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Hazard/Risk Assessment

GENERALIZED CONCENTRATION ADDITION APPROACH FOR PREDICTING MIXTURE TOXICITY

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Abstract: A new mathematical model for analyzing data and predicting the effect of mixtures of toxic substances is presented as a generalized form of the concentration addition model. The proposed method, the generalized concentration addition (GCA) model, can be applied to mixtures with arbitrary strengths of interactions (synergistic or antagonistic). It requires mixture effect data for least 1 exposure concentration of the mixture in which fractions of all components and concentration–response functions for each component are known. The GCA model evaluates the interaction between components by introducing a novel response function, which is independent of the response functions for each individual components, to describe the effect of addition between different components. The GCA method was applied to published mixture toxicity data, and it was found to fit the mixture effect better than both the concentration addition model and the independent action model, the implication being that the proposed approach is widely applicable. *Environ Toxicol Chem* 2016;9999:1–11. © 2016 SETAC

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INTRODUCTION

There is an environmental risk that humans and wild-life will be exposed to multiple substances at the same time. Regulatory frameworks are therefore required to address the issue of mixture effects [1–3]. The development of chemical regulations that take into account the effects of multiple compounds in a scientific way requires statistical or analytical methods to describe and predict the combined effects of mixtures based on toxicity data of individual components and mixtures.

The component-based approach, which is based primarily on the independent action (IA) model or the concentration addition (CA) model, is 1 of the best options, at least for the first or lowest tier of risk assessment. These approaches make it possible to predict the effect of mixtures without additional data of mixture toxicities as long as synergistic (stronger-than-additive) or compensatory (weaker-than-additive) interactions between component chemicals are absent or negligible [4–7].

In addition, knowledge of the mode of action of the components in a mixture may help identify the proper reference model and strengthen this approach, because similarly acting substances with shared modes of action tend to follow the CA model, whereas dissimilarly acting substances with different modes of action tend to follow the IA model [1,8,9]. In general, the CA model has higher predictive power than the IA model for toxicities of mixtures consisting of many chemicals [10], and the discrepancies between the 2 reference models are limited [4,8,11]. The CA model tends to produce slightly overestimated or more protective predictions of adverse responses to mixtures than the IA model [5,9,12]. Thus, the CA model has been advocated as the most general reference model for use in the risk assessment of mixtures, especially at the lowest tier screening level [2,5,13].

However, there are at least 3 issues of concern with respect to reliance on the reference models. First, identifying the appropriate reference model from knowledge of the mode of action is difficult, because the relationship between the similarity of modes of action and the appropriateness of reference models is often ambiguous. In addition, there is seldom enough information about the mode of action of synthetic chemicals [3,14]. At higher tiers of risk assessment, however, more elaborate modeling schemes, which include arbitrary interactive effects [15–18], may be relevant if the proper reference model can be identified and enough toxicity data on mixtures are available. Second, some substances may truly cause synergistic or antagonistic effects with other chemicals. The result of these effects is underestimates or overestimates of mixture toxicity if the estimate is based on the CA or IA model [19–21]. Recent reviews have suggested that only a few experimental studies on mixture toxicities have detected clearly synergistic effects [22], and toxicant concentrations as low as those usually detected in the environment may rarely induce synergistic interactions [13,23]. Nonetheless, these general patterns do not guarantee that synergistic interactions do not occur among substances in the environment. Finally, the strict assumption of the CA model, which is the similarity of the response shape (the shape of a concentration–response function) of all components [24], is referred to as the simple similar action [25] or the dilution principle [1,26]. This assumption may barely be met for some groups of chemicals. Variations of response shapes among components may even explain some of the observed deviations of mixture effects from the CA model [27]. For example, consider an instance in which 1 of the 2 components in a mixture has a much steeper sigmoid response curve than the other component. This also assumes that the mixture of the 2 components, with each concentration equal to half of the median effective concentration (EC50), induces an effect much less than the 50% response, which is predicted by

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the CA model. This deviation from the CA model may not be the result of antagonistic interaction between the components, but may rather occur as a result of the component with the steeper response curve, which may be much more biodegradable and more rapidly excreted from the target organisms than the other component when both are present at small concentrations.

Thus, there is still a need in toxicology for a practical method, analytical or statistical, that can describe and predict the cumulative effects of substances, regardless of whether the effect is interactive (i.e., synergistic or compensatory) or noninteractive (i.e., additive). For practical rather than scientific or heuristic purposes, such methods should not demand detailed information on mixture effects but should require only minimal data on mixture effects in addition to the concentration–response relationships of individual components.

A new analytical method, the generalized concentration addition (GCA) model, is presented in the present study to describe the effect of mixtures of substances. The GCA model is an extension and generalization of the CA concept. The GCA approach conceptually decomposes the cumulative effect of all substances in a mixture into 2 major parts, the effect associated with the accumulation of each substance and the effect associated with mixing of different substances. In this way, the GCA gives a unique estimate of the response shape observable when different components are mixed together, an estimate that is independent of the response shapes observable when each substance is individually accumulated.

