

An Integrated Addition and Interaction Model for Assessing Toxicity of Chemical Mixtures

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The high propensity for simultaneous exposure to multiple environmental chemicals necessitates the development and use of models that provide insight into the toxicity of chemical mixtures. In this study, we developed a mathematical model that combines concepts of concentration addition, response addition, and toxicokinetic chemical interaction to assess toxicity of chemical mixtures. A ternary mixture of acetylcholinesterase inhibiting organophosphates (malathion and parathion) and the P450 inhibitor piperonyl butoxide was used to model toxicity. Concentration-response curves were generated for individual chemicals as well as for mixtures of the chemicals using acute toxicity tests with *Daphnia magna*. The toxicity of binary combinations of malathion and parathion adhered to the principles of concentration addition. The contribution of piperonyl butoxide to mixture toxicity was integrated using a model for response addition. Piperonyl butoxide also modified the toxicity of the organophosphates by inhibiting their metabolic activation. The antagonistic effects of piperonyl butoxide towards the organophosphates were quantified as coefficients of interactions (K-functions) and incorporated into the mixture model. Finally, toxicity of the ternary mixture was modeled at 30 different mixture formulations using three additive models that assumed no interaction (concentration addition, response addition, and integrated addition) and using the integrated addition and interaction (IAI) model. Toxicity of the 30 mixtures was then experimentally determined and compared to model results. Only the IAI model accurately predicted the toxicity of the mixtures. The IAI model holds promise as a means for assessing hazard of complex chemical mixtures.

Key Words: synergy; cumulative toxicity; predictive model; toxicodynamic; hazard assessment; risk assessment.

Surveys of agricultural and urban streams and groundwater have brought public attention to widespread chemical mixture contamination (Battaglin *et al.*, 2003; Kolpin *et al.*, 2002). The infinite number of potential chemical combinations (in terms of both constituents and concentrations of constituents) limits the utility of standard toxicity testing methods for establishing hazard associated with chemical mixtures. Modeling ap-

proaches could augment the standard toxicity testing paradigm when evaluating hazards associated with exposure to chemical mixtures. Chemical constituents of a mixture can elicit similar action, dissimilar action, or interaction (Bliss, 1939; Cassee *et al.*, 1998). Models of mixture toxicity have focused primarily on quantifying the “no-interaction” scenarios, while cases of interaction often appear as qualitative observations (Hertzberg and MacDonell, 2002). Concentration addition (Loewe additivity) and response addition (Bliss independence) (Greco *et al.*, 1992) are commonly used to model the toxicity of non-interacting chemicals within a mixture.

Concentration addition models rely upon the assumption that mixture components contribute to toxicity through a common mechanism of action. Calculating mixture toxicity based upon concentration addition requires assessing the relative contribution of each constituent to the total toxicant pool. The toxicity of this pool is then modeled as a single toxicant. Concentration addition is the basis of the “toxic equivalency” approach commonly used to assess toxicity of chemicals of the same class such as dioxins (Safe, 1990). Ample evidence supports the use of the concentration addition model for assessing mixtures toxicity of like-acting chemicals (Altenburger *et al.*, 2000; Deneer *et al.*, 1988; Könnemann, 1981). The response addition model, also referred to as the independent joint action model, has been used to compute toxicity of mixtures when chemical constituents have different mechanisms of action (Backhaus *et al.*, 2000; Walter *et al.*, 2002). In the response addition model, combined effects of the chemicals are based upon the probability that individual constituents of the mixture will affect the exposed organisms.

The concentration addition and response addition models are limited in their application to complex mixtures in that they do not address chemical interactions. Toxicokinetic interactions can occur between chemicals in which one chemical alters the effective concentration of another (Andersen and Dennison, 2004). Alternatively, toxicodynamic interactions can occur between chemicals in which one chemical influences the response of the organism to another chemical (Andersen and Dennison, 2004). Both toxicokinetic and toxicodynamic interactions can significantly impact the toxicity of chemical mixtures. The importance of addressing chemical interactions

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was highlighted by the US EPA in their recommendations for evaluating risk associated with chemical mixtures (US EPA, 2000).

Recently, Altenburger *et al.* (2005) and Olmstead and LeBlanc (2005) demonstrated that concentration addition and response addition models could be integrated into a comprehensive model for use in evaluating toxicity of non-interacting chemical mixtures. The intent of the present study was to expand this approach to incorporate interactions among chemical constituents when they are predicted to occur. Important issues addressed in this work include: (1) evaluating whether single interaction modifiers can be applied to classes of chemicals and (2) establishing whether clearly defined binary interactions persist in higher order combinations. The strength of the integrated addition and interaction (IAI) model was assessed by comparing model results to experimentally determined toxicity of 30 different derivations of a ternary mixture.

