

Interactive toxicity of simple chemical mixtures of cadmium, mercury, methylmercury and trimethyltin: model-dependent responses

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Received 18 September 2003; accepted 30 May 2004

Abstract

Scientific and societal interest in the analysis of aggregate toxicity derives from the fact that people are seldom exposed to single chemicals, but rather to multiple agents from different sources and even to mixtures of agents from a single source. Many descriptive terms and mathematical, graphical, and statistical models have been used to evaluate the toxicity of simple mixtures. It is not very easy to distinguish clearly the intrinsic differences, distinctions and limitations of these models when applied to characterizing interactive toxicity. A series of experiments were performed to illustrate model-dependent consistencies and differences in interactive toxicity. Cultured murine renal cortical cells, target cells for metal toxicity, were treated with selected concentrations of one metal or binary mixtures of metals to give conditions of dose-additivity, response additivity, or with only one toxic member of the binary mixture. The cytotoxicity was determined at 24 h by lactate dehydrogenase release. The data were analyzed graphically and mathematically by (a) Carter's statistical isobologram, (b) Barton's non-linear, and (c) Kodell and Pounds' linear models to characterize the interaction. These models were compared and contrasted for robustness, and consistency using these common data sets. The models gave generally consistent conclusions, but each model has limitations and strengths for assessing particular mixtures scenarios. This comparison illustrates the complexity of extrapolating conclusions between models, and difficulty of public health assessment from exposures to multiple chemicals in the environment.

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Keywords: Cytotoxicity; In vitro; Mixtures; Cadmium; Mercury; Methylmercury; Trimethyltin; Dose–response; Nonlinear models; Isobologram; Risk assessment

1. Introduction

Historically, health concerns from exposure to single chemicals drive toxicity assessment and criteria derivation procedures. Although such assessments, based on single chemical exposure, enable us to acquire fundamental knowledge about an individual chemical's toxicity and risk to human health they do not mirror real-life exposures. Most of the federal agencies and international organizations such as

ATSDR, USEPA, NIOSH, Health Canada, and the Health Council of the Netherlands use a default assumption of dose or response additivity for the assessment of aggregate toxicity of multiple agents to which human populations are exposed through various environmental media or food (ATSDR, 2002; USEPA, 1986; NIOSH, 1976). However, this assumption does not allow the factoring of chemical interactions into the toxicity assessments. Therefore, an enormous research effort has been expended in the understanding and analyses of chemical interactions that present an assessment challenge to the toxicologists (Yang and Rauckman, 1987; Solana et al., 1987, 1991; Groten et al., 1997; Moser et al., 2003). Thus, the

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interpretation of chemical mixtures experimental data, both in vivo and in vitro, poses a variety of problems (Teuschler et al., 2001).

In addition to the scientific and statistical limitations, the assessment of toxicity of multiple contaminants is a formidable task with respect to expense, experimental design, logistics, data analysis, and interpretation (Meadows et al., 2002). Numerous approaches to the assessment of aggregate toxicity have been developed for pharmacological, toxicological, and epidemiological applications (Carter et al., 1988; Barton et al., 1993; Kodell and Pounds, 1991). These approaches may differ in concept, theory, assumptions, objective and in application. It is clear that experimental measurement of chemical interactions, and the subsequent application of these data to human risk assessment rest on numerous assumptions. Some of these assumptions are well recognized in toxicology, including the appropriateness of animal models for predicting human risk, and differences in response according to sex, age, and phenotype or species variation.

In addition to the complexity of the problem of aggregate toxicity, several problems impair the facile selection, implementation, and application of these approaches. While selecting an appropriate approach, the toxicologist is frequently confounded by the lack of direct comparisons between the available approaches. The number of data, kind of data, nature of the observations, and level of biological organization at which studies are conducted almost always differ as reported in the literature. The lack of an adequate interaction database also hinders scientists who are developing and refining statistical and graphical approaches because these investigators must often use very limited data sets for development of risk assessment tools to define interactions. Thus, sometimes sophisticated methods are developed with the least amount of data available.

The primary goal of this research was to systematically and experimentally investigate the basic concepts and assumptions that are inherent in the statistical models and methods employed for the assessment of simple, defined mixtures of toxicants. The objective of this paper is to compare and contrast selected experimental statistical and graphical approaches (isobologram, nonlinear and linear models) for robustness and consistency using common data sets. Because of the frequent concurrent human exposure to metals in the workplace and environment, studies using toxic metals will be used to illustrate the utility and limitations of some commonly used select models.

2. Experimental methods

2.1. Cell culture

Monkey Kidney cells (ATTC depository LLC-MK2) were cultured in high glucose DMEM medium. Briefly, confluent cultures in 12-well cluster dishes were treated in triplicate with selected concentrations of HgCl_2 , CdCl_2 , CH_3HgCl ,

$(\text{CH}_3)_3\text{SnOH}$, or binary mixtures of these metals for 24 h. All experiments were conducted in D-MEM supplemented with 5% fetal bovine serum, and antibiotic/antimycotic agents. All test chemicals were obtained from Aldrich Chem and were at least 99.99% purity.

The release of the soluble enzyme, lactate dehydrogenase (LDH) was measured kinetically and expressed as the fraction of the total LDH activity released for each culture well. A detailed description of the assay and the calculation is provided by Jernigan et al. (1983). Up to eight concentrations of metal or binary mixture including an untreated control were used to define the concentration-response cytotoxicity. LDH release was selected as the end point to be analyzed because (1) it is a sensitive, reliable measure of irreversible cell injury, and (2) when expressed as a fraction of total LDH released, the data are constrained between 0 and 1 in a manner analogous to LD_{50} , ED_{50} , and EC_{50} , and tumorigenicity data. The fraction of LDH released (cytotoxicity) was transformed to normalize the variances for analysis. The transformations used included the $\ln(p/1-p)$ for the statistical isobole (Carter et al., 1988), $\sin^{-1}(\text{LDH})^{0.5}$ or the Box-Cox transformation for the linear model (Kodell and Pounds, 1991), and the \log_{10} transformation for the nonlinear models (Barton et al., 1993).

2.2. Binary mixture giving dose additivity (Hg & $\text{Hg}_{(2)}$)

Dose addition is biomathematically described as the additive effect resulting from the summation of the doses of the individual agents after adjusting potency-weighted doses for differences among the components of a mixture. Some of the general underlying assumptions of dose addition are that each of the individual agents exerts their effect via the same mechanism at the same site to produce a common response. Clearly, most environmental and occupational toxicants do not fully meet these assumptions. To test this situation, an “artificial metal” was constructed to clearly test dose-additive analysis. An artificial “super” mercury ($\text{Hg}_{(2)}$) was prepared using an Hg stock solution whose actual concentration was twice the nominal concentration. Thus, super mercury or $\text{Hg}_{(2)}$ has the identical physicochemical and toxicological properties as mercuric chloride, but twice the cytotoxic potency. Each “metal” would have identical mechanisms of action and act at identical sites to produce a common response. To evaluate a dose additive mixture, MK2 cultures were exposed in triplicate to $\text{Hg}_{(2)}$, Hg, or $\text{Hg}_{(2)}:\text{Hg}$ in a molar mixture and the cytotoxicity determined at 24 h. For example, the nominal concentration of ‘super’ mercury used for exposure could be labeled 3 μM , while the actual concentration was 6 μM . In this experiment, and other experiments reported herein, the molar mixing ratio was constant.

2.3. Binary mixture giving response additivity (Cd & Hg)

The concurrent exposure to toxicants of dissimilar mechanism of action may describe the most frequent exposure situa-

tion for humans. Some of underlying assumptions of response-additivity models are that individual toxicants may act by different mechanisms and at different sites to produce response(s) which are “additive”. The toxicity of most environmental toxicants is not receptor mediated or nor the result of very specific mechanism(s) of action. In fact, the Safe Drinking Water Committee of the National Academy of Sciences (1989) recommended the use of response additivity models to estimate carcinogenic risks. Cadmium and mercury are very toxic metals to which human populations are often exposed concurrently in the workplace or environment from hazardous waste sites. The primary objective of the following analysis, however, is to illustrate model-dependent analysis of a response-additive mixture, not to provide an analysis of the risk with concurrent exposure to cadmium and mercury. To evaluate a response additive mixture, MK2 cultures were exposed in triplicate to Cd, Hg, or Cd:Hg in a 1:2 mixture and the cytotoxicity determined at 24 h.

