

Toxicological Interactions of Chlorpyrifos and Methyl Mercury in the Amphipod, *Hyalella azteca*

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The mechanism of interaction between chlorpyrifos, an organophosphate insecticide, and methyl mercury, an organometal, was assessed utilizing the amphipod, *Hyalella azteca*. Previous studies have demonstrated that chlorpyrifos and methyl mercury interact additively, with survival as the endpoint. In addition, exposure to chlorpyrifos and methyl mercury increased the accumulation and decreased the elimination of methyl mercury. To elucidate the mechanism responsible for these interactions, biochemical mechanisms indicative of chlorpyrifos and methyl mercury toxicity were assessed in *H. azteca*. Biochemical endpoints that were evaluated include the inhibition of acetylcholinesterase enzyme and indicators of oxidative stress such as glutathione-S-transferase activity, lipid peroxidation, protein oxidation, and glutathione content. Methyl mercury antagonized the effects of chlorpyrifos *in vivo* on acetylcholinesterase inhibition. Methyl mercury did not induce oxidative damage; however, chlorpyrifos decreased glutathione-S-transferase activity. Additional studies demonstrated that methyl mercury did not affect the *in vitro* bioactivation of chlorpyrifos or the subsequent inhibition of acetylcholinesterase enzyme activity. Chemical-chemical interactions were examined utilizing chromatographic techniques. Results of thin layer chromatography suggested the formation of a chlorpyrifos-methyl mercury complex. The formation of this complex may result in increased accumulation of methyl mercury, apparent additive toxicity, and protection against chlorpyrifos mediated acetylcholinesterase inhibition.

Key Words: chemical mixture; *Hyalella azteca*; chlorpyrifos; methyl mercury; acetylcholinesterase; chemical interaction.

Currently, most chemical mixture studies focus on the toxicological interactions of chemicals having similar structure and mechanism. For example, numerous chemical mixture studies have focused on interactions of divalent metals or the interactions of organophosphates (Calabrese, 1991). However, “real world” chemical mixtures have the potential to occur as conglomerates having dissimilar structures and toxicological mechanisms. These mixtures include chemicals such as poly-

aromatic hydrocarbons, pesticides, metals, and polychlorinated biphenyls, among others. Few toxicological studies have addressed the interactions associated with mixtures containing chemicals with dissimilar structures and effects.

The purpose of the current study was to investigate the interactive mechanism of two dissimilar chemicals; an organometal compound, methyl mercury, and an organophosphate insecticide, chlorpyrifos, which have the potential to occur as real-world chemical mixtures. Methyl mercury and chlorpyrifos are dissimilar in structure; however, both are considered to act as neurotoxicants. Methyl mercury occurs widely in sediments, due to its natural occurrence in the earth’s crust, and its environmental concentrations are increasing due to the combustion of fossil fuels and industrial emissions (Lindqvist *et al.*, 1991, U.S. Environmental Protection Agency [EPA], 1998). Risk associated with methyl mercury exists through its potential to bioaccumulate to high concentrations in aquatic organisms and wildlife (U.S. EPA, 1996). Methyl mercury accumulates by way of an L-amino acid transporter and exerts its toxicity by binding to sulfhydryl groups on proteins, depleting cellular stores of the antioxidant glutathione, or by inducing oxidative stress (Clarkson, 1994; Lund *et al.*, 1991; Mokrzan *et al.*, 1995). Methyl mercury has been demonstrated to disrupt cholinergic function at the neuromuscular junction (Eldefrawi *et al.*, 1977; Sager *et al.*, 1982) through the inhibition of acetylcholinesterase (Loua, *et al.*, 1998) and acetylcholinesterase (Petruccioli and Turillazzi, 1991). Chlorpyrifos, a chlorinated organophosphate, is widely used in the United States as an insecticide, with more than 9.5 million kg applied to cropland each year (U.S. Geological Survey, 1998). Chlorpyrifos exerts its toxicity by irreversibly binding to a serine hydroxyl group on the enzyme acetylcholinesterase resulting in cholinergic over-stimulation.

Methyl mercury and chlorpyrifos have the potential for chemical-chemical, toxicokinetic, and toxicodynamic interactions affecting exposure, accumulation, bioactivation, and the toxicological mechanism (Fig. 1). Previous studies in our laboratory have determined that methyl mercury and chlorpyrifos interacted additively on survival of the amphipod, *Hyalella azteca* (Steevens and Benson, 1999). In addition, chlorpyrifos

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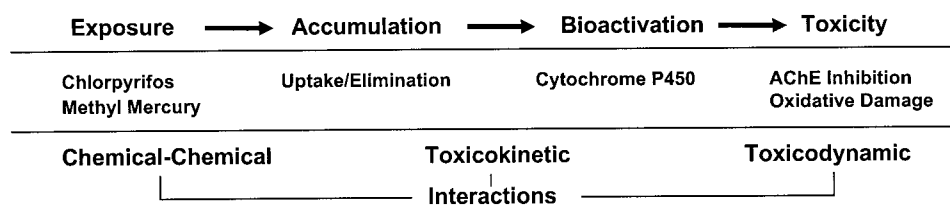


FIG. 1. Loci of potential chlorpyrifos-methyl mercury interactions.

also increased the initial rate of accumulation of methyl mercury and decreased the rate of elimination in bioaccumulation studies. In similar studies, the toxicity of malathion, an organophosphate, was synergistic in combination with methyl mercury (Dieter, 1974). Further studies indicated that malathion and methyl mercury had a greater than additive effect on the inhibition of acetylcholinesterase activity in quail plasma and brain (Dieter and Ludke, 1975). However, no further research has been conducted to evaluate the binary interaction of organophosphates and methyl mercury.

To further understand the interactions previously reported between chlorpyrifos and methyl mercury, the mechanisms of the toxicological interactions were evaluated. Initially, organisms were exposed to chlorpyrifos, methyl mercury, and binary combinations to evaluate biochemical mechanisms of toxicity associated with exposure to chlorpyrifos and methyl mercury. Biochemical indicators of exposure to chlorpyrifos and methyl mercury measured include acetylcholinesterase enzyme activity and measures of oxidative stress such as lipid and protein oxidation, glutathione, and glutathione-S-transferase activity. These measurements indicated potential locations of interaction. Further analysis was conducted *in vitro* to assess the effects of methyl mercury on the chlorpyrifos-mediated inhibition of acetylcholinesterase enzyme. The same experimental design was utilized to evaluate the effects of methyl mercury on the bioactivation of chlorpyrifos. Finally, chemical-chemical interactions were assessed utilizing thin-layer chromatography and gas chromatography with mass spectrophotometric analysis.

