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Original Research

Toxicity Assessment of Environmentally Relevant Pollutant Mixtures Using a Heuristic Model

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ABSTRACT

In this study, 9 chemicals were chosen from a recent report on surface water concentrations of a variety of xenobiotics to test the hypothesis that the toxicity of chemical mixtures could be estimated using a model based on the toxicity of the individual chemicals. Concentration-response curves for the endpoints of lifespan, growth rate, and fecundity were generated for each chemical experimentally using the crustacean, *Daphnia magna*. From this data, a mathematical model for the combined toxicity of these chemicals was generated that merged the concepts of concentration addition and independent joint action. Toxicity of a mixture was modeled at various levels at which the ratio of the chemicals within the mixture was maintained at that reported for median detected environmental levels. Toxicity of the mixture was then determined experimentally and compared to model predictions. The model accurately predicted the most sensitive endpoint, as well as the lowest toxic effect level of the mixture. Results demonstrated that, for this mixture of chemicals, toxicity was not influenced significantly by interactions among the chemicals and a single constituent dominated toxicity. According to model predictions and experimental results, the median detected environmental concentrations of chemicals constituting this mixture provided no margin of safety.

Keywords: Mixture toxicology Mixture model Daphnid Insecticides Pollutants

INTRODUCTION

Human activity results in the deposition of a large variety of xenobiotics into the environment on a continual basis, which effectively ensures human and wildlife exposure to complex mixtures of xenobiotics (Carpenter et al. 1998). Freshwater streams and rivers receive chemical input from many diverse sources of contamination, such as wastewater and industrial discharge, agricultural and urban runoff, landfill seepage, and animal waste overspill. The presence of contaminant mixtures frequently have been found in streams (Kolpin et al. 2002), groundwater (Kolpin et al. 2000), and well water (Stackelberg et al. 2001) in the United States. Although scientists generally have a good understanding of the toxicity of individual chemical pollutants, there is a great need to bridge the gap between our understanding of the toxic effects of exposure to individual xenobiotics and those effects from exposure to mixtures of these chemicals.

Two approaches typically are used to predict the toxicity of chemical mixtures: Dose, or concentration addition, and independent joint action (Drescher and Boedeker 1995). The concept of concentration addition assumes that chemicals share the same mechanism of action for toxicity (Bliss 1939; Drescher and Boedeker 1995; Feron and Groten 2002) and is expressed mathematically as

$$\sum_{n}^{i=1} \frac{C_i}{ECx_i} = 1 \qquad (1)$$

where C_i is the concentration of the ith chemical in a mixture and ECx_i is the concentration of the ith chemical that elicits the same response (x) as the full mixture. This equation serves as the framework for the summation of toxicity of similarly acting chemicals. The concept of independent joint action assumes that the chemicals elicit their effects through different mechanisms of action that have no interaction with each other (Drescher and Boedeker 1995; Feron and Groten 2002). Here the response of the mixture (R_{mix}) is calculated from the combined responses of individual chemicals (R_I), based on probability theory

$$R_{mix} = 1 - \prod_{n}^{I=1} (1 - R_I) \qquad (2)$$

Both approaches have been used to model joint toxicity of chemicals in the aquatic environment. In cases where all the constituents of a mixture were known to possess the same mechanism of action, effects have been predicted accurately by a concentration addition model (Altenburger et al. 2000; Lin et al. 2004). Likewise, when the constituents of a mixture were known to have differing mechanisms of action, the joint toxicity conformed to a model of independent joint action (Backhaus et al. 2000; Walter et al. 2002). Real-world mixtures, however, are made up of chemicals with both similar and dissimilar mechanisms of action. In the present paper, we used a model that integrates both concepts of concentration addition and independent joint action.

Surface waters of the United States are known to harbor complex mixtures of chemicals, though concentrations of individual chemicals commonly exist at levels not considered toxic (Kolpin et al. 2002). However, there is a concern that mixtures of chemicals at which individual constituents are present at low, nontoxic levels may elicit toxicity due to additive or synergistic effects among the constituents (Kortenkamp and Altenburger 1999; Rajapakse et al. 2002). In the present study, 9 chemicals that commonly have been measured in surface waters of the United States (Table 1) were evaluated for toxicity in combination to determine whether the toxicity of a mixture of these chemicals at environmentally relevant levels could be assessed adequately using an additive model, and whether this combination would

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Table 1. Summary of chemicals used in the mixture assessment. The frequency of detection and the median detected concentrations were reported in a survey of U.S. streams (Kolpin et al. 2002). Frequency denotes the percentage of sampled sites at which the chemical was detected. Median concentrations were derived only using measured values that were above the analytical detection limit. Reported (USEPA 2004) toxicity of each chemical to *Daphnia magna* is summarized as median acute toxicity values (24-h EC50, immobilization) and chronic values (21-d no observable effect concentration [NOEC], growth, and reproduction). An asterisk denotes the median 48-h EC50 for chemicals where a 24-h EC50 was not available

Chemical	Frequency (%)	Median concn. (μg/L)	24-h EC50 (μg/L)	21-d NOEC (μg/L)
Bisphenol A	41.2	0.14	15,500	—
Caffeine	70.6	0.10	330,000	—
Carbaryl	16.5	0.04	3.7	6.4
Chlorpyrifos	15.3	0.06	0.41*	0.1
N,N-Diethyl-m-toluamide	74.1	0.06	75,000*	—
Diazinon	25.9	0.07	2.1	0.21
1,4-Dichlorobenzene	25.9	0.09	1,600	350
Fluoranthene	29.4	0.04	196	17
4-Nonylphenol	50.6	0.80	300	74

pose a hazard to aquatic life. We hypothesized that a heuristic model that combined concentration addition and independent joint action would predict accurately the toxicity of this mixture. Toxicity was evaluated by measuring the effects of the chemicals on survival, growth, and reproduction of the freshwater crustacean, *Daphnia magna*. The hypothesis was tested by comparing model predictions to experimental results.