2.4. Binary mixture with only one toxic component (methyl mercury & trimethyltin)

The circumstances when only one member of a binary mixture is toxic can arise when only one of the chemicals produces the toxicity (response) analyzed. For example, a binary mixture could be composed of two toxicants which each produce renal toxicity alone, but only one agent causes neurotoxicity. To evaluate this situation, triplicate cultures of MK2 cells were exposed to Methyl mercury (toxic), and trimethyltin (nontoxic), or a 2:1 mixture and the cytotoxicity determined at 24 h.

2.5. Data analysis

Each model was implemented in SAS V8.2 for Windows (Statistical Analysis Systems, Inc.) using the fraction of LDH released in an individual cell culture dish as the response and the cell culture medium metal concentration as the nominal dose. The three models evaluated are described below.

$$\text{LOGIT} = \log \left[\frac{P}{1-P} \right] = \beta_0 + \beta_1 d_1 + \beta_2 d_2 + \beta_{12} d_1 d_2 \quad (1)$$

2.6. Carter's statistical isobologram

The statistical isobologram, as described by Carter et al. (1988) was implemented and verified using Carter's data. The statistical isobole was fit as using maximum likelihood, where P = fraction of LDH released and d_i ($i = 1, 2$) is the dose (concentration) of toxicant 1 or 2 or the binary mixture). The most important parameter estimated is the interaction term, β_{12} , which is used to statistically define departures from additivity.

2.7. Barton's nonlinear models of null-interaction

Three nonlinear models were implemented in SAS, simple-similar model (approximately equivalent to dose additivity), the independent action model (approximately equivalent to response additivity depending on the shape of the dose–response curve), and the non-additive model (Barton et al., 1993). The published models were edited to accommodate the positive response slope of the data from these experiments and the parameters estimated by nonlinear regression. Model comparison F -tests, visual inspection of residual plots, and visual inspection of the model fit to data were used to select the model and to reject those which did not fit.

The **simple-similar model** (approximately equivalent to Dose/concentration Additivity) is

$$\begin{aligned} \text{SS: } = P = Y_{\min} + & \left(\left[\frac{D_1}{1 + [d_1/\mu_1]^{-\beta}} \right] + \left[\frac{D_2}{1 + [d_2/\mu_2]^{-\beta}} \right] \right. \\ & \left. + \left[\frac{D_{12}}{1 + [d_1/\mu_1 + d_2/\mu_2]^{-\beta}} \right] \right) \\ & \times (Y_{\max} - Y_{\min}) \quad (2) \end{aligned}$$

The **independent action model** (approximately equivalent to Response Additivity) is

$$\begin{aligned} \text{IA: } = P = Y_{\min} + & \left(\left[\frac{D_1}{1 + [d_1/\mu_1]^{-\beta_1}} \right] \right. \\ & + \left[\frac{D_2}{1 + [d_2/\mu_2]^{-\beta_2}} \right] + 1 - \left[1 - \frac{D_{12}}{1 + [d_2/\mu_2]^{-\beta_1}} \right] \\ & \left. \times \left[1 - \frac{D_{12}}{1 + [d_2/\mu_2]^{-\beta_2}} \right] \right) \times (Y_{\max} - Y_{\min}) \quad (3) \end{aligned}$$

The **non-additive model** (interaction) is

$$\begin{aligned} \text{NA: } = P = Y_{\min} + & \left(\left[\frac{D_1}{1 + [d_1/\mu_1]^{-\beta_1}} \right] \right. \\ & + \left[\frac{D_2}{1 + [d_2/\mu_2]^{-\beta_2}} \right] + \left[\frac{D_{12}}{1 + [V_1 d_1/\mu_1]^{-\beta_1}} \right] \\ & \left. \times \left[\frac{D_{12}}{1 + [V_2 d_2/\mu_2]^{-\beta_2}} \right] \right) \times (Y_{\max} - Y_{\min}) \quad (4) \end{aligned}$$

where

$$\begin{aligned} V_1 = 1 + \gamma & \left[\frac{D_2}{1 + [d_2/\mu_2]^{-\beta_2}} \right], \\ V_2 = 1 + \gamma & \left[\frac{D_1}{1 + [d_1/\mu_1]^{-\beta_1}} \right] \quad (5) \end{aligned}$$

In Eqs. (2)–(5), P = fraction of LDH released and d_i ($i = 1, 2$) is the dose (concentration) of toxicant 1 or 2 or the binary mixture, β_{12} is the slope function associated with toxicant 1, 2 or the mixture, Y_{\max} and Y_{\min} are the maximal and minimal

P observed. Other important or useful parameter estimates include μ_i ($i = 1, 2$) as equivalent to the EC_{50} for the individual metal; and gamma as an interaction term that describes the direction (antagonism versus synergism) and magnitude of an interaction.

2.8. Kodell and Pounds' linear models

Kodell and Pounds' Linear Models of null-interaction gives two bench marks for comparison: dose/concentration additivity (approximately equivalent to the simple-similar model of Barton) and response additivity (approximately equivalent to the independent action model of Barton) which are predicted by the dose–response curves of the individual mixture components (Kodell and Pounds, 1991). This approach explicitly addresses the lack of a completely general definition of additivity by using an Envelope of Additivity as the basis of comparison. The use of unspecified functions to describe the dose–response relations prevents this family from being limited to a few families of curve and surface shapes and provides flexibility to be generally applicable to many situations.

2.9. Data transformation

The angle transformation linearizes the dose–response function and makes the variance of the transformed response homogeneous (Finney, 1978). A more refined adaptation of the Kodell and Pounds model has also been proposed (Razzaghi and Kodell, 1992). This method is based on the Box–Cox power transformation to derive the most suitable function of dose to be used in a linear response function obtained from a linear transformation wherein the probability of a toxic response to a dose is estimated by separate regressions for each toxicant by

$$P_i = F_i \left(\alpha_i + \beta_i \frac{d_i^{\lambda_i} - 1}{\lambda_i} \right) \quad (6)$$

Here, λ_1 and λ_2 are parameters to be estimated to provide the most suitable power transformation of dose1 and dose2. The inclusion of the logarithmic transformation is accomplished by noticing that the Box–Cox family is continuous in λ_i . As λ_i approaches zero, this is equivalent to regression on \log -dose $_i$. As λ_i approaches 1, this is equivalent to regression on dose $_i$. By allowing λ_i to vary between 0 and 1, a whole family of transformations is obtained.

Dose Additivity is then predicted from,

$$\begin{aligned} DA &= F_1 \left(\alpha_1 + \beta_1 \frac{(d_1 + \rho d_2)^{\lambda_1} - 1}{\lambda_1} \right) \\ &= F_2 \left(\alpha_2 + \beta_2 \frac{(d_2 + \rho d_1)^{\lambda_2} - 1}{\lambda_2} \right) \end{aligned} \quad (7)$$

Response Additivity is then predicted from,

$$\begin{aligned} RA &= F_1 \left(\alpha_1 + \beta_1 \frac{d_1^{\lambda_1} - 1}{\lambda_1} \right) + F_2 \left(\alpha_2 + \beta_2 \frac{d_2^{\lambda_2} - 1}{\lambda_2} \right) \\ &\quad - F_1 \left(\alpha_1 + \beta_1 \frac{d_1^{\lambda_1} - 1}{\lambda_1} \right) \times F_2 \left(\alpha_2 + \beta_2 \frac{d_2^{\lambda_2} - 1}{\lambda_2} \right) \end{aligned} \quad (8)$$

In practice, the alpha, beta, and lambda parameters estimated by the separate regressions using Eq. (6) are substituted into Eqs. (7) and (8). The observed toxicity of the mixture is then compared to the predicted Dose and Response additivity as calculated and plotted from these two equations. The nature of the mixture toxicity is defined by visual inspection of the observed toxicity versus the two predicted models.

