

Cholinesterase-Inhibiting Pesticides

DOSE-ADDITIVE INHIBITION OF CHINOOK SALMON ACETYLCHOLINESTERASE ACTIVITY BY MIXTURES OF ORGANOPHOSPHATE AND CARBAMATE INSECTICIDES

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Abstract—Organophosphate and carbamate insecticides are widely detected in surface waters of the western United States. These chemicals interfere with acetylcholine-mediated synaptic transmission in the nervous systems of fish and other aquatic animals via the inhibition of AChE (acetylcholinesterase) enzyme activity. Anticholinesterase insecticides commonly co-occur in the environment. This raises the possibility of antagonistic, additive, or synergistic neurotoxicity in exposed fish, including threatened and endangered species of Pacific salmon. We extracted AChE from the olfactory nervous system of chinook salmon (*Oncorhynchus tshawytscha*) and investigated the inhibitory effects of organophosphates (the oxon derivatives of diazinon, chlorpyrifos, and malathion) and carbamates (carbaryl and carbofuran), alone and in two-way combinations. We found that the joint toxicity of anticholinesterase mixtures can be accurately predicted from the inhibitory potencies of individual chemicals within a mixture. This indicates that organophosphate and carbamate insecticides are noninteractive in terms of AChE inhibition and that it might be possible to estimate the cumulative neurotoxicity of mixtures by simple dose addition. Because organophosphates and carbamates are likely to have additive effects on the neurobehavior of salmon under natural exposure conditions, ecological risk assessments that focus on individual anticholinesterases might underestimate the actual risk to salmon in watersheds in which mixtures of these chemicals occur.

Keywords—Salmon Acetylcholinesterase Endangered species Water quality Pesticide

INTRODUCTION

In recent years, systematic monitoring investigations by the U.S. Geological Survey's National Water Quality Assessment (NAWQA) Program have consistently shown that surface waters in many regions of the United States are contaminated with complex mixtures of pesticides and other anthropogenic pollutants [1]. This is particularly true of watersheds with a high degree of urban or agricultural land use. For example, a synthesis of national NAWQA monitoring data found that more than 80% of water samples from urban streams contained two or more pesticides, and some samples (>15%) contained 10 pesticides or more [2]. Mixtures of pesticides are also common in agricultural watersheds. For example, more than half of the surface water samples from certain agricultural drainages in California's Central Valley, USA, contained seven or more pesticides [3]. In California and the Pacific Northwest, USA, these general patterns of pesticide detections are typical of NAWQA monitoring investigations in the San Joaquin [4], Sacramento [5], Willamette [6], Yakima [7], and Puget Sound [8] basins.

Migratory Pacific salmon (*Oncorhynchus* spp.) are a major cultural and economic resource in the western United States. Wild salmon stocks have been declining for years throughout much of their natural range [9,10], and several populations are now listed for federal protection under the U.S. Endangered

Species Act (ESA) [11]. Most of the western river systems that have been a focus of the U.S. Geological Survey's NAWQA surface water monitoring for pesticides also provide freshwater spawning and rearing habitat for anadromous chinook (*Oncorhynchus tshawytscha*), coho (*Oncorhynchus kisutch*), steelhead (*Oncorhynchus mykiss*), or other ESA-listed salmonids. Although exposure to pesticide mixtures might be common for salmon in western watersheds, the potential for cumulative toxicity is poorly understood.

Evaluating the joint toxicity of chemical mixtures is currently a major challenge in aquatic ecotoxicology [12]. In the case of pesticides, the difficulty is due in part to the large numbers of chemicals that enter aquatic systems via various fate and transport pathways [13,14]. To incorporate a degree of ecological realism, laboratory studies of mixture toxicity must contend with the expanding complexity and expense of a factorial experimental design [15,16]. Another problem has been the inconsistent use of terminology, including such terms as synergism, additivity, and antagonism. A key issue is whether studies explicitly define the expected toxicological response, or no interaction, between chemicals in a mixture [17]. The definition of no interaction depends on the expected toxicity of the individual components of a mixture. Where no interaction occurs, the joint toxicity of a mixture is the same as would be expected from the sum of the individual chemicals. This no interaction result is additivity. Synergism and antagonism describe situations in which the toxicological effects of a mixture are greater or less than expected from the sum of the individual components, respectively. However, both terms need to be defined in the context of no interaction to avoid ambiguity [17].

