Published in final edited form as:

Environ Sci Technol. 2011 May 15; 45(10): 4623–4629. doi:10.1021/es200451j.

Hypersalinity Acclimation Increases the Toxicity of the Insecticide Phorate in Coho Salmon (*Oncorhynchus kisutch*)

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Abstract

Previous studies in euryhaline fish have shown that acclimation to hypersaline environments enhances the toxicity of thioether organophosphate and carbamate pesticides. To better understand the potential mechanism of enhanced toxicity, the effects of the organophosphate insecticide phorate were evaluated in coho salmon (*Oncorhynchus kisutch*) maintained in freshwater (<0.5 g/L salinity) and 32 g/L salinity. The observed 96-h LC50 in freshwater fish ($67.34 \pm 3.41 \mu\text{g/L}$) was significantly reduced to $2.07 \pm 0.16 \mu\text{g/L}$ in hypersaline-acclimated fish. Because organophosphates often require bioactivation to elicit toxicity through acetylcholinesterase (AChE) inhibition, the in vitro biotransformation of phorate was evaluated in coho salmon maintained in different salinities in liver, gills, and olfactory tissues. Phorate sulfoxide was the predominant metabolite in each tissue but rates of formation diminished in a salinity-dependent manner. In contrast, formation of phorate-oxon (gill; olfactory tissues), phorate sulfone (liver), and phorate-oxon sulfoxide (liver; olfactory tissues) was significantly enhanced in fish acclimated to higher salinities. From previous studies, it was expected that phorate and phorate sulfoxide would be less potent AChE inhibitors than phorate-oxon, with phorate-oxon sulfoxide being the most potent of the compounds tested. This trend was confirmed in this study. In summary, these results suggest that differential expression and/or catalytic activities of Phase I enzymes may be involved to enhance phorate oxidative metabolism and subsequent toxicity of phorate to coho salmon under hypersaline conditions. The outcome may be enhanced fish susceptibility to anticholinesterase oxon sulfoxides.

1. Introduction

Salmonids and other anadromous and catadromous species of fish routinely move between fresh and saltwater using olfaction to locate critical habitat for spawning. While hypersaline conditions can provide a certain degree of protection to aquatic organisms from metal

intoxication,¹ interactions with organic contaminants such as pesticides which may co-occur in runoff are less clear.² Hypersaline conditions increased mortality resulting from fenthion exposure in rainbow trout and striped bass but not tilapia.³ Similar results have been observed with the thioether anticholinesterase pesticide, aldicarb, which was converted to the more potent inhibitor, aldicarb sulfoxide, under hypersaline conditions.^{4,5}

Phorate (*O,O*-diethyl-*S*-ethylthiomethyl phosphorodithioate) is an organophosphate pesticide (OP) effective against a wide array of insects, mites, and some nematodes.⁶ It has been also classified by the U.S. Environmental Protection Agency as a restricted use pesticide because of its high toxicity to reptiles, birds, and fish.⁷ Upon uptake by organisms, phorate undergoes oxidative biotransformation, under the mediation of cytochrome P450 (CYP450) and flavin-containing monooxygenases (FMO) to primary and secondary metabolites, with enhanced potency to inhibit AChE. Residue analyses in animals and plants indicate the formation of several principal metabolites that include phorate sulfoxide, phorate sulfone, and their correspondent oxons (phorate-oxon, phorate-oxon sulfoxide, and phorate-oxon sulfone)^{8,9} Oxidation to the sulfoxide, the major product of microsomal oxidations, produces a 2-fold enhancement of AChE inhibition in mice. In addition, oxidative desulfuration of the sulfoxide to the oxon sulfoxide increases AChE inhibition approximately 1000-fold.¹⁰