MATERIALS AND METHODS

Daphnid culture. All toxicological experiments were performed with the daphnid *Daphnia magna*. Daphnids were acquired from long-standing cultures in our laboratory that were originally obtained from the US Environmental Protection Agency, Mid-Continent Ecology Division – Duluth, MN. Daphnids were maintained in reconstituted deionized water (192 mg/l CaSO₄·H₂O, 192 mg/l NaHCO₃, 120 mg/l MgSO₄, 8.0 mg/l KCl, 1.0 µg/l selenium and 1.0 µg/l vitamin B₁₂). Cultures were maintained in 1-liter beakers at a density of ~50 daphnids/l medium and culture medium was changed three times per week. Adult daphnids were discarded after three weeks and replaced with neonates. Culture beakers and all experiments were maintained in incubators with a 16/8-h light/dark cycle at a constant temperature of 20°C. Culture daphnids were fed 2.0 ml (1.4 × 10⁸ cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 ml (4 mg dry weight) of Tetrafin fish food suspension (Pet International, Chesterfill, New South Wales, Australia). The *Selenastrum* was cultured in the laboratory using Bold's basal medium.

Acute toxicity assays. Chemicals used in mixture analyses (malathion, parathion, and piperonyl butoxide) were acquired from ChemServices (West Chester, PA). Absolute ethanol was used as the carrier for all of the chemicals. All toxicity assessments were initiated with neonatal (≤24 h old) daphnids. Each treatment consisted of two 50 ml beakers containing 40 ml of exposure medium and 10 neonates. *Selenastrum* (7 × 10⁶ cells) and fish food homogenate (0.2 mg dry weight) were provided to each beaker as food at the start of each exposure. All beakers, including controls, contained 0.01% carrier (ethanol). Beakers were labeled on the bottom and randomly rearranged, so that the exposure concentration in each beaker was not known to the investigator when assessing response of organisms. At 48 h, neonates were evaluated for response. The response endpoint, immobilization, was judged by the inability of the neonate to occupy the water column during 10 s of observation.

Acetylcholinesterase analyses. Acetylcholinesterase activity was measured according to Ellman *et al.* (1961) as modified for use with microtiter plates (Fisher *et al.*, 2000) with minor additional modifications. Exposure groups consisted of three 250 ml beakers containing 200 ml solution and 40 neonates (≤24 h old). Algae (1.4 × 10⁷ cells) and fish food (0.4 mg dry weight) were added to each beaker once per day. Solutions were renewed at 24 h. Following the 48-h exposure period, neonates were transferred to 1.5 ml microfuge tubes. Media was removed from tubes; neonates were rinsed, and homogenized in 35 µl ice cold 0.02 M phosphate buffer, pH 8.0 with 1% Triton-

X-100 using a Teflon pestle. An additional 315 µl phosphate buffer, pH 8.0 without Triton-X-100 was then added and samples were mixed. Samples were centrifuged at 14,000 × g for 4 min at 4°C and supernatant was transferred to a clean pre-cooled microfuge tube. Approximately 100 µl of the supernatant was stored at –20°C for protein analysis. The following solutions were added to each well in a 96-well plate: 100 µl of 8 mM 5,5'-dithio-bis(2-nitrobenzoate) (D-1830 Sigma), 50 µl supernatant (phosphate buffer with 0.1% Triton-X-100 was used for supernatant blanks), 50 µl of 16 mM acetylthiocholine iodide (A-5751 Sigma). Absorbance was measured kinetically for 15 min at 420 nm using a Fusion Universal Microplate Analyzer (PerkinElmer, Boston, MA). Protein was measured according to Bradford (1976) using Bio-Rad Protein Assay dye concentrate (Hercules, CA) and a standard curve generated with bovine serum albumin. The molar extinction coefficient (13,300 M⁻¹·cm⁻¹) (Masson *et al.*, 2004) was used to calculate the amount of yellow anion, 5-thio-2-nitrobenzoate, formed over 15 min and this rate was normalized to the amount of protein added to the assay (nmol/min/mg). Analyses of variance and Tukey-Kramer HSD were used to determine if significant ($p \leq 0.05$) differences existed between treatments.

Individual chemical toxicity. Exposure concentrations for each chemical were selected, based upon preliminary experiments, that would span response levels from 0 to 100%. The percentage response was plotted against exposure concentration on a log scale and fit with a sigmoidal line using Origin software (Microcal Software Inc., Northampton, MA). The logistic equation representing the sigmoidal fit to the data is:

$$R = \frac{1}{1 + \left(\frac{EC50}{C}\right)^p} \quad (1)$$

where R is the response (% immobilization), C is the chemical concentration, p is the power or slope of the curve, and $EC50$ is the exposure concentration eliciting immobilization in 50% of exposed animals. These individual concentration-response curves were subsequently used in mixture modeling as described below.

