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Interactive Neurobehavioral Toxicity of Diazinon, Malathion, and Ethoprop to Juvenile Coho Salmon

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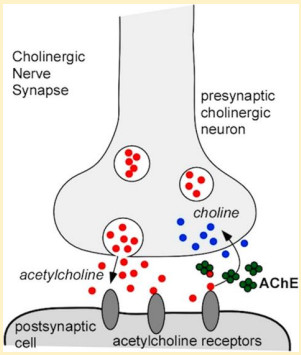
Supporting Information

ABSTRACT: In western North America, mixtures of current use pesticides have been widely detected in streams and other aquatic habitats for threatened and endangered Pacific salmon and steelhead (*Oncorhynchus* sp.). These include organophosphate insecticides that inhibit acetylcholinesterase (AChE) enzyme activity in the salmon nervous system, thereby disrupting swimming and feeding behaviors. Several organophosphates have been shown to interact as mixtures to produce synergistic AChE inhibition at concentrations near or above the upper range of surface water detections in freshwater systems. To evaluate potential synergism at lower concentrations (near or below 1 part per billion), juvenile coho (*Oncorhynchus kisutch*) were exposed to a range of mixtures of diazinon-malathion and ethoprop-malathion below a cumulative 0.05 of the predicted EC_{50} for AChE inhibition, as determined from single chemical concentration−response curves. Brain enzyme inhibition was concentration-dependent, with a 90% reduction and a significant decrease in spontaneous swimming speed at the highest binary mixture concentrations evaluated (diazinon-malathion at 2.6 and 1.1 μg/L, respectively; ethoprop-malathion at 2.8 and 1.2 μg/L, respectively). Brain enzyme activity gradually recovered over six weeks. Our findings extend earlier observations of organophosphate synergism in salmon and reveal an unusually steep concentration−response relationship across a mere 2-fold increase in mixture concentration.



INTRODUCTION

Organophosphate (OP) insecticides have been used as biological control agents worldwide for decades. In the United States, there are at present 27 OP active ingredients registered for use in hundreds of different pesticide products (for current registrations see http://www.epa.gov/oppsrrd1/reregistration/status_op.htm). The OPs share a common mechanism of toxic action. Specifically, they inhibit cholinesterases, including acetylcholinesterase (AChE), in insects, fish, birds, mammals, and other animals. AChE is localized to cholinergic synapses throughout the central and peripheral nervous systems. The enzyme hydrolyzes the neurotransmitter acetylcholine (ACh) and is therefore essential for neurotransmission within cholinergic networks. In humans and nontarget wildlife, AChE inhibition leads to a variety of adverse neurobehavioral effects. In fish for example, ACh is a key transmitter at neuromuscular junctions, and muscle paralysis, hyperactivity, and loss of equilibrium are canonical symptoms of anticholinesterase intoxication.[1]

Historically, advances in OP risk evaluation have largely occurred in the context of human health. For example, in the United States, the National Academy of Science reviewed pesticide risks to children in the early 1990s. The panel made several recommendations specific to aggregate risk (exposure to pesticides from multiple sources) as well as risk from cumulative exposure to pesticides that share a common mechanism of toxicity.[2] The National Academy report spurred new legislation in the form of the 1996 Food Quality Protection Act (FQPA), which directed the Environmental Protection Agency (EPA) to review the aggregate and cumulative risks of all pesticides within the following decade, beginning with the OPs. Ensuing EPA assessments for children found unacceptable aggregate risks for diazinon and chlorpyrifos, leading to the phase out of residential uses of both OPs between 2000 and 2004. Residential exposures have since declined for children,[3] as have the number of reported human exposure incidents more generally.[4] Measured concentrations of these two OPs have also fallen sharply in urban rivers and streams.[5]

Although organophosphate pesticides share a common mechanism of action,[6] assessing cumulative risk from exposures to OP mixtures remains a challenge. The EPA currently recommends a dose (or concentration) addition approach that

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assumes no interaction between chemicals in a mixture. Cumulative toxicity is calculated as the sum of individual toxicities, typically defined in terms of AChE inhibition.[7] However, in mammals, it has long been known that interactions do occur within certain OP mixtures, yielding greater than additive or synergistic toxicity.[8] Malathion is a particularly important synergist.[8−10]