The aquatic invertebrate, *Hyalella azteca*, was utilized as an animal model to elucidate the mechanism of toxicity associated with chlorpyrifos and methyl mercury mixtures. *H. azteca* is a small (<5 mm length) amphipod that lives primarily in the surface of freshwater sediments where the concentration of methyl mercury and chlorpyrifos are greatest. *H. azteca* is routinely utilized as an invertebrate animal to assess the toxicity and bioaccumulation of sediment contaminants and can be cultured in large numbers (U.S. EPA, 1991). The large number of organisms is advantageous to meet the statistical criteria for comprehensive chemical mixture analysis (Calabrese, 1991).

MATERIALS AND METHODS

Chemicals. Analytical grade chlorpyrifos (99.2% pure) and chlorpyrifos-oxon (99.0% pure) were obtained from Chem Service (Westchester, PA). Methyl mercuric chloride (CH_3HgCl , 97% pure) was obtained from Phaltz and

Bauer, Inc (Waterbury, CT). Standard chemicals for biochemical and analytical procedures were obtained from both Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Company (St. Louis, MO). Saturated solutions of the toxicants were prepared for use in water-only experiments in order to eliminate the need for solvent carriers. Briefly, an excess of each chemical, $\times 5$ the water solubility, was added to 1 L of deionized water in a silanized amber glass jar. The mixture was vigorously stirred for 24 h at room temperature. The resulting mixture was filtered utilizing a 0.45- μm Whatman glass-fiber filter. The filtrate was stored at room temperature in amber glass jars and continuously mixed. Concentrations of the resulting solutions were verified by chemical analysis prior to use.

Animal model. *Hyalella azteca* culture was established at the University of Mississippi in 1994, with organisms originally obtained from the USGS Biological Resources Division, Environmental and Contaminants Research Center (Columbia, MO). Species identity has been verified by a genetic differentiation study (Duan *et al.*, 1997). Organisms were cultured in flow-through, dechlorinated tap water, and fed flake food (Aquatic Ecosystems) and hard-maple tree leaves. Dechlorinated tap water used for culturing and experiments was well water from the University of Mississippi. Water characteristics were: 10 to 20 ppm hardness (as CaCO_3), 50 to 60 μMHOS conductivity, 6.5 to 6.8 pH, alkalinity of 20 to 30 ppm, total ammonia less than 0.05 ppm, and temperature $23^\circ\text{C} \pm 1^\circ\text{C}$. Adult organisms were collected by sieving with a # 25 U.S. standard sieve and acclimated to experimental conditions for 24 h prior to all experiments.

Chemical analysis. Chlorpyrifos was analyzed utilizing a Hewlett-Packard 5890 gas chromatograph (GC) with dual electron capture detectors (ECD) as outlined by EPA Method 608 (CFR 40, Part 136). Water (200 ml) in glass bottles with Teflon caps was held at 4°C for less than 7 days prior to extraction, and 30 days prior to analysis. Anhydrous sodium sulfate (4 g) was added to each water sample and triple extracted with 20 ml of pentane. Pentane extracts were purified utilizing an X-400-mm chromatography column packed with 6 g of 60- to 100-mesh florisil and eluted with 10% ethyl ether in hexane. Resulting eluate was evaporated under nitrogen to 0.5 ml and placed into 1.0-ml autosampler vials for analysis. The GC was equipped with a J&W DB-1 60-meter capillary column with 0.25- μm diameter and 0.25- μm film. A Hewlett-Packard Vectra 25 GC data station with Hewlett-Packard Chemstation software was utilized for programmed autosampler operation. The program started with an initial temperature of 200°C and held for 5 min, then increased at $5^\circ\text{C}/\text{min}$ to a final temperature of 250°C . Inlet temperature was 280°C and detector temperature was 310°C . Integrations of eluted peaks were determined, based on peak area, and elution times based upon known standards, verified with internal calibration verification. Acceptable test conditions were verified by less than 10% variance of internal calibration verification. The detection limit for chlorpyrifos was 3 nM in water.

Total mercury was determined following a method outlined in ASTM (1993). Water (50 ml) in glass bottles with teflon caps was acidified with 1 ml concentrated nitric acid and stored at 4°C for less than 30 days prior to analysis. Water samples were oxidized for 24 h with the addition of 10 ml of 5% potassium persulfate and 10 ml of 5% potassium permanganate. The oxidation was terminated by addition of 10 ml of a 12% sodium chloride, 12% hydroxylamine hydrochloride solution. Prior to analysis, 50 μl of antifoam-B reagent was added to each sample. Tissue (25–50 mg) in high density polyethylene vials was stored at 0°C for less than 30 days. Total mercury concentration in water was determined utilizing a Varian Spectra AA-20 atomic

absorption spectrophotometer and VGA-76 vapor generation system. The detection limit for mercury was 5 nM in water.

In vivo exposures to chlorpyrifos and methyl mercury. Adult *H. azteca* were exposed to chemicals in water using a modification of methods outlined by U.S. EPA (1991). Concentrations used in exposures were previously determined in pilot 4-day toxicity experiments. Water quality, feeding, and observations for surviving organisms were conducted every 24 h throughout the 4-day exposure. Water quality parameters monitored include dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity. Exposure chambers consisted of 1000-ml glass beakers containing 100 adult organisms and 800 ml of test water. One hundred organisms were utilized for each replicate to obtain sufficient amounts of tissue for biochemical analyses. Exposure water was renewed every 12 h. Chamber water was sampled daily for chemical analysis prior to and following renewal. At termination of the exposures, surviving organisms were counted, dried, weighed, placed in vials, and stored in a -80°C freezer until biochemical analysis.