CYP and FMO have been identified in fish and catalyze the sulfoxidation and bioactivation of several organophosphate and carbamate insecticides.^{11,12} A unique feature of FMO and CYP3A regulation within the salmonid, rainbow trout, is the up-regulation of expression and catalytic activity under hypersaline conditions.^{11,13} Hypersaline conditions increased the formation of the more potent anticholinesterase, fenoxon, from the organophosphate biocide, fenthion, and the even more potent fenoxon sulfoxide from the oxon, indicating biotransformation may contribute to the increased toxicity of these compounds in hypersaline-acclimated animals.¹¹ In fish and mammals, biotransformation enzymes are primarily found in the liver, although extra-hepatic tissues are also involved in biotransformation of xenobiotics and include the gut, kidneys, gills, and the olfactory system.^{14–18} Despite extensive investigation on the role of phase I enzymes in liver and gills, little is known about the activity of these enzymes in fish olfactory tissues which have fundamental importance in salmonid migration.¹⁹

Because it is important to understand the impact of hypersaline conditions to phorate biotransformation and toxicity in euryhaline fish, this study was designed to better characterize phorate toxicity in freshwater- and hypersaline-acclimated coho salmon (*Oncorhynchus kisutch*). To this end, 96-h LC₅₀ values of phorate were measured in fish under freshwater (<0.5 g/L salinity) and hypersaline (32 g/L salinity) conditions. Enzymatic pathways involved in detoxification or activation of phorate and its metabolites were also evaluated in microsomes isolated from liver, gills, and olfactory tissues from animals acclimated to different salinities (<0.5 g/L salinity as freshwater, 8 g/L salinity as low dose, 16 g/L salinity as medium dose, and 32 g/L salinity as high dose) (the experiments were done in spring season and the fish were maintained at 8–13 °C). To evaluate the toxicity of phorate and each of its metabolites, the *in vitro* inhibition of AChE was evaluated using coho salmon brain homogenates and also electric eel (EE) and human recombinant (HR) AChE for comparison.

2. Material and Methods

2.1. Organisms

Juvenile coho salmon (*Oncorhynchus kisutch*) (21.6 ± 1.7 cm length and 100.7 ± 28.7 g weight) were obtained from the Nimbus Hatchery (Gold River, CA), collected and

maintained in freshwater. Organisms were maintained in a flow-through living-stream system at the University of California Riverside, with dechlorinated carbon-filtered municipal water at 11–13 °C and acclimated for a minimum of 2 months before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT).

2.2. Experiment 1. Hypersalinity Acclimation and Phorate Exposure

Fish were transferred and sequentially acclimated for 2 d to hypersaline water at 4, 8, 16, and 32 g/L saline concentrations (CrystalSea Marine Mix, Marine Enterprises International, Baltimore, MD) in 24-L tanks. All fish were left for 1 week at the specific salinity regime prior to pesticide exposure. After salinity acclimation, fish from both groups (freshwater-acclimated fish as <0.5 g/L and hypersaline-acclimated fish as 32 g/L) were exposed to four different nominal concentrations (2, 20, 40, 80 µg/L) of phorate dissolved in ethanol and mortality was measured every 6 h for 280 h. Phorate concentrations were based on 96-h LC50 values for other salmonid species (*O. mykiss* and *O. clarki*).²⁰ The control group was exposed only to the carrier solvent (ethanol, 0.01% v/v). Three replicates with 8 animals each (total 24 animals) were used for each experimental group. Water was replaced and fresh phorate dissolved in ethanol was added every day. At hour 1, 75, and 96, 50 mL of water was collected and phorate concentrations were evaluated after extraction and concentration.

2.3. Experiment 1. Phorate Determination in Water

Water was extracted through a SPE C18 cartridge (1 g, 6 mL) (Sep-Pak, Waters, Milford, MA) that had been sequentially preconditioned with methanol (4 mL) and Milli-Q water (8 mL). After the sample was loaded (50 mL), cartridges were eluted with 5 mL of methanol, and the eluate was dried, resuspended in 100 µL of 60% acetonitrile in water, and 40 µL (injection loop was 50 µL) was injected into a reverse phase HPLC system. *R*-methyl-*p*-tolyl sulfoxide (0.04 mM) was added to each 50-mL sample prior to extraction as an internal standard. The recovery was 92–96%.