MATERIALS AND METHODS

Daphnid culture

Daphnids (*D. magna*) were cultured in incubators at a density of 40 adults in 1 L of medium at a temperature of 20°C and 16:8-h light:dark photoperiod. Algae (*Selenastrum capricornutum*) were cultured in Bold's Basal medium and were used as a food source for the daphnid cultures and experiments. Algae (1.4×10^8 cells) were provided to each 1-L culture twice daily, and daphnids were transferred to fresh medium 3 times weekly. Daphnids also were provided with a fish food homogenate, prepared as described previously (Baldwin and LeBlanc 1994), and provided to cultures at 4 mg (dry wt) twice daily. Daphnids were discarded after 3 weeks in culture. This crustacean species can reproduce either sexually or asexually (parthenogenesis). Under these culture conditions, daphnids were all female and reproduced asexually.

Toxicity characterization of individual chemicals

All experiments were initiated with individual daphnids (<24-h-old) placed in 40 ml of medium. Algae (3.5×10^6 cells) and fish food (0.1 mg dry wt) were provided to each beaker twice daily for the 1st week, after which these amounts were doubled. Medium was changed 3 times weekly and biological endpoints were measured once every 24 h. Mortality was defined as occurring when no discernible movement was visible with the naked eye during 30 s of observation. Growth rates were determined in a manner described previously (Olmstead and LeBlanc 2001). Briefly, the first 4 molted exoskeletons of each daphnid were measured in length from the base of the shell spine to the top of the carapace using an ocular micrometer under the microscope (×4 or ×10 magnification). Molt lengths were plotted against molt numbers and the results fitted with linear regression to yield a slope that was taken as the growth rate. Offspring were removed and counted daily. Experiments were ended once all daphnids had released 3 broods of offspring (17–19 d of exposure).

The 9 chemicals used in this study were chosen from a survey of freshwater streams in the United States (Kolpin et al. 2002). Criteria for selection included frequency of detection, median detected levels, toxicity of the chemicals, and the mechanisms of action for toxicity. Chemicals selected were present at >10% of the sites sampled and, when detected, were found at median levels between 0.04–0.14 μg/L (Table 1). These chemicals all were presumed to have differing mechanisms of toxicity except for the pesticides carbaryl, diazinon, and chlorpyrifos, which all are inhibitors of cholinesterase. Carbaryl, chlorpyrifos, diazinon, and N,N-diethyl-m-toluamide (DEET) were obtained from Chem Service (West Chester, PA, USA). Bisphenol A, fluoranthene, 1,4-dichlorobenzene, and caffeine were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 4-nonylphenol was acquired from Fluka Chemika (Milano, Italy).

The experimental design used for the toxicity assessments of individual chemicals was described previously (Olmstead and LeBlanc 2001). Each experiment consisted of 50 different exposure concentrations. The concentrations of chemicals in the exposure treatments were determined by starting with the lowest discerned acutely lethal level based upon preliminary experiments, or 1,000 μg/L, whichever was lower, as the highest treatment. Subsequent treatments were prepared at concentrations 90% of the next highest treatment level. A single female daphnid was exposed continuously to each treatment. Experimental conditions ensured that these females would reproduce asexually (parthenogenesis). All treatment solutions within an experiment contained the same concentration of ethanol (≤0.01%) that was used to deliver the chemicals. Ten control daphnids exposed to the appro-

priate amount of ethanol were monitored with each experiment.

Data from each toxicity assessment was transformed to a 0 to 100% scale to normalize results among experiments and to allow for the same concentration-response fit to be performed on all endpoints. Survival raw data were transformed using the following equation:

$$S = 100\left(\frac{18 - M}{17}\right) \tag{3}$$

where S is the percent lifespan reduction and M is the day on which the daphnid died. Percent lifespan reduction represents the degree to which a chemical or mixture reduced the lifespan of a daphnid from a maximum of 18 d. Percent lifespan reduction among control daphnids typically was 0. The growth and reproductive raw data was transformed by dividing each treatment response by the average control response and multiplying by 100%. Data then were graphed and fit with a sigmoidal line using Origin™ software (Microcal™ Software, Northampton, MA, USA) with the following equation:

$$R = \frac{100}{1 + \left(\frac{C_m}{C}\right)^{\rho}} \tag{4}$$

where R is the endpoint response and C is the concentration of the chemical. The power or slope of the curve (ρ) and the center of the sigmoid curve (C_m) were determined from fits of the experimental data and used to generate model predictions of mixture toxicity as described below. Concentrations of a given chemical expected to yield a 5% response (EC05) and a 50% response (EC50) were interpolated from these fits, with 95% confidence intervals calculated using a bootstrap approach (Efron and Tibshirani 1993) with SAS® 9.1 software (SAS institute, Cary, NC, USA). The center of the curve (C_m) corresponds to the EC50 of a given chemical.