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Additive toxicity is a possible outcome for salmon exposed to pesticide mixtures, particularly if two or more chemicals in a mixture share a common mode of action. The organophosphate and carbamate classes of insecticides, which target the enzyme AChE (acetylcholinesterase) in the central and peripheral nervous systems of vertebrates, are prominent examples. Anticholinesterase insecticides are widely used in the western United States, and correspondingly, they are frequently detected in salmon habitats. For example, a NAWQA investigation detected the organophosphates diazinon, chlorpyrifos, ethoprop, fonofos, malathion, dimethoate, and azinphosmethyl and the carbamates carbaryl and carbofuran in filtered surface water samples from Oregon's Willamette Basin, USA [18]. The cumulative effects of mixtures containing these or other anticholinesterase insecticides have not been investigated in terms of the health of anadromous salmon or steelhead.

Acetylcholinesterase hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses [19], and AChE inhibition is generally accepted as a biomarker of exposure to organophosphate and carbamate insecticides in fish [20]. Organophosphates and carbamates bind to the AChE active site and serve as surrogate substrates for the enzyme. The resulting phosphorylation or carboxylation, respectively, prevents neurotransmitter binding and hydrolysis [21]. The major distinction between the two classes of insecticides is the duration of AChE inhibition. Whereas organophosphate-induced inhibition is effectively irreversible, inhibition by carbamates is reversible, which leads to a faster rate of recovery from enzyme inhibition [22].

To test the hypothesis that organophosphates and carbamates produce additive neurotoxicity in salmon, we evaluated the inhibitory effects of five insecticides (or their active metabolites) on the activity of AChE extracted from the olfactory epithelium and olfactory forebrain of chinook salmon. We focused on the olfactory nervous system because anticholinesterases have previously been shown to impair olfaction [23,24] and olfactory-mediated behaviors [25–27] in salmonids and other fish species (e.g., [28]). In this study, inhibitory dose-response relationships were determined for individual pesticides. The data for all five pesticides were subsequently combined and fit with a single nonlinear regression. The resulting curve was used to explicitly define the concept of no interaction among components of a pesticide mixture. We then screened all possible binary (i.e., two-way) combinations of pesticide mixtures for interactive toxicity (antagonism or synergism) or noninteractive toxicity (addition). We found that simple mixtures of organophosphate and carbamate insecticides are strictly additive in terms of their effects on chinook AChE activity. These findings are discussed in the context of salmon health and ecological risk assessment for threatened or endangered species.

MATERIALS AND METHODS

Animals

Adult chinook salmon (two to three years of age; $n = 68$) were collected as they returned to the University of Washington hatchery facility (Seattle, WA, USA) to spawn. The ratio of females to males was approximately 4:1, and the fish had an average fork length of 75.8 ± 6.1 cm and a weight of 526 ± 139 g, (mean \pm 1 SD, $n = 61$). The sizes of seven fish were not recorded. Tissues were collected from fish that were killed as part of normal hatchery operations for the fall spawning season. The heads were removed and stored on ice for up to

6 h. Subsequently, the olfactory rosettes (the supporting structure for the peripheral sensory epithelium), olfactory bulb, and telencephalon were removed, washed in 10 mM PBS (phosphate-buffered saline; pH 7.4), and stored individually at -80°C .

Cholinesterase extractions

A sequential extraction technique (modified from Younkin et al. [29]) was used to separate cholinesterase fractions on the basis of solubility. Samples were pooled by tissue before homogenizing on ice in a low-salt buffer (10 mM PBS, pH 7.4). The homogenate was subsequently passed through a 15- μm ground glass filter and sonicated for 30 s to produce a primary homogenate. A sequence of three different buffers was then used to extract three distinct solubility fractions from the primary homogenate. To produce a low-salt fraction, the primary homogenate was centrifuged at 120,000 g for 30 min at 4°C , and the supernatant was removed. To produce a high-salt fraction, the resulting pellet was homogenized in a high-salt buffer (10 mM PBS containing 1 mM NaCl, pH 7.4) and centrifuged as above, and the supernatant was removed. Finally, the pellet was homogenized in high-salt buffer containing 1% Triton X-100 (Sigma, St. Louis, MO, USA) and centrifuged to produce a high-salt + detergent fraction. For each tissue, the primary homogenate and the different fractions were aliquoted and stored at -80°C .

Cholinesterase enzyme assays

Cholinesterase enzyme activity was determined by the Ellman method [30] with modifications for a microtiter plate reader [31]. The assay measures the hydrolysis of acetylthiocholine iodide as indicated by the reduction of a chromogen, DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]). Chinook tissue extracts (500 mg/L for the olfactory rosette and 50 mg/L for the olfactory bulb and telencephalon) were incubated in 10 mM PBS containing 0.7 mM DTNB (Sigma), pH 7.4, for 15 min at 25°C . Acetylthiocholine iodide (3 mM, Sigma) was added, and the samples were vortexed and transferred to a 96-well microtiter plate. To monitor the consistency of the experimental procedure, each aliquot was assayed three times in separate wells. Measurements from the three wells then were averaged to provide a single value for enzyme activity. Because the tissue homogenates were pooled from many animals, replicated data (from independent observations) were not collected.