This type of modeling, although neither empirically verified nor theoretically established, is expected to give 1 of the most statistically robust estimates of mixture effects regardless of the interactions between the components. Furthermore, the GCA approach is also expected to provide 1 of the most general and parsimonious links between the component-based approach and the whole-mixture approach.

THEORETICAL CONSIDERATIONS

In this discussion we briefly explain a concept that links the interacting effect of substances and concentration–response (or dose–response) curves in general. This concept forms the theoretical basis of the GCA approach.

Toxic interaction rule for toxicants

Substances usually have interacting effects, regardless of whether the interactions are between the same or different substances. The only exception is the special case in which responses are linearly associated with concentrations or doses of substances [26,28]. The interacting effect of substances in this broad sense may be illustrated by the concentration–response (or dose–response) curve (Figure 1), in which the response slope usually changes with exposure concentrations or dose levels if not transformed properly (e.g., into the logarithmic or the probit scale) to ensure linearity of responses. It follows that the increase of response as a result of an additional amount of the substance depends on the accumulated mass of the substance (Figure 1). The interaction between molecules of the increased substance and molecules already accumulated in the body (or at the target site) is regarded as the only factor that brings about the difference in the toxic effect of the same amount of the substance between the cases with different amounts of the previously exposed substances. In this way, the nonlinearity of the concentration–response function conceptually represents the strength of interaction within substances. In this section we focus on a single substance, to derive a general definition of chemical interaction.

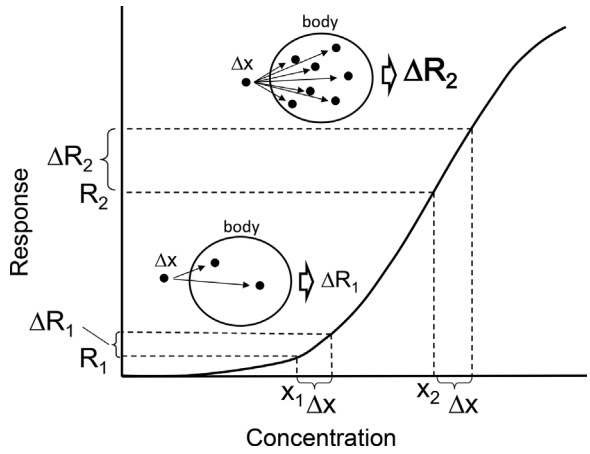

Figure 1. Schematic diagram of the association between the interacting effects of substances and the concentration–response curve. As long as responses to a substance follow a nonlinear concentration–response curve, the responses, ΔR_1 and ΔR_2 as a result of an increase in the substance by the same amount Δx, depend on previously exposed or dosed levels of the substance, x_1 and x_2. The difference in the unit increase of responses, $\Delta R/\Delta x$, reflects the concentration-dependent interaction within the same substance. The 2 pictures in the graph illustrate the concept that the effect of a substance changes with masses of molecules (closed dots) already taken into the body if there are interactions between molecules of the substance in eliciting adverse effects.

The scheme of concentration-dependent effect of interaction between molecules of a toxicant, which governs unit increases of response throughout the entire range of toxicant concentrations, is in the present study referred to as the "toxic interaction rule" (TIR). The TIR is denoted by the change of response ΔR associated with a small increase of the toxicant concentration Δc, and is a function of the previously exposed level c of the toxicant: $\Delta R(\Delta c|c) = R(c + \Delta c) - R(c)$, in which $R(c)$ is the concentration–response function. If there is no interaction between toxicant molecules (the effect is strictly additive), the change of response does not change with exposure concentration ($\Delta R/\Delta c$ is constant for all c), and the response function must be linear. The constancy of the ratio $\Delta R/\Delta c$ represents the strict additivity. On the other hand, if the response function is sigmoid, as in the usual case, the per-unit increase of a toxicant induces a smaller effect than the strict additivity when the exposure concentration is much lower than the median effect concentration (the inflection point in the concentration–response function), whereas it induces a larger effect than the strict additivity when the exposure concentration is close to the median effect concentration.

The TIR can be mathematically defined as $\Delta R(\Delta c|c)/\Delta c$ across the entire relevant range of concentrations. It follows that $\mathrm{TIR}(c)$ is equivalent to the response slope and converges to the derivative when $\Delta c \to 0$, $\mathrm{TIR}(c) = \frac{dR(c)}{dc}$. Thus, the TIR determines the response function: $R(c) = \int_0^c \mathrm{TIR}(z)dz + R_0$, if the initial condition R_0 is specified for a reference concentration such as the EC50. In other words, the shape of a concentration–response function represents a TIR.

The concept of TIR will be extended to include the addition between different substances in a mixture for the GCA approach. Thus, when we combine 2 different substances to make a mixture, there can be 3 different TIRs: TIR_1 for the first component, TIR_2 for the second component, and TIR_m for

mixing these substances (TIR_1 or TIR_2 determines the concentration–response function for either substance).