Mixture toxicity modeling

The toxicity of a mixture of the 9 chemicals was modeled with each chemical present in the mixture at the median concentration measured in those waters where the chemical was detected (Kolpin et al. 2002; Table 1). The toxicity of this mixture was modeled at 50 different levels representing dilutions or fortifications of the base level (median detected concentration). Mixture levels were designated by their percentage of the base level concentrations. For example, at 200%, all chemicals were present at twice the base level and, at 50%, all chemicals were present at half of the base level. The toxicity of all mixture levels was modeled with respect to reduced lifespan, growth rate, and fecundity.

Toxicity of the mixture was modeled by combining the concepts of concentration addition and independent joint action. Chemicals were assigned to cassettes based upon their presumed mechanisms of action. Chemicals having the same mechanism of action were assigned to the same cassette. The acetylcholinesterase inhibitors diazinon, chlorpyrifos, and carbaryl all were placed in the same cassette. The other 6 chemicals all were presumed to have different mechanisms of action and were assigned to separate cassettes. The joint toxicity of chemicals within a cassette was calculated using the concentration addition approach, while the joint toxicity of different cassettes was calculated using the independent joint action paradigm. All parameters used in the models were derived from the toxicity evaluations of the individual chemicals and are presented in Table 2.

The toxicity or response (R) to chemicals within the same cassette was calculated by concentration addition. Equation 4 was rearranged to

$$C = \frac{C_m}{\left(\frac{1}{R} - 1\right)^{1/\rho}} \tag{5}$$

where R is the response of a mixture and C is equivalent to ECx in Equation 1. The right side of Equation 5 can be substituted into Equation 1 to yield

$$\sum_{n}^{i=1} \frac{C_i \cdot \left(\frac{1}{R} - 1\right)^{1/\rho}}{C_{m,i}} = 1 \tag{6}$$

The average power (ρ') for the individual chemicals within a cassette was used in place of ρ_i because the powers of the concentration-response curves for chemicals having the same mechanism of toxicity are comparable (USEPA 1986). Solving this equation for R yields

Table 2. Parameters derived from toxicity evaluations of the individual chemicals that were used in the mixture model. These parameters were used in Equation 7 in order to calculate expected responses from mixtures of chemicals. Values are represented as the parameter estimate plus or minus the error of that estimate. The average power (ρ') is reported for the 3 cholinesterase inhibitors. An NA indicates that the respective chemical did not exhibit a response in the given endpoint at the concentrations examined and, therefore, would not contribute to the toxicity of the mixture. Caffeine, N,N-diethyl-m-toluamide, and dichlorobenzene did not exhibit any toxicity at the concentrations tested and were assumed to not contribute to any toxic effects of the mixtures

	Lifespan		Growth		Fecundity	
Chemical	C_m (μg/L)	ρ	C_m (μg/L)	ρ	C_m (μg/L)	ρ
Bisphenol A	NA	NA	365,000 ± 648,000	0.663 ± 0.255	7,310 ± 330	7.96 ± 2.96
Carbaryl	10.4 ± 0.5	3.37 ± 0.86	13.6 ± 1.4	4.21 ± 1.14	9.18 ± 0.76	4.10 ± 1.30
Chlorpyrifos	0.190 ± 0.018	3.37 ± 0.86	NA	NA	NA	NA
Diazinon	0.522 ± 0.036	3.37 ± 0.86	NA	NA	NA	NA
Fluoranthene	NA	NA	194 ± 11	1.85 ± 0.17	85.9 ± 6.0	3.64 ± 0.88
4-Nonylphenol	195 ± 0	361 ± 0	205 ± 14	5.66 ± 1.72	NA	NA

$$R = \frac{100}{1 + \left(\sum_{n}^{i=1} \frac{1}{\frac{C_i}{C_{m,i}}} \right)^{\rho'}} \quad (7)$$

where $C_{m,i}$ and C_i are the center and concentration of the ith chemical, respectively.

The combined toxicity (R_{mix}) of cassettes that comprised the mixture was modeled using independent joint action (Eqn. 2) for each endpoint (reduced lifespan, growth, and fecundity) at each mixture level. Effect levels (EL05 or EL50) were calculated from the model. The EL05 is the mixture level calculated to elicit a 5% response and was used as an estimate of the lowest level of the mixture at which toxicity would be evident (i.e., lowest observed effect level). The EL50 is the mixture level calculated to elicit a 50% response and provides a characterization of the toxicity of the mixture with the greatest statistical confidence.

Mixtures toxicity assessment

The toxicity of the chemical mixture was measured experimentally at 50 levels using the same methods as described for measuring the toxicity of the individual chemicals. The mixture was considered as a single chemical with levels used as concentrations. Data were used to generate level-response lines for each endpoint using Equation 4 (Origin software). From this fit, EL05 and EL50 values, as well as 95% confidence limits, were calculated. Model predictions were compared to experimental data using coefficients of determination (r^2; Zar 1996). A high r^2 indicated that the experimental data were well represented by the model prediction.

