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Mechanisms of fenthion activation in rainbow trout (*Oncorhynchus mykiss*) acclimated to hypersaline environments

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Abstract

Previous studies in rainbow trout have shown that acclimation to hypersaline environments enhances the toxicity to thioether organophosphate and carbamate pesticides. In order to determine the role of biotransformation in this process, the metabolism of the thioether organophosphate biocide, fenthion was evaluated in microsomes from gills, liver and olfactory tissues in rainbow trout (*Oncorhynchus mykiss*) acclimated to freshwater and 17‰ salinity. Hypersalinity acclimation increased the formation of fenoxon and fenoxon sulfoxide from fenthion in liver microsomes from rainbow trout, but not in gills or in olfactory tissues. NADPH-dependent and independent hydrolysis was observed in all tissues, but only NADPH-dependent fenthion cleavage was differentially modulated by hypersalinity in liver (inhibited) and gills (induced). Enantiomers of fenthion sulfoxide (65% and 35% *R*- and *S*-fenthion sulfoxide, respectively) were formed in liver and gills. The predominant pathway of fenthion activation in freshwater appears to be initiated through initial formation of fenoxon which may be subsequently converted to the most toxic metabolite fenoxon *R*-sulfoxide. However, in hypersaline conditions both fenoxon and fenthion sulfoxide formation may precede fenoxon sulfoxide formation. Stereochemical evaluation of sulfoxide formation, cytochrome P450 inhibition studies with ketoconazole and immunoblots indicated that CYP3A27 was primarily involved in the enhancement of fenthion activation in hypersaline-acclimated fish with limited contribution of FMO to initial sulfoxidation.

Keywords

Fenthion; Fenoxon; Sulfoxidation; Hypersalinity; Cytochrome 450; *Oncorhynchus mykiss*

Introduction

Fenthion [*O,O*-dimethyl-*O*-(4-methylmercapto)-3-methylphenylthio-phosphate] is an organophosphate pesticide (OP) widely used throughout the world as a broad-spectrum insecticide for numerous crops and also as an ectoparasiticide on farm animals (Roberts and Hutson, 1999). OP insecticides exert their principal biological effects by phosphorylation and inhibition of the enzyme acetylcholinesterase (AChE), resulting in subsequent accumulation of acetylcholine and continuous stimulation of the nervous system (Costa, 2006). Fenthion has been also classified by the U.S. Environmental Protection Agency as a restricted use pesticide because of its toxic effects in birds, reptiles and fish. Toxic effects such as structural changes in the testes of gobiid fish, reduction in larval production in sand

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fiddler crabs, chronic toxicity in hens, acute toxicity in birds, reptiles, and fish have been reported (Mullie et al., 1999; Tuler and Bowen, 1999; Schoor et al., 2000; Zutshi and Murthy, 2001). The relationship between the toxicity and metabolism of fenthion has been widely examined in human neuroblastoma cell lines, birds, and fish, and either an increase or a decrease in toxicity as a result of metabolism has been found (Cova et al., 1995; Roux et al., 1995; Kitamura et al., 2000).

Upon uptake by organisms, fenthion undergoes oxidative metabolism, under the mediation of cytochrome P450 (CYP) and flavin-containing monooxygenases (FMO) to primary and secondary metabolites, with either enhanced or reduced potency to inhibit AChE. Enzymatic reactions include oxidative desulfuration of the phosphorothioate and sulfoxidation of the distal thioether group (Kitamura et al., 2000; Furnes and Schlenk, 2004) (Fig. 1). *In vitro* and *in vivo* studies demonstrated that fenthion is biotransformed to fenthion sulfoxide and fenoxon in fish and rats (Kitamura et al., 2003). As shown in Fig. 1, residue analyses in animals and plants indicate the formation of several principal metabolites and include fenthion sulfoxide, fenoxon, fenoxon sulfoxide, the cleavage product 3-methyl-4-(methylthio)-phenol (MMTP) and the corresponding sulfones (Tsuda et al., 1996; Cavanna and Molinari, 1998). Fenthion also undergoes nonenzymatic transformation including photodegradation to the sulfoxide, a relatively stable oxidation product in the environment (Hirahara et al., 2001). *S*-oxygenation of fenthion creates a chiral center with *S*- and *R*-oxides of fenthion diminishing AChE inhibition (Gadepalli et al., 2007). Although the stereoselective sulfoxidation of fenthion to *R*-fenthion sulfoxide by FMO1 represents a detoxification pathway, the subsequent oxidative desulfuration of the *R*-oxide may represent a critical bioactivation pathway, resulting in the production of *R*-fenoxon sulfoxide, a more potent AChE inhibitor (IC_{50} for *R*-fenthion sulfoxide is $>1000 \mu M$, IC_{50} for *R*-fenoxon sulfoxide is $6.5 \pm 0.2 \mu M$) (Gadepalli et al., 2007).

A unique feature of FMO regulation within rainbow trout is the up-regulation of expression and catalytic activity under hypersaline conditions (Rodriguez-Fuentes et al., 2008). Hypersaline runoff into surface water from agricultural practices is common in arid climates and landscapes (Vengosh, 2004). 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 (Dwyer et al., 1992), interactions with organic contaminants such as pesticides which may co-occur in runoff are less clear (Hall and Anderson, 1995). Hypersaline conditions increased mortality resulting from fenthion exposure in rainbow trout and striped bass but not tilapia (Bawardi et al., 2007). Similar results have been observed with another thioether anti-cholinestase pesticide, aldicarb, which was converted to the more potent inhibitor, aldicarb sulfoxide, under hypersaline conditions (El-Alfy and Schlenk, 1998; El-Alfy et al., 2001).

The current study was designed to better characterize fenthion metabolism in different target tissues and evaluate the effect of hypersalinity on biotransformation in juvenile rainbow trout. To this end, key enzymatic pathways involved in detoxification or activation of fenthion were investigated in microsomes isolated from liver, gills and olfactory tissues from freshwater- and hypersaline-acclimated fish.

Material and methods

Chemicals

Fenthion (99.9%), fenoxon, and racemic fenthion sulfoxide were purchased from Chem Service (West Chester, PA). MMTP and tetraethylpyrophosphate (TEPP) were purchased from Sigma (Milwaukee, WI). *R*- and *S*-fenthion sulfoxides and fenoxon sulfoxides were

synthesized as described previously (Gadepalli et al., 2007). [^{14}C] Testosterone was purchased from Perkin-Elmer (Waltham, MA). Methanol, ethanol, ethyl acetate, acetonitrile, *n*-hexane and isopropanol were of analytical grade (Fisher, Pittsburgh, PA).

Fish treatment

Juvenile rainbow trout (*Oncorhynchus mykiss*) (age approximately 5 months, 16 ± 3 cm), without discernible gonadal morphology indicative of gender, were obtained from Jess Ranch Fish Hatchery (Apple Valley, CA). Organisms were maintained in a flow-through living-stream system with dechlorinated carbon-filtered municipal water at $13\text{--}15^\circ\text{C}$ and acclimatized for 2 months before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT). After tank acclimation, fish were transferred and sequentially acclimated for 2 days to hypersaline water at concentrations of 4 g/L, followed by 8, 12, and 17 g/L (ppt) saline concentration (CrystalSea Marine Mix, Marine Enterprises International, Baltimore, MA). At 17 ppt, they were left for 1 week. After acclimation animals were euthanized using tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA), and the liver, gills and olfactory tissue were dissected and frozen in liquid nitrogen and stored at -80°C . These conditions were used in previous studies with rainbow trout and enhanced the biotransformation and toxicity of aldicarb (Wang et al., 2001) and the toxicity of fenthion (Bawardi et al., 2007).