In parallel to human health, OP mixture toxicity remains an important if poorly understood consideration for the conservation of nontarget, nonmammalian species. This is particularly true for aquatic systems, and research defining the impacts of pesticide mixtures on aquatic species continues to be a national priority.[11] In western North America, Pacific salmon and steelhead (*Oncorhynchus* sp.) spawn and rear in large river basins with mixed urban, residential, and agricultural land uses. These different land uses correspond to varying spatial and temporal patterns of pesticide use. Many salmon stocks are at historic lows, and 28 distinct population segments are presently listed as threatened or endangered under the U.S. Endangered Species Act (for current listings, see http://www.nmfs.noaa.gov/pr/species/fish/). Degraded water quality is one of many factors that can limit the recovery of most ESA-listed stocks. Numerous regional surface water monitoring studies dating back to the 1990s have shown that mixtures of OPs and other pesticides are common in salmon habitats within watersheds where these chemicals are routinely applied [e.g., ref 12]. Sublethal exposure to individual anticholinesterase insecticides inhibits brain AChE activity in fish[13,14] and impairs behaviors including feeding,[14,15] swimming,[16,17] prey capture,[18] and predator avoidance.[19] However, the combinatorial effects of OP mixtures on the neurobiology and behavior of salmonids have not been widely investigated.

We have previously shown that binary OP mixtures cause both noninteractive (additive) and interactive (synergistic) AChE inhibition in juvenile coho salmon (*O. kisutch*), depending on the specific chemical combination and relative exposure concentration.[20] Mixture interactions were evaluated using individual concentration−response curves normalized to the EC_{50} (the concentration producing 50% brain AChE inhibition) for each of three OPs (diazinon, malathion, and chlorpyrifos). This approach allowed for equitoxic OP pairings based on a default assumption of no-interaction (predicted 0.5 EC_{50}, 0.1 EC_{50}, etc.). However, the earlier study did not establish a NOEC (no observed effect concentration) for the potent synergistic combination of diazinon and malathion, a time course for sublethal AChE inhibition and recovery, or a link between AChE inhibition and behavior. The current investigation addressed these information gaps and also assessed the interactive toxicity of another potent OP combination (ethoprop and malathion).

METHODS

Fish. For diazinon-malathion (D-M) exposures, juvenile coho salmon were obtained from the University of Washington hatchery (Seattle, WA). For the ethoprop-malathion (E-M) exposures, juvenile coho were obtained from the Northwest Fisheries Science Center's hatchery (NWFSC, Seattle, WA). Both hatcheries use adult coho spawners (brood stock) from the same Puget Sound watershed, and we expected no source or age-specific differences among fish. Fish were transported from these hatcheries to Washington State University's Puyallup Campus (Puyallup, WA) where they were maintained for the duration of the study. Fish were held in recirculating tanks of dechlorinated municipal water (hatchery water; temperature 11−12 °C, pH 7.0−8.0, dissolved oxygen 85−90%, and total hardness as $CaCO_3$ 120 mg/L) on a 12:12 light-dark photoperiod and fed commercial salmon pellets (Bio-Oregon, Warranton, OR) daily. Fish used in the D-M exposures were 5−6 months old, with an average size (±SD) of 5.8 ± 0.5 cm and 2.0 ± 0.5 g, while fish used in the E-M exposures were 6−7 months old with an average size (±SD) of 7.1 ± 0.7 cm and 3.7 ± 0.1 g. Animal husbandry and experimental procedures were in accordance with guidelines established by Washington State University's Institutional Animal Care and Use Committee.

Pesticide Exposures. Diazinon (*O*,*O*-diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate; CAS #333-41-5; 99.5% pure), ethoprop (1-(ethoxy-propylsulfanylphosphoryl)sulfanylpropane; CAS #13194-48-4; 98.4% pure), and malathion (diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate; CAS #121-75-5; first lot 99.5% pure, second lot 98.7% pure) were purchased from Chem Service (West Chester, PA). Exposure concentrations (measured values; Table 1) were based on previously established single-chemical EC_{50} values for AChE inhibition in juvenile coho of a similar size and age.[20,21] These mixtures yielded relatively high, medium, and low binary exposure concentrations. However, all pairings were at pesticide levels below 1/20th (0.05) of the predicted EC_{50} for brain AChE inhibition based on concentration−response relationships for individual chemicals. At these very low exposure concentrations, an assumption of no-interaction (concentration-addition) would predict no measurable AChE inhibition in response to any of the binary pesticide combinations.