Mixture Modeling

Concentration addition. According to Olmstead and LeBlanc's (2005) integrated addition model, like acting chemicals are assigned to a common cassette (i.e., grouping). Toxicity associated with the cassette is then calculated using a concentration addition approach. Accordingly, malathion and parathion were assigned to a common cassette, the organophosphate (OP) cassette. To establish whether the toxicity of the chemicals within the OP cassette conformed to a concentration addition model, five ratios (Table 2) of the chemicals (malathion:parathion) were each tested at six different concentrations. Parathion concentrations were expressed in terms of malathion equivalents. All five ratios were equitoxic based upon characterization of the toxicity of the individual OPs. The six concentrations of each binary mixture used in the experiments were selected to define the concentration-response curve for the mixture. The joint toxicity of these binary mixtures of like-acting chemicals was computed using the following equation (Olmstead and LeBlanc, 2005):

$$R = \frac{1}{1 + \left(\frac{1}{\sum_{i=1}^n \frac{C_i}{EC50_i}}\right)^{p'}} \quad (2)$$

where R is the response to the mixture, C_i is the concentration of chemical i in the mixture, $EC50_i$ is the concentration of chemical i that causes a 50% response, and p' is the average power associated with the chemicals in the cassette. The average power was used because chemicals within a cassette should have similar slopes, as was the case with malathion and parathion. Concentration-response results from each binary mixture were then used to calculate $EC50$ values as described for individual chemicals. Analyses of

variance were performed to detect significant ($p \leq 0.05$) differences among the five ratios using SAS 8.2 software (SAS Institute, Cary, NC).

Response addition. The concept of response addition was used by Olmstead and LeBlanc (2005) to compute the joint toxicity associated with the different chemical cassettes within a mixture. The response addition model was used because each cassette is assumed to elicit a response through different mechanisms. The response addition model can be depicted as:

$$R = 1 - \prod_{i=1}^n (1 - R_i) \quad (3)$$

where R represents the response to the mixture and R_i is the response to chemicals in cassette i .

Equations 2 and 3 were integrated to establish the response associated with individual cassettes within a mixture and to sum the responses associated with the cassettes (Olmstead and LeBlanc, 2005). The resulting equation is a combination of concentration and response addition equations:

$$R = 1 - \prod_{j=1}^N \left\{ 1 - \frac{1}{1 + \left(\frac{\sum_{i=1}^n C_i}{\sum_{i=1}^n EC50_i} \right)^p} \right\} \quad (4)$$

Chemical interactions. The ability of one chemical in the mixture to modify the effective concentration of another was defined by coefficients of interactions or K-functions (Finney, 1942; Mu and LeBlanc, 2004). Specifically, K-functions, defined the degree to which the concentration of PBO in the mixture altered the effective concentration (i.e., oxon metabolite) of either organophosphate in the mixture. K-functions were described by experimentally deriving the effect of concentrations of PBO on the EC50 values derived for each organophosphate. K-functions were calculated for each of the PBO concentrations with the following equation:

$$K = \frac{EC50_{OP}}{EC50_{OP+PBO_x}} \quad (5)$$

where $EC50_{OP}$ is the concentration of organophosphate that immobilized 50% of the exposed animals and $EC50_{OP+PBO_x}$ is the EC50 of the organophosphate when exposure occurred in the presence of x concentration of PBO. These K-functions were then plotted against the concentration of PBO from which they were derived. The logistic equation that defined this relationship was used to calculate K-functions when modeling mixture toxicity. K-functions were integrated into this model to describe toxicokinetic interactions between PBO and the organophosphates:

$$R = 1 - \prod_{j=1}^N \left\{ 1 - \frac{1}{1 + \left(\frac{\sum_{i=1}^n k_{a,i}(C_a) \times C_i}{\sum_{i=1}^n EC50_i} \right)^p} \right\} \quad (6)$$

where $k_{a,i}$ represents a function describing the extent to which chemical a (PBO) present in the mixture at concentration C_a alters the effective concentration of chemical i (malathion or parathion).

The response to thirty combinations of the three chemicals was computed using the concentration addition model (Equation 2), the response addition model (Equation 3), the integrated addition model (Equation 4), and the IAI model (Equation 6). In addition, the actual toxicity of the 30 mixtures was measured and results were compared to the four model results. The 30 mixture formulations were designed so that the ratio of the three chemicals varied among the mixture formulations. Model predictions were compared to experimental data using coefficients of determination (r^2 ; Zar, 1996). An r^2 value of 0.70 or greater was considered a good fit of the observed data to the model (Quality America, 2004).