Acetylcholinesterase activity. Acetylcholinesterase activity was measured utilizing a microplate method to assess the activity of the enzyme in a tissue homogenate (Day and Scott, 1990). The assay utilized Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid)], which reacts with sulfhydryl groups of proteins. *H. azteca* were homogenized in 52.0 mM sodium phosphate buffer pH 7.4 with 1% Triton X-100. The homogenate was centrifuged at $10,000 \times g$ for 10 min. The pellet was discarded and remaining homogenate utilized for enzyme analysis. Fifty μl of the sample was diluted 1:1 with 50 μl of sample buffer. DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)] solution (100 μl) was added to each well. DTNB solution was prepared by adding 3.96 mg of DTNB to 1 ml of NaPO_4 buffer pH 7.0 and adding 1.5 mg of NaHCO_3/ml . The mixture was heated gently to dissolve all components. The DTNB solution was diluted 30-fold prior to use. The enzyme reaction was initiated by addition of 50 μl 2.6 mM acetylthiocholine in buffer and the formation of DTNB-thiol complex measured at 405 nm. Reduced glutathione ranging from 3 to 50 nM was used to generate a standard curve for the production of DTNB-reactive compounds. Protein concentration of tissue homogenates was determined by microanalysis modification of the Bradford method (1976) utilizing Coomassie blue stain.

Glutathione-S-transferase. Glutathione-S-transferase (GST) activity, measured as total sulfotransferase activity, was determined utilizing a spectrophotometric method modified for microplates (James *et al.*, 1979). *H. azteca* were homogenized in 0.5 ml 0.2 M HEPES [N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]] with 1.15% KCl pH 7.4 for 30 s on ice with a tissue tearor. The homogenate was centrifuged for 10 min at $9,500 \times g$. Supernatant was diluted 1:50 to reach a protein concentration of 0.1 mg/ml for use in the assay. One hundred μl of the diluted homogenate, 25 μl of 1.0 M HEPES pH 8.0, and 25 μl of glutathione (5 mM final concentration) was added to each well in the microplate. To initiate the enzymatic reaction, 50 μl of 4 mM CDNB [1-chloro 2,4 dinitrobenzene] in 4% ethanol was added to the wells. The formation of a glutathione conjugate to CDNB was measured spectrophotometrically at 340 nm, and activity quantified using an extinction coefficient of $9600 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Lipid peroxidation. Lipid peroxidation was measured using a modified version of the thiobarbituric acid reactive substances (TBARS) assay as described by Jentzsch (1996). The TBARS assay was modified to utilize 96-well microtiter plates, and products were measured using a spectrofluorometer. Whole *H. azteca* were homogenized in 0.01 M NaPO_4 buffer pH 7.4, then centrifuged at 3000 rpm for 30 min. Tissue homogenate (200 μl), 15% trichloroacetic acid (200 μl), and 0.11 M thiobarbituric acid in 0.1 M NaOH (25 μl) was added to a microcentrifuge tube, thoroughly vortexed, and incubated for 30 min at 70°C . The incubation mixture was cooled, and lipids were extracted with 500 μl of n-butanol and 50 μl of saturated NaCl added. The butanol-incubation mixture was vortexed and centrifuged at 6500 rpm for 10 min. The n-butanol layer (250 μl) was measured fluorometrically with excitation at 530 nm and emission at 590 nm. Malondialdehyde was used for a standard curve and the Fenton reaction utilized as a positive control.

Glutathione. Total glutathione was determined spectrophotometrically utilizing a variant of the Ellman method (Nigra and Huxtable, 1992). Tissue homogenates from whole organisms were prepared by homogenizing 25 to 50 adults in 50 mM Tris buffer (pH 7.4) with 1 mM EDTA and centrifuged at $10,000 \times g$ for 10 min. An equal volume of 5 percent sulfosalicylic acid was added, the mixture vortexed, and centrifuged at $10,000 \times g$ for 10 min. Fifty μl of the supernatant was added to a microplate well and 200 μl of diluted (1:10) Ellman reagent (39.6 mg DTNB in 10 ml ethanol) was added to the supernatant. The microplate was read at 405 nm and total glutathione determined using an extinction coefficient of $14521 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and verified with a standard curve ranging from 0.04 to 0.005 μmol .

Protein oxidation. The oxidation of proteins, resulting in the formation of carbonyl groups, was determined utilizing an immunoblot modification of a spectrophotometric method (Keller *et al.*, 1993; Levine *et al.*, 1990). Ten adult *H. azteca* were homogenized in 500 μl of 10 mM sodium phosphate buffer (pH 7.4) and centrifuged at $10,000 \times g$ for 15 min at 4°C . The supernatant was removed and protein concentration analyzed and adjusted to 1 mg/ml. One hundred μl of 1 mg/ml sample was derivatized with 50 μl of 25% SDS and 300 μl of 20 mM dinitrophenyl hydrazine in 15% trifluoroacetic acid. Following incubation at 25°C for 30 min, the reaction was terminated with 675 μl of 2 M Tris base in 30% glycerol. Five hundred μl of the mixture was transferred to a nitrocellulose membrane utilizing a Bio-Dot SF microfiltration apparatus (Biorad; Hercules, CA). The resulting blot was rinsed thrice with phosphate-buffered saline (PBS) then blocked with 2% bovine serum albumin in 100 ml PBS. After blocking, the blot was rinsed twice with PBS and incubated for 1 h with a monoclonal anti-dinitrophenyl (DNP) antibody conjugated alkaline phosphatase (clone SPE-7 from purified mouse immunoglobulin) diluted 1:10,000. Following the incubation, the blot was rinsed twice with PBS and twice with Tris/Saline pH 7.4. The blot was developed utilizing alkaline phosphatase reagents, BCIP [5-bromo-4-chloro-3-indolylphosphate] and NBT [nitro blue tetrazolium], in alkaline phosphatase buffer. The developed blot was scanned utilizing a Visioneer Paperport 6000A scanner and density analyzed by Scion Image version Beta 2 (Scion Corp, Frederick, MD).

Surrogate bioactivation of chlorpyrifos. *In vitro* bioactivation of chlorpyrifos was achieved utilizing a surrogate rat-liver microsome containing cytochrome P450 enzymes. Lobster hepatopancreas microsomes and rat liver microsomes were utilized to bioactivate chlorpyrifos. American lobster-hepatopancreas microsomes were prepared following procedures outlined by James (1990). Rat liver microsomes were prepared from adult male Sprague-Dawley rats as described by Guengerich (1994) and assayed utilizing the reduced-CO difference spectrum (Omura and Sato, 1964). For the bioactivation, 85 μl of rat or lobster microsomes (1.84 nmoles P450/ml), 85 μl of *H. azteca* homogenate in 0.01 M NaPO_4 pH 7.4, with 0.2 mM PMSF, 20 μl 20 mM NADPH in 0.01 sodium phosphate, pH 7.4, was incubated with 10 μl of chlorpyrifos in 10% ethanol for 15 min at 37°C . NADPH cytochrome P450 reductase was not added to the lobster microsomal preparation as previously described in published methods.