Table 1. Measured Pesticide Mixture Exposure Concentrations (μg/L)[a]

	high	medium	low
diazinon	2.6 ± 0.22	2.2 ± 0.12	1.2 ± 0.04
malathion	1.1 ± 0.25	0.6 ± 0.15	0.4 ± 0.04
ethoprop	2.8 ± 0.15	n/a	0.9 ± 0.10
malathion	1.2 ± 0.12	n/a	0.4 ± 0.02

[a]Measured exposure concentrations (means ± SE of n = 3 tanks) for binary pairings of pesticides in equitoxic ratios. For example, the high D-M treatment combined 2.6 μg/L diazinon and 1.1 μg/L malathion. n/a indicates not assessed.

Pesticide-containing stock solutions were prepared in methanol and added in either 100 μL or 1 mL aliquots to 25 L of hatchery water in 30-L glass aquaria. Final carrier concentration in exposure tanks was ≤0.004% of the total volume. Pesticides were mixed in equitoxic ratios (e.g., 0.015 EC_{50} diazinon + 0.015 EC_{50} malathion = cumulative 0.03 EC_{50} D-M mixture). For each treatment, fish were exposed in three replicate tanks (n = 6 fish each) for 96 h on a 24-h static renewal schedule. Fish were not fed during the exposure interval. Spontaneous swimming speed (described below) was recorded for individual fish immediately following exposures. After behavior trials, fish were terminally anaesthetized by immersion in MS-222 (tricaine methanesulfonate, 5 g/L; Sigma, St. Louis, MO) until gill activity ceased. Brains were immediately removed, placed in chilled plastic microcentrifuge tubes, and stored cryogenically (−80 °C) for subsequent analysis of AChE enzymatic activity. There was no mortality among fish in any of the exposures.

Time to Effect and Recovery. Aliquots of diazinon and malathion stock solutions were added to 50 L of hatchery water in a single 60-L glass aquarium to yield concentrations of 2.6 and 0.7 μg/L, respectively, corresponding to a cumulative exposure concentration of approximately 0.03 EC_{50}. Sixty fish were exposed for 96 h with 24-h static renewals. Immediately following exposure, fish were transferred to a 200-L recovery tank filled with clean hatchery water and allowed to recover for 42 days. Fish were not fed during the 96-h pesticide exposure interval but were fed daily during the recovery interval. Six fish were subsampled weekly for AChE activity analysis.

Spontaneous Swimming Behavior. Spontaneous swimming speed was measured as described previously.[14] Behavioral trials were conducted in a 30-L glass aquarium (observation tank) filled with 25 L of hatchery water. The observation tank was positioned behind black plastic walls to shield fish from visual disturbances. Two sides and the bottom of the observation tank were opaque, and an overhead fluorescent light provided uniform lighting. Two small fans circulated air to keep the tank free of condensation. A small aquarium pump was submerged in the observation tank to circulate water. After exposure to OP mixtures for 96 h, individual fish were transferred to the observation tank and allowed to acclimate for 30 min. Following acclimation, spontaneous swimming was recorded for 3 min.

The movements of the fish were monitored using orthogonally positioned digital cameras (Fire-I, Unibrain, San Ramon, CA) connected to a laptop computer (PowerBook, Apple Computer, Cupertino, CA). One camera was positioned to view the front of the tank, while the second camera viewed the left side. A custom software program acquired simultaneous frames from the cameras every 2 s. Image analysis software (ImageJ, http://rsb.info.nih.gov/ij/) was used to locate the fish in both two-dimensional views. The data were corrected for refraction and used to triangulate the three-dimensional position of the fish over time.