2.4. Experiment 2. Hypersalinity Acclimation

After tank acclimation, fish were transferred and sequentially acclimated for 2 days to hypersaline water at a concentration of 4 g/L, followed by 8, 16, and 32 g/L saline concentration in 24-L aquaria. At selected salinities, they were left for 1 week. Five animals and 3 replicates (total 15 animals) were used per experimental group. After acclimation to each salinity regime, animals were weighed, measured, and euthanized using tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) with subsequent removal of the liver, gills, and olfactory tissue, which were frozen in liquid nitrogen and stored at –80 °C (between 1 and 5 days as maximum). All experiments were done in accordance with animal welfare regulations in the U.S.

2.5. Experiment 2. Subcellular Fractionation

Livers, gills, and olfactory tissues were selected due to their physiological roles in biotransformation (liver), osmoregulation (gill), and behavior (olfactory tissues). The subcellular fractionation was obtained by differential centrifugation and the full method is described in the Supporting Information.

2.6. Experiment 2. Phorate Biotransformation

Phorate biotransformation was evaluated as described in Henderson et al.²¹ with minor modifications. A wide range of phorate concentrations, microsomal protein content, and incubation times were used to optimize conditions. Concentrations of phorate ranged from 0.5 µM to 1 mM for kinetic evaluations in liver microsomes from coho salmon. Formation

of metabolites was linear up to 800 μg of protein and up to 45 min of incubation. For optimal incubation comparisons, 10 μM of phorate, 500 μg of microsomal protein, and 30 min of incubation were chosen. Liver, gill, and olfactory tissue microsomal fractions were incubated in 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.6 (prepared at 25 °C) with 10 μM of phorate and its metabolites and 100 μM NADPH in a total volume of 500 μL . The reaction was initiated by the addition of substrate and was incubated for 30 min at 25 °C. The reaction was stopped by placing the tube on ice and the addition of 1 mL of ethyl acetate to extract the incubation mixture (extracted 2 times using 1 mL of ethyl acetate). An internal standard, *R*-methyl-*p*-tolyl sulfoxide (0.04 mM final concentration) was added to each sample after incubation in order to determine recovery rates. The extract was removed, transferred to glass HPLC vials, and evaporated under nitrogen flow to dryness. Negative controls consisted of identical additives with the exception of NADPH or adding boiled proteins. Water/acetonitrile (40/60 v/v) (100 μL) was used to reconstitute the samples for analysis and 40 μL (injection loop was 50 μL) was injected into a reverse phase LC-ESI-MS system (3 injection replicates for each sample). The HPLC and LC-ESI-MS analyses are described in the Supporting Information.

2.7. Acetylcholinesterase (AChE) Inhibition Studies

The in vitro inhibition of AChE by phorate and its metabolites was evaluated using coho salmon brain homogenates and also electric eel (EE) and human recombinant (HR) AChE for comparison. AChE activity was evaluated according to Nillos et al.²² with minor modifications. The method is described in the Supporting Information.

2.8. Statistical Procedures

Statistical significance was assessed using a Student's *t* test between two groups and one-way ANOVA to evaluate differences between multiple groups, with the use of GraphPad Prism version 5.00 for MacOS X (GraphPad Software, San Diego, CA). A *p*-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's multiple range tests were performed. Samples showing levels below the detection limits were considered as having 50% of the minimal values detectable for statistical comparisons. All data were analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests. Kinetic parameters (K_m , V_{max} , and catalytic efficiency) of enzymatic activities were calculated using the same software package.