Cholinesterase activity was measured at 25°C on an Opti-max plate reader (Molecular Devices, Sunnyvale, CA, USA) with the emission spectra (λ) set at 412 nm. The assay duration, pH, incubation temperature, and substrate concentration were optimized on the basis of preliminary experiments (data not shown). Each plate contained tissue and substrate blanks, and rates of activity were corrected for the spontaneous hydrolysis of acetylthiocholine iodide and the nonspecific reduction of DTNB by tissue extracts. In addition, an untreated aliquot (no inhibitors or pesticides) was included on each plate to determine maximal or 100% enzyme activity for the appropriate homogenate.

Pharmacological inhibitors were used to distinguish between the different cholinesterases that might be present in chinook olfactory tissue extracts [32]. These included the AChE inhibitor 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW284c51) and the BChE (butyrylcholinesterase) inhibitor iso-OMPA (tetraisopropylpyro-

phosphoramidate). Extracts were also treated with physostigmine (eserine), a general inhibitor of serine esterases. Inhibitors were coapplied with DTNB during the 15-min incubation that preceded the addition of substrate to the reaction mixture. All three inhibitors were obtained from Sigma.

To determine the inhibitory effects of insecticides on chinook olfactory cholinesterase, tissue extracts were treated with two carbamates (carbaryl and carbofuran) and the oxon metabolites of three organophosphates (chlorpyrifos-oxon, diazinon-O-analog, and malaoxon). For the organophosphates, oxon derivatives were used because these metabolites, and not their respective parent compounds (chlorpyrifos, diazinon, and malathion), are primarily responsible for cholinesterase inhibition in vertebrates [33]. For clarity, we subsequently refer to the oxon derivatives as chlorpyrifos-oxon, diazinon-oxon, and malathion-oxon. As with the pharmacological inhibitors, pesticides were applied (alone or in binary combinations) during the incubation step of the Ellman assay. Nominal treatment concentrations were as follows: carbaryl (0–23,560 $\mu\text{g/L}$, $n = 18$ concentrations); carbofuran (0–5,890 $\mu\text{g/L}$, $n = 20$); chlorpyrifos-oxon (0–236 $\mu\text{g/L}$, $n = 12$); diazinon-oxon (0–10,720 $\mu\text{g/L}$, $n = 12$); malathion-oxon (0–104 $\mu\text{g/L}$, $n = 12$). Analytical grade pesticides and pesticide metabolites were purchased from Chem Services (West Chester, PA, USA).

Data analysis

Curve fits of the dose–response data for cholinesterase activity were performed with Lev-Mar nonlinear regressions (KaleidaGraph, Synergy Software, Reading, PA, USA) to a sigmoid logistic function.

$$\text{Activity} = \frac{\text{Upper} - \text{Lower}}{1 + \left(\frac{\text{Concentration}}{\text{Half}}\right)^{\text{Slope}}} + \text{Lower}$$

In this equation, Lower is the minimum activity as concentration ($\mu\text{g/L}$) approaches $+\infty$ (fixed at 0%), Upper is the maximum activity as concentration approaches 0 (fixed at 100%), Half is the concentration that yields activity halfway between Upper and Lower, and Slope is the steepness of the curve in the “linear” region around Half. When Lower is 0% and Upper is 100%, Half is equivalent to the concentration producing 50% enzyme inhibition (IC_{50}).

RESULTS

Acetylcholinesterase is expressed in the chinook olfactory epithelium and forebrain

The primary homogenates from all three tissues contained a basal cholinesterase activity. This was evident from the hydrolysis of the substrate acetylthiocholine iodide and the complete inhibition of enzyme activity by 9.5 μM eserine (data not shown). Sequential extractions with low-salt, high-salt, and high salt + detergent buffers revealed a different pattern of molecular forms in the olfactory rosette compared with the olfactory bulb and the telencephalon in Figure 1. The large majority (90.8%) of the cholinesterase activity in the rosette separated with the low-salt fraction. Because the low-salt buffer extracts soluble, globular forms of the enzyme (reviewed in Massoulié et al. [32]), these appear to be the dominant forms in the peripheral olfactory tissue of salmon. All three tissues showed a small but detectable amount of cholinesterase activity in the high-salt extracts, which contain the asymmetric forms of AChE. Although a substantial portion of the cholin-