Subcellular fractionation

Livers, gills and olfactory tissues were selected due to their physiological roles in biotransformation (liver), osmoregulation (gill), and behaviour (olfactory tissues). The subcellular fractionation was performed according to Lavado et al. (2004) with minor modifications. Briefly, after weighing each tissue, it was rinsed in ice-cold 1.15% KCl and homogenized in 1:5 w/v of cold 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing 100 mM KCl, 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM 1,10-phenanthroline. Olfactory tissues were pooled from 8 different individuals, but gills and liver were analyzed from individual organisms. Homogenates were centrifuged at 500 g for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000 g for 20 min. The 12,000 g supernatant was further centrifuged at 100,000 g for 60 min to obtain microsomal fractions. Microsomal pellets were resuspended in a small volume of 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing 100 mM KCl, 20% (w/v) glycerol, 1 mM EDTA, 0.1 mM PMSF and 0.1 mM 1,10-phenanthroline. Protein concentrations were determined by the Coomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL) using bovine serum albumin as a standard.

Fenthion, fenoxon and fenthion sulfoxide metabolism

A wide range of fenthion concentrations, microsomal protein content and incubation times were used to optimize conditions. Concentrations of fenthion ranged from $1\text{ }\mu\text{M}$ to 1 mM for kinetic evaluations in liver microsomes from freshwater-acclimated (FA) fish and hypersaline-acclimated (HA) fish. Fenthion, fenoxon and fenthion sulfoxide (*S*- and *R*-) metabolism was linear up to 1.2 mg protein and up to 60 min of incubation. For the samples, 100 μM of fenthion, 600 μg of microsomal protein and 60 min of incubation were chosen. Liver, gill and olfactory tissue microsomal fractions (0.6 mg protein) were incubated in 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.6 with 100 μM of substrate (fenthion, fenoxon, *R*-fenthion sulfoxide or *S*-fenthion sulfoxide) and 400 μM NADPH in a total volume of 500 μL . The reaction was initiated by the addition of substrate and samples were incubated for 60 min at 25°C . The reaction was stopped by placing the tube on ice and the addition of 1 mL ethyl acetate to extract the incubation mixture. An internal standard, *R*-methyl(*p*)tolyl sulfoxide (0.04 mM final concentration) was added to each sample after incubation was

concluded 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 boiled proteins. Positive control studies with recombinantly expressed human FMO1 were carried out using conditions previously described (Furnes and Schlenk, 2004). Isopropanol (50 μ L) was used to reconstitute the samples for analysis and 30 μ L (injection loop was 50 μ L) was injected into a HPLC system (3 injection replicates for each sample). HPLC analyses were performed on a SCL-10AVP Shimadzu HPLC system equipped with a 250 mm $\times$ 4.6 mm Chiralcel OJ-H chiral column (Daicel Chemical Industries, Fort Lee, NJ). Separation of fenthion and fenthion metabolites (MMTP, fenoxon, *R*-fenthion sulfoxide, *S*-fenthion sulfoxide, *R*-fenoxon sulfoxide and *S*-fenoxon sulfoxide) was performed using an HPLC gradient system elution at a flow rate of 1 mL/min with a mobile phase composed of (A) 24% isopropanol and 76% hexane and (B) 100% isopropanol. The run consisted of a 3 min linear gradient from 100% A to 92% A, and 3–35 min linear gradient to 80% A. Peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 237 nm and quantified by integrating the area under the peaks and identified with co-elution of authentic standards (as it is shown in Fig. 2, the retention times observed were 7.2 for MMTP, 10.1 for fenoxon, 12.9 min for fenthion, 13.6 for *S*-fenoxon sulfoxide, 14.1 for *S*-fenthion sulfoxide, 14.7 for *R*-fenoxon sulfoxide and 16.2 for *R*-fenthion sulfoxide; and the recovery of each varied from 95.1 to 98.7%). The detection limit of each metabolite was 0.3 pmol/min/mg protein.

CYP inhibition studies were performed by co-incubation of microsomal fractions with ketoconazole (500 μ M) and FMO was inhibited by the co-incubation of methimazole (500 μ M). Inhibitors were added immediately after NADPH with the subsequent addition of fenthion after 2–3 min. Microsomal fractions of liver, gills and olfactory tissues from FA fish were also incubated *in vitro* with different concentrations of tetraethylpyrophosphate (TEPP), a general carboxylesterase inhibitor (Ross et al., 2006) in order to evaluate the contribution of carboxylesterases to MMTP production. TEPP has been recently shown to significantly inhibit the cleavage of the pyrethroid, permethrin in rainbow trout hepatocytes (Schlenk et al. unpublished).

CYP1A1, CYP2M1, CYP2K1 and CYP3A27 immunoblot evaluations

CYP1A, CYP2K1, CYP2M1 and CYP3A27 protein levels were determined by Western blot as described in Lavado et al. (2004) with minor modifications. Briefly, microsomal fractions were boiled for 5 min in SDS-PAGE buffer (Laemmli, 1970), and 40 μ g of protein were separated by electrophoresis using 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. They were probed using a 1:1000 dilution (v/v) of primary mouse anti-rainbow trout monoclonal CYP1A antibodies from Biosense (Bergen, Norway), 1:500 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP2M1, 1:500 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP2K1 and 1:1000 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP3A27 antibodies provided by Dr. D. R. Buhler, Oregon State University. Blots were incubated at room temperature overnight and rinsed three times with Tris-buffered saline containing 0.2% Tween 20 (v/v) and 0.5% gelatin (w/v). The membrane was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG, the excess of secondary antibody was removed, and immunoreactive bands were visualized by incubation with the substrates *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate from a commercial alkaline phosphatase conjugation kit (BioRad, Hercules, CA). Semi-quantification by densitometry was carried out using Quantity One (Biorad) in a Molecular Imager Gel Doc XR System (Biorad) image analyzer and are presented as optical density units/mg protein.

[¹⁴C]Testosterone hydroxylase activity

Testosterone hydroxylation activities were measured as described in Martin-Skilton et al. (2006). Briefly, 0.4 mg hepatic microsomal protein was incubated with 2 μ M [¹⁴C]testosterone (150 μ Ci/ μ mol; 97.6% purity) and 300 μ M NADPH in a final volume of 0.25 mL of 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4. Samples were incubated for 1 h at 25 °C. Incubations were stopped by adding 250 μ L of acetonitrile and after centrifugation (10,000 g; 10 min), 200 μ L of supernatant was injected onto a reverse-phase HPLC column. HPLC analyses were performed on a SCL-10AVP Shimadzu HPLC system equipped with a 250 $\times$ 4.6 mm Atlantis C18 (5 μ m) reverse-phase column (Waters, Milford, MA). Separation of testosterone metabolites employed an HPLC gradient system elution at a flow rate of 1 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile. The run consisted of a 40 min linear gradient from 100% A to 100% B, 40–45 min 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a radioflow detector β -ram Model 3 (INUS Systems Inc., Tampa, FL) using In-Flow 2:1 (INUS Systems Inc.) as scintillation cocktail. Metabolites (6 β - and 16 β -hydroxytestosterone) were identified by co-chromatography with authentic standard compounds and quantified by integrating the area under the radioactive peaks (recovery was from 98.9% to 99.7% for each metabolite and the detection limit was 0.2 pmol/min/mg protein).

Statistical analyses

Statistical significance was assessed using a one-way ANOVA to evaluate differences between groups, with the use of a SPSS v15.0 software package. 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 test or Student's *t*-test were performed to determine differences between groups. All data was analysed prior to statistical analysis to meet the homocedasticity and normality assumptions of parametric tests. If data failed these initial tests, non-parametric tests were utilized (Kruskal–Wallis test and two-tailed multiple comparison Dunn's test).

Results

Microsomal oxidation of fenthion to the oxon and sulfoxide was observed in liver and gills but not in olfactory tissues (Table 1). All metabolites were stable throughout the 60 min incubation with no observed hydrolysis of sulfoxides or fenoxon observed (data not shown). When liver microsomes from FA fish were incubated with fenthion, fenoxon production was lower compared to total production of fenthion sulfoxide (Table 1). In gills, fenoxon and fenthion sulfoxide production were up to 2.2-fold lower compared to liver (*P*<0.01). In olfactory tissues, no sulfoxidation of fenthion was observed. In all tissues, NADPH-independent and NADPH-dependent cleavage of fenthion was observed (Table 1). NADPH-independent production of MMTP from fenthion was higher in gills and olfactory tissues than in liver (*P*<0.01). NADPH-dependent cleavage of fenthion was significantly higher in olfactory tissues than in liver and gills (*P*<0.01).