3. Results

3.1. Toxicity Testing

Average concentrations of phorate determined in the experimental tanks during the duration of exposure were similar to nominal concentrations, viz. 1.3 ± 0.5 , 14.3 ± 9.7 , 32 ± 11.4 , and 79.1 ± 20.3 $\mu\text{g/L}$ (values are mean $\pm$ standard deviation of three different sampling days and different time after dosing). Phorate was not detected in the control tank (<0.1 $\mu\text{g/L}$). As shown in Figure 1A and B, all survival curves of freshwater- and hypersaline-acclimated fish fit a nonlinear regression ($r^2 = 0.93$ to $r^2 = 0.99$). A significant difference was observed in salinity-enhanced toxicity of phorate. The estimated 96-h LC50 of phorate in freshwater-acclimated salmon, determined using the measured concentrations, was 67.3 ± 3.4 $\mu\text{g/L}$ (95% Confidence Interval 53.4 to 81.2). Salmon exposed to phorate in a hypersaline environment experienced up to 32-fold higher toxicity ($p < 0.01$) with a 96-h LC50 of 2.1 ± 0.2 $\mu\text{g/L}$ (95% CI 1.1 to 3.1). Survival curves with respect to LC50 are shown in the Supporting Information as Figure S1.

3.2. Phorate Biotransformation

Liver—The kinetics of phorate metabolism were characterized in liver microsomes from freshwater-acclimated fish to optimize the assay for comparative studies. Conversion to different metabolites indicated varied catalytic efficiencies. NADPH-dependent phorate-oxon production showed the highest apparent K_m ($121.71 \pm 11.75 \mu\text{M}$) and the lowest V_{max} . The apparent K_m for phorate-oxon production was up 20.4 times higher than that for phorate sulfoxide production ($5.95 \pm 1.79 \mu\text{M}$) but the V_{max} for the oxon was 12.3 times lower compared to the sulfoxide production ($64.5 \pm 2.84 \text{ pmol/min/mg protein}$). As a consequence, higher catalytic efficiency was observed for the formation of the phorate sulfoxide (10.85) relative to the oxon (0.04). Phorate sulfone was not detected ($<0.024 \text{ pmol/min/mg protein}$). Hypersaline conditions significantly increased total phorate biotransformation by liver microsomes in coho salmon (Table 1). When microsomes were incubated with phorate, rates of phorate-oxon were not enhanced in hypersaline-acclimated fish, but the rate of phorate sulfone was significantly enhanced ($p < 0.05$) in medium and high salinity groups. Only phorate-oxon sulfoxide was enhanced in a salinity-dependent manner. Hypersalinity acclimation significantly reduced ($p < 0.05$) the production of phorate sulfoxide in salinity-acclimated groups. Microsomal incubations with phorate-oxon led to the formation of phorate-oxon sulfoxide in a salinity-dependent manner. Phorate sulfone production was not observed in phorate-oxon incubations ($<0.024 \text{ pmol/min/mg protein}$). When liver microsomes were incubated with phorate sulfoxide, phorate-oxon sulfoxide and phorate sulfone production were enhanced by salinity acclimation ($p < 0.05$).

Gills—In microsomal fractions isolated from gills of hypersaline-acclimated animals incubated with phorate, the rates of all metabolite production were significantly lower than liver microsomes (Table 2). Phorate-oxon production was enhanced in a salinity-dependent manner. As in liver microsomes, phorate sulfoxide production was decreased in hypersaline animals, with significant differences in the medium and high salinity groups ($p < 0.01$) relative to freshwater exposure. Sulfone production was not detected ($<0.024 \text{ pmol/min/mg protein}$) with incubations of phorate, phorate sulfoxide, or phorate-oxon. In contrast to liver microsomes, biotransformation of phorate sulfoxide was not observed in gill microsomes in any treatment group.

Olfactory tissues—In olfactory tissues the same trends with respect to salinity-dependent phorate biotransformation in liver and gills were observed (Table 3). Interestingly, higher biotransformation rates were observed compared to gills, but lower than in liver. In freshwater-acclimated animals higher phorate-oxon sulfoxide (up to 3.4-fold) and phorate sulfoxide (up to 4.9-fold) production were observed compared to gills. In olfactory tissues, hypersaline acclimation significantly increased oxon and oxon sulfoxide production ($p < 0.05$) but reduced sulfoxide production ($p < 0.01$) with phorate incubations. Sulfone formation was not detected in any incubation, and no detectable biotransformation was observed with incubations of phorate sulfoxide. With incubations of phorate-oxon, formation of phorate-oxon sulfoxide increased significantly ($p < 0.05$) with hypersaline acclimation in the medium (16 g/L) and high (32 g/L) salinity groups.