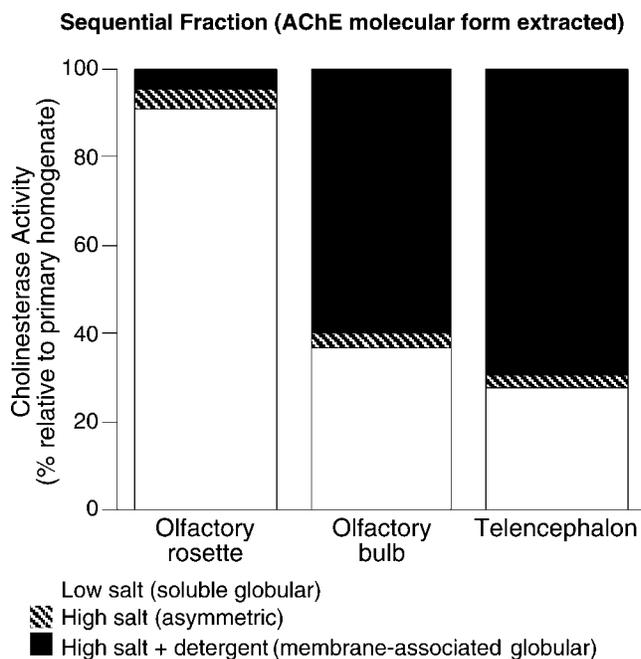


Fig. 1. Chinook salmon olfactory tissues contain distinct molecular forms of the AChE (acetylcholinesterase) enzyme. Primary homogenates from the olfactory rosette, olfactory bulb, and telencephalon were sequentially treated with buffers containing low-salt, high-salt, and high-salt–containing detergent to extract soluble globular, asymmetric, and membrane-associated globular forms of AChE, respectively [32]. The proportion of AChE activity in each fraction is expressed relative to the activity of the primary homogenate.

esterase activity from the olfactory bulb and telencephalon was also present in low-salt extracts (36.5 and 27.6%, respectively), for these two tissues, the majority of the activity separated with the high salt + detergent fraction (59.9 and 69.3%, respectively). This latter fraction typically contains detergent-soluble (i.e., membrane-associated), globular forms of the enzyme [32].

To evaluate the response of chinook cholinesterase activity to pharmacological inhibitors, tissue homogenates were processed with a single buffer (high salt + detergent) to extract most or all of the molecular forms that might be present. To verify that molecular forms of AChE, and not BChE, were the source of the observed activity, tissue extracts were preincubated with inhibitors before the cholinesterase assay. These included a selective inhibitor of AChE (BW284c51), a selective inhibitor of BChE (iso-OMPA), and the nonselective inhibitor eserine. As shown for the telencephalon in Figure 2A, cholinesterase activity was inhibited in a dose-dependent fashion by eserine and BW284c51 but not iso-OMPA. This pattern was also observed in extracts from the sensory epithelium and olfactory bulb (data not shown). The inhibitory dose–response curves for BW284c51 were very similar for extracts from all three tissues (Fig. 2B). Consequently, the cholinesterase measurements from peripheral and central regions of the chinook nervous system appear to reflect the activity of AChE with little or no contribution from BChE.

Individual pesticides inhibit chinook AChE

The inhibitory effects of five pesticides (two carbamates and three organophosphates) on the AChE activity of telencephalic fractions extracted with high salt + detergent are shown in Figure 3. Parameters describing the inhibitory dose–