When microsomes were incubated with fenoxon (Table 1), total sulfoxidation to fenoxon sulfoxide was higher in liver and gills when compared to sulfoxidation of fenthion (up to 1.8-fold). This activity was also higher in liver compared to gills. Sulfoxidation was not observed in olfactory tissues. As observed for fenthion, NADPH-independent and dependent cleavage of fenoxon was detected. Higher NADPH-independent MMTP production from fenoxon was observed in gills. In olfactory tissues the NADPH-dependent activity was 1.7-fold and 2.2-fold higher compared to liver and gill tissues (*P*<0.01), respectively.

When each fenthion sulfoxide enantiomer was used as a substrate (*S*- or *R*-), minimal production of the corresponding fenoxon sulfoxide was observed in microsomal fractions from liver and gills (see Table 1). The rate of fenoxon sulfoxide formation was lower than the oxidation of fenthion or fenoxon but still 2 to 3-fold higher in liver than in gills. There were no significant stereoselective differences in fenoxon sulfoxide production from the individual sulfoxide enantiomers.

In terms of stereoselective sulfoxidation of fenthion, the ratios of each enantiomer formed in liver and gill microsomes from FA fish favoured the formation of the *S*-fenthion sulfoxide. The observed ratio in both tissues was 65% *S*-fenthion sulfoxide and 35% *R*-fenthion sulfoxide. When microsomes isolated from both tissues were incubated with fenoxon, the observed ratio towards its fenoxon sulfoxides was different from the ratio for fenthion (74% *S*-fenoxon sulfoxide and 26% *R*-fenoxon sulfoxide; $P < 0.05$).

As a positive control, biotransformation of fenthion and its metabolites was evaluated using recombinant human FMO1. When incubated with 100 μM fenthion, only *R*-fenthion sulfoxide production was observed (204.2 ± 34.6 pmol/min/mg protein). When fenoxon was used as substrate, the *R*-fenoxon sulfoxide was the only metabolite produced and the rate of conversion to fenoxon sulfoxide was significantly higher (293.6 ± 15.4 pmol/min/mg protein) than the conversion from fenthion to fenthion sulfoxide. Fenoxon sulfoxides were not produced from fenthion sulfoxide.

NADPH-independent or dependent cleavage of fenthion or related metabolites was not observed in cytosolic incubations isolated from liver, gills and olfactory rosettes, suggesting that no cytosolic enzymes (i.e. cytosolic esterases) were involved in detoxification pathways of this pesticide (data not shown).

The kinetics of fenthion metabolism was characterized in liver microsomes from FA fish and HA fish. As shown in Table 2, conversion to different metabolites indicated varied catalytic efficiencies. NADPH-independent MMTP production showed the highest apparent K_m (292.0 ± 66.6 μM), and the lowest V_{max} . The apparent K_m for fenthion sulfoxide production was 1.8 times higher than that for fenoxon production. In FA fish, higher catalytic efficiency was observed for the formation of NADPH-dependent MMTP followed by fenoxon production. However, in HA fish, a dramatic shift was observed to the formation of sulfoxides with efficiency of *S*-sulfoxide formation being approximately 3-fold higher than the *R*-sulfoxide. The efficiency of *R*-sulfoxide formation was 2-fold greater than fenoxon.

Hypersaline conditions significantly increased total fenthion biotransformation by liver microsomes in rainbow trout (Table 3). Rates of fenoxon production were enhanced more than 1.5-fold in HA fish. The rate of fenthion sulfoxide production (*S*- and *R*-) was also enhanced nearly 3-fold in HA fish relative to FA fish. The formation of fenoxon sulfoxides (*S*- and *R*-) from fenthion was observed in liver microsomes from HA fish but not from FA fish. The NADPH-dependent production of MMTP was reduced up to 7-fold in liver microsomes of HA fish when compared to FA fish ($P < 0.01$). In contrast, hypersaline conditions failed to alter the NADPH-independent cleavage of fenthion. Incubations with fenoxon, produced significantly higher rates of sulfoxide formation in HA fish (up to 2.4-fold and 2.8-fold for *S*- and *R*-fenoxon sulfoxide, respectively). Also, the production of *S*- and *R*-fenoxon sulfoxides from fenthion sulfoxide increased with hypersalinity. When liver microsomes from HA fish were incubated with fenoxon, the NADPH-dependent production of MMTP was significantly reduced (up to 14-fold) compared with FA fish ($P < 0.01$), and the NADPH-independent activity remained unchanged.

In microsomal fractions isolated from gills of HA fish incubated with fenthion, fenoxon production remained unchanged compared to freshwater-treated fish, and *S*- and *R*-fenthion sulfoxidation was significantly reduced by 1.8-fold and 2-fold, respectively ($P<0.01$) (Table 4). The same trend was observed when gill microsomes from HA fish were incubated with fenoxon with *S*- and *R*-fenoxon sulfoxide production significantly reduced 2-fold and 2.2-fold, respectively ($P<0.01$). The respective production of *S*- and *R*-fenoxon sulfoxides from *S*- and *R*-fenthion sulfoxides was also reduced by hypersaline conditions to levels below detection (<0.3 pmol/min/mg protein). As in liver microsomal fractions, NADPH-independent production of MMTP remained unchanged in HA fish, but the NADPH-dependent formation of MMTP was significantly enhanced ($P<0.01$).

In olfactory tissues, sulfoxidation of fenthion and related metabolites was not detected in HA fish (data not shown). MMTP was produced through NADPH-independent and NADPH-dependent pathways from fenthion and fenoxon, but was unaffected by hypersaline exposure ($P>0.05$).

In terms of stereoselective sulfoxidation of fenthion, the synthesis ratios of each enantiomer remained unchanged (65% and 35%, *S*-fenthion sulfoxide and *R*-fenthion sulfoxide, respectively) in microsomes from liver and gills of HA fish relative to FA fish. In addition, hypersaline conditions failed to alter the stereoselectivity of fenoxon sulfoxidation in either liver or gill.

In liver microsomes, fenoxon formation was significantly reduced by co-incubation with ketoconazole in FA fish and HA fish, 67% and 60%, respectively (Table 5). Ketoconazole (500 μ M) also inhibited testosterone hydroxylase below detection (<0.2 pmol/min/mg protein; data not shown). Water treatments did not cause fish to alter stereoisomer production. Ketoconazole failed to alter fenthion sulfoxide enantiomeric ratios when reactions were performed with microsomes isolated from FA or HA fish. Co-incubation with methimazole failed to alter fenoxon formation, but reduced total sulfoxide formation 28% and 35% in FA and HA fish, respectively. In contrast to ketoconazole, enantiomeric ratios were significantly altered from 65% *S*-fenthion sulfoxide to 75% *S*-fenthion sulfoxide specifically through the reduction of the *R*-sulfoxide in HA fish. Ratios were unchanged in FA fish. NADPH-independent and NADPH-dependent MMTP production was unaltered by exposure to ketoconazole or methimazole.

In all three tissues from FA fish, 100 μ M TEPP completely inhibited the NADPH-independent cleavage of fenthion to MMTP (<0.3 pmol/min/mg protein) (Table 6). NADPH-dependent MMTP production was not altered at this TEPP concentration. However, co-incubation with 500 μ M of TEPP led to a range of 46.4–57.3% inhibition of NADPH-dependent hydrolysis in microsomes of each tissue. Co-incubation with 1 mM TEPP reduced NADPH-dependent MMTP production to levels below detection all three tissues (Table 6).