AChE Enzyme Assays. Quantification of AChE activity followed the Ellman method[22] as previously modified for juvenile salmon.[20] Briefly, whole brains were homogenized at 50 mg/mL in 0.1 M sodium phosphate buffer with 0.1% Triton X-100. Homogenates were centrifuged, and 15 μL of the supernatant was combined with 685 μL of 10 mM phosphate buffered saline, 50 μL of 6 mM DTNB (5,5′-dithio-bis(2-nitrobenzioc acid)), and 30 μL of 75 mM acetylthiocholine iodide. All chemicals were purchased from Sigma (St. Louis, MO). Triplicate 200 μL samples were transferred to a 96-well plate, and the change in absorbance at 412 nm was measured at 12 s intervals for 5 min at 25 °C on an Optimax plate reader (Molecular Devices, Sunnyvale, CA). AChE activity is reported as either μmol/min/g tissue or as a percentage of the average enzyme activity for fish from an unexposed control treatment (% control).

Analytical Chemistry. Triplicate water samples were collected in prewashed 500 mL amber glass bottles from exposure tanks immediately following pesticide addition at the beginning of the 96-h exposure (0 h). For the D-M exposures, water samples were also collected one day later, prior to the first static-renewal (24 h) to estimate pesticide loss over a 24-h period. To improve chemical stability by minimizing aqueous hydrolysis, water samples were acidified with 4−5 drops of a 50% acetic acid solution to lower the pH and then stored at 4 °C until analysis. All analyses were conducted at Washington State University's Food and Environmental Quality Laboratory (Richland, WA). The D-M analyses were conducted using gas chromatography mass spectrometry (GC-MS) with a limit of quantification of 0.5 μg/L for each compound. The E-M analyses were carried out by gas chromatography with pulsed flame photometric detection (GC-PFPD) with a limit of quantification of 0.4 μg/L for each compound. The extraction method used was modified from methodology specified in SW-846 EPA method 3510C, Rev. Three (1996).

RESULTS

Synergistic Acetylcholinesterase Inhibition. Single-chemical EC_{50} concentrations (50% AChE inhibition) were previously determined for juvenile coho salmon of a similar size and age to the fish used in this study. The single chemical concentration−response curves yielded calculated EC_{50} values of 145 μg/L for diazinon,[20] 74 μg/L for malathion,[20] and 91 μg/L for ethoprop.[21] In the present study, several single chemical exposures were conducted to confirm these predicted EC_{50} estimates (Supporting Information, Figure S1). The average brain enzyme activities of control coho exposed to clean water or a carrier (methanol) only were not significantly different (ANOVA, $p > 0.05$, Tukey's post hoc).

As described previously,[20] to distinguish between concentration-addition and pesticide interaction (e.g., synergism), we normalized single-chemical concentration−response curves (Supporting Information, Figure S1, panels A-C) to their respective EC_{50}s and collectively fit the combined data with a single regression. This curve was subsequently used to determine the presence or absence of interactions within binary pesticide mixtures. For example, in a mixture containing two pesticides each at 0.015 EC_{50}, concentration-addition occurs if the cumulative AChE inhibition is equivalent to 0.03 EC_{50}. This result would fall on the curve (Supporting Information, Figure S1, panel D). Synergism would be evident from mixture results falling significantly below the curve - i.e., much greater AChE inhibition than expected from individual toxicities.

The D-M and E-M pesticide combinations both produced synergistic neurotoxicity to juvenile coho as indicated by much greater brain AChE inhibition than predicted by concentration-addition (Figure 1A). The low D-M combination was comparable to controls, but AChE activity markedly decreased at the middle D-M treatment group (Figure 1A). This corresponds to modest concentration increases from 1.2 to 2.2 μg/L (diazinon) and 0.4 to 0.6 μg/L (malathion). Brain AChE activity was significantly decreased to <10% in the high D-M mixture (2.6 μg/L diazinon and 1.1 μg/L malathion; ANOVA, $p < 0.05$, Tukey's post hoc). Thus, a small (2- to 3-fold) increase in diazinon and malathion concentrations within a binary mixture was sufficient to drive AChE activity from control rates to >90% inhibition. Moreover, this high degree of inhibition occurred in response to diazinon and malathion levels that were more than 50-fold lower than those expected to produce 50% AChE inhibition based on concentration−response curves for the two pesticides individually. We did not observe coho mortality, consistent with a previous study where juvenile coho survived OP mixture exposures at slightly higher concentrations.[20] For the E-M combinations (Figure 1A), the low treatment group (0.9 μg/L ethoprop and 0.4 μg/L malathion) produced marginal AChE inhibition that increased to >90% for the high E-M mixture (2.8 μg/L ethoprop and 1.2 μg/L malathion).