RESULTS

Toxicity of individual chemicals

Four of the 9 chemicals evaluated reduced lifespan of the daphnids at the concentrations evaluated (Figure 1). Carbaryl, chlorpyrifos, and diazinon each elicited a concentration-dependent reduction in lifespan with EC50s at 10, 0.19, and 0.52 µg/L, respectively (Figure 1A–C). The 4-nonylphenol caused mortality on the 1st day of exposure at concentrations above 200 µg/L, but no additional mortality occurred at later times (Figure 1D). Bisphenol A, caffeine, 1,4-dichlorobenze, DEET, and fluoranthene did not reduce lifespan at the exposure concentrations evaluated. The EC05 and EC50 values derived for lifespan reduction for each chemical are presented in Table 3.

Exposure to 4 of the 9 chemicals resulted in reduced growth rates at concentrations lower than 1,000 µg/L (Figure 2). Bisphenol A elicited a slight negative effect on growth rates over a wide range of concentrations (Figure 2A). A higher range of exposure concentrations corroborated this finding and both data sets are included in Figure 2A. Carbaryl and 4-nonylphenol both adversely impacted growth rates, but at concentrations that also reduced lifespan (Figure 2B and D). Fluoranthene negatively affected growth rate at exposure concentrations that had no effect on lifespan (Figure 2C). Caffeine, diazinon, DEET, 1,4-dichlorobenzene, and chlorpyrifos had no effect on growth rate at the concentrations evaluated. The EC05 and EC50 values derived for growth rate reduction for each chemical are presented in Table 3.

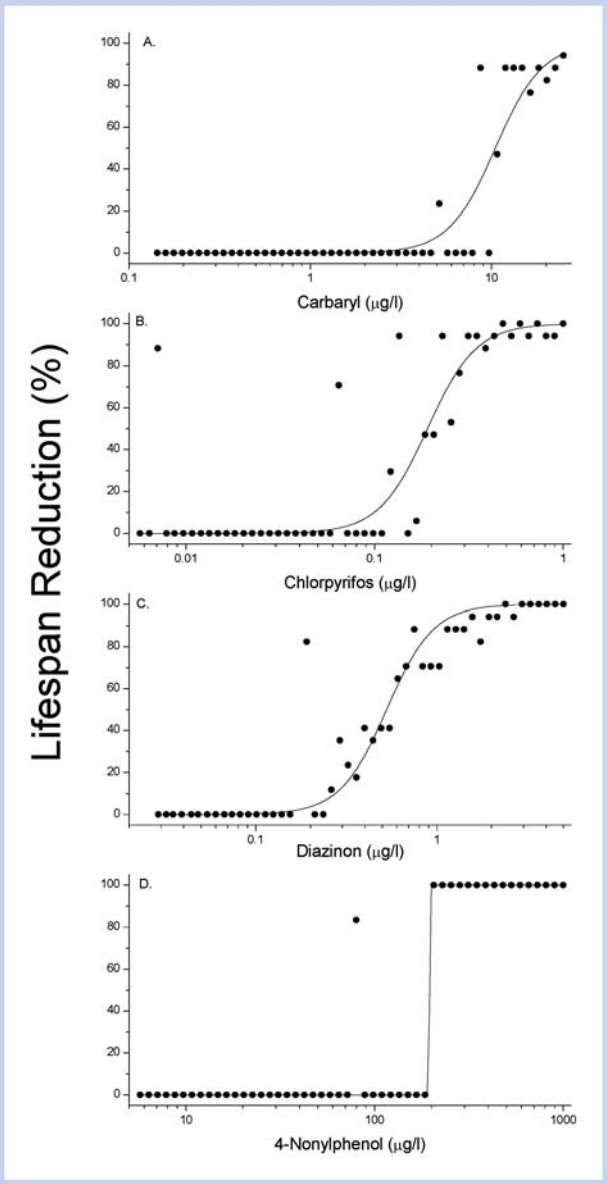

Figure 1. Lifespan reduction of daphnids exposed to concentrations of individual chemicals. Each data point represents the percentage of time that the lifespan of 1 individual daphnid was reduced based on a total assessment period (i.e., lifespan) of 18 d. The line represents a sigmoidal fit to the data (Eqn. 4). Sigmoidal fits in panels A to C were derived using the average power ($\rho' = 3.37$) of the individual concentration-response curves ($\rho_i = 2.08$–4.99) as was used in the mixture assessment. (**A**) Carbaryl, (**B**) Chlorpyrifos, (**C**) Diazinon, and (**D**) 4-Nonylphenol.

Of the 9 chemicals used in this study, bisphenol A, fluoranthene, and carbaryl specifically reduced fecundity (Figure 3). This reduction in fecundity occurred over a range of concentrations similar to those that reduced growth rates. 1,4- Dichlorobenzene, 4-nonylphenol, diazinon, chlorpyrifos, caffeine, and DEET did not adversely affect fecundity at the concentrations used in these assessments. The EC05 and EC50 values derived for fecundity reduction for each chemical are presented in Table 3.