Immunoblots of microsomes from liver showed that CYP1A levels were reduced 2-fold in HA fish ($P<0.01$) (Figs. 3 and 4). CYP2K1 and CYP2M1 content remained unchanged in liver microsomes from animals from both exposure groups. Expression of hepatic CYP3A27 was significantly increased ($P<0.01$) in HA fish. The formation of 6 β -hydroxytestosterone and 16 β -hydroxytestosterone from testosterone was also increased (up to 2.6-fold and up to 1.5-fold, respectively) in liver microsomes from HA fish (Fig. 5).

Discussion

Freshwater conditions

Liver microsomes from FA fish were more effective in the oxidative biotransformation of fenthion relative to the gill and olfactory tissues. Few studies have explored olfactory tissue as a target for biotransformation of pesticides, even though pesticides clearly disrupt olfaction and resultant behaviour in salmonids (Scholtz et al., 2000). Neither fenoxon nor fenthion was converted to sulfoxide or oxon metabolites in olfactory tissue, although CYP isoforms (CYP1A, CYP2K1, CYP2M1, CYP3A27) and FMO activities were previously identified in olfactory tissues of Coho salmon (*Oncorhynchus kisutch*) (Matsuo et al., 2008). *S*-oxidation by FMO has been reported in olfactory tissue in mammals at levels comparable to liver (Genter et al., 1995). Although neither *S*-oxidation nor oxidative desulfuration of the phosphorothionate were detected in olfactory tissues from rainbow trout, hydrolytic cleavage of fenthion and fenoxon was observed, which may allow detoxification in this tissue through the elimination of AChE inhibition.

Fenthion has been shown to be readily hydrolyzed in plants and mammals (Huang and Mabury, 2000). In the current study, microsomal NADPH-independent and NADPH-dependent esterases were involved in the cleavage of fenthion and fenoxon, to the metabolite, MMTP. Hydrolysis was not observed in cytosolic incubations (data not shown). Esterase activity was observed in each of the three tissues examined and incubations with ketoconazole, a CYP inhibitor, in liver microsomes did not reduce conversion indicating CYP may not be a likely candidate for the catalytic activity. NADPH-independent activity was highest in gill but NADPH-dependent activity was higher in olfactory tissue than in liver and gills. The differential inhibition of NADPH-independent and NADPH-dependent production of MMTP due to the carboxylesterase inhibitor TEPP suggests that NADPH-independent fenthion cleavage could be due to classic microsomal carboxylesterases. In mammals, the nature of microsomal esterases in liver have been extensively studied (Huang et al., 1996; Jewell et al., 2007), and research on a limited number of ester substrates indicates that fish have measurable esterase activity (Barron et al., 1999). Non CYP esterase activity through NADPH-dependent catalysis has been described in mammals, where microsomal carboxylesterases hydrolyzed pyrrolidizine alkaloids in a NADPH-dependent manner (Tang et al., 2007).

In addition to esterase cleavage, sulfoxidation of thioether anticholinesterase pesticides may also allow detoxification as the sulfoxide metabolites are less potent inhibitors of cholinesterases (Furnes and Schlenk, 2003; Henderson et al., 2004). However, formation of the sulfoxide metabolites of fenthion generates a chiral structure such that two enantiomeric sulfoxides (*R*- and *S*-) are created. Subsequent oxidation of the sulfoxide to the oxon sulfoxide could actually lead to bioactivation as fenoxon *R*-sulfoxide was shown to be significantly more potent than the *S*-enantiomer or fenoxon (Gadepalli et al., 2007). Although there was no significant difference in catalytic efficiency ($V_{\max}/K_m$) between oxon and sulfoxide formation from fenthion in liver microsomes from FA fish, the oxidation of fenthion sulfoxide to fenoxon sulfoxide was lower than the formation of the same metabolite from fenoxon indicating that fenthion bioactivation may occur through initial oxidation of fenthion to the oxon with subsequent sulfoxidation to the fenoxon sulfoxide in freshwater animals. Because there was no significant difference in the formation of fenoxon sulfoxide between incubations with fenthion *R*-sulfoxide or fenthion *S*-sulfoxide, a stereoselective preference for oxon sulfoxide did not appear to occur in rainbow trout tissues.

In a reconstituted system with human recombinant FMO1, it stereoselectively converted fenthion to the *R*-sulfoxide with much higher turnover than FMO3, which formed the *S*-sulfoxide in 75% excess (Furnes and Schlenk, 2003; Furnes and Schlenk, 2004). In trout,

stereoselective metabolism was found with approximately 65% *S*-sulfoxide and 35% *R*-sulfoxide formed in liver and gills. These results are consistent with other studies in liver microsomes of rainbow trout where a 65:35 ratio was observed with fenthion and another thioether, *p*-tolyl sulfide (Schlenk, et al., 2004; Bawardi et al., 2007). In liver microsomes from freshwater rainbow trout, co-incubation with the FMO inhibitor, methimazole failed to alter this ratio although a 28% reduction in overall sulfoxidation was observed indicating that FMOs may have some role in the biotransformation of fenthion to sulfoxides.

FMOs are generally limited to the formation of sulfoxides from thioethers (Hajjar and Hodgson, 1980), whereas CYP enzymes 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 (Dauterman, 1971). A study on oxidative organophosphate metabolism by CYP and FMO isoforms found sulfoxidation of several thioether pesticides in human liver microsomes to be mainly CYP-driven (85–90%), with the remainder accounted for by FMO (Usmani et al., 2004). In goldfish liver, sulfoxidation turnover was relatively low and only weakly affected by both inhibiting CYP and FMO isoforms, suggesting a role for both systems in fenthion sulfoxidation (Kitamura et al., 2003). In the current study, ketoconazole significantly inhibited the formation of oxon and sulfoxides in liver microsomes of FA and HA fish indicating CYP may contribute more to fenthion sulfoxidation than FMO. However, absolute inhibition of desulfuration and sulfoxidation activity was not observed indicating the possible contribution of additional CYPs that are resistant against ketoconazole or the contribution of FMO to at least sulfoxidation. While ketoconazole is an effective inhibitor of several known isoforms of CYP in trout (Miranda et al., 1998), it may not inhibit all CYP forms. Additional studies to identify additional CYP orthologs should help in determining the contributions of each form to the oxidative metabolism of fenthion as well as other substrates. In summary, fenthion appears to primarily undergo hydrolytic cleavage in liver, gill and olfactory tissues of FA fish with some activation to the fenoxon primarily in the liver. Sulfoxidation is also catalyzed in liver and gill microsomes and, like hydrolysis, appears to be a detoxification strategy.

Hypersaline activation

Fenthion and other thioether anticholinesterases are more acutely toxic in several fish species, such as rainbow trout, that have been acclimated to hypersaline conditions. A unique feature of FMO within rainbow trout is its induction during acclimation to hypersaline conditions which require organic osmolytes such as trimethylamine N-oxide to counter increases in osmotic pressure as well as intracellular urea (Schlenk, 1998; Larsen and Schlenk, 2001). 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 (Rodriguez-Fuentes et al., 2008). The transcript was not observed in the gill. Chemicals which are biotransformed to more toxic intermediates by FMO tend to be more toxic in species that up-regulate FMO in response to hypersaline conditions like rainbow trout and Japanese medaka (El-Alfy and Schlenk, 1998; El-Alfy et al., 2001; Wang et al., 2001). 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 anticholinesterase (Wang et al., 2001). Although enhanced toxicity was previously observed in rainbow trout exposed to fenthion under hypersaline conditions, relationships with stereoselective sulfoxidation was not observed (Bawardi et al., 2007). Unfortunately, the previous study failed to examine the contribution of other bioactive metabolites, such as fenoxon and fenoxon sulfoxide, or the biotransformation of fenthion in other tissues.