3.3. AChE Inhibition

The IC₅₀ of the individual phorate metabolites and the parent compound are provided in Table 4. Of the five compounds tested phorate had the highest IC₅₀ in coho salmon brain. Phorate sulfoxide exhibited a 3-fold increase in AChE inhibition in coho salmon brain as compared to phorate. Phorate-oxon and phorate sulfone were shown to be more potent with over 140-fold and 340-fold increases, respectively. Phorate-oxon sulfoxide was the most potent inhibitor of the five compounds tested for coho salmon brain with over a 450-fold increase. This pattern of increased inhibition from phorate, to phorate sulfoxide, phorate-

oxon, phorate sulfone, and last phorate-oxon sulfoxide was also seen in human recombinant AChE. The inhibition by phorate and phorate sulfoxide was similar. There was a 370-fold increase in potency from phorate to phorate-oxon sulfoxide in human recombinant AChE. This pattern of inhibition was relatively similar in electric eel AChE. There was over a 220-fold increase in inhibition from phorate to phorate-oxon sulfoxide.

4. Discussion

Acclimation to hypersaline conditions significantly alters the physiology of euryhaline aquatic organisms. In slow osmoconforming species, such as salmonids, up-regulation of flavin-containing monooxygenases (FMOs) occurs producing organic osmolytes such as trimethylamine *N*-oxide countering increases in osmotic pressure as well as intracellular urea in hypersaline environments.^{12,23} The expression of an FMO transcript that encodes a protein (hFMO) that is approximately 50% identical to mammalian FMO1 and FMO5 was enhanced in primary rainbow trout hepatocytes treated with NaCl and the osmotic hormone, cortisol.¹³ Cortisol also up-regulates the expression of CYP3A which has been observed in salmonids undergoing osmotic acclimation.¹¹ Thus, it has been hypothesized that chemicals which are biotransformed to more toxic intermediates by FMO and CYP3A (i.e., thioether pesticides) may be more toxic in species that up-regulate these monooxygenases in response to hypersaline conditions. This hypothesis has been previously tested and confirmed with thioether pesticides: aldicarb in several species of fish^{4,5,24} and fenthion in rainbow trout.^{3,11} The toxicity of aldicarb was greatly enhanced in rainbow trout acclimated to hypersaline conditions which caused induction of FMO leading to the subsequent enhanced production of aldicarb sulfoxide which was a significantly more potent cholinesterase inhibitor.²⁴ Hypersaline conditions also dramatically increased the toxicity of the organophosphate fenthion in rainbow trout and striped bass, reducing the 96-h LC50 6- and 4.7-fold, respectively.³ In the case of fenthion, *S*-oxygenation creates a chiral center with *S*- and *R*-oxides diminishing AChE inhibition.²⁵ However, the subsequent oxidative desulfuration of the *R*-fenthion sulfoxide to *R*-fenoxon sulfoxide by CYP3A and FMO was a critical bioactivation pathway, resulting in the production of *R*-fenoxon sulfoxide, a more potent AChE inhibitor.²⁵

In liver microsomes from coho salmon, phorate was biotransformed to phorate-oxon and phorate sulfoxide, with higher catalytic efficiency ($V_{\max}/K_m$) toward the sulfoxide than toward the oxon (up to 270-fold higher). The biotransformation of phorate in liver microsomes of mammals has been shown to be almost entirely oxidative, with ester cleavage being a minor pathway for phorate.²⁶ Biotransformation of phorate-oxon to phorate-oxon sulfoxide was observed in microsomes from each of the three previously observed in mammals^{9,10,21} Since the reaction was NADPH-dependent, CYP or FMO monooxygenases are likely involved in the reaction. FMOs are generally limited to the formation of sulfoxide from thioethers,²⁷ whereas CYPs are involved in both oxidative desulfuration of the phosphorothionate to form the oxon as well as oxidation of the thioether group to the corresponding sulfoxide and sulfone of various organophosphates.²⁸ Sulfoxidation of several thioether pesticides in human liver microsomes are mainly CYP-driven (85–90%), with the remainder accounted for by FMO.²⁹