Mixtures toxicity modeling

The model predicted that, at the mixture levels evaluated, lifespan of daphnids would be reduced in a level-dependent manner with a 100% reduction in lifespan (i.e., death within 24 h) at mixture levels ≤10 times that containing median

Table 3. Chemical concentrations (μg/L) determined to elicit a 5 (EC05) or 50% (EC50) effect for each of the three endpoints evaluated. Values were derived from the concentration-response curves generated for each chemical and endpoint. Where applicable, 95% confidence intervals are listed in parentheses. Experiments typically were not performed at exposure concentrations of >1,000 μg/L. Bisphenol A was evaluated at concentrations as high as 8,200 μg/L in an effort to corroborate slight effects discerned at 1,000 μg/L

	Lifespan		Growth rate		Fecundity	
Chemical	EC05	EC50	EC05	EC50	EC05	EC50
Bisphenol A	>8,200	>8,200	>8,200	>8,200	5,000 (3,300–6,400)	7,300 (6,300–7,800)
Caffeine	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
Carbaryl	5.7 (3.1–6.7)	10.4 (9.3–11.2)	6.8 (4.6–7.8)	>11	4.5 (3.4–6.8)	9.2 (8.1–11)
Chlorpyrifos	0.072 (0.019–0.10)	0.19 (0.15–0.22)	>0.25	>0.25	>0.15	>0.15
N,N-Diethyl-m-toluamide	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
Diazinon	0.127 (0.054–0.16)	0.52 (0.40–0.56)	>0.55	>0.55	>0.26	>0.26
1,4-Dichlorobenzene	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
Fluoranthene	>200	>200	39 (32–49)	>180	38 (23–54)	86 (73–99)
4-Nonylphenol	190 (110–200)	200 (140–200)	120 (78–140)	>190	>170	>170

detected concentrations of each chemical (Figure 4A). The model also predicted that neither growth nor fecundity would be impacted by the mixture at levels at which daphnids had survived the exposure (Figure 4B and C). The model predicted that the mixtures level representative of median detected concentrations of the individual chemicals (100% level) would elicit a significant adverse effect on the population (12% lifespan reduction; Figure 4A) and the threshold level, defined as the EL05, would be 70% of the median detected level (Table 4).

Mixture toxicity determination

Lifespan of daphnids was reduced in a level-dependent manner over the range of mixture levels evaluated (Figure 4A). The model accurately predicted the toxicity of the mixture (r^2 = 0.976). The EL05 and EL50 values derived from the mixtures exposure was highly consistent with those predicted from the model (Table 4). No level-response relationships were discerned for either growth or fecundity of surviving daphnids over the range of mixture levels evaluated (Figure 4B and C). These results compare favorably with the model prediction that mixture levels at which daphnids survived would affect neither growth rate nor fecundity (Figure 4B and C).

Further analyses of the modeling results suggested that toxicity of the mixture largely was due to the concentration of chlorpyrifos in the mixture. Indeed, modeling the concentration-response relationship for lifespan reduction based solely upon the concentration of chlorpyrifos in the mixture levels yielded a line that only slightly underpredicted the toxicity of the mixture (r^2 = 0.955). Therefore, an additional series of experiments were conducted to determine whether toxicity of the mixture could be predicted in the absence of this major toxic constituent.

For this series, the toxicity of a mixture having the same proportions of the constituents as in the previous experiments, but with no chlorpyrifos, was modeled and determined experimentally. Model results predicted that lifespan again would be the most-sensitive endpoint, despite the absence of chlorpyrifos (Figure 5A). However, the impact of the mixture on lifespan was not predicted to be as great as with the mixture containing chlorpyrifos. Again, the model predicted that neither growth nor fecundity would be impacted by the mixture at the levels assessed, assuming that daphnids had survived the exposure (Figure 5B and C).

The model was effective in predicting the toxicity of this mixture of 8 chemicals (r^2 = 0.934). Though the model overpredicted the toxicity of this mixture, the EL05 and EL50 values predicted by the model and determined experimentally differed by less than 2-fold (Table 5). In the absence of chlorpyrifos, the EL05 for this mixture both was predicted and determined experimentally to be greater than the mixture of median detected environmental concentrations of the chemicals.

DISCUSSION

Results of this study indicate that toxicity of chemical mixtures can be evaluated accurately using a heuristic modeling approach that integrates algorithms for concentration addition and independent joint action. This modeling approach requires that the toxicity of the individual constituents of the mixture is known and described by a concentration-response relationship. Three toxicological endpoints were used in this evaluation: Survival, growth, and reproduction. For purposes of generating concentration-response curves for these endpoints, the data were presented as percent lifespan reduction, percent growth-rate reduction, and percent reduction in fecundity. Other toxicological endpoints also would be amenable to this modeling approach provided the results can be formatted to describe a concentration-response relationship.