In contrast to earlier studies in rainbow trout and Japanese medaka with the anticholinesterase thioether pesticide, aldicarb, hypersaline conditions failed to enhance sulfoxidation of fenthion in the gill. In fact, hypersaline conditions caused overall reductions in sulfoxidation and increased esterase cleavage of fenthion in gill microsomes. Hypersaline conditions also failed to alter biotransformation in olfactory tissues. However in liver, hypersaline conditions led to significant increases in the rates and catalytic efficiencies of oxidative biotransformation of sulfoxides as well as activated metabolites, fenoxon and fenoxon sulfoxide, and a reduction in the detoxifying cleavage reaction. Significant increases in the catalytic efficiency for sulfoxide formation under hypersaline conditions indicates sulfoxidation may not be a detoxification pathway. These data coupled with the failure of hypersaline conditions to increase fenoxon production suggests fenthion sulfoxide formation may precede fenoxon sulfoxide production and that sulfoxidation may be a critical step in the bioactivation of fenthion under hypersaline conditions in salmonids.

As observed in FA fish liver microsomes, co-incubation with the FMO inhibitor, methimazole caused a general reduction in overall sulfoxidation. However, in microsomes from HA fish, a more pronounced and stereoselective reduction of the *R*-sulfoxide was observed with methimazole co-incubation changing the *S*:*R* ratio to 75%. FMO1 stereoselectively catalyzed the formation of the *R*-sulfoxide of fenthion (Furnes and Schlenk 2003, 2004; and positive control this study). Neither enantiomer of fenoxon sulfoxide was observed in FA fish liver microsomal reactions. However, both enantiomers were observed after incubation in liver microsomes from HA fish. As fenoxon *R*-sulfoxide is the most potent anticholinesterase metabolite, one or more forms of hepatic FMO may contribute to the bioactivation of fenthion in hypersaline conditions likely through the sulfoxidation of fenoxon. It should also be noted that co-incubation with ketoconazole also diminished fenoxon sulfoxide formation below detection indicating both enzymes may contribute to fenoxon sulfoxide formation.

Hypersaline conditions increased the rates and efficiency of fenoxon formation as well as fenoxon sulfoxide formation from incubations using fenoxon as starting substrate. This rate of fenoxon sulfoxide formation was higher than the rates of formation when incubations used fenthion sulfoxide as the initial substrate. Formation of the fenoxon and the sulfoxide in liver microsomes from HA fish was more dramatically reduced after co-incubation of liver microsomes with ketoconazole relative to methimazole. These results indicate CYP may play more of a role in the bioactivation of fenthion not only through the formation of the fenoxon sulfoxide through sulfoxidation, but also by fenoxon sulfoxide formation through phosphothiolate desulfuration of fenthion sulfoxide under hypersaline conditions.

In order to identify the specific CYP isoform(s) potentially responsible for the enhanced catalysis of fenthion, expression of three CYP isoforms were evaluated by western blotting. Expression and catalytic activity of CYP3A27 was enhanced in HA fish and CYP1A was diminished. CYP3A expression is highly correlated with cortisol levels (Celander, 1999) which are augmented following hypersalinity acclimation (McLean et al., 1997; Arjona et al., 2007). The glucocorticoid, dexamethasone inhibited CYP1 protein in rainbow trout liver (Lee et al., 1993) and cortisol has also been shown to repress CYP1A in fish (Jorgensen et al., 2001). Enhanced expression of CYP3A27 under hypersaline conditions was confirmed by augmented testosterone hydroxylase activities which are catalyzed by CYP3A27 (Miranda et al., 1989). In human liver, CYP3A4 catalyzed the highest turnover of fenthion relative to other CYPs and FMO (Buratti et al., 2006). Expression of CYP3A4, CYP3A7, and CYP3A5 were all enhanced by hypertonicity in human derived cell lines and human primary colonic cells (Kosuge et al., 2007). Thus, enhanced expression of CYP3A27 in rainbow trout may explain the higher conversion of fenoxon to fenoxon sulfoxide or the conversion of fenthion sulfoxide to fenoxon sulfoxide in hypersaline animals. Since these

metabolites are more potent cholinesterase inhibitors (specifically the *R*-sulfoxide enantiomer of fenoxon), this may be the putative pathway of fenthion activation under hypersaline conditions.

In summary, hypersaline conditions increased the formation of fenoxon from fenthion and fenoxon sulfoxide from fenoxon, and reduced fenthion esterase cleavage in liver microsomes from rainbow trout. This could be an important mechanism of increasing toxicity since fenoxon and the *R*-enantiomer of fenoxon sulfoxide are the most potent inhibitors of cholinesterases relative to fenthion and its metabolites (Gadepalli et al., 2007). In gills, hypersalinity acclimation reduced the formation of sulfoxides and increased fenthion esterase activity. In olfactory tissues, sulfoxidation was not detected, but higher fenthion esterase activity was observed. The predominant pathway of fenthion activation by hypersaline conditions appears to be initiated either through formation of fenoxon which is an active metabolite or fenthion sulfoxide which may be further oxygenated to the most toxic metabolite *R*-fenoxon sulfoxide. Accordingly, fenoxon itself may also be subsequently converted to the most toxic metabolite fenoxon *R*-sulfoxide. Similar to FMO, hypersalinity acclimation increased the content and catalytic activity of CYP3A27 in liver microsomes. These results indicate that CYP3A27 as well as FMO may contribute to enhanced fenthion oxidative metabolism and subsequent toxicity of fenthion to rainbow trout under hypersaline conditions.

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References

- Arjona FJ, Vargas-Chacoff L, Ruiz-Jarabo I, del Rio MPM, Mancera JM. Osmoregulatory response of Senegalese sole (*Solea senegalensis*) to changes in environmental salinity. *Comp Biochem Physiol A, Mol Integr Physiol* 2007;148:413–421. [PubMed: 17604667]
- Barron MG, Charron KA, Stott WT, Duvall SE. Tissue carboxylesterase activity of rainbow trout. *Environ Toxicol Chem* 1999;18:2506–2511.
- Bawardi O, Rimoldi J, Schlenk D. Impacts of hypersaline water on the bio-transformation and toxicity of fenthion rainbow trout (*Oncorhynchus mykiss*), striped bass (*Morone saxatilis*×*Morone chrysops*) and tilapia (*Oreochromis mossambicus*). *Pest Biochem Physiol* 2007;88:321–327.
- Buratti FM, Leoni C, Testai E. Foetal and adult human CYP3A isoforms in the bioactivation of organophosphorothionate insecticides. *Toxicol Lett* 2006;167:245–255. [PubMed: 17110060]
- Cavanna S, Molinari GP. Residues of fenthion and trichlorofon in olives and olive oil after olive tree treatments. *Food Addit Contam* 1998;15:518–527. [PubMed: 9829035]
- Celander, M. Impact of stress on animal toxicology. In: Balm, PHM., editor. *Stress Physiology in Animals*. Sheffield Academic Press; Sheffield, UK: 1999. p. 246-279.
- Costa LG. Current issues in organophosphate toxicology. *Clin Chim Acta* 2006;366:1–13. [PubMed: 16337171]
- Cova D, Perego R, Nebuloni C, Fontana G, Molinari GP. *In vitro* cytotoxicity of fenthion and related metabolites in human neuroblastoma cell lines. *Chemosphere* 1995;30:1709–1715. [PubMed: 7757428]
- Dauterman WC. Biological and non-biological modifications of organophosphorus compounds. *Bull World Health Organ* 1971;44:133. [PubMed: 4938021]
- Dwyer FJ, Burch SA, Ingersoll CG, Hunn JB. Toxicity of trace element and salinity mixtures to striped bass (*Morone saxatilis*) and *Daphnia magna*. *Environ Toxicol Chem* 1992;11:513–520.