Phorate-oxon sulfone formation was not detected in any of the three tissues evaluated contrasting studies in mice¹⁰ and the fall armyworm (*O. frugiperda*) where phorate-oxon sulfone formation from the desulfuration of phorate sulfone was observed.³⁰ These data indicate fish may have unique biotransformation pathways that preclude additional *S*-oxidation of oxon sulfoxide metabolites toward sulfone metabolites. The outcome may be enhanced fish susceptibility to anticholinesterase oxon sulfoxides.

Phorate was converted to oxon, oxon sulfoxide, and sulfoxide metabolites with higher rates of turnover than in gills indicating monooxygenase-catalyzed bioactivation to more potent anticholinesterases in olfactory tissues. CYP isoforms (CYP1A, CYP2K1, CYP2M1, and CYP3A27) as well as FMO activities have been identified in olfactory tissues of coho salmon.¹⁵ Previous studies evaluating chlorpyrifos, fenthion, and parathion failed to show any oxidative biotransformation, but esterase cleavage was observed.¹⁸ Pesticides clearly disrupt olfaction and resultant behavior in salmonids.^{31–33} Whether the disruption of olfaction is due to cholinesterase inhibition is unclear, but additional studies with the oxon and oxon sulfoxide metabolites may provide some insight toward this mechanism.

In contrast to earlier studies in rainbow trout and Japanese medaka with the anticholinesterase thioether aldicarb,^{4,24} hypersaline conditions failed to enhance overall sulfoxidation of phorate in the liver, gills, and olfactory tissues of coho salmon. In fact, hypersaline conditions caused overall reductions in sulfoxide formation but increased oxidative desulfuration in microsomes from the three selected tissues. In liver microsomes from rainbow trout incubated with fenthion, hypersaline conditions also led to significant reductions in sulfoxide formation and esterase cleavage but increases in fenoxon and fenoxon sulfoxide formation shifting pathways from detoxification to bioactivation.¹¹ The enhanced production of the oxon and oxon sulfoxide metabolites under hypersaline conditions presents a similar scenario where much more potent cholinesterase inhibitors are being created, shifting the metabolic pathway away from detoxification to bioactivation.

Hypersaline conditions increased the rates of phorate-oxon, phorate-oxon sulfoxide, and phorate sulfone in microsomes isolated from liver using phorate as a starting substrate. The rates of phorate-oxon sulfoxide production were similar using either phorate or phorate-oxon as a substrate, but 2-fold lower when the substrate was phorate sulfoxide, indicating that formation of phorate-oxon may predominate over phorate sulfoxide as the initial activation step in the bioactivation of phorate to phorate-oxon sulfoxide in coho salmon. The results of AChE inhibition by phorate and four of its metabolites in coho salmon brain homogenates, as well as electric eel and human recombinant AChE, followed a general trend observed with other AChE inhibitors. From previous studies, it was expected that phorate and phorate sulfoxide would be less potent inhibitors than phorate-oxon, with phorate-oxon sulfoxide being the most potent of the compounds tested.¹⁰ This trend was confirmed in the current study.

Phorate sulfone was a potent inhibitor of AChE, with an IC₅₀ between that of phorate-oxon and phorate-oxon sulfoxide. Although phorate sulfone has previously been shown as an active inhibitor, the sulfone of disulfoton has not been shown to be a potent AChE inhibitor.^{10,34} Both phorate and disulfoton are organophosphate insecticides that have phosphothiolate and thioether moieties.²¹ Disulfoton has a two carbon unit separation between the thioether and the highly electronegative atoms of the phosphothiolate. Since phorate lacks the second carbon unit seen in disulfoton, it is possible that the thioether sulfur in phorate has a higher partial positive charge due to its close proximity to the highly electronegative atoms present in this compound. A stronger partial positive charge would enable it to bind more readily to the cationic site of AChE as compared to disulfoton, causing it to be a more potent inhibitor. Because phorate sulfone is one of the more stable metabolites of phorate in the environment,^{8,35} fish and other aquatic organisms may be more affected by this metabolite than the parent compound.