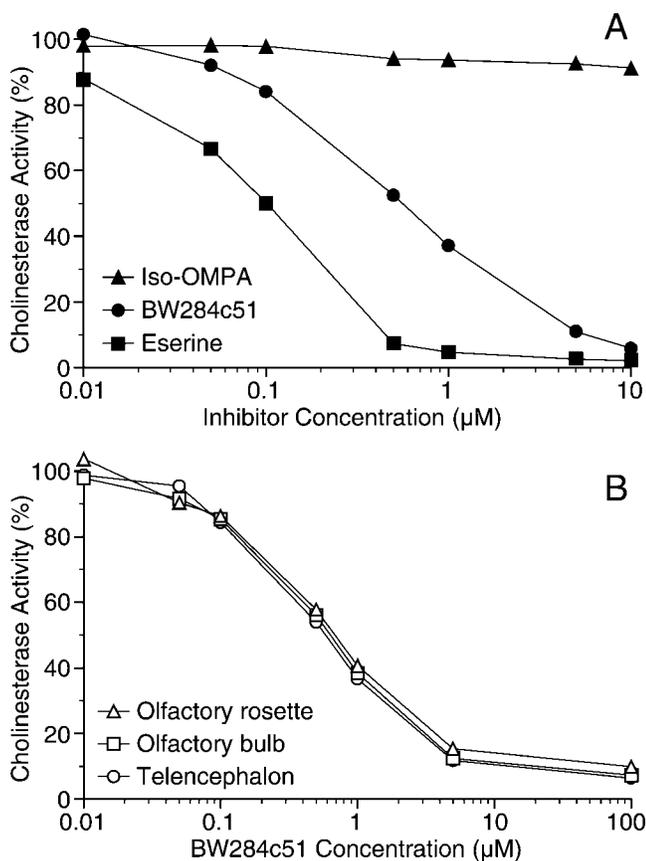


Fig. 2. Basal cholinesterase activity in the chinook nervous system is attributable to AChE (acetylcholinesterase). (A) Cholinesterase activity in the telencephalic extract (see the *Materials and Methods* section) was inhibited in a dose-dependent fashion by eserine (a general inhibitor of serine esterases) and the AChE-specific inhibitor BW284c51 (1,5-bis[4-allyldimethyl-ammoniumphenyl]pentan-3-one dibromide). By contrast, the butyrylcholinesterase-specific inhibitor iso-OMPA (tetraisopropylpyrophosphoramidate) had no effect on enzyme activity. (B) The inhibitory dose-response curves for BW284c51 were very similar for all three tissues examined.

response relationship for each chemical are shown in Table 1. As expected for pesticides that share a common mode of action, the slopes of the individual curves were similar (range 1.03–1.37). The relative position of the individual curves along the *x*-axis (the variation in IC₅₀s) reflects differences in the inhibitory potencies of individual pesticides. This is likely because of differences in binding affinity for AChE [34].

Table 1. Parameters of the curve fits of the dose-response data shown in Figure 3. See *Materials and Methods* section for a description of the equation and parameters. The combined data pooled from all five pesticides shown in Figure 3B were fit with half fixed at 1, because the individual pesticides were normalized to their respective IC₅₀ values (the concentration producing 50% enzyme inhibition) before being pooled

Pesticide	<i>r</i> ²	Slope		Half (IC ₅₀) (μg/L)	
		Best fit value	SE	Best fit value	SE
Carbaryl	0.9987	1.19	0.04	66	2
Carbofuran	0.9967	1.03	0.05	14.7	0.8
Diazinon-oxon	0.9970	1.34	0.07	525	22
Malathion-oxon	0.9939	1.26	0.09	4.1	0.3
Chlorpyrifos-oxon	0.9960	1.37	0.08	8.0	0.4
Combined	0.9957	1.20	0.04		