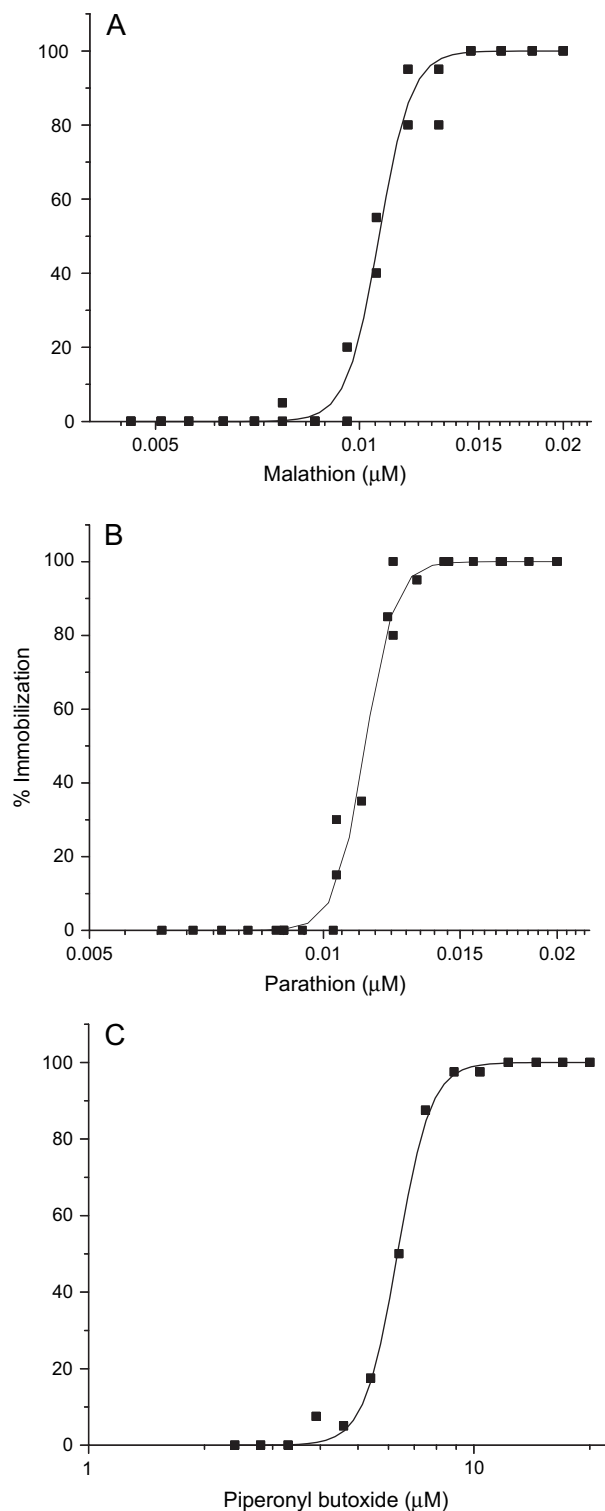


FIG. 1. Concentration-response profiles of the individual chemicals used in the mixtures toxicity assessment: malathion (A), parathion (B), and piperonyl butoxide (C). Data points represent the percentage of immobilized daphnids. Data were fit using a logistic equation (Equation 1).

TABLE 1
Concentration-Response Parameters of Individual Chemicals Used in Mixture Toxicity Modeling

Chemical	EC50 (μM)	95% Confidence interval (μM)	Power (p)
Malathion	0.0107	0.0105–0.0108	18
Parathion	0.0113	0.0112–0.0115	23
Piperonyl butoxide	6.34	6.24–6.44	10

Note. Toxicity of chemicals was assessed in 48-h acute toxicity tests measuring immobilization in *Daphnia magna*. The EC50, 95% confidence interval, and power were calculated from a logistic fit to the concentration-response data.

RESULTS

Individual Chemical Toxicity Analyses

The IAI model requires toxicity description for the individual chemicals within a mixture. Concentration-response curves were generated for malathion, parathion, and piperonyl butoxide (Fig. 1) from which EC50 values and corresponding 95% confidence intervals, and power of the curves (p) were derived (Table 1). The logistic equation provided a good fit to the malathion ($r^2 = 0.987$), parathion ($r^2 = 0.987$), and piperonyl butoxide ($r^2 = 0.998$) concentration-response data. The two organophosphates exhibited similar toxicity characteristics. Piperonyl butoxide was considerably less toxic as compared to the organophosphates and had a power approximately one-half that of the organophosphates.

Cassette Assignment

According to the IAI model, the organophosphates would be assigned to the same cassette and toxicity associated with the cassette would be assessed using a concentration addition approach. The validity of using concentration addition to model the toxicity associated with the organophosphate

TABLE 2
Concentration-Response Toxicity Evaluation of Five Ratios of Malathion:Parathion Predicted to be Equitoxic Based Upon Concentration Addition Modeling

Ratio	EC50 (μM)	95% Confidence interval (μM)
1:0	0.00889	0.00843–0.00939
2:1	0.00876	0.00844–0.00910
1:1	0.00874	0.00737–0.0102
1:2	0.00905	0.00865–0.00949
0:1	0.00867	0.00737–0.0102

Note. EC50s and 95% confidence intervals were calculated from a logistic fit to the concentration-response data generated for each ratio. Concentrations are expressed in malathion equivalents.