Thin-layer chromatography. Chemical-chemical interaction analysis of chlorpyrifos and methyl mercury was determined by thin layer chromatography (TLC). Chlorpyrifos (0.01 M) and methyl mercury (0.01 M) were allowed to react in ethyl acetate or deionized water under slow mixing for 24 h at 23°C . The reaction mixture was triple extracted into hexane and reduced to 1 ml of volume under nitrogen. One hundred μl of the extract was spotted onto Analtech GF silica gel 250- μm plates and eluted with 30% ethyl acetate in hexane. Chlorpyrifos and methyl mercury were detected utilizing iodine. After detection of a chlorpyrifos-methyl mercury product, total mercury was determined in the mixture lane by scraping 2-cm by 1-cm sections and analyzing as previously described.

Gas chromatography-mass spectrophotometric analysis. To verify the formation of a chlorpyrifos-methyl mercury product, the extract utilized for TLC analysis was analyzed utilizing a Hewlett-Packard Model 6890 Series II GC with 5973 mass selective detector. The GC was equipped with a J&W DB-5 30-meter capillary column with 0.25 μm diameter and 0.25- μm film. The automated program started with an initial temperature of 70°C and held for

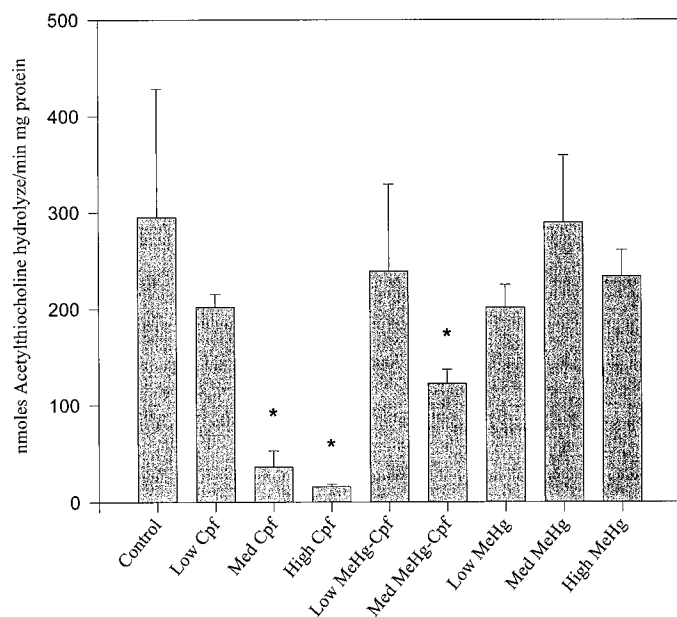


FIG. 2. Acetylcholinesterase activity in *Hyalella azteca* following 48-h exposure to methyl mercury (MeHg), chlorpyrifos (Cpf), and methyl mercury and chlorpyrifos. Treatments ($n = 3-5$) were as follows: low methyl mercury (30 nM), medium methyl mercury (150 nM), high methyl mercury (350 nM), low chlorpyrifos (0.04 nM), medium chlorpyrifos (0.14 nM), and high chlorpyrifos (0.4 nM). Statistically significant results indicated (*) as determined by ANOVA where $p < 0.05$. Medium MeHg-Cpf was significantly different from the Control, Med Cpf, and High Cpf.

2 min, then increased at $10^{\circ}\text{C}/\text{min}$ to a final temperature of 250°C . Inlet temperature was 250°C and detector temperature was 250°C . Integrations of eluted peaks were assessed with Enhanced Chemstation software and identified utilizing the 1998 National Institute of Standards and Technology library software.

Statistical analysis. Statistical analysis was conducted using Sigma Stat[®] version 2.03 statistical software (Jandel Scientific). Biochemical analyses were evaluated utilizing one-way analysis of variance (ANOVA) with Bonferroni pairwise comparisons. Where tests for normality ($p = 0.01$) and equal variance ($p = 0.01$) failed, Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's pairwise comparisons were utilized. All data presented represent the mean and standard deviation.

RESULTS

In Vivo Effects of Chlorpyrifos and Methyl Mercury

Acetylcholinesterase activity was evaluated in *H. azteca* following exposure to chlorpyrifos (0.04, 0.14, and 0.4 nM) and methyl mercury (30, 150, and 350 nM) alone and in binary combination. Basal level of acetylcholinesterase enzyme activity, in control organisms, was 295 ± 133 nmol/min/mg protein. Acetylcholinesterase activity in *H. azteca* exposure to chlorpyrifos and methyl mercury for 48 h is shown in Figure 2. Chlorpyrifos inhibited acetylcholinesterase activity in a statistically significant and dose-dependent manner. Methyl mercury did not significantly decrease acetylcholinesterase activity. However, binary combinations of methyl mercury and chlor-

pyrifos decreased enzyme inhibition as compared to chlorpyrifos alone. The combined exposure to 0.14-nM chlorpyrifos and 150-nM methyl mercury resulted in statistically significant decreased enzyme inhibition compared to control and 0.14 nM chlorpyrifos treatments. In a similar manner, exposure to 0.04-nM chlorpyrifos and 30-nM methyl mercury for 96 h also resulted in decreased enzyme inhibition (data not shown).

Oxidative stress was evaluated in *H. azteca* following exposure to chlorpyrifos and methyl mercury. Measures of oxidative stress included lipid peroxidation, protein oxidation, glutathione, and glutathione-S-transferase. Lipid peroxidation, determined by measuring the production of TBARS was evaluated following exposure to chlorpyrifos (0.04, 0.14, and 0.28 nM) and methyl mercury (50, 390, and 590 nM). Background concentrations of TBARS in control samples were 8.9 ± 4.4 nM TBARS/mg protein. There were no statistically significant differences in TBARS following exposure to chlorpyrifos or methyl mercury for 48 and 96 h at these exposure concentrations.