In summary, hypersaline conditions increased phorate toxicity 32-fold in coho salmon (*O. kisutch*) and increased the formation of phorate-oxon, phorate sulfone, and phorate-oxon sulfoxide from phorate in microsomes from liver, gill, and olfactory tissues from coho salmon. This could be an important mechanism of increasing toxicity since the oxon,

sulfone, and the oxon sulfoxide are more potent inhibitors of AChE relative to phorate.¹⁰ The predominant pathway of phorate activation by hypersaline conditions appears to be through the formation of phorate-oxon, which may also be subsequently converted to the more toxic metabolite phorate-oxon sulfoxide. These results suggest that differential expression and/or catalytic activities of CYPs and/or FMOs may be involved to enhance phorate oxidative metabolism and subsequent toxicity of phorate to coho salmon under hypersaline conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We appreciate the assistance of Kristy Richardson for her valuable help with LC-ESI-MS, and Dr. Kristy L. Forsgren and Prof. Dr. Graham Young from the University of Washington for supplying coho salmon brain tissue. This work was supported from funds of the University of Washington Superfund Basic Research Program [NIEHS P42ES04696].

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Figure 1. Survival curves of coho salmon (*Oncorhynchus kisutch*) acclimated to freshwater, <0.5 g/L, (A) and hypersaline water, 32 g/L, (B) exposed to 4 different doses of phorate. Data are presented as mean $\pm$ standard deviation (n = three replicates with eight animals each for each experimental group).

Table 1
Effects of Hypersaline Conditions on the in Vitro Biotransformation of Phorate in
Microsomes of Liver from Coho Salmon (*Oncorhynchus kisutch*)^a

substrate (10 μ M)	treatment			
metabolite	freshwater (<0.5 g/L)	low salinity (8 g/L)	medium salinity (16 g/L)	high salinity (32 g/L)
phorate				
phorate-oxon	0.49 $\pm$ 0.18 ^a	0.40 $\pm$ 0.14 ^a	0.39 $\pm$ 0.08 ^a	0.39 $\pm$ 0.15 ^a
phorate-oxon sulfoxide	5.32 $\pm$ 1.89 ^a	9.49 $\pm$ 3.03 ^a	11.17 $\pm$ 1.97 ^b	16.14 $\pm$ 2.88 ^c
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide	36.14 $\pm$ 5.83 ^a	31.29 $\pm$ 2.46 ^a	24.91 $\pm$ 4.68 ^b	19.43 $\pm$ 3.08 ^b
phorate sulfone	0.19 $\pm$ 0.03 ^a	0.26 $\pm$ 0.04 ^{ab}	0.38 $\pm$ 0.10 ^{bc}	0.43 $\pm$ 0.07 ^c
phorate-oxon				
phorate-oxon sulfoxide	7.56 $\pm$ 2.74 ^a	16.00 $\pm$ 8.86 ^{ab}	21.38 $\pm$ 7.36 ^{bc}	21.96 $\pm$ 4.79 ^{bc}
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide				
phorate-oxon sulfoxide	2.17 $\pm$ 1.28 ^a	3.52 $\pm$ 2.80 ^{ab}	5.86 $\pm$ 2.26 ^b	6.45 $\pm$ 1.33 ^b
phorate sulfone	0.19 $\pm$ 0.09 ^a	0.29 $\pm$ 0.23 ^{ab}	0.39 $\pm$ 0.11 ^{ab}	0.47 $\pm$ 0.04 ^b

^aData are expressed as pmol/min/mg protein and as mean $\pm$ standard deviation ((*n* = three replicates with five animals each for each experimental group).

Different letters indicate significant differences (*p* < 0.05; One-way ANOVA, Tukey's test).

bdl: below detection limit (< 0.024 pmol/min/mg protein).