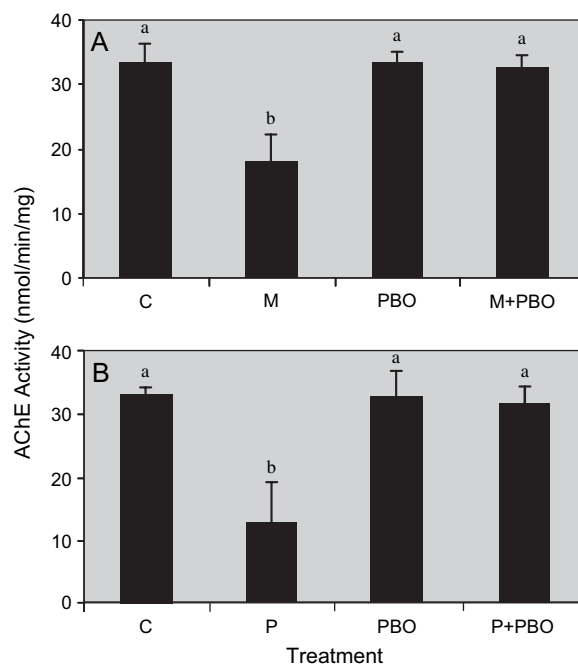


FIG. 2. Acetylcholinesterase activity in daphnids following exposure to mixture constituents. Treatment abbreviations: C, control; M, malathion; P, parathion; PBO, piperonyl butoxide. (A) Malathion was evaluated at 0.01 μM and PBO at 0.1 μM. (B) Parathion was evaluated at 0.01 μM and PBO at 0.1 μM. Bars represent the mean and SD for 3 replicate treatments. Treatments with the same letter were not significantly different (analyses of variance and Tukey-Kramer HSD, $p \leq 0.05$).

cassette was determined using several combinations of the two organophosphates deemed to be equitoxic based upon concentration additivity. Indeed, the concentration-response assessments of these binary mixtures were statistically indistinguishable (Table 2). Therefore, the contributions of malathion and parathion to the toxicity of the final mixtures were modeled as a single organophosphate cassette.

The common mode of action of the organophosphates—the inhibition of acetylcholinesterase activity—was confirmed experimentally (Fig. 2). In contrast, piperonyl butoxide did not inhibit acetylcholinesterase activity. Piperonyl butoxide was, therefore, assigned to its own cassette where the toxicity of this mixture component was integrated into the toxicity of the mixture using the response addition model.

Chemical Interaction

We hypothesized that piperonyl butoxide would interact with the constituents of the organophosphate cassette in a manner that would modify the toxicity associated with this cassette. The ability of piperonyl butoxide to abrogate the acetylcholinesterase-inhibiting potential of each organophosphate was demonstrated directly (Fig. 2). The antagonistic effect of piperonyl butoxide on the toxicity of the organophosphates was further demonstrated by the progressive shifting of the concentration-response curves for malathion (Fig. 3A) and

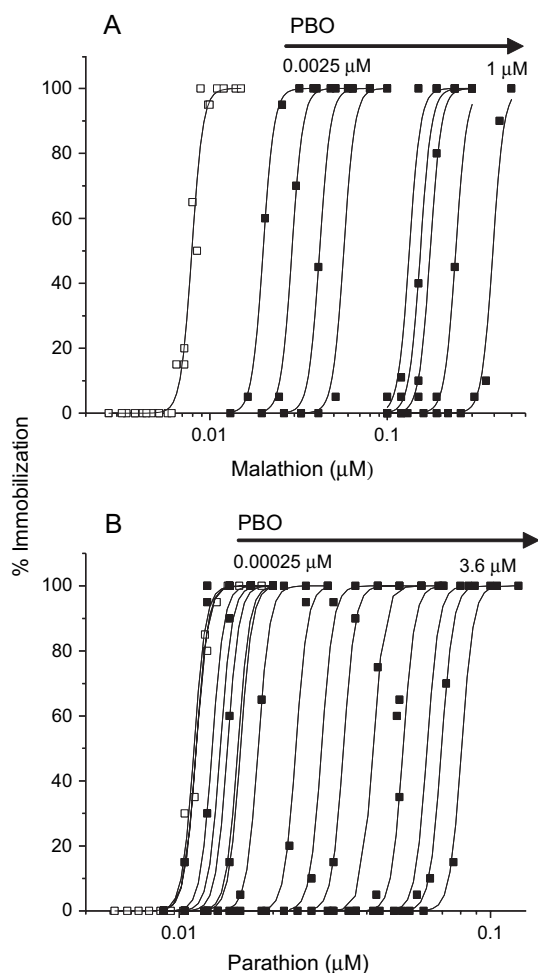


FIG. 3. Impact of piperonyl butoxide on organophosphate concentration-response curves. Concentration-response curves were generated for malathion (A) and parathion (B) alone (\square) and in combination with increasing concentrations of piperonyl butoxide (\blacksquare). Data points represent the percentage of immobilized daphnids. Data were fit using Equation 1. All piperonyl butoxide concentrations tested were below the *no observed effect level* for piperonyl butoxide alone.

parathion (Fig. 3B). This modifying effect of piperonyl butoxide was quantified as concentration-dependent K-functions (Fig. 4). These K-functions were used in the final IAI model to modify the effective concentrations of malathion and parathion as dictated by the concentration of piperonyl butoxide in the mixture.