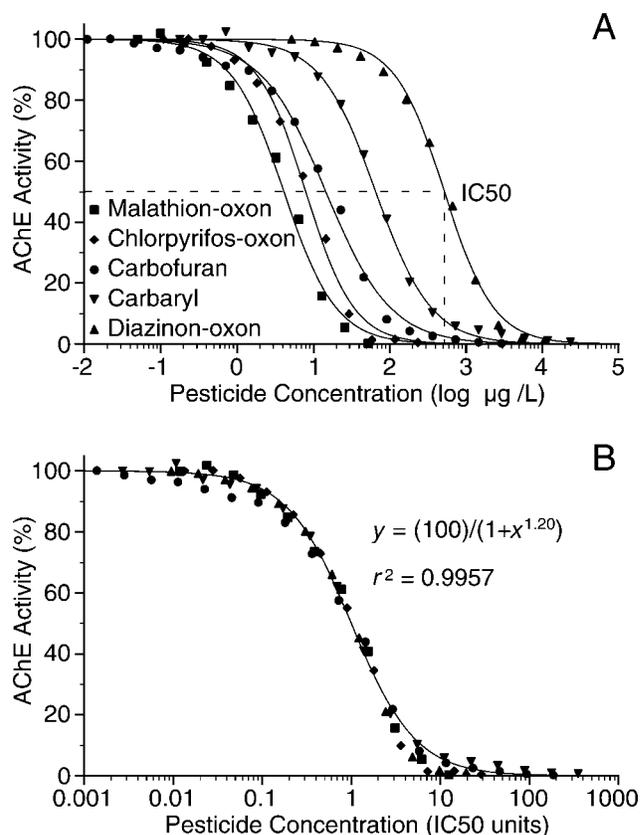


Fig. 3. The slopes of the dose-response curves for chinook AChE (acetylcholinesterase) inhibition by individual organophosphate and carbamate pesticides are similar. (A) Solid lines show the best fit sigmoid function for the inhibitory effects of carbaryl, carbofuran, and the oxon derivatives of chlorpyrifos, diazinon, and malathion. The individual curves have similar slopes but vary in position along the *x*-axis. (B) The dose-response data for each pesticide were replotted after dividing each treatment concentration by the respective concentration producing 50% enzyme inhibition (IC₅₀) for that particular chemical (an example for diazinon-oxon is shown in panel A). The solid line shows a single sigmoid function fit to the pooled, IC₅₀-normalized data (*p* < 0.001).

Pesticide mixtures produce additive AChE inhibition

To explicitly define an outcome of no interaction among individual components of a pesticide mixture (i.e., additive AChE inhibition), the data from Figure 3A were combined [35]. First, the dose-response curve for each pesticide was normalized to the IC₅₀ for that particular chemical. As shown

in Figure 3B, IC50 normalization effectively collapsed the five dose–response curves to a single curve centered around a value of one IC50 unit on the x -axis. Although one IC50 unit represents a different absolute concentration for each pesticide (Table 1), it also represents the relative concentration that produces an equivalent degree of AChE inhibition (50%). On an IC50-normalized scale, a value of 0.4 corresponds to a concentration that is 40% of a pesticide's IC50, whereas a value of 1.5 corresponds to a concentration that is 150% of the same IC50. A single regression was fit to the normalized and pooled data for all five pesticides (Fig. 3B, parameters in Table 1). The significance of the fit ($r^2 = 0.9957$; $p < 0.001$) is consistent with the five chemicals sharing a common mode of action [35].

The dose–response curve in Figure 3B served as the empirical basis for evaluating mixtures of organophosphate and carbamate pesticides for additive AChE inhibition. The curve describes the prediction of no interaction, or additivity. The three possible responses to a binary mixture (addition, antagonism, or synergism) are illustrated as a hypothetical example in Figure 4A. In this example, a mixture containing two pesticides at concentrations of 0.4 and 0.6 respective IC50 units (open circles) should have a cumulative effect on chinook AChE activity that is equivalent to 50% inhibition if the two chemicals are noninteractive. This outcome is addition, and the result from the combined exposure (filled circles) should fall on the curve derived from Figure 3B. Conversely, if the two pesticides interact at the level of the target enzyme, the combined effect could be either antagonism or synergism. If the result falls above the curve (<50% inhibition in this example), the interaction would be antagonistic. If the result falls below the curve (>50% inhibition), the interaction would be synergistic. The curve therefore serves to predict the extent of AChE inhibition caused by any binary mixture (and at any cumulative IC50 unit) where the response to the two chemicals is additive.

The joint inhibitory effects of all possible binary combinations of carbaryl, carbofuran, chlorpyrifos-oxon, diazinon-oxon, and malathion-oxon ($n = 10$ combinations) on chinook salmon AChE activity are shown in Figure 4B. For each binary pair (e.g., carbofuran plus carbaryl), pesticides were assayed individually over a range of concentrations and together as a single mixture. The dose–response relationships for individual chemicals were used to calculate IC50s for the purpose of normalizing the data in Figure 4B. The results for the individual treatments (open circles in Fig. 4B) and the possible binary mixtures (closed circles in Fig. 4B) were then compared with the curve derived from the larger IC50-normalized data set (Fig. 3B). Each possible mixture was tested at two different concentrations (10 combinations \times 2 concentrations = 20 mixtures plotted). The aim was to produce two sets of mixtures with cumulative IC50 units of 0.5 and 1.0, which is why the measured rates of AChE inhibition cluster around these values in Figure 4B. The curve was a very close fit for both of the individual pesticide results ($r^2 = 0.987$) and the results of the binary mixtures ($r^2 = 0.959$). Therefore, mixtures of organophosphate and carbamate insecticides are noninteractive and additive in terms of their inhibition of chinook salmon AChE enzyme activity.