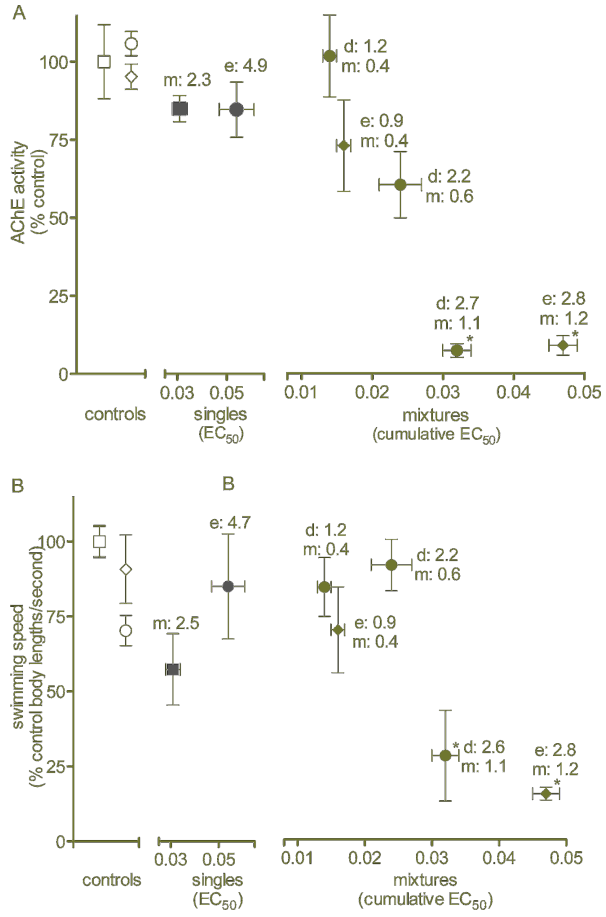

Figure 1. Reductions in both acetylcholinesterase (AChE) activity (A) and spontaneous swimming speed (B) followed exposure to mixtures of diazinon-malathion (filled circles) and ethoprop-malathion (filled diamonds). Unexposed controls are shown as open circles (diazinon-malathion) and open diamonds (ethoprop-malathion); methanol carrier controls are shown as open squares; and single-chemical exposures to ethoprop and malathion are shown as gray squares and gray circles, respectively. Values are the mean ± SE (n = 3 replicate tanks of 6 fish each). Exposure concentrations are measured levels relative to their single or cumulative EC_{50} and are means ± SE (n = 3 replicate tanks). The * indicates a significant difference in brain AChE activity or swimming speed from all other treatment groups.

Reduced Swimming Speed. Anticholinesterase intoxication produces lethargy in juvenile salmon,[14] as evidenced by a reduction in swimming speed. As with AChE inhibition, the effects of the D-M and E-M mixtures occurred relatively abruptly over a very small increase in pesticide concentration from the lowest to the highest mixtures tested (Figure 1B). This steep concentration–response relationship suggests synergistic effects on coho behavior, with toxicity thresholds slightly above 1.2, 0.4, and 0.9 μg/L for diazinon, malathion, and ethoprop, respectively, in binary mixtures. The absolute swimming speeds for all treatment groups are shown in Tables 2 and 3. The high D-M and E-M exposures both reduced average swimming speeds to less than 1 cm/s (n = 3 replicate tanks of 6 fish each). This was significantly lower than their respective controls as well as for fish from all other exposure concentrations (ANOVA, $p < 0.05$, Tukey's posthoc). Swimming speeds of water-only and methanol controls within each exposure were not significantly different (ANOVA, $p > 0.05$, Tukey's posthoc). However, swimming speeds of control fish from D-M exposures were lower than E-M control fish, possibly due to a difference in cohort age.