Mixtures Toxicity Assessment

The toxicity of 30 combinations of the ternary mixture (Table 3) was experimentally determined and compared to predicted toxicity using the concentration addition model (Equation 2), the response addition model (Equation 3), the integrated addition model (Equation 4), and the IAI model (Equation 6). Neither the concentration addition, response addition nor integrated addition models accurately described

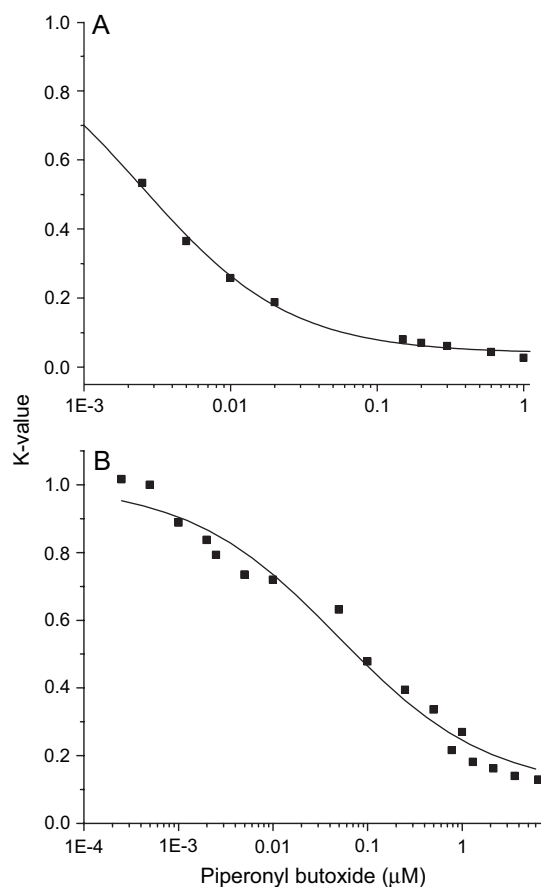


FIG. 4. The relationship between piperonyl butoxide concentrations and modifying effects on malathion (A) and parathion (B) as defined by K-functions. K-functions were derived for each concentration of PBO evaluated (Fig. 3) using Equation 5. Parameters used in Equation 5 were derived from the concentration-response curves depicted in Figure 3. A curve was fit to the plot using Equation 1.

the toxicity of the mixtures ($r^2 < 0.10$). Rather, all models grossly overestimated mixture toxicity (Figs. 5A–5C). However, the IAI model provided a good ($r^2 = 0.716$) assessment of the toxicity of the various mixture formulations (Table 3, Fig. 5D). Toxicity was accurately estimated within a factor of 2 for 83% of the mixture formulations.

DISCUSSION

The results of this study demonstrate that toxicokinetic interactions can be incorporated into an integrated addition model to assess mixture toxicity. Recent studies have shown that concentration and response addition models can be used in combination to create a comprehensive additive model to calculate the toxicity of non-interacting chemical mixtures (Altenburger *et al.*, 2005; Olmstead and LeBlanc, 2005; Teuschler *et al.*, 2004). Here, we build upon that modeling framework by incorporating toxicokinetic interactions between mixture constituents.