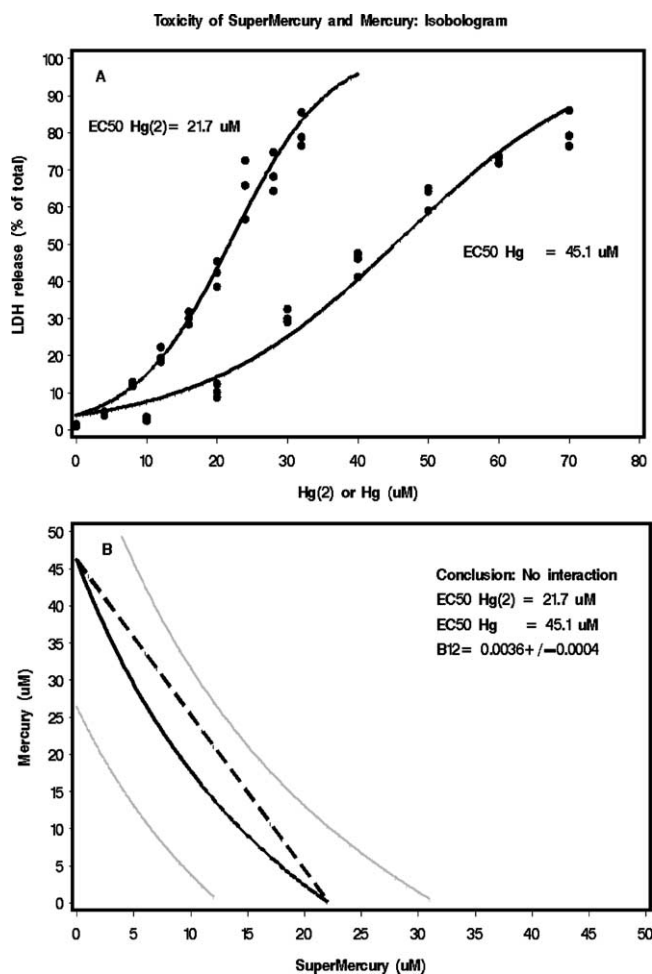


Fig. 1. Carter's isobolographic analysis of the individual cytotoxicity of Hg(2) and Hg in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (—) line represents the model prediction from nonlinear regression of Eq. (1) (panel A). Carter's isobolographic analysis of the interactive cytotoxicity of Hg(2) and Hg in MK2 monkey kidney cells (thick dashed line) represents line of no interaction for EC₅₀ (thick line) represents predicted EC₅₀ isobole; (thin line) represents predicted EC₂₀ and EC₈₀ isoboles from nonlinear regression of Eq. (1) (panel A).

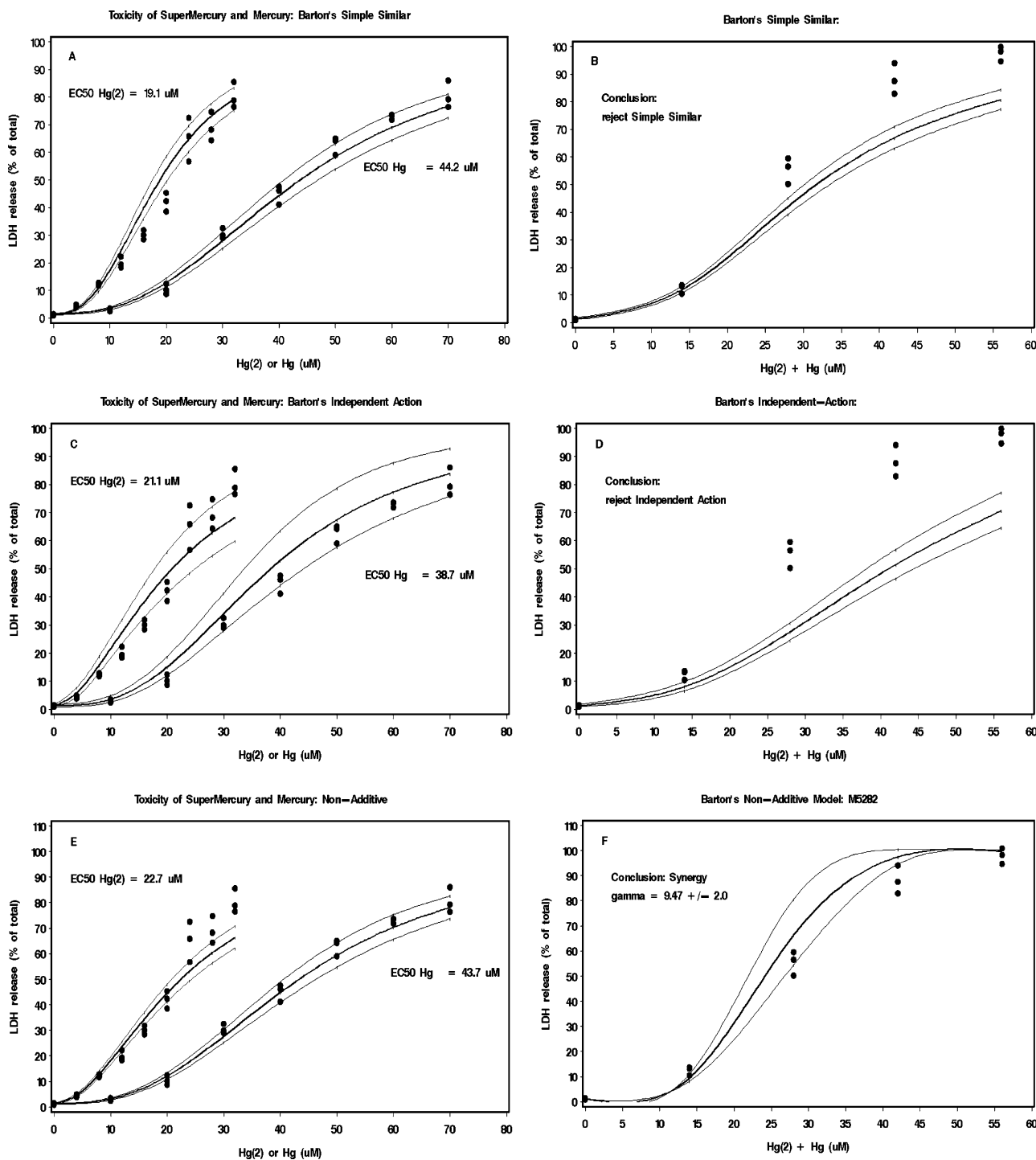


Fig. 2. Barton's nonlinear analysis of the individual cytotoxicity of Hg₍₂₎ and Hg in MK2 monkey kidney cells by the Simple panels A and B show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Simple Similar model (Eq. (2)) for the response to the each metal alone and the mixture. Panels C and D show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture. Panels E and F show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture.

Interaction is defined by the absence of Dose or Response additivity.

3. Results

3.1. Analysis of putative dose-additive mixture (Hg & Hg₍₂₎)

The results of the analyses of the Hg & Hg₍₂₎ experiment are shown in Figs. 1–3 and summarized in Tables 1 and 2. All three models identified the mixture of Hg and Hg₍₂₎ as synergistic although each approach provided slightly different insights into the experimental data. Carter's isobologram was very effective at modeling the dose–response of the individual metals (Fig. 1A). The “interaction term” was small, but statistically different from zero ($\beta_{12} = 0.0036$; $P = <0.0004$). Thus, the interaction is defined as synergistic. The statistical conclusion is supported by graphic analysis (Fig. 1B) showing the ED₅₀ isobole as a curved line contrasting to

straight line theoretical EC₅₀ isobole. In this particular experiment, Barton's nonlinear models did not fit the individual dose–response data for Hg₍₂₎ as well as they did for Hg (Fig. 2C and E). Statistical analysis (Table 1) and graphical comparisons (Fig. 2B, D and F) of Barton's three nonlinear models clearly show that the Simple Similar (Dose Additivity) and Independent Action (Response Additivity) models were poor visual fits to the observed toxicity the Hg₍₂₎ and Hg mixture and that the Non-Additive model was the best fit, showing a synergistic response. The statistical parameter, R^2 , was deceptively good (Table 2) illustrating the importance of both visual and statistical evaluation. Kodell and Pounds linear model fit the individual data quite well (Fig. 3). Statistical analysis (Table 1) and graphical analysis (Fig. 3) support the conclusion of synergy for the mixture. The observed potency-dependent interaction (synergy) observed here has implications for risk assessment of certain classes of chemicals with similar mechanisms of action such as dioxin, polycyclic aromatic hydrocarbons, etc.