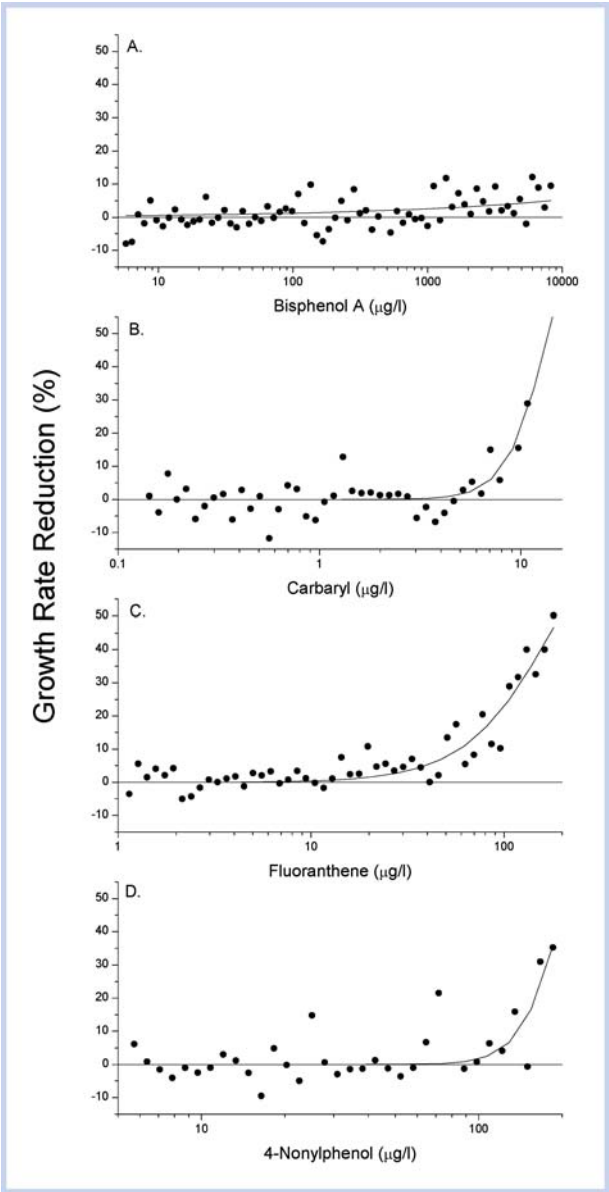

Figure 2. Growth-rate reduction among daphnids exposed to concentrations of individual mixture constituents. Each data point represents the percentage growth rate reduction of a single daphnid when compared to the mean growth rate of 10 control daphnids. The horizontal line represents the performance of the control organisms. The fitted line was generated using Equation 4. (**A**) Bisphenol A, (**B**) carbaryl, (**C**) fluoranthene, and (**D**) 4-nonylphenol.

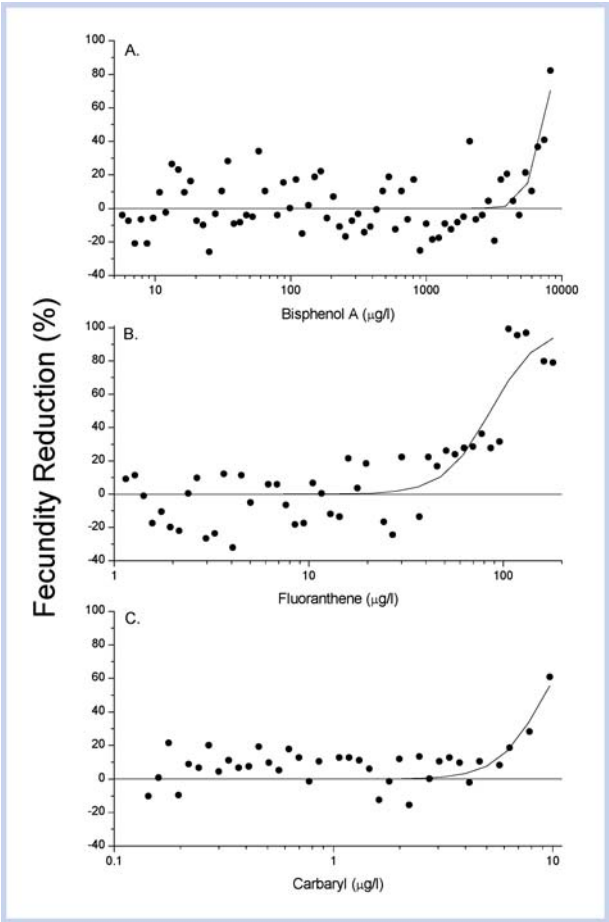

Figure 3. Fecundity reduction in daphnids exposed to concentrations of individual mixture components. Each data point represents the percentage reduction in the number of offspring produced by a single daphnid when compared to the mean offspring production of 10 control daphnids. The horizontal line represents the performance of the control organisms. The fitted line was generated using Equation 4. (**A**) Bisphenol A, (**B**) fluoranthene, and (**C**) carbaryl.

Evaluations of the chronic toxicity of chemicals using standard testing protocols typically do not yield data that define a concentration (or dose)-response relationship. Rather, these assays often are designed to identify the highest concentration of the toxicant at which no effect is observed (i.e., Benchmark dose, etc.; Cunny and Hodgson 2004; LeBlanc 2004) and often yield an all-or-none–type response (i.e., significant vs nonsignificant response). The experimental approach used in the present study to define chronic toxicity allowed for the definition of a concentration-response relationship from which the lowest effect level of the chemical can be interpolated statistically as the EC05 value (i.e., the concentration of the chemical that elicits a 5% response). We and others have used this approach successfully in other applications (Stephan and Rogers 1985; Hoekstra and Van Ewijk 1993; Olmstead and LeBlanc 2001; Chevre et al. 2002; Mu and LeBlanc 2002; Olmstead and LeBlanc 2003; Mu and LeBlanc 2004). In addition to describing the lowest effect level of the chemical, this approach allows for the calculation of the power of the concentration-response curve, as well as the EC50 value for the relationship. These descriptors then are used in the mixtures modeling.