Glutathione-S-transferase activity and glutathione content were evaluated in adult *H. azteca* exposed to chlorpyrifos (0.04, 0.14, and 0.28 nM) and methyl mercury (50, 390, and 590 nM) for 48 and 96 h. Basal levels of GST activity in control organisms was 0.830 mM GSH conjugated/min/mg protein. In the current experiment, there were no statistically significant differences in GST activity following a 48-h exposure to chlorpyrifos, methyl mercury, and binary combinations. After 96 h, there was a significant decrease in GST activity of *H. azteca* exposed to 0.2 nM chlorpyrifos (Fig. 3). There were no statistically significant differences in GST activity in *H. azteca* exposed to methyl mercury or the combination as compared to the control. An insufficient quantity of tissue was available for analysis after 96 h of exposure for treatment groups including 390- and 590-nM methyl mercury and the combination of 390-nM methyl mercury and 0.14-nM chlorpyrifos. Basal glutathione levels in control samples were below the lowest standard on the curve ($0.005 \mu\text{M}$). Protein concentrations of whole organism homogenates were greater than 2 mg/ml.

Protein oxidation was evaluated (Fig. 4) following a second exposure to chlorpyrifos (0.15, 0.75, and 1.5 nM), methyl mercury (80, 430, and 700 nM), and binary combinations of chlorpyrifos and methyl mercury for 48 and 96 h. No statistically significant differences were observed. Protein oxidation in treatments receiving 430- and 700-nM methyl mercury were not measured due to the insufficient quantity of tissue available as a result of *H. azteca* mortality.

In Vitro Effects of Chlorpyrifos and Methyl Mercury

The effects of chlorpyrifos and methyl mercury on acetylcholinesterase were evaluated *in vitro* utilizing *H. azteca* tissue homogenate (Table 1). Initially, incubation of chlorpyrifos with *H. azteca* tissue homogenate did not result in significant acetylcholinesterase inhibition at concentrations up to $25 \mu\text{M}$. Utilizing surrogate lobster hepatopancreas microsomes to bio-

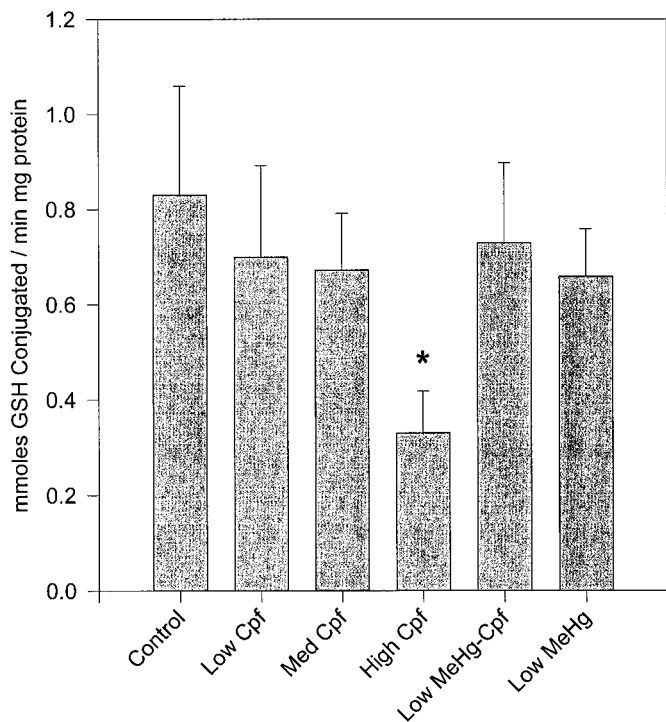


FIG. 3. Glutathione-S-transferase activity in *Hyalella azteca* following 48-h exposure to methyl mercury (MeHg), chlorpyrifos (Cpf), and methyl mercury and chlorpyrifos. Treatments ($n = 3-5$) were as follows: low methyl mercury (30 nM), low chlorpyrifos (0.04 nM), medium chlorpyrifos (0.14 nM), and high chlorpyrifos (0.4 nM). High chlorpyrifos treatment, as determined by ANOVA, was significantly different from control ($p < 0.05$).

activate chlorpyrifos in the *H. azteca* tissue homogenate also did not inhibit the activity of acetylcholinesterase enzyme. However, incubation of chlorpyrifos with rat liver microsomes and *H. azteca* tissue resulted in a 50% inhibition concentration (IC_{50}) value of 140 nM. The IC_{50} value for chlorpyrifos-oxon was 10-fold lower than chlorpyrifos, which had been bioactivated utilizing rat liver microsomes.

Experiments were conducted to assess acetylcholinesterase inhibition with chlorpyrifos-oxon and chlorpyrifos in the presence of methyl mercury. Methyl mercury did not affect the chlorpyrifos-oxon inhibition of acetylcholinesterase following incubation with the tissue homogenate, chlorpyrifos-oxon, or simultaneous incubation with chlorpyrifos-oxon and tissue homogenate. Methyl mercury did not affect the bioactivation of chlorpyrifos following a 1-h incubation with the rat liver microsomes. In addition, methyl mercury did not significantly affect the bioactivation of chlorpyrifos following a 1-h incubation with chlorpyrifos.

Chemical-Chemical Interaction of Chlorpyrifos and Methyl Mercury

Results of thin layer chromatography of chlorpyrifos, and a chlorpyrifos-methyl mercury mixture are shown in Figure 5.

Chlorpyrifos in the second lane eluted with an R_f value of 0.93. Under the same conditions, methyl mercury in the fourth lane eluted with an R_f value of 0.71. The first and third lanes represent the separation of the extract from the aqueous mixture incubation. A chlorpyrifos spot was detected with both iodine and UV light in the mixture lane; however, methyl mercury was not visually detected. An additional unknown compound associated with the chlorpyrifos-methyl mercury mixture was detected with iodine and shown to have an R_f value of 0.04. Further analysis of the detected compounds for total mercury, utilizing atomic absorption spectroscopy, demonstrated that methyl mercury migrated 5.2 cm from the origin (Fig. 6). The majority of mercury was associated with the area on the TLC plate from 0 to 2 cm from the origin and corresponds to the location of the unknown compound detected with iodine. TLC also was conducted, utilizing an extract from a reaction of chlorpyrifos and methyl mercury in ethyl acetate. However, the formation of a complex, in the absence of water, was not detected. Analysis of the aqueous chlorpyrifos-methyl mercury mixture extract, utilizing gas-chromatography mass-spectroscopy, detected methyl mercury and chlorpyrifos. However, the additional compound resolved by TLC was not detected.