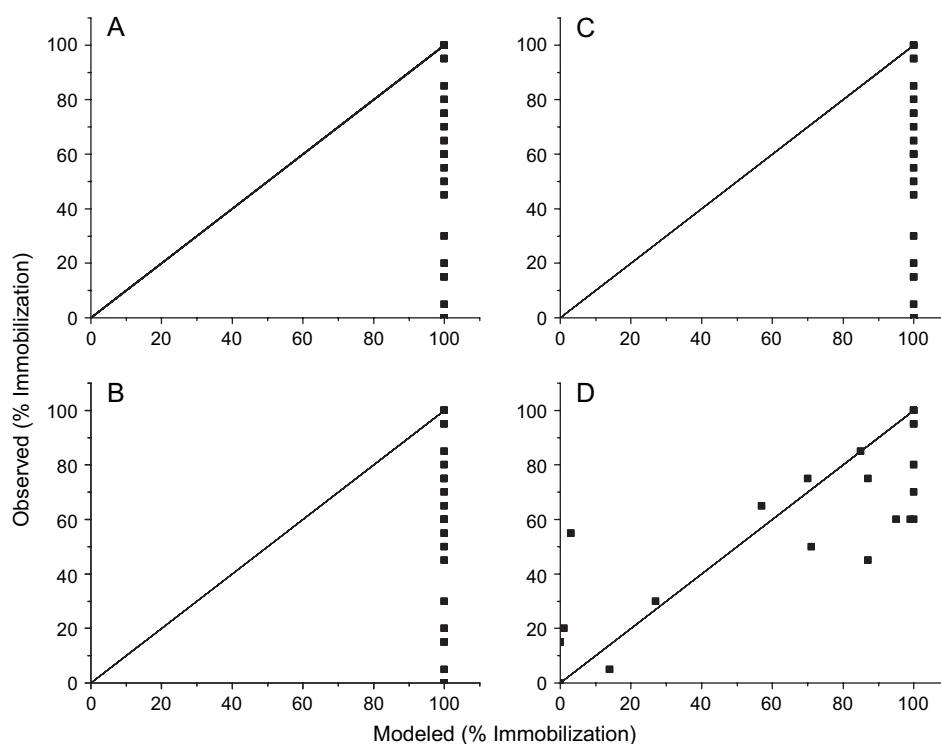


FIG. 5. Comparison of observed data to results generated from concentration addition (A), response addition (B), integrated addition (C), and integrated addition and interaction models (D). The solid line represents a 1:1 relationship between modeled and predicted data. Observed data were generated in toxicity tests with *Daphnia magna* using thirty concentrations of the ternary mixture of malathion, parathion, and piperonyl butoxide described in Table 3. Concentration addition, response addition, integrated addition, and integrated addition and interaction results were calculated with Equations 2, 3, 4, and 6 respectively.

By definition, chemical interactions represent a deviation from simple additivity when modeling mixture toxicity. To quantify these interactions, the expected additive toxicity of the mixture must first be determined. Choosing the appropriate model to assess additivity is essential for accurate interpretation of interaction results. US EPA guidelines for assessing mixture toxicity suggest a default model of concentration addition (2000). This recommendation is based on a tendency towards more conservative estimates of mixture toxicity with concentration addition than with response addition modeling (Drescher and Boedecker, 1995). However, indiscriminate application of concentration addition lacks a sound mechanistic basis and therefore increases the uncertainty associated with predicting mixture toxicity. The integrated addition model described in recent works (Altenburger *et al.*, 2005; Olmstead and LeBlanc, 2005) provides a mechanism-based alternative to assessing mixture toxicity. Initially, chemicals with similar mechanisms of action are placed into groups, or cassettes. The toxicity within each cassette is modeled with concentration addition and overall toxicity of the different cassettes is then modeled with response addition (Fig. 6). The integrated addition models presented by Altenburger *et al.* (2005) and Olmstead and LeBlanc (2005) are conceptually equivalent and differ only slightly in their methods of calculation. The

integrated addition model represents a significant advance in assessing toxicity of non-interacting chemical mixtures. This model, however, is not equipped to manage interactions among chemicals that impact toxicity of the mixture.

The possibility of significant synergistic interactions occurring between two or more chemicals in the environment is perhaps the most compelling reason to study mixture toxicity. Well-defined examples of synergy include enhanced hepatotoxicity of carbon tetrachloride with pre-exposure to kepone (Klingensmith and Mehendale, 1982) and interactions involving hormone receptor antagonists and hormone synthesis inhibitors (Mu and LeBlanc, 2004). Interactions often can be predicted based on mechanisms of action of constituent chemicals. For example, the P450 inhibitor piperonyl butoxide used in the present study was hypothesized to antagonize the toxicity of malathion and parathion by decreasing their metabolic activation. However, some interactions will not be apparent from constituent mechanisms of action. The integrated addition model has the potential to identify these unexpected interactions. In effect, significant deviation of experimental results from model predictions implies interaction. Once the source of the interaction is identified, either through inference or experimentation, quantification and incorporation of the interaction into the model follow.

TABLE 3
Formulations of the 30 Ternary Mixtures of Malathion, Parathion, and Piperonyl Butoxide (PBO) Used to Model (IAI Model; Equation 6) and Measure (with *D. magna*) Mixture Toxicity

Mixture (#)	Malathion (μM)	Parathion (μM)	PBO (μM)	Response (% immobilization)	
				Modeled	Measured
1	0.0271	0.0193	1.81	0	0
2	0.0190	0.0305	1.90	0	0
3	0.0350	0.0161	2.00	0	0
4	0.0158	0.0395	2.11	0	0
5	0.0444	0.0119	2.22	0	0
6	0.0117	0.0500	2.34	14	5
7	0.0553	0.0066	2.46	0	0
8	0.0065	0.0623	2.59	85	85
9	0.0681	0.0000	2.72	0	0
10	0.0000	0.0767	2.87	99	60
11	0.0453	0.0323	3.02	0	0
12	0.0318	0.0510	3.18	27	30
13	0.0585	0.0268	3.34	0	15
14	0.0264	0.0659	3.52	95	60
15	0.0741	0.0198	3.71	0	15
16	0.0195	0.0835	3.90	100	70
17	0.0924	0.0110	4.11	1	20
18	0.0108	0.1041	4.32	100	80
19	0.1138	0.0000	4.55	3	55
20	0.0000	0.1281	4.79	100	95
21	0.0756	0.0540	5.04	87	45
22	0.0531	0.0852	5.31	100	60
23	0.0978	0.0448	5.59	71	50
24	0.0441	0.1101	5.88	100	95
25	0.1238	0.0331	6.19	57	65
26	0.0326	0.1395	6.52	100	100
27	0.1543	0.0184	6.86	70	75
28	0.0181	0.1738	7.22	100	100
29	0.1900	0.0000	7.60	87	75
30	0.0000	0.2140	8.00	100	100