Initially, it was surprising to us that all three models agreed that the joint toxicity of mercury with itself as supermercury was synergistic rather than dose additive. However, upon reflection, the dose-dependent kinetics of metal uptake and accumulation as well as the dose-dependent manifestation of metal toxicity is well established for mercury, lead, cadmium, and other metals (for review see ATSDR Toxicity Profiles for these metals or other toxicants). Therefore, as the cellular dose of mercury increases different cellular and molecular processes will be targeted. For example, inhibiting of DNA or protein repair processes or inhibition of cellular homeostasis is likely to enhance the toxic damage on other cellular processes. This speculation is supported by examination of Figs. 2B, D and 3B wherein the joint toxicity at the lowest total metal concentration tested (~14 μM) is modeled by the models of additivity, while the joint toxicity at higher concentrations is not.

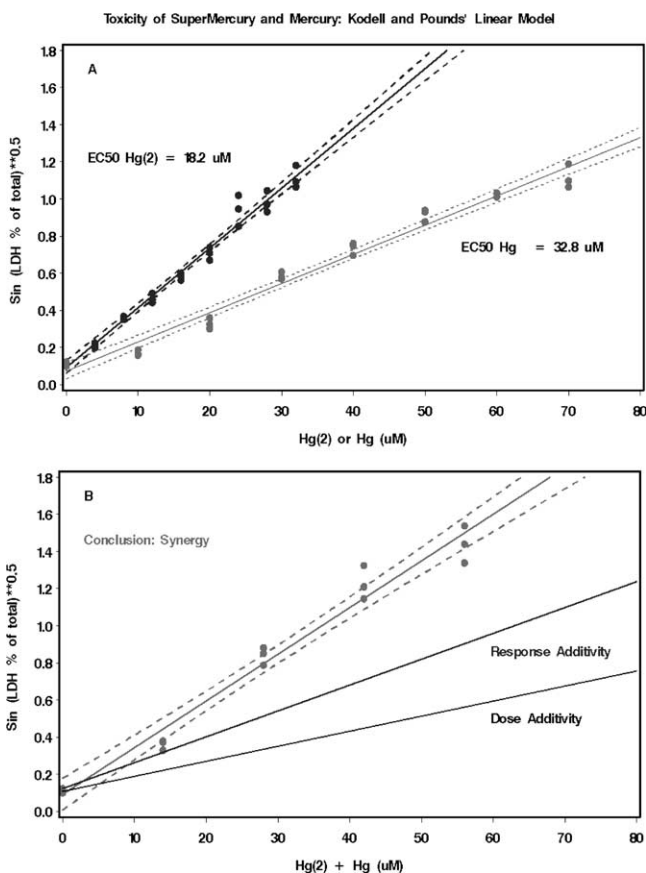


Fig. 3. Kodell and Pounds analysis of the individual cytotoxicity of HgCl₂ and Hg in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (—) line represents the best fit from nonlinear regression of Eq. (4) (panel A). Kodell and Pounds's linear model for the interactive cytotoxicity of Hg₍₂₎ and Hg in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (thick dashed line) represents model predicted dose additivity, (thick line) represents model predicted response additivity from Eqs. (7) and (8), respectively (panel B).

3.2. Analysis of a putative response-additive mixture (Cd & Hg)

The results of the analyses of the Cd and Hg experiment are shown in Figs. 4–6 and summarized in Tables 1 and 2. Carter's isobologram provided very good fits to the dose–response of the individual metals (Fig. 4). The “interaction term”, β_{12} , is a negative number (-0.0088 ± 0.0014) statistically less than zero. Thus, the interaction is defined as synergistic. The statistical conclusion is supported by graphic analysis (Fig. 4B) showing the ED₅₀ isobole as a curved line deviating from the line of no interaction (additivity). Barton's nonlinear models of null-interaction were less effective at modeling the dose–response of cadmium and mercury (Fig. 5C and E) individual metals. Inspection of residual plots (data not shown) reinforce the conclusion of imperfect fits. Interpretation of the dose–response curve of the metal mixture is complicated by the ‘over-fitting’ of the model predicted curves (Fig. 5B, D and F). The complexity of these predicted

Table 1
Summary of model parameter estimates

Isobologram	$\beta_{12} \pm \text{S.E.}$		<i>P</i> -value	EC ₅₀₋₁	EC ₅₀₋₂	<i>R</i> ²	<i>F</i> -value
Hg ₍₂₎ & Hg	-0.0036 ± .0004		0.0001	21.7	45.1	0.938	281
Cd & Hg	-0.0088 ± .0014		0.0001	19.5	35.9	0.979	344
MM & TMT	-0.0001 ± .0003		0.6309	16.2	140	0.972	229
Barton Hg(2) & Hg:	<i>Y</i> _{min}	<i>Y</i> _{max}	Gamma	EC ₅₀₋₁	EC ₅₀₋₂	<i>R</i> ²	<i>F</i> -value
Simple-similar	1.33	100.0	n/a	19.1	44.2	0.961	4625
Independent action	1.14	100.0	n/a	21.1	38.7	0.949	1504
Non-additive	1.21	100.0	9.47	22.7	43.7	0.989	1190
Barton (Cd & Hg)							
Simple-similar	1.43	80.1	n/a	18.1	32.5	0.955	1076
Independent action	1.05	80.0	n/a	25.8	29.4	0.964	695
Non-additive	1.21	81.6	16.8	30.2	34.3	0.979	759
Barton MM & TMT							
Simple-similar	8.2	100.0	n/a	17.2	156	0.972	15873
Independent action	7.4	100.0	n/a	15.1	32006	0.963	6220
Non-additive	7.8	100.0	29.4	17.2	736	0.990	10925
Kodell	λ	λ_1	λ_2	EC ₅₀₋₁	EC ₅₀₋₂	<i>R</i> ²	<i>F</i> -value
Hg ₍₂₎ & Hg	0.99	1.00	0.99	16.9	32.8	0.982	233
Cd & Hg	0.99	1.00	0.95	24.0	36.6	0.963	16.7
MM & TMT	1.00	1.00	1.00	24.1	132	0.943	889

fits reduces confidence in evaluation of the models by visual inspection. However, the gamma interaction term is different than zero (16.2 ± 5.9) indicating synergy. Kodell's linear model of null-interaction suitably described the toxicity of the individual metals (Fig. 6A). The joint toxicity observed was not dose or response additive, but was generally within the bounds of the predicted dose and response additivity. The conclusion to be drawn is that the joint toxicity is ad-

ditive but cannot be specifically defined by dose or response additivity.