DISCUSSION

Previous studies have shown that methyl mercury and chlorpyrifos interact additively (Steevens and Benson, 1999). Results of the current study indicate that chlorpyrifos and methyl mercury do not interact directly at the acetylcholinesterase

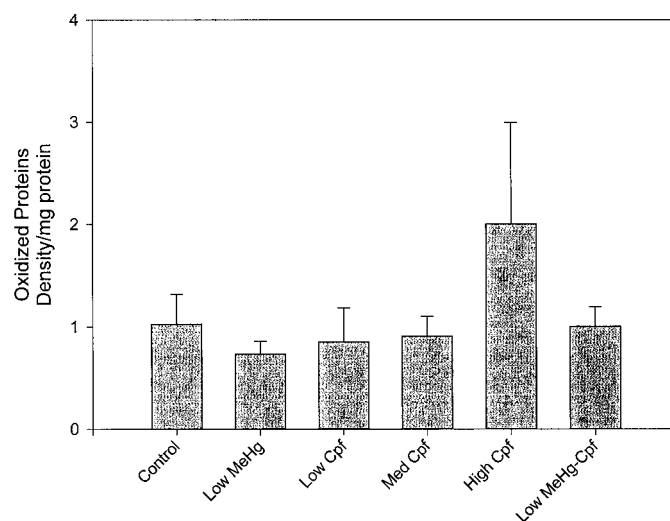


FIG. 4. Protein oxidation in *Hyalella azteca* following 96 h exposure to methyl mercury (MeHg), chlorpyrifos (Cpf), and methyl mercury with chlorpyrifos. Treatments ($n = 3-5$) were as follows: low methyl mercury (80 nM), low chlorpyrifos (0.15 nM), medium chlorpyrifos (0.75 nM), and high chlorpyrifos (1.5 nM). There were no statistically significant differences by Kruskal-Wallis ANOVA ($p = 0.058$).

TABLE 1
In vitro Acetylcholinesterase Inhibition (IC_{50} values) in *Hyalella azteca* Tissue Homogenate following Exposure to Chlorpyrifos and Methyl Mercury

Treatment	IC_{50} Values for Chlorpyrifos			
	Control	1 μ M CH_3Hg	0.1 μ M CH_3Hg	0.01 μ M CH_3Hg
Chlorpyrifos with tissue homogenate	>25 μ M	NA	NA	NA
+ lobster microsomes	>25 μ M	NA	NA	NA
+ rat liver microsomes	140 nM (118–167) ^a	NA	NA	NA
Chlorpyrifos-oxon with tissue homogenate				
Incubate chlorpyrifos-oxon and methylmercury for 1 h prior to addition to tissue homogenate	18.1 nM (11.8–27.8)	15.1 nM (9.2–24.8)	10.3 nM (7.6–14.0)	20.7 nM (13.2–32.4)
Incubate methyl mercury and tissue homogenate for 1 h prior to addition of chlorpyrifos-oxon	18.3 nM (11.6–28.7)	10.2 nM (6.7–15.3)	13.0 nM (7.3–23.3)	13.6 nM (8.7–21.3)
Incubate methylmercury, chlorpyrifos-oxon, and tissue homogenate for 15 min	20.2 nM (19.5–21.0)	19.9 nM (19.2–20.6)	20.8 nM (19.2–22.6)	21.2 nM (19.2–23.4)
Chlorpyrifos bioactivated with rat liver microsomes (RLM):				
Incubate methyl mercury and RLM for 1 h prior to addition of chlorpyrifos and tissue homogenate.	243 nM (222–266)	245 nM (228–265)	241 nM (224–261)	232 nM (218–246)
Incubate methyl mercury and chlorpyrifos for 1 h prior to addition to RLM and tissue homogenate	140 nM (118–167)	146 nM (132–162)	143 nM (125–165)	158 nM (146–171)

^a Values represent 50 percent inhibition concentration (IC_{50}) and 95 percent confidence interval calculated from 6 to 7 point sigmoidal variable slope dose response curve (3 measurements/data point).

enzyme or through the bioactivation of chlorpyrifos. In addition, the interaction does not appear to act through methyl mercury-induced oxidative damage. Results of the previous bioaccumulation study and TLC suggest the formation of a chlorpyrifos-methyl mercury complex.

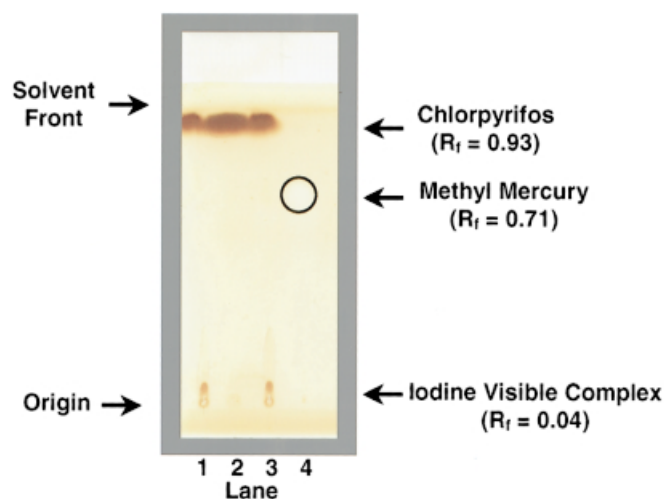


FIG. 5. Thin layer chromatography of chlorpyrifos (lane 2), methyl mercury (lane 4), and chlorpyrifos-methyl mercury mixture (lane 1 and 3) following an incubation in water at room temperature for 24-h. The mixture was resolved on silica plates utilizing 30% ethyl acetate/ 70% hexanes and visualized by exposure to iodine and ultraviolet (UV) light.

Acetylcholinesterase activity was protected in *H. azteca* exposed to chlorpyrifos and methyl mercury as compared to chlorpyrifos alone. These results do not correspond to the previously characterized dose-additive interaction with survival as the endpoint. Acetylcholinesterase enzyme activity in control organisms was 295 ± 133 nmol/min/mg protein. Day and Scott (1990) reported basal acetylcholinesterase levels in *H. azteca* of 74.8 ± 5.8 nmol/min/mg protein. A significant dose-dependent decrease in acetylcholinesterase activity was observed after 24- and 48-h exposures to chlorpyrifos. After 96 h of exposure, there were no surviving organisms in the highest chlorpyrifos treatment. However, there was a significant decrease in enzyme activity for the 0.04 and 0.12 nM chlorpyrifos treatments. Key and Fulton (1993) reported a significant decrease in acetylcholinesterase activity in grass shrimp exposed to 4.6 nM chlorpyrifos for 6 h. The value is higher than that observed in *H. azteca*; however, the duration of exposure for grass shrimp was shorter compared to the 48-h exposure. Exposure to methyl mercury did not result in significant decreases in acetylcholinesterase activity for the 24-, 48-, or 96-h exposure periods. Although no inhibition was observed in *H. azteca* under these conditions, methyl mercury has been demonstrated to inhibit acetylcholinesterase in mammalian models (Petruccioli and Turillazi, 1991). During the development of the acetylcholinesterase-activity assay, methyl mercury interfered with the colorimetric product (DTNB) at con-