The chemicals selected for this mixture assessment represent 9 out of 82 organic contaminants detected in surface waters in a survey of 139 streams (Kolpin et al. 2002). Toxicity characterization of these individual chemicals, generated in this study, was consistent with results of previous characterizations (compare 21-d NOEC values in Table 1 to lowest EC05 values in Table 3). A comparison of median levels of these contaminants at sites from which they were detected with reported toxicity of the materials suggest that the chemicals, individually, were present at levels that would pose no risk to daphnids and similarly susceptible species (compare median concentrations in Table 1 to EC05 values in Table 3). However, predictions of joint toxicity using our heuristic model indicated that median detected levels of these chemicals in combination would elicit toxicity and this prediction was confirmed experimentally. Of the 9 constituents of the mixture, chlorpyrifos posed the greatest

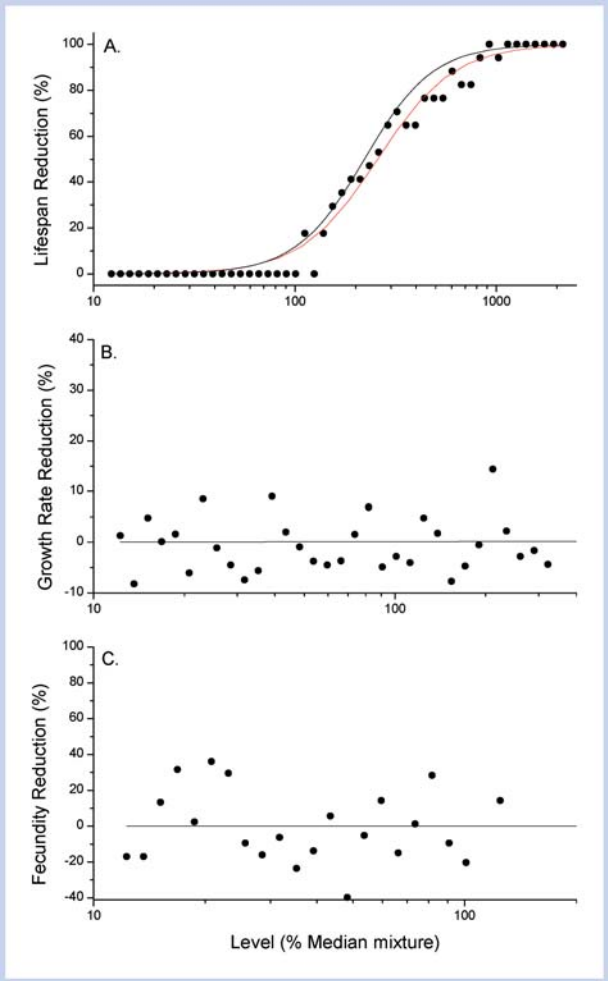

Figure 4. Modeled and measured performance of daphnids exposed to various levels of the 9-chemical mixture. The black line represents the model prediction of the organisms' response to the mixture based upon the characterized toxicities of the individual constituents (Eqn. 5). Levels are expressed as a percentage of the mixture consisting of all 9 chemicals at their respective median detected environmental concentrations. (**A**) Lifespan: Each data point represents the percentage of time that the lifespan of 1 individual daphnid was reduced based on a total assessment period (i.e., lifespan) of 18 d. The red line represents a sigmoidal fit to the data (Eqn. 4). (**B**) Growth rate: Each data point represents the percentage growth rate reduction of a single daphnid when compared to the mean growth rate of 10 control daphnids. (**C**) Fecundity: Each data point represents the percentage reduction in the number of offspring produced by a single daphnid when compared to the mean offspring production of 10 control daphnids.