DISCUSSION

Acetylcholinesterase plays a critical role in the vertebrate nervous system by terminating acetylcholine-mediated neurotransmission. Given the wide distribution of cholinergic net-

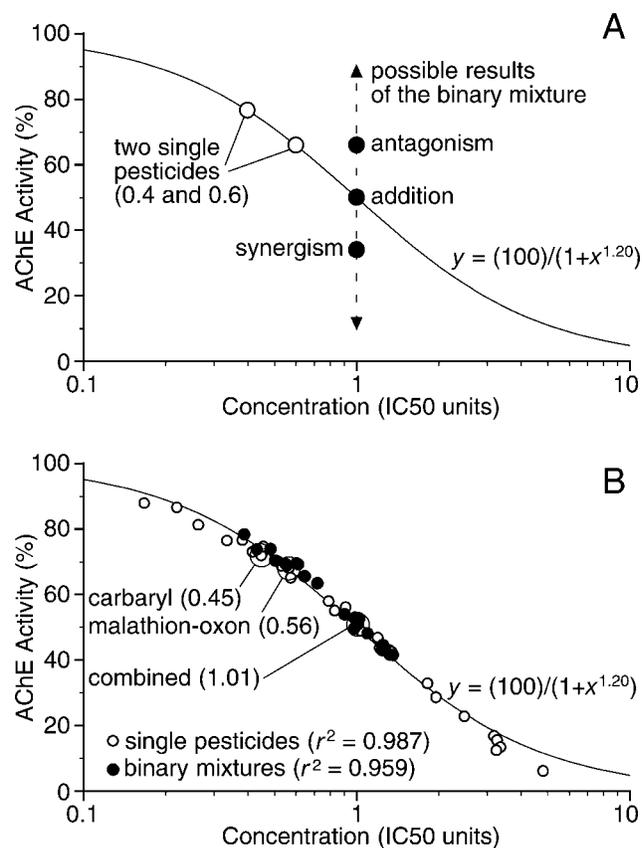


Fig. 4. Binary mixtures of organophosphates and carbamates produce additive inhibition of Chinook acetylcholinesterase (AChE) activity. (A) Hypothetical basis for defining no interaction among individual components of a pesticide mixture. Open circles denote two pesticides presented singly at IC50 units of 0.4 and 0.6. The vertical dashed line represents the possible effects of the binary mixture of the two pesticides (a combined IC50 unit of 1.0). A result of strict addition will fall on the curve generated in Figure 3B (in this 50%), while antagonism or synergism will be somewhere above or below the curve, respectively. (B) Empirical data for five pesticides are presented individually (open circles) and as binary mixtures (closed circles; see the Results section). Note that the data for individual pesticides are different from those shown in Figure 3 and that not all data are shown. The three large circles highlight a specific pairing of pesticides. The mixtures produced results that fell along the curve generated from the data in Figure 3B, consistent with addition.

works in the vertebrate brain [19], environmental agents that interfere with AChE activity have the potential to disrupt many different neurobehavioral processes. For salmonids, the toxicological effects of anticholinesterase pesticides have generally been evaluated on a chemical-by-chemical basis. This includes, for example, recent studies on chlorpyrifos [24,31,36], diazinon [25,37], malathion [37], carbaryl [38], and carbofuran [23]. By contrast, the cumulative toxicity of mixtures containing organophosphate and carbamate insecticides has not been explored. Here, we show that combinations of these five insecticides (or their active metabolites) produce additive inhibition of chinook salmon AChE in vitro. It is therefore likely that the individual components of anticholinesterase pesticide mixtures are noninteractive and that the joint toxicity of such mixtures can be explained by simple dose addition.

In this study, we focused on in vitro preparations of chinook salmon AChE. Thus, our parameter estimates for the inhibitory potencies of individual pesticides (Table 1) might not accu-

rately reflect dose–response relationships for AChE inhibition *in vivo*. This is because of potential differences in the uptake of chemicals from aquatic habitats, as well as differences in the toxicokinetics and toxicodynamics of individual pesticides in the intact animal. It is worth noting, however, that previous studies in other vertebrate species have shown that *in vitro* and *in vivo* rates of AChE inhibition are often correlated [39].

Molecular forms of AChE in the chinook olfactory system

In the chinook olfactory forebrain (olfactory bulb and telencephalon), the membrane-associated, globular forms of AChE were predominant. This is consistent with the distribution of these forms in other fish species [40], and also with AChE's classical role in terminating neurotransmission at cholinergic synapses in the central nervous system [32].

Interestingly, the enzyme activity associated with the chinook olfactory epithelium can be attributed largely to the soluble, globular form of AChE. In other species, this form is not typically associated with cholinergic synapses, and it has also been found in nonneuronal tissues [32]. The role of this soluble enzyme is currently unclear but might include non-synaptic, nonneuronal, or even noncatalytic functions. Several studies have shown that *in vivo* exposure to organophosphates or carbamates inhibits AChE activity in the olfactory rosettes of fish (e.g., [23]). However, there are no known cholinergic synapses in the peripheral sensory epithelium, and it is possible that AChE is expressed in cell types other than the ciliated receptor neurons that mediate olfactory signal transduction. Jarrard et al. [23] have hypothesized, for example, that AChE plays a role in epithelial mucus secretion, and that this, in turn, could limit the delivery of odorants to receptor cells.