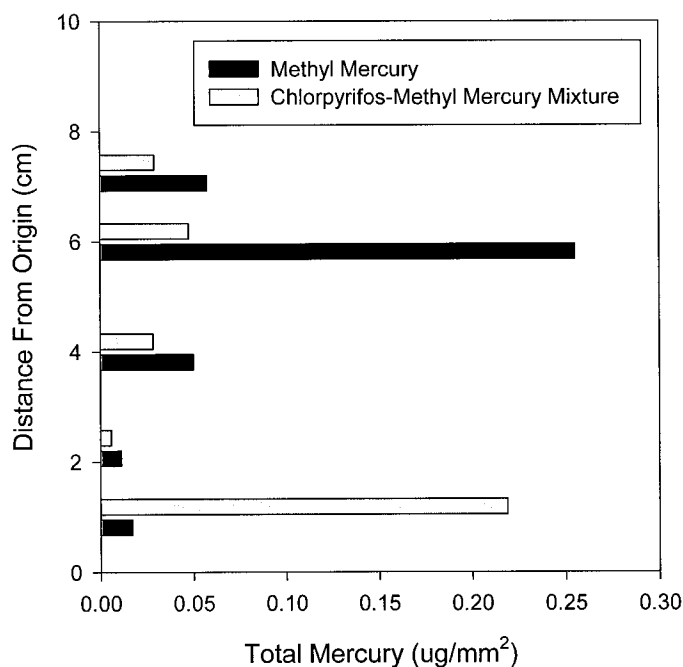


FIG. 6. Total mercury analyzed from thin layer chromatography of methyl mercury and methyl mercury-chlorpyrifos mixture. Sections scraped from the plate were associated with visualized spots from the thin layer chromatography plate shown in Figure 5.

centrations greater than 1 μM . Therefore, clear evidence of inhibition of acetylcholinesterase activity following exposure to greater than 1 μM methyl mercury, reported in previous studies, may be artifactual rather than the result of specific enzyme inhibition. In the current study, methyl mercury appeared to protect acetylcholinesterase activity. Results of the *in vivo* experiments would suggest an antagonism of chlorpyrifos at the enzyme or by decreasing the bioactivation of chlorpyrifos.

Methyl mercury did not appear to induce oxidative stress in *H. azteca* as measured by lipid peroxidation, protein oxidation, or glutathione-S-transferase (GST) activities. Although methyl mercury did not induce oxidative stress, chlorpyrifos alone decreased GST activity following exposure to 0.4 nM for 48 h. GST is an important metabolic enzyme responsible for detoxifying compounds including chlorpyrifos and methyl mercury. Specifically, chlorpyrifos is, in part, metabolized through conjugation of glutathione by GST (Ecobichon, 1996). Exposure to chlorpyrifos has been demonstrated to induce GST in chickens and rats (Vodela and Dalvi, 1995) as well as chlorpyrifos resistance in roaches (Hemingway *et al.*, 1993). The observed decrease in GST activity is similar to that observed in rats, where a mixture of organophosphates decreased cellular metabolism (Lodovici *et al.*, 1994). The mechanism responsible for the decrease in GST activity is not known and results of the GST activity measurements in the current experiment cannot differentiate between enzyme inhibition and decreased expression of the enzyme protein.

Initial experiments, conducted to assess the effects of chlorpyrifos and methyl mercury *in vitro*, were not successful. Inhibition of acetylcholinesterase in *H. azteca* tissue homogenate alone was not produced at concentrations as high as 25 μM chlorpyrifos. This concentration was much higher than that required to inhibit acetylcholinesterase *in vivo*, suggesting that chlorpyrifos was not bioactivated by the tissue homogenate. Therefore, the inhibition of acetylcholinesterase was assessed utilizing the bioactivated form of chlorpyrifos, chlorpyrifos-oxon. Chlorpyrifos-oxon inhibited acetylcholinesterase at concentrations ranging from 18 to 20 nM, verifying that chlorpyrifos was not bioactivated in the tissue homogenate. It is likely that during the preparation of the whole organism tissue homogenate, the cytochrome P450, responsible for the oxidative desulfuration of chlorpyrifos, was degraded or available at concentrations too low to be effective. Previous studies in invertebrates have demonstrated the difficulty associated with isolating intact and bioactive cytochrome P450 enzymes *in vitro* (James and Boyle, 1998). Cytochrome P450 enzymes are susceptible to degradation by proteases found in high concentrations within the hepatopancreas. During homogenization, these proteases are available to the cytochrome P450 enzymes, resulting in their degradation. In addition, NADPH cytochrome P450 reductase, necessary for the transfer of electrons to cytochrome P450, has been demonstrated to be cleaved from the mitochondrial membrane (James, 1990). To bioactivate chlorpyrifos *in vitro*, a surrogate cytochrome P450 system analogous to that for bioactivation of compounds in cell cultures, was utilized (Barber *et al.*, 1998).

Two different surrogate systems, American lobster hepatopancreas and rat liver microsomes, were utilized to bioactivate chlorpyrifos in *H. azteca* tissue homogenate. The lobster microsomal surrogate system was preferred due to species similarity. However, the lobster microsomal preparation did not bioactivate chlorpyrifos, potentially due to inactive cytochrome P450 enzymes. Therefore, rat liver microsomes were utilized to bioactivate chlorpyrifos. Chlorpyrifos, in the bioactivation system was approximately 10-fold less potent than chlorpyrifos-oxon. These differences may be the result of incomplete bioactivation of chlorpyrifos, rapid degradation of the chlorpyrifos-oxon by esterases within the rat liver homogenate, or binding of chlorpyrifos-oxon to esterases within the rat liver homogenate.

Methyl mercury did not significantly affect the chlorpyrifos-oxon inhibition of acetylcholinesterase enzyme or result in a chemical-chemical interaction with chlorpyrifos-oxon. These results demonstrate that methyl mercury does not antagonize chlorpyrifos-oxon, specifically at the enzyme. In addition, there were no chemical-chemical interactions that decreased the ability of chlorpyrifos-oxon to bind to the enzyme. These results suggest that the antagonism observed *in vivo* exists through alteration in bioactivation or uptake and elimination of chlorpyrifos.