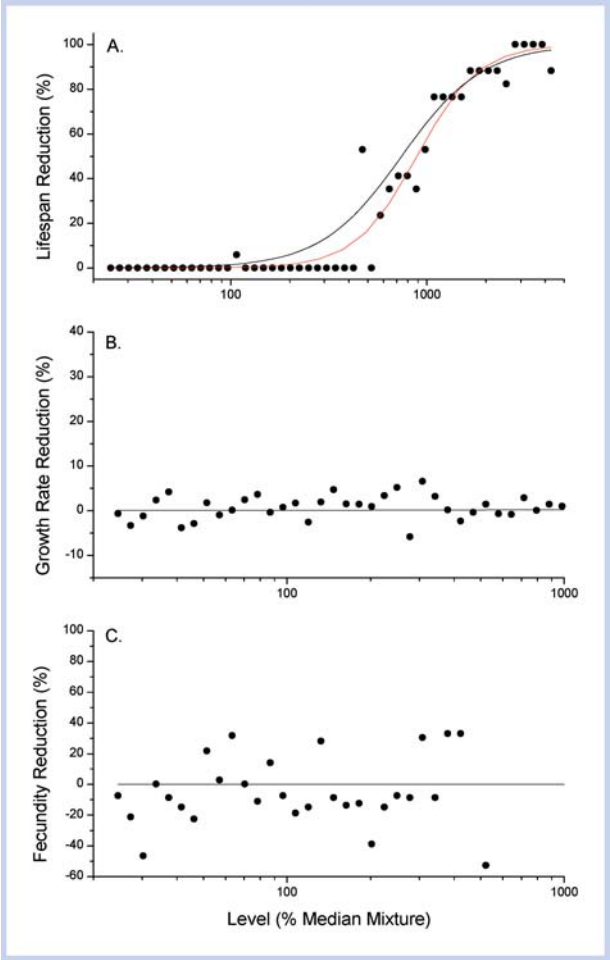

Figure 5. Modeled and measured performance of daphnids exposed to various levels of the mixture in the absence of chlorpyrifos. The black line represents the model prediction of the response of the organisms to the mixture based upon the characterized toxicities of the individual constituents (Eqn. 5). Levels are expressed as a percentage of the mixture consisting of all 8 chemicals at their respective median detected environmental concentrations. (**A**) Lifespan: Each data point represents the percentage of time that the lifespan of 1 individual daphnid was reduced based on a total assessment period (i.e., lifespan) of 18 d. The red line represents a sigmoidal fit to the data (Eqn. 4). (**B**) Growth rate: Each data point represents the percentage growth rate reduction of a single daphnid when compared to the mean growth rate of 10 control daphnids. (**C**) Fecundity: Each data point represents the percentage reduction in the number of offspring produced by a single daphnid when compared to the mean offspring production of 10 control daphnids.

Table 4. Mixture levels calculated to elicit a 5 (EL05) or 50% (EL50) response for each of the 3 endpoints evaluated based upon model predictions or experimental derivation. Model predictions are based on an additive model incorporating data from the 9 individual chemical toxicity evaluations. Experimental values were derived from a sigmoidal fit to the data (Figure 4). Where appropriate, 95% confidence intervals are listed in parentheses. Levels are expressed as a percentage of the mixture consisting of all 9 chemicals at their respective median detected environmental concentrations

	EL05		EL50	
Endpoint	Model	Experimental	Model	Experimental
Lifespan	70	72 (63–83)	220	260 (250–270)
Growth	>320	>2,100	>320	>2,100
Fecundity	>120	>2,100	>120	>2,100

Table 5. Levels of the mixture containing all constituents except chlorpyrifos calculated to elicit a 5 (EL05) or 50% (EL50) response on each of the 3 endpoints evaluated based upon model predictions or experimental derivation. Model predictions are based on an additive model incorporating data from the eight individual chemical toxicity evaluations. Experimental values were derived from a sigmoidal fit to the data (Figure 5). Where appropriate, 95% confidence intervals are listed in parentheses. Levels are expressed as a percentage of the mixture consisting of all 8 chemicals at their respective median detected environmental concentrations

	EL05		EL50	
Endpoint	Model	Experimental	Model	Experimental
Lifespan	180	310 (220–360)	750	890 (800–940)
Growth	>4,300	>980	>4,300	>980
Fecundity	>4,300	>520	>4,300	>520

risk of toxicity as judged by the low margin of safety (lowest EC05/median environmental concentration = 1.2). Indeed, we demonstrated that chlorpyrifos primarily was responsible for the toxicity associated with the mixture. However, removal of chlorpyrifos did not eliminate toxicity. Toxicity was still predicted and demonstrated with the mixture, although the concentration-response curve had shifted, denoting some reduced toxicity of the mixture. For this mixture formulation, toxicity could be explained largely through the actions of the new dominant toxicant, diazinon (data not shown). These results suggest that toxicity of mixtures often may be predicted by the toxicity of the major contributor to toxicity alone. Using mixtures of groundwater contaminants, Heindel et al. (1995) reported that the overall toxicity of these mixtures was approximated by the toxicity of the most toxic constituent. Reducing mixtures toxicity assessments to the assessment of single or a few constituents of the mixture would simplify greatly the hazard characterization process; however, further studies are necessary to determine under what conditions such an approach is legitimate or when more inclusive considerations must be given to the mixtures.

In the approach used, possible interactions between chemicals were not modeled, but rather, we assumed that combined toxicity of the mixture constituents entirely was due to additivity. Our assumption proved correct with this mixture. However, when evaluating the toxicity of chemical mixtures, consideration must be given to synergistic or antagonistic interactions among chemicals when such interactions are indicated by deviations from a zero-interaction model. Both prospective and retrospective approaches can be used to identify interactions. The prospective approach involves predicting interactions based upon known toxicodynamics of the chemicals. Retrospective analyses involve searching for interactions when the strict additive model fails to predict toxicity accurately. Regardless of the approach used to identify interactions, these interactions must be quantified by evaluating the degree to which the modifying chemical alters the toxicity of the affected chemical. The modifying effects of 1 chemical constituent upon another can be described by coefficients of interaction (Finney 1942), which can be incorporated into an additive model to account for interactions as we have described previously (Mu and LeBlanc 2004).

In conclusion, a heuristic model incorporating concentration addition and independent joint action can be used to characterize hazard of some environmentally relevant mixtures of chemicals. As demonstrated with the 9 chemicals used in the present assessment, this model can be effective in characterizing the toxicity of a mixture, identifying the most-sensitive endpoint, and providing a means for predictive interpolation to various modifications to the mixture. Further studies using this modeling approach may reveal means to simplify the mixtures assessment process (i.e., identifying when it is appropriate to assess hazard based solely upon the dominant toxicant), as well as increase accuracy of the assessment (i.e., identifying when and how chemical interactions need to be integrated into the assessment).

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