3.3. Analysis of a binary mixture with only one toxic member (MM & TMT)

The results of the analyses of the MM and TMT experiment are summarized in Figs. 7–9 and in Tables 1 and 2. The

Table 2
Summary of model conclusions

Analysis model	Conclusion	Comment
Putative dose-additive mixture—Hg & Hg ₍₂₎		
Isobologram	Synergy	Good fit to toxicity of individual metals; statistical parameters and visual inspection support synergy
Nonlinear models	Synergy	Poor fit to toxicity of individual metals; statistical parameters and visual inspection support synergy
Linear model	Synergy	Good fit to toxicity of individual metals; statistical parameters and visual inspection unambiguously support synergy
Putative response-additive mixture—Cd & Hg		
Isobologram	Synergy	Good fit to toxicity of individual metals; statistical parameters and visual inspection support synergy; visual inspection does not provide information related to mixing ratio-dependent response
Nonlinear models	Additivity at low concentrations, synergy at high	Good fit to toxicity of mercury, variable fit to toxicity of cadmium; experimental design with multiple mixing ratios complicated comparison of model fits by visual inspection; statistical analysis supports synergy
Linear model	Additivity	Good fit to toxicity of cadmium, adequate fit to toxicity of mercury; visual comparison of observed joint toxicity suggests additivity.
Binary mixture with only one toxic member—methylmercury and trimethyltin		
Isobologram	Dose additivity	Adequate fit to toxicity of individual metals; statistical parameters and visual inspection unambiguously support additivity
Nonlinear models	No-interaction	Good fit to toxicity of individual metals; visual inspection of plots supports additivity at low total metal concentrations; statistical and graphic analysis supports synergy only at the highest concentrations tested
Linear model	Response additivity	Good fit to toxicity of TMT; adequate fit to toxicity of MM; poor fit to toxicity of mixture (poorly linearized by the transformation); visual inspection supports dose additivity to 25 uM, response additivity to 40 uM, and synergy above 40 uM; statistical analysis supports response additivity

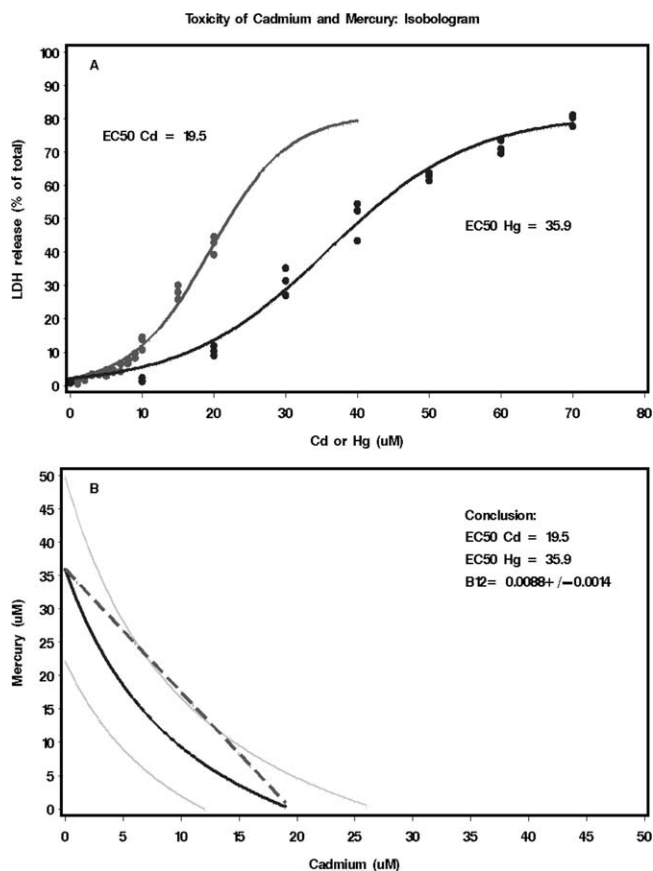


Fig. 4. Carter's isobolographic analysis of the individual cytotoxicity of Cd and Hg in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (—) line represents the model prediction from nonlinear regression of Eq. (1) (panel A). Carter's isobolographic analysis of the interactive cytotoxicity of Cd and Hg in MK2 monkey kidney cells. (thick dashed line) represents line of no interaction for EC₅₀ (thick line) represents predicted EC₅₀ isobole; (thin line) represents predicted EC₂₀ and EC₈₀ isoboles from nonlinear regression of Eq. (1) (panel A).

three models give different conclusions. The Statistical Isobol gave adequate fit to the observed toxicity of the individual metals (Fig. 7A). Little confidence is placed in the estimated EC₅₀ for TMT as the value of 1401 μM is well beyond the observed data. Both the interaction term (0.0001 ± 0.0003) and visual inspection of the response clearly support no interaction (additivity) for the mixture of MM and TMT. Barton's non-linear models provided reasonable fits to the toxicity of the individual metals (Fig. 8A, C and E) although little confidence is placed in the estimated EC₅₀ for TMT. At total metal concentrations (MM + TMT) of 30 μM or less, all three models provided an excellent fit to the observed toxicity of the mixture (Fig. 8B, D and F). At higher total metal concentrations, the Non-additive model provided the best fit. Gamma was 29.4 ± 10.9 supporting a conclusion of synergy.

The ability of a model to adequately fit the individual data will affect the conclusions pertaining to the mixture (Pounds et al., 1998). It is not certain to what degree the disparate conclusions among models is a result of intrinsic differences

in their ability to model this situation or poor modeling of the interactions due to inconsistent modeling of the individual data.

4. Discussion

These studies illustrate the complexity and difficulty of making public health assessment decisions from exposures to multiple chemicals. Metals were selected for these joint action studies because they have similar, but not identical, physicochemical, biochemical, and toxicological properties. The methods of analysis employed in this study differ subtly, but importantly in their mathematical and statistical assumptions, implementation, and in the formation of the conclusions. It is not surprising that the conclusions drawn are partially dependent upon the method of analysis. Rather, these studies emphasize the necessity for the investigator and the risk assessor to understand the limitations of the various models available for the integrated assessment of the joint toxic action of chemicals.

4.1. Models of interaction

Models of interaction generally estimate an interaction parameter which is derived to represent the direction and degree of interaction once the model has been fit to the data. The 95% confidence interval for the estimate of the interaction parameter is inspected for deviation from zero (or one depending on the model, which represents the null hypothesis of no interaction, or Additivity). The graphical or statistical Isobologram and the Median-effect Principle are widely used to characterize the nature of the interaction among drugs and chemicals administered jointly and are used as part of this project.

4.2. Models of null-interaction

Models of null-interaction are used to predict the responses of mixtures, from the individual dose-response curves, expected if there is no interaction. Observed responses are then compared to the predicted null-interactive response to see if, and the how, they differ. Observed responses to mixtures, which are greater than the predicted null-interaction (additive) are interpreted as synergistic (greater than additive) by default, and responses less than predicted as antagonistic (less than additive) by default.

4.3. Barton's nonlinear models of null-interaction

Barton's Nonlinear Models of Null-interaction are a relatively new derivation using nonlinear regression models for evaluating additivity, synergism, and antagonisms of mixtures. This approach provides several advantages over commonly used methods which involve linear regression with logits or probits. For example, a single model fit is performed