Table 2
Effects of Hypersaline Conditions on the in Vitro Biotransformation of Phorate in
Microsomes of Gills from Coho Salmon (*Oncorhynchus kisutch*)^a

substrate (10 μ M)	treatment			
metabolite	freshwater (<0.5 g/L)	low salinity (8 g/L)	medium salinity (16 g/L)	high salinity (32 g/L)
phorate				
phorate-oxon	0.16 $\pm$ 0.02 ^a	0.17 $\pm$ 0.09 ^{ab}	0.39 $\pm$ 0.18 ^{bc}	0.61 $\pm$ 0.19 ^c
phorate-oxon sulfoxide	0.16 $\pm$ 0.08 ^a	0.14 $\pm$ 0.04 ^a	0.16 $\pm$ 0.07 ^a	0.15 $\pm$ 0.05 ^a
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide	0.46 $\pm$ 0.12 ^a	0.47 $\pm$ 0.15 ^a	0.26 $\pm$ 0.04 ^b	0.24 $\pm$ 0.03 ^b
phorate sulfone	bdl	bdl	bdl	bdl
phorate-oxon				
phorate-oxon sulfoxide	0.88 $\pm$ 0.30 ^a	0.60 $\pm$ 0.11 ^a	1.05 $\pm$ 0.49 ^a	0.52 $\pm$ 0.21 ^a
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide				
phorate-oxon sulfoxide	bdl	bdl	bdl	bdl
phorate sulfone	bdl	bdl	bdl	bdl

^aData are expressed as per Table 1.

bdl: below detection limit (< 0.024 pmol/min/mg protein).

Table 3
Effects of Hypersaline Conditions on the in Vitro Biotransformation of Phorate in
Microsomes of Olfactory Tissues from Coho Salmon (*Oncorhynchus kisutch*)^a

substrate (10 μ M)	treatment			
metabolite	freshwater (<0.5 g/L)	low salinity (8 g/L)	medium salinity (16 g/L)	high salinity (32 g/L)
phorate				
phorate-oxon	0.19 ± 0.03^a	0.25 ± 0.01^a	0.58 ± 0.09^b	0.47 ± 0.04^b
phorate-oxon sulfoxide	0.55 ± 0.06^a	0.87 ± 0.01^b	0.92 ± 0.04^b	0.86 ± 0.02^b
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide	2.25 ± 0.10^a	1.38 ± 0.05^b	1.46 ± 0.15^b	1.13 ± 0.12^b
phorate sulfone	bdl	bdl	bdl	bdl
phorate-oxon				
phorate-oxon sulfoxide	5.81 ± 0.52^a	9.26 ± 1.25^{ab}	13.14 ± 1.81^b	13.31 ± 1.87^b
phorate-oxon sulfone	bdl	bdl	bdl	bdl
phorate sulfoxide				
phorate-oxon sulfoxide	bdl	bdl	bdl	bdl
phorate sulfone	bdl	bdl	bdl	bdl

^aData are expressed as per Table 1.

bdl: below detection limit (<0.024 pmol/min/mg protein).

Table 4
In Vitro Acetylcholinesterase Median Inhibitory Concentration IC₅₀ (μM) of Phorate and Its Metabolites in Coho Salmon Brain (*Oncorhynchus kisutch*), Electric Eel AChE (*Electrophorus electricus*; EE-AChE), and Human Recombinant AChE (HR-AChE)^a

compound	IC ₅₀ (μM)		
	EE-AChE	HR-AChE	coho salmon brain
phorate	311.4 ± 43.3	185.0 ± 26.8	5692.0 ± 872.7
phorate sulfoxide	395.1 ± 47.4	144.9 ± 20.8	1767.1 ± 306.3
phorate sulfone	2.9 ± 0.2	0.7 ± 0.1	16.6 ± 1.3
phorate-oxon	2.6 ± 0.1	1.3 ± 0.1	39.7 ± 4.7
phorate-oxon sulfoxide	1.4 ± 0.3	0.5 ± 0.1	12.5 ± 1.4

^aValues are presented as mean ± standard deviation ($n = 4$).