Table 2. AChE Activities and Swimming Speeds of Fish from Diazinon-Malathion Exposures[a]

treatment	AChE activity (μmol/min/g tissue)	AChE activity (% control)	swimming speed (cm/s)	swimming speed (body lengths/s)
water control	123.1 ± 4.5	108 ± 9.1	2.4 ± 0.2	0.42 ± 0.03
methanol control	116 ± 14	100 ± 11.8	3.4 ± 0.1	0.58 ± 0.03
low mixture	113.1 ± 14.6	97.6 ± 9.4	2.8 ± 0.3	0.46 ± 0.05
medium mixture	67.2 ± 11.8	57.4 ± 7.2	3.1 ± 0.2	0.50 ± 0.05
high mixture	8.1 ± 2.5*	7.6 ± 3.1*	0.9 ± 0.5*	0.15 ± 0.08*

[a]Values are means ± SE of 3 replicate tanks of 6 fish each (2 to 9 fish for some water and methanol control tanks). The * indicates a significant difference in brain enzyme activity or swimming speed from all other treatment groups (ANOVA, $p < 0.05$, Tukey's post hoc).

Table 3. AChE Activities and Swimming Speeds of Fish from Ethoprop-Malathion Exposures[a]

treatment	AChE activity (μmol/min/g tissue)	AChE activity (% control)	swimming speed (cm/s)	swimming speed (body lengths/s)
water control	94.5 ± 4.0	95.2 ± 4.0	5.4 ± 0.6	0.77 ± 0.10
methanol control	99.2 ± 1.1	100 ± 1.1	5.6 ± 0.3	0.85 ± 0.05
low mixture	69.2 ± 13.9	69.7 ± 14.0	4.1 ± 0.8	0.57 ± 0.12
high mixture	8.4 ± 3.0*	8.5 ± 3.0*	0.9 ± 0.1*	0.12 ± 0.02*
ethoprop alone	84.1 ± 8.8	84.7 ± 15.5	4.5 ± 1.0	0.7 ± 0.15
malathion alone	84.3 ± 4.1	85.0 ± 7.3	3.1 ± 1.0	0.5 ± 0.10

[a]AChE activities are presented as means ± SE of 3 replicate tanks of 6 fish each. Swimming speeds are means ± SE of 3 replicate tanks of 3 fish each for controls, 3 replicate tanks of 6 fish each for mixtures, and 2 replicate tanks of 6 fish each for single pesticides. The * indicates a significant difference in brain enzyme activity or swimming speed from all other treatment groups (ANOVA, $p < 0.05$, Tukey's post hoc).

Time to Effect and Recovery. Juvenile coho (n = 6 fish per time point) showed a gradual inhibition of AChE activity over the course of a 96-h exposure to a D-M mixture at an approximate cumulative 0.03 EC_{50} (2.6 μg/L diazinon and 0.7 μg/L malathion), with a decrease in mean enzyme activity (±SE) from 8.1 (±0.14) to 2.6 (±0.61) μmol/min/g tissue (Figure 2). When fish were subsequently transferred to clean water, brain enzyme activity steadily increased over a six-week interval. However, AChE activity was still significantly reduced relative to control fish after 42 days of recovery in clean water (7.7 ± 0.3 μmol/min/g tissue, n = 12 means of 2 to 8 fish each, t test, $p < 0.05$).

Analytical Chemistry. Overall, the observed biological effects on coho brain AChE and swimming behavior occurred over a low and very narrow range of OP mixture concentrations near the analytical limit of quantitation. For each exposure, measured pesticide levels relative to nominal concentrations are shown in Table S1 (Supporting Information). Analysis of water samples collected at the end of a 24-h static-renewal interval indicated a modest insecticide loss with an average (±SD) loss of 38 ± 12% for diazinon and 28 ± 18% for malathion.

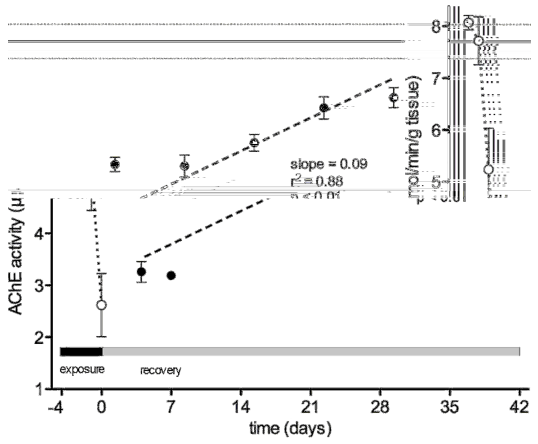

Figure 2. Brain acetylcholinesterase (AChE) activity decreased in juvenile coho salmon over the course of a 96-h exposure to a diazinon-malathion mixture (2.6 and 0.7 μg/L, respectively; open circles). Activity subsequently increased over a six-week recovery period in clean water (closed circles). Each symbol is the mean ± SE of $n = 6$ juvenile coho. Horizontal bar and associated dashed lines represents the mean ± SE AChE activity for unexposed fish ($n = 12$ tanks of 2 to 8 fish each).