- El-Alfy A, Schlenk D. Potential mechanisms of the enhancement of aldicarb toxicity to Japanese medaka, *Oryzias latipes*, at high salinity. *Toxicol Appl Pharmacol* 1998;152:175–183. [PubMed: 9772213]
- El-Alfy AT, Grisle S, Schlenk D. Characterization of salinity-enhanced toxicity of aldicarb to Japanese medaka: sexual and developmental differences. *Environ Toxicol Chem* 2001;20:2093–2098. [PubMed: 11521840]
- Furnes B, Schlenk D. Expression and functional characterization of novel human flavin-containing monooxygenase 1 (FMO1) variants. *Drug Metab Rev* 2003;35:202.
- Furnes B, Schlenk D. Evaluation of xenobiotic *N*- and *S*-oxidation by variant flavin-containing monooxygenase 1 (FMO1) enzymes. *Toxicol Sci* 2004;78:196–203. [PubMed: 14976351]
- Gadepalli R, Rimoldi JM, Fronczek FR, Nillos M, Gan J, Deng X, Rodriguez-Fuentes G, Schlenk D. Synthesis of fenthion sulfoxide and fenoxon sulfoxide enantiomers: effect of sulfur chirality on acetylcholinesterase activity. *Chem Res Toxicol* 2007;20:257–262. [PubMed: 17253727]
- Genter MB, Deamer NJ, Blake BL, Wesley DS, Levi PE. Olfactory toxicity of methimazole: dose–response and structure–activity studies and characterization of flavin-containing monooxygenase activity in the Long–Evans rat olfactory mucosa. *Toxicol Pathol* 1995;23:477–486. [PubMed: 7501959]
- Hajjar NP, Hodgson E. Flavin adenine dinucleotide-dependent monooxygenase —its role in the sulfoxidation of pesticide in mammals. *Science* 1980;209:1134–1136. [PubMed: 7403873]
- Hall LW, Anderson RD. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. *Crit Rev Toxicol* 1995;25:281–346. [PubMed: 7576155]
- Henderson MC, Krueger SK, Siddens LK, Stevens JF, Williams DE. *S*-Oxygenation of the thioether organophosphate insecticides phorate and disulfoton by human lung flavin-containing monooxygenase 2. *Biochem Pharmacol* 2004;68:959–967. [PubMed: 15294458]
- Hirahara Y, Ueno H, Nakamuro K. Comparative photodegradation study of fenthion and disulfoton under irradiation of different light sources in liquid- and solid-phases. *J Health Sci* 2001;47:129–135.
- Huang JP, Mabury SA. Hydrolysis kinetics of fenthion and its metabolites in buffered aqueous media. *J Agric Food Chem* 2000;48:2582–2588. [PubMed: 10888588]
- Huang TL, Shiotsuki T, Uematsu T, Borhan B, Li QX, Hammock BD. Structure–activity relationships for substrates and inhibitors of mammalian liver microsomal carboxylesterases. *Pharm Res* 1996;13:1495–1500. [PubMed: 8899840]
- Jewell C, Ackermann C, Payne NA, Fate G, Voorman R, Williams FM. Specificity of procaine and ester hydrolysis by human, minipig, and rat skin and liver. *Drug Metab Disp* 2007;35:2015–2022.
- Jorgensen EH, Celander M, Goksoyr A, Iwata M. The effect of stress on toxicant-dependent cytochrome p450 enzyme responses in the Arctic charr (*Salvelinus alpinus*). *Environ Toxicol Chem* 2001;20:2523–2529. [PubMed: 11699778]
- Kitamura S, Kadota T, Yoshida M, Jinno N, Ohta S. Whole-body metabolism of the organophosphorus pesticide, fenthion, in goldfish, *Carassius auratus*. *Comp Biochem Physiol C, Pharmacol Toxicol Endocrinol* 2000;126:259–266.
- Kitamura S, Suzuki T, Kadota T, Yoshida M, Ohashi K, Ohta S. *In vitro* metabolism of fenthion and fenthion sulfoxide by liver preparations of sea bream, goldfish, and rats. *Drug Metab Disp* 2003;31:179–186.
- Kosuge K, Chuang AI, Uematsu S, Tan KP, Ohashi K, Ko BCB, Ito S. Discovery of osmosensitive transcriptional regulation of human cytochrome P4503As by the tonicity-responsive enhancer binding protein (nuclear factor of activated T cells 5). *Mol Pharmacol* 2007;72:826–837. [PubMed: 17600221]
- Laemmli UK. Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* 1970;227:680. [PubMed: 5432063]
- Larsen BK, Schlenk D. Effect of urea and low temperature on the expression and activity of flavin-containing monooxygenase in the liver and gill of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol Biochem* 2001;25:19–29.
- Lavado R, Thibaut R, Raldua D, Martin R, Porte C. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol Appl Pharmacol* 2004;196:247–257. [PubMed: 15081271]

- Lee PC, Yoon HI, Haasch ML, Lech JJ. Negative control of cytochrome P450 1A1 (CYP1A1) by glucocorticoids in rainbow trout liver. *Comp Biochem Physiol C, Pharmacol Toxicol Endocrinol* 1993;104:457–461.
- Martin-Skilton R, Lavado R, Thibaut R, Minier C, Porte C. Evidence of endocrine alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. *Environ Pollut* 2006;141:60–68. [PubMed: 16203071]
- Matsuo AYO, Gallagher EP, Trute M, Stapleton PL, Lavado R, Schlenk D. Characterization of phase I biotransformation enzymes in Coho salmon (*Oncorhynchus kisutch*). *Comp Biochem Physiol C, Toxicol Pharmacol* 2008;147:78–84. [PubMed: 17826357]
- McLean E, Devlin RH, Byatt JC, Clarke WC, Donaldson EM. Impact of a controlled release formulation of recombinant bovine growth hormone upon growth and seawater adaptation in Coho (*Oncorhynchus kisutch*) and Chinook (*Oncorhynchus tshawytscha*) salmon. *Aquaculture* 1997;156:113–128.
- Miranda CL, Wang JL, Henderson MC, Buhler DR. Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. *Arch Biochem Biophys* 1999;268:227–238. [PubMed: 2536262]
- Miranda CL, Henderson MC. Evaluation of chemicals as inhibitors of trout cytochrome P450s. *Toxicol Appl Pharmacol* 1998;148:237–244. [PubMed: 9473531]
- Mullie WC, Diallo AO, Gadjji B, Ndiaye MD. Environmental hazards of mobile ground spraying with cyanophos and fenthion for quelea control in Senegal. *Ecotoxicol Environ Saf* 1999;43:1–10. [PubMed: 10330314]
- Roberts, TR.; Hutson, DH. Insecticides and fungicides. In: Roberts, TR.; Hutson, DH.; Lee, PW.; Nichols, PH.; Plimmer, JR., editors. *Metabolic Pathways of Agrochemicals*. The Royal Society of Chemistry; Cambridge, UK: 1999. p. 326-331.
- Rodriguez-Fuentes G, Aparicio-Fabre R, Li Q, Schlenk D. Osmotic regulation of a novel flavin-containing monooxygenase in primary cultured cells from rainbow trout (*Oncorhynchus mykiss*). *Drug Metab Disp* 2008;36:1212–1217.
- Ross MK, Borazjani A, Edwards CC, Potter PM. Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. *Biochem Pharmacol* 2006;71:657–669. [PubMed: 16387282]
- Roux D, Jooste S, Truter E, Kempster P. An aquatic toxicological evaluation of fenthion in the context of finch control in South-Africa. *Ecotoxicol Environ Saf* 1995;31:164–172. [PubMed: 8521783]
- Schlenk D. Occurrence of flavin-containing monooxygenases in non-mammalian eukaryotic organisms. *Comp Biochem Physiol C, Toxicol Pharmacol* 1998;121:185–195.
- Schlenk D, Yeung C, Rettie A. Unique monooxygenation pattern indicates novel flavin-containing monooxygenase in liver of rainbow trout. *Mar Environ Res* 2004;58:499–503. [PubMed: 15178073]
- Scholtz NL, Truelove NK, French BL, Berejikian BA, Quinn TP, Casillas E, Collier TK. Diazinon disrupts antipredator and homing behaviors in Chinook salmon (*Oncorhynchus tshawytscha*). *Can J Fish Aquat Sci* 2000;57:1911–1918.
- Schoor WP, Middaugh DP, Marcovich DT. Effects of aerially applied fenthion on survival and reproduction of the panacea sand fiddler, *Uca panacea*, in laboratory habitats. *Arch Environ Contam Toxicol* 2000;38:327–333. [PubMed: 10667930]
- Tang J, Akao T, Nakamura N, Wang ZT, Takagawa K, Sasahara M, Hattori M. *In vitro* metabolism of isoline, a pyrrolizidine alkaloid from *Ligularia duciformis*, by rodent liver microsomal esterase and enhanced hepatotoxicity by esterase inhibitors. *Drug Metab Dispos* 2007;35:1832–1839. [PubMed: 17639025]
- Tsuda T, Kojima M, Harada H, Nakajima A, Aoki S. Accumulation and excretion of fenthion, fenthion sulfoxide and fenthion sulfone by killifish (*Oryzias latipes*). *Comp Biochem Physiol C, Pharmacol Toxicol Endocrinol* 1996;113:45–49.
- Tuler SM, Bowen JM. Chronic fenthion toxicity in laying hens. *Vet Hum Toxicol* 1999;41:302–307. [PubMed: 10509433]
- Usmani KA, Karoly ED, Hodgson E, Rose RL. *In vitro* sulfoxidation of thioether compounds by human cytochrome P450 and flavin-containing monooxygenase isoforms with particular reference to the CYP2C subfamily. *Drug Metab Dispos* 2004;32:333–339. [PubMed: 14977868]

