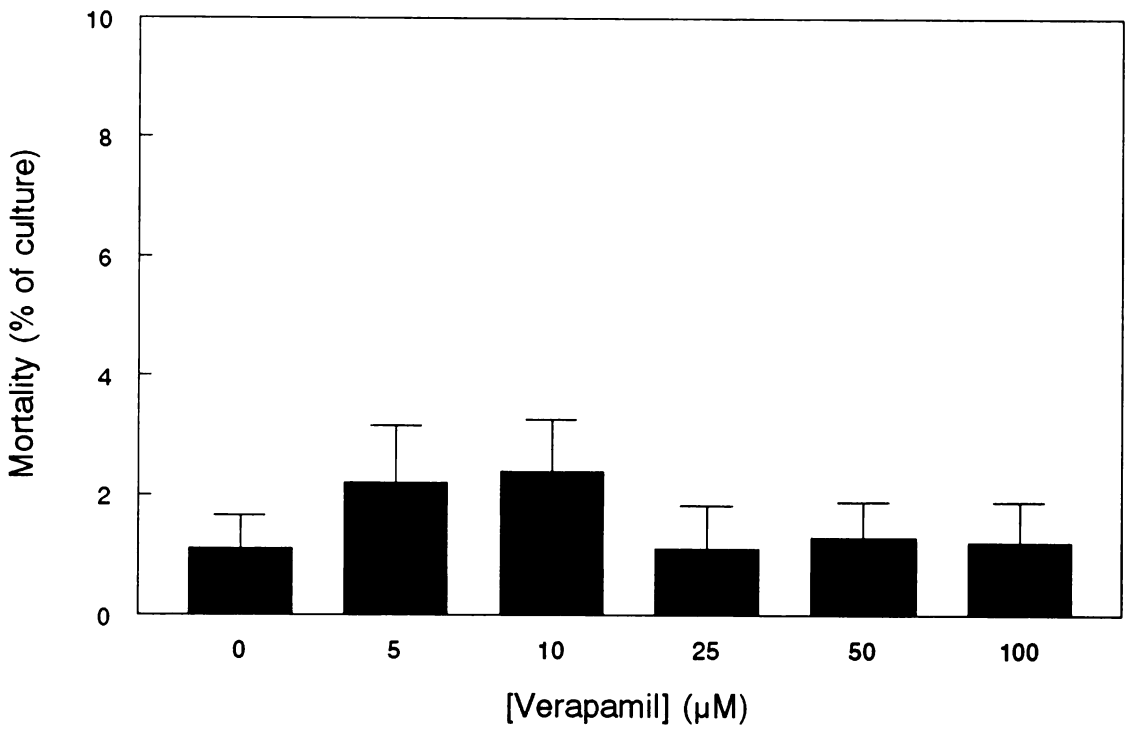


Figure A.5. Mortality of *C. elegans* as a function of 24 h verapamil exposure.

Concentration response of organismal death in *C. elegans* exposed to the L-type Ca VGCC blocker verapamil for 24 h. There was no significant difference between control and any verapamil concentration tested (n = 7-9).

FIGURE A.5.