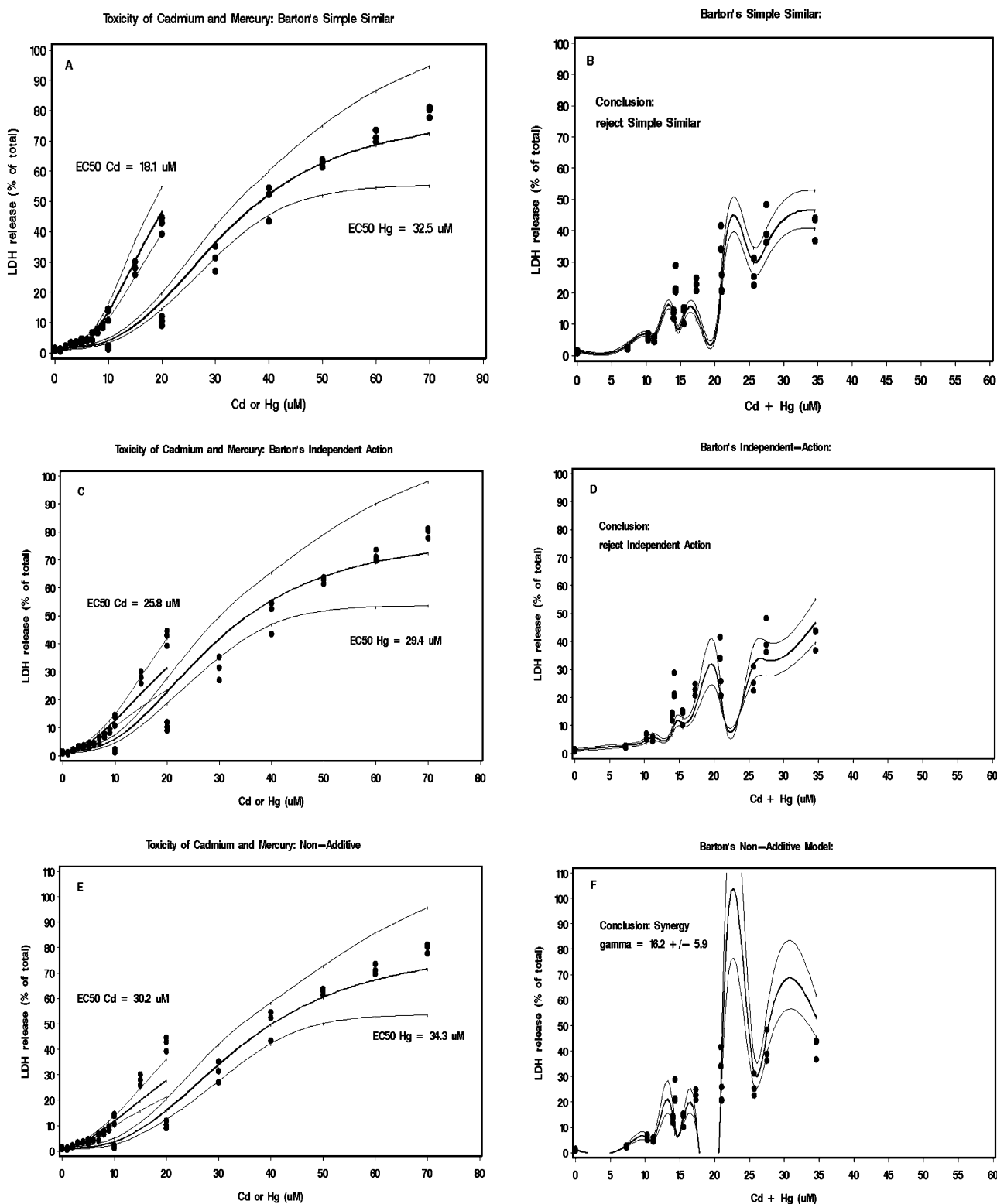


Fig. 5. Barton's nonlinear analysis of the individual cytotoxicity of Cd and Hg in MK2 monkey kidney cells. Panels A and B show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Simple Similar model (Eq. (2)) for the response to the each metal alone and the mixture. Panels C and D show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture. Panels E and F show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture.

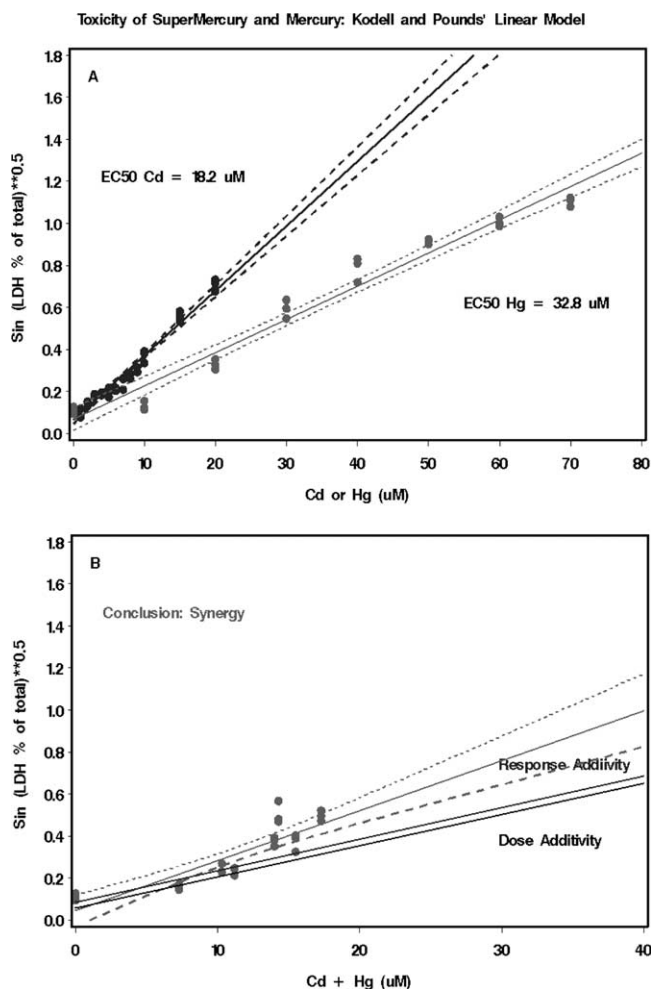


Fig. 6. Kodell and Pounds analysis of the individual cytotoxicity of Cd and Hg in MK2 monkey kidney cells. (●) represent the individual responses of triplicate cultures; (—) line represents the best fit from nonlinear regression of Eq. (4) (panel A). Kodell and Pounds's linear model for the interactive cytotoxicity of Cd and Hg in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (thick dashed line) represents model predicted dose additivity, (thin line) represents model predicted response additivity from Eqs. (7) and (8), respectively (panel B).

to fit the data for the individual toxicants and for the mixture simultaneously, rather than as a multi step procedure. The analyses are performed in the natural response metric, facilitating interpretation of results. Also, the nonlinear regression approach allows the use of the same general analysis methods with quantal data and continuous data.

4.4. Kodell and Pounds' linear models

Kodell and Pounds' Linear Models of Null-interaction give two benchmarks for comparison: Dose/concentration Additivity (approximately equivalent to the simple-similar model of Barton) and Response Additivity (approximately equivalent to the independent action model of Barton) which are predicted by the dose response curves of the individual mixture components. This approach explicitly ad-

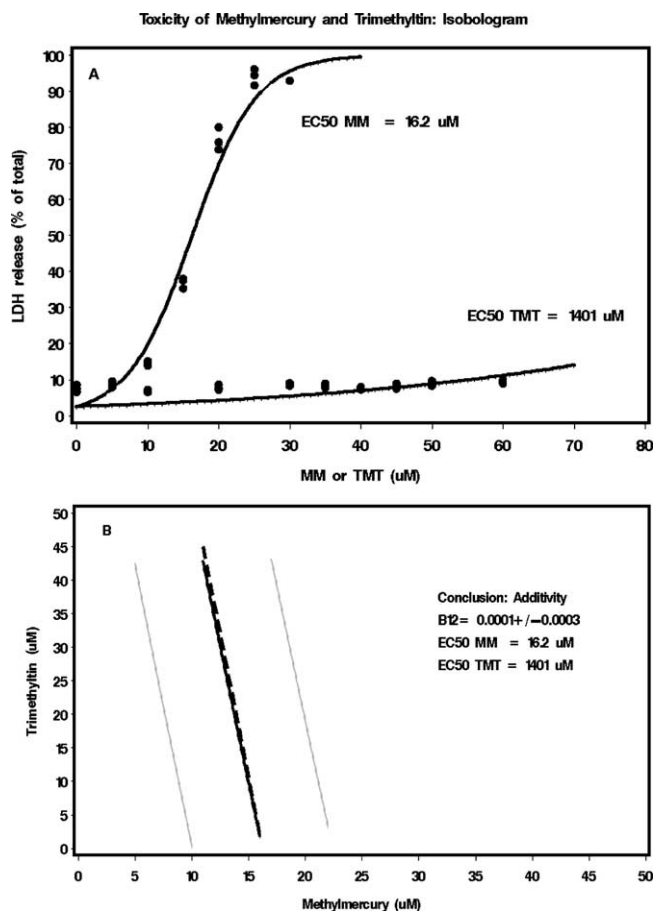


Fig. 7. Carter's isobolographic analysis of the individual cytotoxicity of TMT and MM in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (—) line represents the model prediction from nonlinear regression of Eq. (1) (panel A). Carter's isobolographic analysis of the interactive cytotoxicity of TMT and MM in MK2 monkey kidney cells. (thick dashed line) represents line of no interaction for EC₅₀ (thick line) represents predicted EC₅₀ isobole; (thin line) represents predicted EC₂₀ and EC₈₀ isoboles from nonlinear regression of Eq. (1) (Panel B).

dresses the lack of a completely general definition of additivity by using an Envelope of Additivity as the basis of comparison. The use of unspecified functions to describe the dose–response relations prevents this family from being limited to a few families of curve and surface shapes and provides flexibility to be generally applicable to many situations.