Ethoprop loss was not measured but was expected to be comparable to the loss of diazinon and malathion over time based on the physical properties of the three pesticides. Due to this loss, the average actual exposure concentrations were likely lower (by no more than approximately 30%) than reported values. Stock solutions used in the diazinon-malathion mixture exposures averaged (±SD) 103 ± 13% and 92 ± 7% of nominal, respectively. The stock solutions from the ethoprop-malathion exposures averaged 146% and 102% of nominal, respectively.

■ DISCUSSION

It is generally expected that mixtures of chemicals having a common mechanism of action (e.g., AChE inhibition) will be noninteractive, thereby producing concentration-additive toxicity.[23] An earlier study showed that binary mixtures of the organophosphate insecticides diazinon and malathion diverge from this paradigm, causing greater-than-expected (i.e., synergistic) toxicity to juvenile salmon.[20] Our current findings confirm D-M synergism and define a steep concentration–response relationship for exposures in the range of a part per billion. Moreover, malathion acts synergistically with ethoprop at similarly low concentrations. Interactive toxicity was evident for biochemical and behavioral end points alike, and brain AChE activity was not fully restored after a six-week recovery in clean water.

The timecourse for AChE inhibition during the 96-h exposure interval is similar to previous reports for single-chemical exposures in fish.[24] Therefore, OP mixtures appear to enhance the severity but not the rate of onset of AChE inhibition. The protracted and gradual recovery of enzyme activity over several weeks is also consistent with the findings of earlier, single-chemical studies.[13] This extended recovery interval suggests that oxon metabolites, alone or in mixtures, bind irreversibly to AChE, thereby necessitating new gene expression and protein targeting to synapses to restore cholinergic homeostasis. As noted previously,[25] this indicates that short-term (i.e., pulsatile) pesticide exposures may have sublethal effects on juvenile salmon health that persist long after the exposure window (carry-over toxicity; e.g., ref 26), at least at a biochemical scale. Although not assessed here, additional exposures to OPs during the recovery window for AChE activity may have disproportionately greater impacts on cholinergic brain function relative to a one-time pesticide exposure. This raises the possibility of synergistic toxicity in response to OP pulses that are separated in time and space in salmon habitats.

For both binary mixtures, the concentration–response relationships for AChE inhibition and swimming impairment were surprisingly steep. An incremental increase in exposure concentration (e.g., from 0.02 to 0.03 of the predicted cumulative EC_{50}) yielded abruptly large changes in both toxicity end points, with fish in the highest treatments showing the expected qualitative signs of anticholinesterase intoxication (lethargy and loss of orientation). Thus, juvenile coho are very sensitive to individual pesticide concentration shifts of only ∼1 part per billion (or less) when these shifts take place within a mixture.

As expected from previous studies with single OPs,[14] behavioral impairment tracked closely with AChE inhibition, albeit with greater variability among individual animals. Behavioral indicators of anticholinesterase toxicity in fish, including reductions in spontaneous swimming,[16,17] prey capture,[18] and predator avoidance[19] are generally more variable than AChE inhibition. Also, the more variable effects on swimming in the present study may be due in part to the sensitivity of the coho cholinergic system to slight differences in mixture concentrations. Nevertheless, both mixtures had pronounced neurobehavioral effects on juvenile coho at individual pesticide concentrations predicted to yield no detectable AChE inhibition based on concentration–response curves for single chemicals.

Two classes of esterases, carboxylesterases (CaEs) and butyrylcholinesterases (BChEs), are likely to play detoxifying or otherwise protective roles against anticholinesterase poisoning in salmon. Both types of enzymes bind OP parent compounds, thereby reducing the biologically available fraction for metabolic conversion to the more potent AChE-inhibiting oxon metabolites.[27] Moreover, CaEs detoxify oxon derivatives via hydrolysis.[28] It has long been known from mammalian models that CaE inhibition potentiates AChE inhibition in OP-exposed animals,[8,29] and OP insecticides have been shown to inhibit CaE activity in the livers of salmonids and other fish.[13,30,31] While the toxicodynamic mechanism(s) underlying D-M and E-M synergism have yet to be determined for juvenile coho, future studies should assess the likely role of non-AChE esterases and, in particular, whether malathion inhibits CaE-mediated hydrolysis, thereby enhancing the inhibitory effects of diazinon-oxon and ethoprop on brain AChE activity.