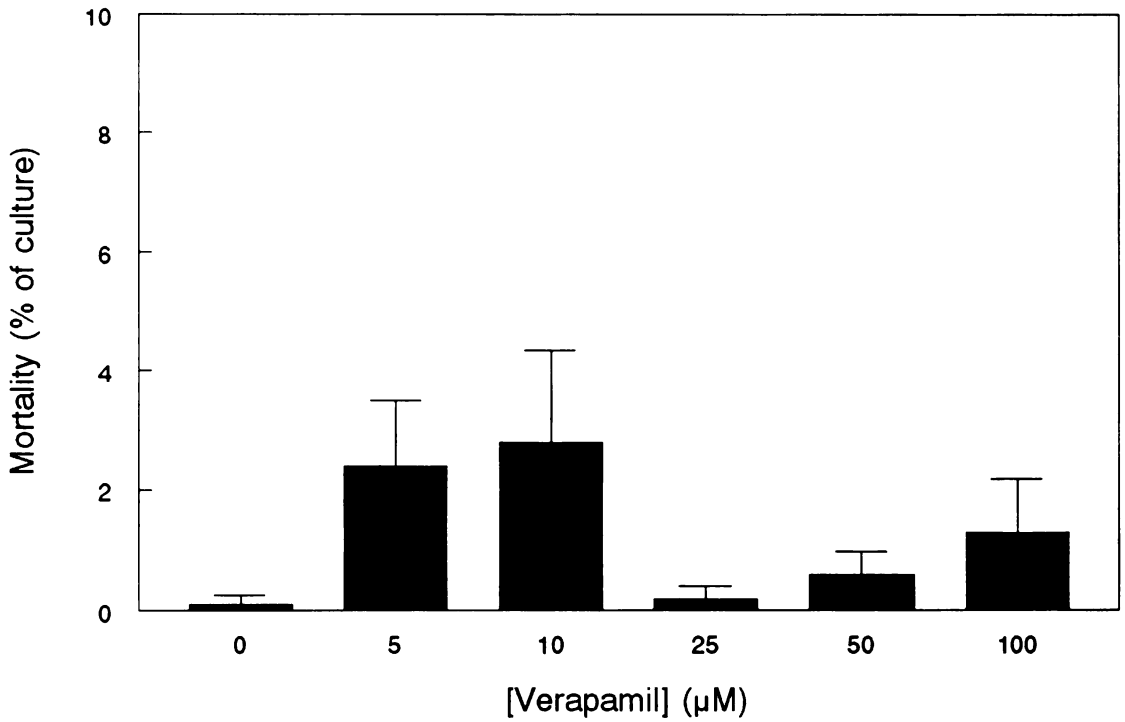


Figure A.6. Body length of *C. elegans* as a function of verapamil exposure for 12 h.

Body length of *C. elegans* exposed to the L-type VGCC blocker verapamil for 12 h. Body length was not significantly different between control and any verapamil concentration (n = 9).

FIGURE A.6.

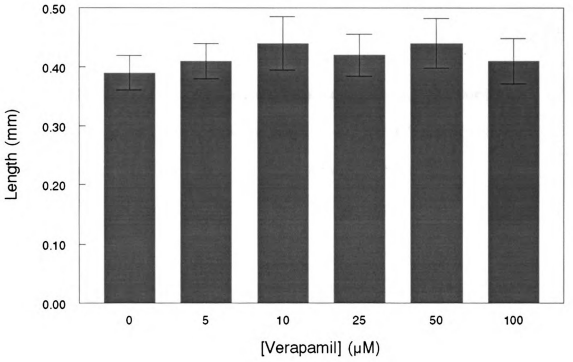


Figure A.7. Body length of *C. elegans* as a function of verapamil exposure for 24 h.

Body length of *C. elegans* exposed to the L-type VGCC blocker verapamil for 24 h. Body length was not significantly different between control and any verapamil concentration (n = 8-9).

FIGURE A.7.

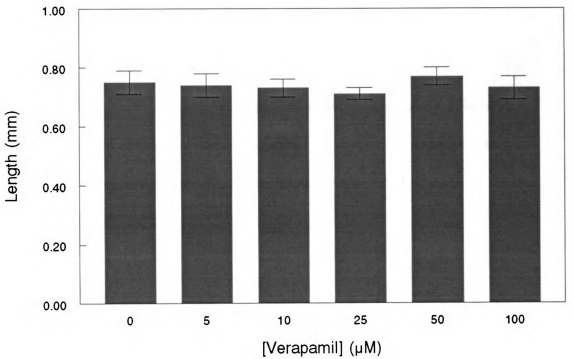
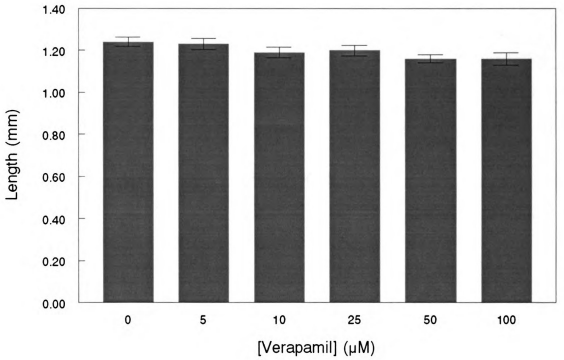


Figure A.8. Body length of *C. elegans* as a function of verapamil exposure for 48 h.

Body length of *C. elegans* exposed to the L-type VGCC blocker verapamil for 48 h. Body length was not significantly different between control and any verapamil concentration (n = 17-21).

FIGURE A.8.


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Length (mm)
0
5
10
25
50
100
[Verapamil] (μM)

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