- Vengosh, A. Salinization and saline environments. In: Holland, H.; Turekian, KK., editors. Treatise on Geochemistry. Elsevier; Amsterdam, Netherlands: 2004. p. 333-365.
- Wang J, Grisle S, Schlenk D. Effects of salinity on aldicarb toxicity in juvenile rainbow trout (*Oncorhynchus mykiss*) and striped bass (*Morone saxatilis* × *chrysops*). Toxicol Sci 2001;64:200–207. [PubMed: 11719702]
- Zutshi B, Murthy PS. Ultrastructural changes in testis of gobiid fish *Glossogobius giuris* (Ham) induced by fenthion. Indian J Exp Biol 2001;39:170–173. [PubMed: 11480215]

Fig. 1. Metabolites produced from fenthion in microsomes isolated from different tissues in rainbow trout (modified from Gadepalli et al., 2007) (FMO: Flavin monooxygenase; CYP: cytochrome P450).

Fig. 2.

Elution profile of metabolites produced from fenthion in microsomes isolated from different tissues in rainbow trout recorded at 237 nm. (1) 3-Methyl-4-(methylthio)-phenol (MMTP); (2) fenoxon; (3) fenthion; (4) *S*-fenoxon sulfoxide; (5) *S*-fenthion sulfoxide; (6) *R*-fenoxon sulfoxide and (7) *R*-fenthion sulfoxide.

Fig. 3.

CYP1A, CYP2K1, CYP2M1 and CYP3A27 protein determined by Western blot in microsomal fraction isolated from liver from freshwater- and hypersaline-acclimated rainbow trout (*Oncorhynchus mykiss*). Data expressed as mean $\pm$ SD ($n=4-6$ individuals with 2 replicates for each one). Significant differences with respect to freshwater-treated rainbow trout indicated by ** ($P<0.01$; Student's t -test).

Fig. 4. Western blots of CYP1A1 (A), CYP2K1 (B), CYP2M1 (C) and CYP3A27 (D)-like proteins in liver microsomal fractions from freshwater- (FAF) and hypersaline (HAF)-acclimated rainbow trout (*Oncorhynchus mykiss*). Protein load: 40 μ g/lane. Molecular weight of bands is shown by arrows.

Fig. 5. Testosterone hydroxylase activity (CYP3A27-related) in microsomal fractions isolated from liver in freshwater- and hypersaline-acclimated rainbow trout (*Oncorhynchus mykiss*). Data expressed as mean±SD ($n=8$ individuals with 3 replicates for each one). Significant differences with respect to freshwater-acclimated rainbow trout indicated by * ($P<0.05$; Student's t -test) and ** ($P<0.01$; Student's t -test).

Table 1

Biotransformation of fenthion and related metabolites in microsomal fractions isolated from liver, gills and olfactory tissues in freshwater-acclimated rainbow trout (*Oncorhynchus mykiss*)

Substrate (100 μ M)	Metabolite	Tissue		
		Liver	Gills	Olfactory tissues
Fenthion	Fenoxon	25.1 $\pm$ 4.5	11.7 $\pm$ 2.1	bdl
	S-fenthion sulfoxide	57.1 $\pm$ 7.9	21.2 $\pm$ 1.2	bdl
	R-fenthion sulfoxide	29.8 $\pm$ 0.4	18.0 $\pm$ 0.5	bdl
	S-fenoxon sulfoxide	bdl	bdl	bdl
	R-fenoxon sulfoxide	bdl	bdl	bdl
	MMTP (NADPH-independent)	7.5 $\pm$ 3.2	133.5 $\pm$ 5.8	71.2 $\pm$ 11.1
	MMTP (NADPH-dependent)	145.9 $\pm$ 4.4	117.5 $\pm$ 9.5	240.2 $\pm$ 28.0
Fenoxon	S-fenoxon sulfoxide	112.1 $\pm$ 5.6	60.5 $\pm$ 3.4	bdl
	R-fenoxon sulfoxide	38.4 $\pm$ 0.9	21.7 $\pm$ 6.3	bdl
	MMTP (NADPH-independent)	7.2 $\pm$ 1.2	129.9 $\pm$ 4.3	84.5 $\pm$ 9.1
	MMTP (NADPH-dependent)	158.3 $\pm$ 12.0	123.4 $\pm$ 6.2	269.3 $\pm$ 21.5
S-fenthion sulfoxide	S-fenoxon sulfoxide	6.1 $\pm$ 0.2	2.3 $\pm$ 0.2	bdl
R-fenthion sulfoxide	R-fenoxon sulfoxide	5.2 $\pm$ 1.1	2.9 $\pm$ 0.1	bdl

Data are presented as pmol/min/mg protein and as mean $\pm$ SD ($n=8$ individuals for liver and gill; $n=3$ replicates for each pooled sample for olfactory tissue).

MMTP: 3-methyl-4-(methylthio)-phenol.

bdl: below detection limit (<0.3 pmol/min/mg protein).

Table 2

Enzymatic kinetic characterization of fenthion metabolites in microsomal fractions isolated from liver from freshwater- and hypersaline-acclimated rainbow trout (*Oncorhynchus mykiss*) and incubated with fenthion

Metabolite	Freshwater			Hypersaline		
	K_m (μ M)	V_{max} (pmol/min/mg protein)	V_{max}/K_m	K_m (μ M)	V_{max} (pmol/min/mg protein)	V_{max}/K_m
MMTP (NADPH-independent)	292.0 $\pm$ 66.6	53.7 $\pm$ 4.8	0.18	652.0 $\pm$ 221.1	59.8 $\pm$ 10.5	0.09
MMTP (NADPH-dependent)	86.6 $\pm$ 37.8	199.2 $\pm$ 24.4	2.06	136.4 $\pm$ 23.1	35.1 $\pm$ 1.9	0.25
Fenoxon	115.7 $\pm$ 21.1	82.8 $\pm$ 4.6	0.72	187.2 $\pm$ 25.2	130.0 $\pm$ 6.1	0.69
S-fenthion sulfoxide	264.4 $\pm$ 38.2	173.4 $\pm$ 9.6	0.66	103.8 $\pm$ 9.4	355.7 $\pm$ 9.4	3.42
R-fenthion sulfoxide	293.3 $\pm$ 48.6	78.6 $\pm$ 5.2	0.27	142.0 $\pm$ 26.3	201.1 $\pm$ 11.9	1.41
S-fenoxon sulfoxide	ND	bdl	ND	114.5 $\pm$ 31.5	18.4 $\pm$ 1.1	0.16
R-fenoxon sulfoxide	ND	bdl	ND	132.6 $\pm$ 33.3	7.7 $\pm$ 0.6	0.06

Data are presented as mean $\pm$ SD ($n=8$ individuals with 2 replicates for each one).

MMTP: 3-methyl-4-(methylthio)-phenol.