The results of this research program to date clearly support the hypothesis that method of analysis clearly modifies the conclusions regarding the interactive and null-interactive toxicity of the same set of experimental data. Thus, these results emphasize the importance of experimental design and method of analysis in the use of data from mixture experiments and illustrate the complexity of extrapolating conclusions between models, and the difficulty of public health assessment from exposure to multiple chemicals in the environment. These concepts and conclusions should be extended to in vivo studies and to the assessment of the aggregate toxicity of other classes of toxicants. It is important for these

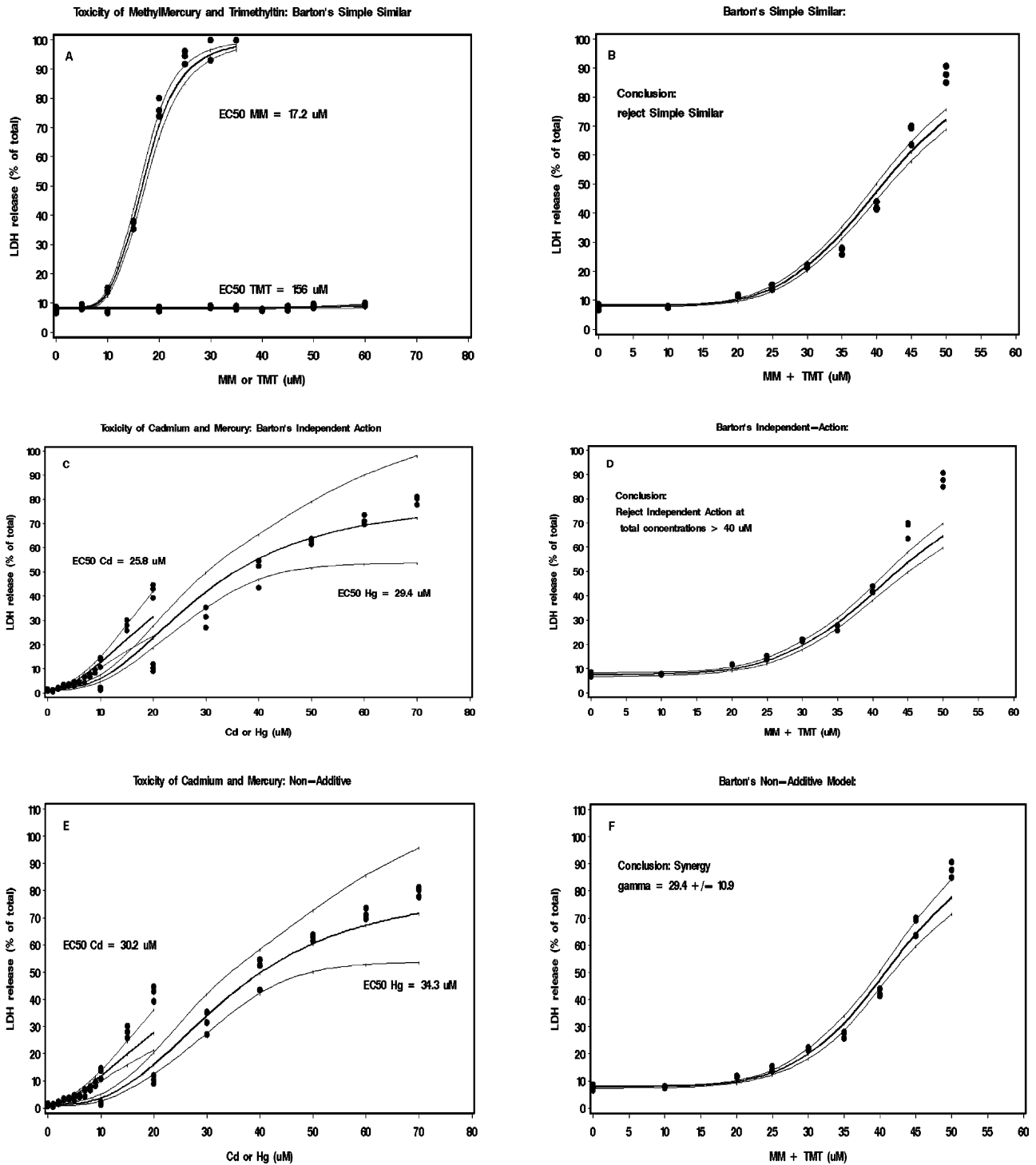


Fig. 8. Barton's nonlinear analysis of the individual cytotoxicity of TMT and MM in MK2 monkey kidney cells. Panels A and B show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Simple Similar model (Eq. (2)) for the response to the each metal alone and the mixture. Panels C and D show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture. Panels E and F show the observed responses of individual cultures (●) and the best fit from nonlinear regression of the Independent Action model (Eq. (3)) for the response to the each metal alone and the mixture.

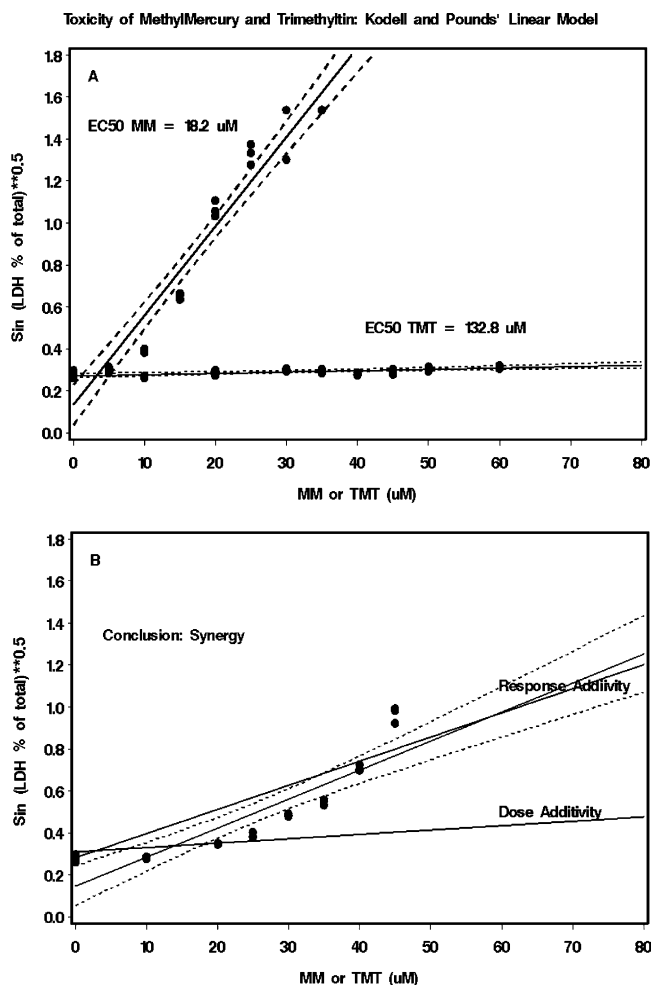


Fig. 9. Kodell and Pounds analysis of the individual cytotoxicity of TMT and MM in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures (Panel A); (—) line represents the best fit from nonlinear regression of Eq. (4). Kodell and Pounds's linear model for the interactive cytotoxicity of TMT and MM in MK2 monkey kidney cells. (●) Represent the individual responses of triplicate cultures; (thick dashed line) represents model predicted dose additivity, (thick line) represents model predicted response additivity from Eqs. (7) and (8), respectively (panel B).

results to be extended to experimental studies in animals and in humans.