Toxicokinetic interactions can be incorporated into mixture assessments via a qualitative “weight of evidence” approach or a quantitative approach. The two approaches are conceptually quite similar in that both modify the effective concentrations of chemicals in an effector concentration-dependent manner. However, the approaches differ significantly in their application. The “weight of evidence” approach (Mumtaz and Durkin, 1992; modified by Hertzberg *et al.*, 1999) is currently recommended in the EPA mixture toxicity guidelines (2000). Briefly, interaction terms that define the effect of one chemical upon another are generated based upon the predicted magnitude of interaction (experimentally determined or default value) as a function of the concentrations of the interacting chemicals. Hazard quotients (exposure level divided by reference dose or reference concentration) of individual chemicals in the mixture are multiplied by the interaction term. The modified hazard quotients are then summed to arrive

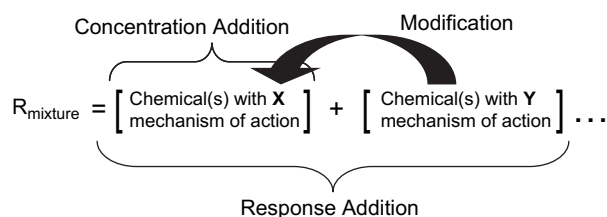


FIG. 6. Schematic representation of the integrated addition and interaction model. R_{mixture} represents the response to the mixture. Similar-acting chemicals are placed in a cassette. Toxicity associated with the cassette is calculated using the concentration addition model. Toxicokinetic interactions between chemicals are incorporated as modifiers of concentrations of chemicals within the cassettes. Total mixture toxicity associated with all cassettes is calculated using the response addition model.

at the hazard index of the mixture (Hertzberg and MacDonell, 2002). The hazard index is dimensionless and simply provides a general estimate of the hazard associated with the mixture. It is useful for identifying potentially hazardous mixtures, but it does not provide an accurate calculation of mixture toxicity. Alternatively, a strictly quantitative approach was described by Mu and LeBlanc (2004), which is based on the concept of k-values, or K-functions, first introduced by Finney (1942). This approach involves quantification of the progressive shift in the concentration-response curve of a chemical elicited by increasing concentrations of the effector chemical.

The primary goal of this work was to establish whether modifying functions (i.e., K-functions) could be used to augment the integrated addition model to account for chemical interactions that impact toxicity of mixture constituents. A secondary aim of this work was to increase our understanding of how mechanism-based classes of chemicals, or cassettes, function in mixtures. For example, evidence suggests that certain classes of chemicals display consistent patterns of interaction (Durkin *et al.*, 1995). Such consistency raises the possibility that K-functions could be generated that describe the effect of one cassette of chemicals upon another cassette. However, displaying the same type of interaction does not imply that the chemicals exhibit the same magnitude of interaction. In the present work, piperonyl butoxide demonstrated substantial antagonism with both malathion and parathion; however, the degree of antagonism was significantly different between the two organophosphates necessitating the generation of K-functions specific to each organophosphate. Application of K-functions based on malathion/piperonyl butoxide interactions to the entire organophosphate cassette significantly underestimated mixture toxicity (data not shown). Further, some organophosphates (e.g., dichlorvos) do not require metabolic activation, but are detoxified by P450s. These compounds might appropriately be assigned to the organophosphate cassette to calculate joint organophosphate toxicity, but they would require K-functions that describe a synergistic, and not antagonistic, interaction with piperonyl butoxide.

The three concepts describing mixture behavior originally identified by Bliss (1939) over 60 years ago are mathematically integrated in the IAI model. The IAI model provided reasonable predictions of the toxicity of a ternary mixture tested at thirty unique formulations. The model represented a significant improvement over basic addition models. The variability that did exist between observed and modeled results may be due to several factors. Inherent biological variability resulting in different responses of organisms between assays may have contributed to some of the observed variability. The assumption that K-functions derived in binary exposures are unaffected when used with higher order chemical mixtures may not be entirely correct. Further testing of the IAI model with increasingly complex mixtures will help to elucidate basic principles and limitations associated with K-function application.

This model is relatively simple in its application and requires input parameters that are typically available from standard concentration-response analyses. However, quantification of interactions among chemicals requires rigorous experimentation. Future studies may reveal whether limited but targeted experimentation can provide the information required to quantify interactions. Additional studies also are required to develop means of describing interactions where the response to a chemical modifies the organism's response to another chemical in the mixture. Such toxicodynamic interactions are less common (Hertzberg and McDonnell, 2002), but may still be important contributors to mixture toxicity. The IAI model holds promise to increase the accuracy of hazard and risk assessments of chemical mixtures by reducing uncertainty in estimating mixture toxicity.

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