The effects of methyl mercury on the bioactivation of chlor-

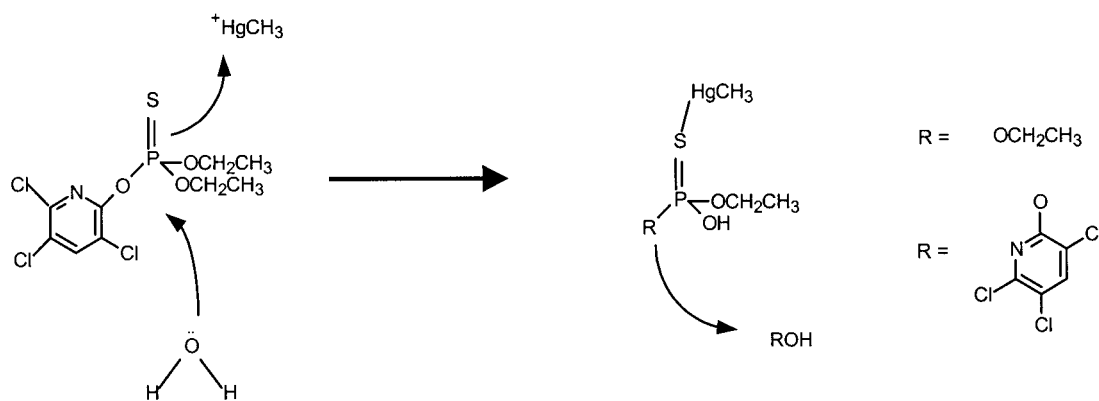


FIG. 7. Formation of chlorpyrifos-methyl mercury complex and hydrolysis products.

pyrifos to chlorpyrifos-oxon were assessed utilizing a surrogate rat-liver-microsome system. Methyl mercury did not decrease the bioactivation of chlorpyrifos by inhibiting cytochrome P450. In addition, the results demonstrate that methyl mercury does not form a complex with chlorpyrifos that results in decreased bioactivation. These results further indicate that chlorpyrifos and methyl mercury interact prior to bioactivation.

Methyl mercury is known to form complexes with sulfur and sulfur-containing compounds. Biological compounds such as proteins and DNA that contain thiol moieties form a weak complex with mercury (Katz and Samitz, 1973; Yamamoto *et al.*, 1994; Yasutake *et al.*, 1990). The formation of thiol-mercury complexes have been demonstrated to alter the accumulation (Gottofrey and Tjalve, 1991; Hirayama, 1985) of mercury as well as the function of the thiol containing biochemical molecule (Clarkson, 1994). Mercury also has the potential to form complexes with non-biological sulfur-containing molecules such as pesticides. Wan *et al.* (1994) demonstrated the enhanced hydrolysis of organophosphorus pesticides in the presence of inorganic mercury (HgCl_2). Inorganic mercury enhanced the hydrolysis of phosphorothioate pesticides; however, it did not promote the hydrolysis of a pesticide (dichlorvos) that did not contain a sulfur group. These results demonstrate the ability of mercury to preferentially form weak complexes with sulfur. Further studies by Zeinali and Torrents (1998) demonstrated that mercury acts as an electrophile to the sulfur of methyl parathion, which is structurally similar to chlorpyrifos. In addition, the mercury-sulfur complex formed with compounds such as methyl parathion had a relatively low dissociation energy that was susceptible to photodecay (Stromberg, 1991).

In the current study, we have investigated the potential of a chemical-chemical interaction between chlorpyrifos and methyl mercury. Chromatographic analysis of an aqueous reaction mixture suggested the formation of a chlorpyrifos-methyl mercury complex. Mercury analysis of sections of a TLC plate demonstrates mercury that was associated with the mixture complex. Previous studies (Gottofrey and Tjalve, 1991; Hirayama, 1985) that observed an increased accumula-

tion of mercury in the presence of thiol molecules suggested that the mercury-sulfur complex was more lipophilic. The TLC results suggest that the product is more polar, as evidenced by the relatively low R_f value and short distance of migration. Although the mercury-sulfur complex is more polar, there may be biological mechanisms that actively transport the complex across membrane barriers. Methyl mercury has been demonstrated to complex with the amino acid, cysteine, to form a complex that mimics methionine and is actively transported across membranes (Clarkson, 1994). However, the more polar mercury-containing complex may also be more bioavailable to the organism, due to increased water solubility.

Further analysis of the methyl mercury-mixture by GC did not indicate the formation of a complex product. However, due to the weakness and low energy of the mercury-sulfur bond, high temperature conditions (250°C) in the GC may have resulted in its degradation. Although the complex was not detected by GC, TLC analysis of the mixture clearly indicates the formation of a polar chlorpyrifos-methyl mercury complex. The formation of the chlorpyrifos-methyl mercury complex (Fig. 7) potentially occurs through electrophilic attack by methyl mercury in the presence of water. Following the formation of the mercury-sulfur bond, hydrolysis of a side chain occurs. The two ethyl side chains and a chlorinated ring would act as potential leaving groups during hydrolysis. The chlorinated ring is more energetically stable and is therefore a better leaving group than the two ethyl side chains. Once hydrolyzed, chlorpyrifos does not have the capacity to inhibit acetylcholinesterase enzyme. Therefore, dissociation of the weak methyl mercury-sulfur complex releases a hydrolyzed inactive metabolite of chlorpyrifos.

In conclusion, the methyl mercury and chlorpyrifos mixture resulted in a chemical-chemical interaction and increased toxicity and accumulation of mercury. The mechanism of toxicity resulting from the additive interaction may be associated with the complex or through increased accumulation and toxicity of methyl mercury. The formation of the complex enhances the hydrolysis and decreases the subsequent toxicity of chlorpyrifos. Therefore, methyl mercury decreases the *in vivo* inhibition

of acetylcholinesterase activity by chlorpyrifos. *In vitro*, methyl mercury did not affect the toxicity associated with chlorpyrifos-oxon due to the absence of the sulfur moiety. Methyl mercury did not affect the bioactivation of chlorpyrifos *in vitro*. The current study demonstrates the potential for methyl mercury and chlorpyrifos to interact at the chemical-chemical level and result in a toxicological effect that would not be predicted from single chemical toxicological studies.

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