These concepts should be extended to in vivo studies and to the assessment of the aggregate toxicity of other classes of toxicants. Moreover, the concepts of interactive toxicity, and the experimental approach developed by this research should have broad application to other short-term assays for organ system toxicity, mutagenicity, carcinogenicity, and teratogenicity.

First, there are certain general limitations pertaining to the complexity of mixture toxicity. The complexity of the metal interactions underlying the joint actions cannot be underestimated. The multitude of factors affecting the measurement of toxicity in vitro, the extrapolation to in vivo situations, and further extrapolation to the human toxicants acting alone are compounded when considering the joint toxicity. Some

of these factors include route of exposure, dose and duration of exposure, age, sex, species, etc. This complexity contributes to the difficulty in applying joint action analyses to human health decisions and emphasizes some of the limitations specifically addressed in this paper. The complexity of the problem however, does not excuse toxicologists from developing appropriate approaches and understanding of the problems of exposure to multiple toxicants.

Metals, as do other toxicants, induce many important non-lethal changes in cell function, whether in vitro or in vivo. Although, cytotoxicity does not adequately represent the range of toxic responses to metals or other toxicants, cell death can be measured both in vitro and in vivo and is a convincing, quantitative measure to toxicity. Thus, understanding the joint toxic action of metals on cytotoxicity is a reasonable beginning for understanding the joint action of metals on nonlethal responses. The third limitations pertaining to extrapolations of results and concepts to in vivo situations assumes that the mechanisms of action and moreover, the mechanisms of joint action are the same in vitro and in vivo. The validity of this assumption will require much more work. The usefulness of the concepts and conclusions developed by this research do not depend upon the validity of this assumption, rather this will provide information which will make the testing of the assumption possible.

Unfortunately, it is premature for several reasons to conclude that one model is more appropriate to assess mixture toxicity than other models. First, the results reported herein did not evaluate many other statistical and graphical approaches. Secondly, we used one type data that was theoretically constrained between 0 and 1 (fraction of total LDH released). The models evaluated could be more or less appropriate to other numerical data types or to the data transformations employed. The results of these experiments to emphasize the importance of using both statistical and graphic evaluation.

The concepts developed in this investigation provide some useful insights into the biological and methodological limitations of extrapolating from limited data to generalized human situations that may not parallel the experimental data. Establishment of relationships between exposure to multiple environmental pollutants and adverse health outcomes uses input from a variety of areas of health sciences including toxicology, epidemiology, and surveillance studies. Once a relationship is established assessing health effects and attendant health risks posed by such pollutants needs strategic planning and systematic use of statistical methods available for the analysis of toxicologic data. Data analysis should include *a priori* triaging of available methods from the most simplest available through a logical progression to the most appropriate methods. This triaging is needed to obtain an unbiased and realistic solution, since different methods yield different results as has been shown from the mixtures data analysis presented in this paper. Often existent data cannot address all the issues currently faced by health risk assessors. In such situations, experimental studies, the driving force of

cause–effect relationships, need to be designed to address the health risk assessors needs. Such studies should be part of targeted research that include an efficient experimental design that would yield results to fill specific data gaps that can be analyzed by known statistical methods. Just applying advanced statistical methods and models does not solve the problems unless the data structure, experimental design, and method of analysis are appropriately matched. Indeed, efficient experimental designs and testing methods are needed to generate data that can be analyzed by appropriate statistical methods to draw conclusions that are appropriate for human health assessment and for mitigation of potential health effects that can be caused by the ever present chemicals in today's world. A team approach needs to be taken to address mixtures health concerns wherein, from the very start, experimental scientists, model or methods developers, health assessors and regulators work together to understand the challenges and concerns of each discipline to achieve their common goal of protecting human health.

Acknowledgments

The authors thank Drs. Curtis Barton, H.W. Carter, Ralph Kodell and Medi Razzaghi for helpful discussion. The authors also thank journal and ATSDR reviewers for many helpful and thought provoking comments. Supported by ATSDR U61/ATU 581481 and NIEHS P30ES06639.

References

- Agency for Toxic Substances and Disease Registry (ATSDR), 2002. Guidance manual for the assessment of joint toxic action of chemical mixtures. Draft for public comment. Available at <http://www.atsdr.cdc.gov>.
- Barton, C.N., Braunberg, R.C., Friedman, L., 1993. Nonlinear statistical models for the joint action of toxins. *Biometrics* 49, 95–105.
- Carter Jr., W.H., Gennings, C., Staniswalis, J.G., Campbell Ed, White Jr., K.I., 1988. A statistical approach to the construction and analytical analysis of isobolograms. *J. Am. Coll. Toxicol.* 7, 963–973.
- Finney, D.J., 1978. *Statistical Method in Biological Assay*, third ed. Griffin, London (Chapter 3).
- Groten, J.P., Schoen, E.D., van Balderen, P.J., Kuper, C.F., van Zorge, J.A., Feron, V.J., 1997. Subacute toxicity of a mixture of nine chemicals in rats: detecting interactive effects with a fractioned two-level factorial design. *Fundam. Appl. Toxicol.* 36, 15–29.
- Jernigan, J., Pounds, J.G., Harbison, R.D., 1983. Potentiation of chlorinated hydrocarbon toxicity by 2,5-hexanedione in primary cultures of adult rat hepatocytes. *Fundam. Appl. Toxicol.* 3, 22–26.
- Kodell, R.L., Pounds, J.G., 1991. Assessing the toxicity of mixtures of chemicals. In: Krewski, D., Franklin, C. (Eds.), *Statistics in Toxicology*. Gordon and Breach, New York.
- Meadows, S.L., Gennings, C., Carter Jr., W.H., Bae, D.-S., 2002. Experimental design for mixtures of chemicals along fixed ratio rays. *Environ. Health Perspect.* 110 (Suppl. 6), 979–983.
- Moser, V.C., MacPhil, R.C., Gennings, C., 2003. Neurobehavioral evaluations of mixtures of trichloroethylene, heptachlor, and di(2-ethylhexyl)phthalate in a full-factorial design. *Toxicology* 188, 125–137.
- National Academy of Sciences 1989. Risk assessment of mixtures of systemic toxicants in drinking water. *Drink. Water Health* 9, pp. 121–132.
- National Institute for Occupational Safety and Health (NIOSH), 1976. Criteria for a Recommended Standard of Occupational Exposure to Methylene Chloride. National Institute for Occupational Health Safety and Health, Cincinnati, Ohio.
- Pounds, J.G., Chen, D.G., Mumtaz, M.M., 1998. Importance of Model Fitting in Assessment of Chemical Mixtures Toxicity. Annual Meeting of the Society of Toxicology, Seattle, WA.
- Razzaghi, M., Kodell, R.L., 1992. Box-Cox transformation in the analysis of combined effects of mixtures of chemicals. *Environmentrics* 3, 319–334.
- Solana, R.P., Chinchilli, V.M., Carter Jr., W.H., Wilson, J.D., Carchman, R.A., 1987. The evaluation of biological interactions using response surface methodology. *Cell Biol. Toxicol.* 3, 263–277.
- Solana, R.P., Gennings, C., Carter Jr., W.H., Anderson, D., Lennox, W.J., Carchman, R.A., Harris, L.W., 1991. Efficacy comparison of two cholinolytics, scopolamine and azapropfen, when used in conjunction with physostigmine and pyridostigmine for protection against organophosphate exposure. *J. Am. Coll. Toxicol.* 10, 215–222.
- Teuschler, L.K., Groten, J.P., Hertzberg, R.C., Mumtaz, M.M., Rice, G., 2001. Environmental Chemical Mixtures Risk Assessment: Current Approaches and Emerging Issues. *Comments Toxicol.* 7 (5–6), 453–493.
- U.S. Environmental Protection Agency (USEPA), 1986. Guidelines for the health risk assessment of chemical mixtures. *Fed Reg* 51(185):34014–34025.
- Yang, R.S.H., Rauckman, E.J., 1987. Toxicological studies of chemical mixtures of environmental concern at the National Toxicological Program: health effects of groundwater contaminants. *Toxicology* 47, 15–34.