ND: not determined.

bdl: below detection limit (<0.3 pmol/min/mg protein).

Table 3

Effects of hypersaline conditions on the *in vitro* biotransformation of fenthion in microsomes of liver from rainbow trout (*Oncorhynchus mykiss*)

		Treatment	
Substrate	Metabolite	Freshwater	Hypersaline
Fenthion (100 μM)			
	Fenoxon	20.5±2.2	42.2±4.1**
	<i>S</i> -fenthion sulfoxide	59.6±4.5	298.1±22.4**
	<i>R</i> -fenthion sulfoxide	27.6±2.5	138.3±12.4**
	<i>S</i> -fenoxon sulfoxide	bdl	8.2±0.3**
	<i>R</i> -fenoxon sulfoxide	bdl	3.4±0.4**
	MMTP (NADPH-independent)	7.4±2.9	6.4±2.3
	MMTP (NADPH-dependent)	132.2±10.3	21.1±10.5**
Fenoxon (100 μM)			
	<i>S</i> -fenoxon sulfoxide	112.1±5.6	268.7±4.0**
	<i>R</i> -fenoxon sulfoxide	38.4±0.9	106.8±2.2**
	MMTP (NADPH-independent)	7.2±1.2	9.2±3.4
	MMTP (NADPH-dependent)	158.3±12.0	10.5±6.1**
<i>S</i> -fenthion sulfoxide (100 μM)			
	<i>S</i> -fenoxon sulfoxide	6.1±0.2	12.2±3.2*
<i>R</i> -fenthion sulfoxide (100 μM)			
	<i>R</i> -fenoxon sulfoxide	5.2±1.1	9.2±0.3*

Data are expressed as pmol/min/mg protein and as mean±SD ($n=8$). Significant differences with respect to freshwater-acclimated rainbow trout indicated by ^{*} ($P<0.05$; Student's *t*-test) and ^{**} ($P<0.01$; Student's *t*-test).

MMTP: 3-methyl-4-(methylthio)-phenol.

bdl: below detection limit (<0.3 pmol/min/mg protein).

Table 4

Effects of hypersaline conditions on the *in vitro* biotransformation of fenthion in microsomes of gills from rainbow trout (*Oncorhynchus mykiss*)

		Treatment	
Substrate	Metabolite	Freshwater	Hypersaline
Fenthion (100 µM)			
	Fenoxon	11.7±2.1	12.1±0.2
	<i>S</i> -fenthion sulfoxide	21.5±1.2	11.8±0.8**
	<i>R</i> -fenthion sulfoxide	18.0±0.5	9.9±0.8**
	<i>S</i> -fenoxon sulfoxide	bdl	bdl
	<i>R</i> -fenoxon sulfoxide	bdl	bdl
	MMTP (NADPH-independent)	133.5±5.8	127.4±8.1
	MMTP (NADPH-dependent)	117.5±9.5	213.9±40.4*
Fenoxon (100 µM)			
	<i>S</i> -fenoxon sulfoxide	60.4±3.4	29.1±1.4**
	<i>R</i> -fenoxon sulfoxide	21.7±6.3	9.8±3.3*
	MMTP (NADPH-independent)	51.6±1.2	68.0±7.4
	MMTP (NADPH-dependent)	123.4±6.1	209.3±11.9**
<i>S</i> -fenthion sulfoxide (100 µM)			
	<i>S</i> -fenoxon sulfoxide	2.1±0.1	bdl
<i>R</i> -fenthion sulfoxide (100 µM)			
	<i>R</i> -fenoxon sulfoxide	2.9±0.1	bdl

Data are expressed as pmol/min/mg protein and as mean±SD ($n=8$ individuals with 3 replicates for each one). Significant differences with respect to freshwater-acclimated rainbow trout indicated by * ($P<0.05$; Student's *t*-test) and ** ($P<0.01$; Student's *t*-test).

MMTP: 3-methyl-4-(methylthio)-phenol.

bdl: below detection limit (<0.3 pmol/min/mg protein).

Table 5

The effects of the FMO inhibitor (methimazole, 500 μ M) and CYP inhibitor (ketoconazole, 500 μ M) on the biotransformation of fenthion in microsomal fractions isolated from liver from freshwater- and hypersaline-acclimated rainbow trout (*Oncorhynchus mykiss*)

Exposure	Metabolite	NADPH+ fenthion	NADPH+ methimazole+ fenthion	NADPH+ ketoconazole+ fenthion
Freshwater				
	Fenoxon	20.5 $\pm$ 2.2	23.0 $\pm$ 1.9	6.9 $\pm$ 1.3**
	S-Fenthion sulfoxide	59.6 $\pm$ 4.5	42.8 $\pm$ 1.7*	33.7 $\pm$ 2.7*
	R-Fenthion sulfoxide	27.6 $\pm$ 2.5	20.5 $\pm$ 1.1*	16.7 $\pm$ 1.7**
	MMTP (NADPH-independent)	7.4 $\pm$ 2.9	6.9 $\pm$ 2.3	5.3 $\pm$ 2.1
	MMTP (NADPH-dependent)	132.2 $\pm$ 10.3	132.1 $\pm$ 2.3	121.2 $\pm$ 15.3
Hypersaline				
	Fenoxon	42.2 $\pm$ 4.1	47.0 $\pm$ 3.7	16.8 $\pm$ 2.4**
	S-Fenthion sulfoxide	298.1 $\pm$ 22.4	214.1 $\pm$ 8.5*	168.4 $\pm$ 13.2*
	R-Fenthion sulfoxide	138.3 $\pm$ 12.4	72.5 $\pm$ 5.4**	83.4 $\pm$ 8.4**
	MMTP (NADPH-independent)	6.4 $\pm$ 2.3	10.1 $\pm$ 2.9	4.5 $\pm$ 1.1
	MMTP (NADPH-dependent)	21.1 $\pm$ 10.5	18.3 $\pm$ 2.8	13.2 $\pm$ 4.5

Data are presented as pmol/min/mg protein and as mean $\pm$ SD ($n=4$ individuals with 3 replicated for each one). Significant differences with respect to freshwater-acclimated rainbow trout indicated by * ($P<0.05$; Student's t -test) and ** ($P<0.01$; Student's t -test).

MMTP: 3-methyl-4-(methylthio)-phenol.

bdL: below detection limit (<0.3 pmol/min/mg protein).

Table 6

MMTP [3-methyl-4-(methylthio)-phenol] formation from fenthion and TEPP (tetraethylpyrophosphate) inhibition in microsomal fractions isolated from liver, gills and olfactory tissues in freshwater-acclimated rainbow trout (*Oncorhynchus mykiss*)

Tissue	Inhibitor	MMTP Formation	
		NADPH-independent	NADPH-dependent
Liver	Control	7.5±3.2 ^a	145.9±4.4 ^a
	100 µM TEPP	bdl ^b	138.4±10.3 ^a
	500 µM TEPP	bdl ^b	65.4±11.1 ^b
	1000 µM TEPP	bdl ^b	bdl ^c
Gills	Control	133.5±5.8 ^a	117.5±9.5 ^a
	100 µM TEPP	bdl ^b	105.3±8.9 ^a
	500 µM TEPP	bdl ^b	74.3±10.1 ^b
	1000 µM TEPP	bdl ^b	bdl ^c
Olfactory tissue	Control	71.2±11.1 ^a	240.2±28.0 ^a
	100 µM TEPP	bdl ^b	254.3±18.1 ^a
	500 µM TEPP	bdl ^b	100.2±13.3 ^b
	1000 µM TEPP	bdl ^b	bdl ^c

Data are presented as pmol/min/mg protein and as mean±SD in pmol/min/mg protein ($n=3-5$ individuals with 3 replicates for each one (liver and gill); 2 replicates for each one of the 3 pooled samples (olfactory tissue)). Different letters mean significant differences between groups and its own control ($P<0.01$; One-way ANOVA, Tukey's test).

bdl: below detection limit (<0.3 pmol/min/mg protein).