Pesticide mixtures and salmon health

Surface water monitoring studies in salmon habitats [3–8] have generally detected anticholinesterase insecticides at concentrations that are well below those likely to cause acute fish kills (i.e., median lethal concentrations for rainbow trout). Consequently, from a natural resource management perspective, there are two primary concerns for salmonids. The first is whether these chemicals, alone or in combination, reach concentrations in the environment that are sufficient to inhibit AChE activity in the nervous systems of fish. A related concern is whether enzyme inhibition corresponds to sublethal physiological effects that might ultimately reduce the survival, distribution, or reproductive success of exposed salmon or steelhead.

Relatively few studies have explored the effects of organophosphate and carbamate insecticides on salmon AChE activity at low, environmentally realistic exposure concentrations. As a consequence, quantitative thresholds for sublethal AChE inhibition have not been determined for most chemicals. One exception is the organophosphate chlorpyrifos. Sandahl and Jenkins [31] measured AChE inhibition in the brains of juvenile steelhead after laboratory exposures to chlorpyrifos at concentrations representative of the upper range detected in surface waters of Pacific Northwest watersheds. Similarly, in juvenile coho, benchmark concentrations (statistical departure values) for chlorpyrifos-induced AChE inhibition in brain and muscle were recently shown to be at or below 0.5 $\mu\text{g/L}$ [36]. Although comparable thresholds for other anticholinesterase insecticides are not known, there is a clear potential for mixtures of these agents to produce measurable AChE inhibition in field-exposed fish [41].

For salmonids, the relationship between AChE inhibition and higher order neurobehavioral effects has not been widely explored. Sandahl et al. [36] described the effects of low-level chlorpyrifos exposures on coho brain and muscle AChE activity *in vivo* and compared these with sublethal impairments of spontaneous swimming and feeding behaviors. Chlorpyrifos reduced both endpoints at environmentally relevant concentrations, and these effects were significantly correlated. Thus, in certain western river systems, chlorpyrifos alone could reach levels sufficient to cause both AChE inhibition and neurobehavioral effects in salmon. More work is needed to determine comparable *in vivo* physiological effect thresholds for other organophosphate and carbamate insecticides, alone and in combination.

Pesticide mixtures and ecological risk assessment

Several federal statutes have been enacted in the United States to protect nontarget species from the potentially harmful effects of pesticides. These include the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Clean Water Act (CWA), and the ESA. The first two statutes are chemical specific, in that they address the registration or reregistration of individual pesticides and the development of water quality criteria for single chemicals, respectively. Both are focused on the sources, transport, and fate of individual chemicals, as well as their potential effects on fish and other species. The U.S. Environmental Protection Agency administers FIFRA and the CWA and conducts ecological risk assessments in both regulatory contexts. At present, U.S. Environmental Protection Agency risk assessments do not formally assess the ecological effects of pesticide mixtures [15].

The ESA, by contrast, is a species-specific statute. For ESA-listed salmonids, the underlying question of risk attempts to define the contribution of habitat degradation (among other stressors) to the decline of distinct population segments [10]. The distribution and biological requirements of listed species are therefore important considerations for ecological risk assessment, as is the condition of the different ecosystems that support salmon. Because pesticide mixtures are frequently detected in salmon habitats [3–8], assessments that focus exclusively on single chemicals might underestimate the actual risk to threatened and endangered species under real-world exposure conditions. This is particularly true for the anticholinesterase insecticides, as well as other classes of pesticides that share a common mode of action.

Reducing uncertainty surrounding the ecotoxicological effects of pesticide mixtures is likely to pose a considerable scientific challenge for the foreseeable future [15]. We have shown here that the joint inhibitory effects of simple mixtures of organophosphate and carbamate insecticides on chinook salmon AChE activity are dose-additive *in vitro*. That is, the effect (AChE inhibition) can be estimated directly from the sum of the individual concentrations that make up the mixture. It should therefore be possible in future studies to empirically determine the relative potencies of individual anticholinesterase insecticides as well as thresholds for AChE inhibition *in vivo*; estimate the aggregate effects of organophosphate and carbamate mixtures on salmon AChE activity by dose addition; and relate cumulative AChE inhibition to behaviors that underlie the growth and survival of at-risk salmon and steelhead. Ecological risk assessments for threatened and endangered species would benefit considerably from such information.

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