Our mixture results have implications for certain single-chemical, deterministic ecological risk assessments. For example, risk assessments to support pesticide registration or reregistration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) set exposure levels of concern (LOCs) for acute risk at a fraction of the LC_{50} for nontarget taxa. If the species is endangered, as are 28 population segments of Pacific salmon and steelhead that inhabit waters of Idaho, California, Washington, and Oregon, the LOC is derived by dividing the LC_{50} by a factor of 20. There is a presumption of no risk for acute exposures to endangered species when estimated environmental concentrations are less than the

endangered species LOC. For salmonids (*Oncorhynchus spp.*), the 96 h LC_{50} for ethoprop ranges from 700 to 13,800 μg/L, with an average of 5,808 μg/L (n = 6 rainbow trout tests; U.S. EPA AQUIRE database: http://cfpub.epa.gov/ecotox/). However, when paired with malathion, ethoprop produces severe anticholinesterase intoxication at an exposure concentration (2.8 μg/L) that is more than 1000-fold lower than the average LC_{50} and more than 2 orders of magnitude below the conventional LOC for an endangered species − i.e., 5,808 μg/L divided by 20, or 290 μg/L. Notably, while intoxicated animals are technically alive from the standpoint of an LC_{50}, salmon that are unable to effectively swim are unlikely to survive under natural conditions.

Synergistic interactions among environmental mixtures of organophosphates also have implications for probabilistic risk assessments. Probabilistic analyses refine the exposure-response relationship in part by examining a range of possible pesticide exposure concentrations to estimate a likely frequency for exceeding one or more thresholds for adverse toxicological impacts to aquatic species. In practice, probabilistic assessments have focused on individual chemicals (e.g., chlorpyrifos[32]). For salmon, however, a modest change in the exposure concentration probability for a single OP (e.g., diazinon) may be much less consequential biologically than the likelihood of a coexposure to the same chemical and one or more synergists (e.g., malathion). This is important because single chemical probabilistic assessments may yield type two errors (false predictions of no toxicity) for aquatic habitats where OP insecticides are likely to co-occur. In the U.S., pesticide mixtures in surface waters are more common than not, as evidenced by extensive monitoring in river basins with different degrees of urban, residential, and agricultural land cover.[33] Recent risk analyses based on monitoring data from salmon habitats and the AChE-inhibiting potencies of individual OPs in isolation [34] do not account for OP mixture interactions and are therefore likely to underestimate toxicity to ESA-listed salmonids.

Given factorial experimental design considerations, it would be difficult to evaluate large numbers of OP mixture combinations in salmon. Prior to the current study, however, the interaction between ethoprop and malathion was identified in a preliminary screen using early life stages of zebrafish (*Danio rerio*; data not shown). Subtle interspecific differences notwithstanding, zebrafish offer a rapid and high-throughput model system for identifying synergistic OP interactions in binary and more complex mixtures. High hazard combinations could then be validated in native species of concern such as salmon. Relative to coho, zebrafish also provide an advantageous experimental context for determining specific mechanisms of OP synergism, for example by targeted genetic knockdown of metabolic esterases using antisense morpholinos (e.g., ref 35).

Lastly, our findings emphasize the importance of chemical mixtures in salmon habitats. In terms of chemical-chemical interactions, we have evaluated only one group of current-use pesticides and only in simple binary pairings. Nonetheless, we have clearly shown that OP mixtures produce strikingly synergistic toxicity and depressions in swimming speed at very low concentrations. Our results shed light on the complexities of pesticide mixtures and their subsequent effects on exposed salmon and highlight the need for pesticides to continue to receive increased attention as a potential limiting factor to salmon recovery in the western United States.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 shows single chemical concentration−response curves from previous studies together with single chemical exposures from the present study (A-C) as well as the collective fit of single-chemical data normalized to their respective EC_{50} concentrations (D). Table S1 lists the percent recovery of pesticide residues in exposure waters. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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