Concentrations of the 2 components in a mixture can be added together to give the mixture effect because the assumptions for CA are met if the response shapes are similar between the 2 components (TIR_1 is equal to TIR_2) and if mixing between the 2 components also elicits similar responses (TIR_m is also equal to TIR_1 and TIR_2). The implication is that the concentration–response curve of the first component can be made identical to that of the second component by linear scale transformation. In addition, the 2 components can be regarded as the same substance after 1 of them is diluted to the proper extent (the dilution principle [26]), because the TIR is common to both components and to the mixture (Figure 2). This concept is the basis for the CA model, 1 of the leading reference models in mixture studies [1,5,6,25,29]. The CA model is then based on the assumption that the combined effect of all components in a mixture reflects the fact that all components share the same TIR. (The irregular shape of the response curve depicted in Figure 2 emphasizes that the concept of CA holds with an arbitrary TIR as long as it is common among all components and the mixture.)

The general mathematical expression of CA or the Loewe additivity [30] is

$$\sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} = 1 \qquad (1)$$

where n is the number of components in the mixture, c_i is the concentration of the ith component, and EC_{Xi} is the concentration of the ith component that induces X% effect. Conventionally, the effect of a mixture above or below the effect that is compatible with this equation is usually regarded as synergistic or antagonistic (or compensatory). This standard definition of nonzero interaction is not compatible with the previous explanation based on strict additivity. Because synergism is a comparative statement [6], the zero or no interaction is defined as the baseline interaction within the same substance or between different substances in a mixture in which they appear to simply dilute one another [26,28,31,32].

GCA approach

The GCA approach allows addition between concentrations of different substances with dissimilar response shapes and arbitrary interactions. The GCA model starts from the following general formulation of adverse responses to toxicants as a function of exposure concentration c:

$$R(c) = F\left[\left(\frac{c}{\theta}\right)^{\beta}\right] \qquad (2)$$

where θ is the scale of concentration corresponding to a certain toxic response, such as the EC50 or no-observed-effect concentration, and β is the response shape, which determines the nonlinearity of responses. $F(Z)$ denotes a scale-independent and parameter-free concentration–response function that relates the standardized concentrations, $Z = (c/\theta)^{\beta}$, to responses R, and is referred to as the response mother function. All model parameters used in the GCA approach are listed in Table 1.

The response mother function can take any specific functional form such as the logit model, the probit (the cumulative log-normal) model, the Weibull model, or the threshold model. The response $R(c)$ measures the fractional decreases of biologically important performance, for example, survivorship and fecundity during specific time intervals ($R = 0$ denotes no response, and $R = 1$ denotes the full response). For simplicity, we assume that all components in a mixture and the additive effect of components follow identical response mother functions, although this assumption can be relaxed with additional mathematical complexities. The typical response mother functions are $F(Z) = 1 - \frac{1}{1+\exp(\log_{10} Z)}$ (the logit model),

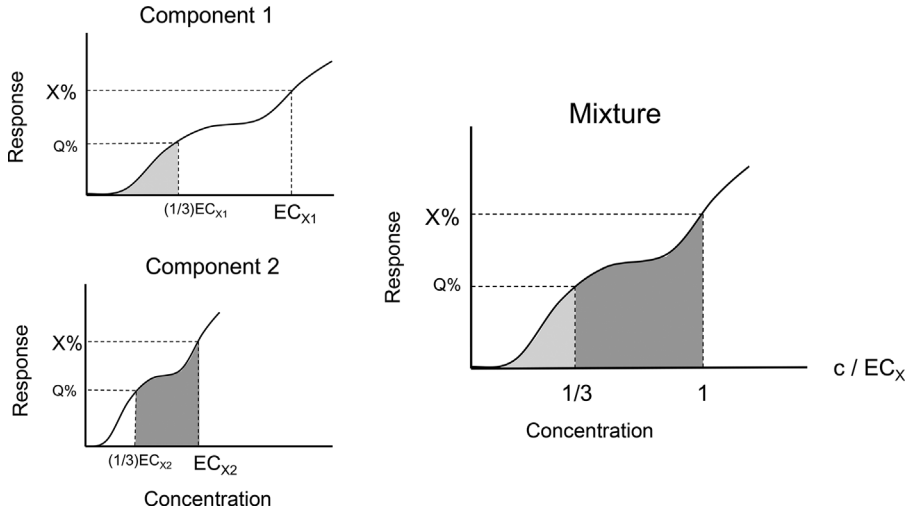

Figure 2. The principle of concentration addition between substances. Two substances with a similar action, components 1 and 2, can be added together to generate the mixture effect predictable from the CA model. Addition of a particular amount of component 2, say $\frac{2}{3}EC_{X2}$ (EC_{X2}: the concentration of component 2 eliciting X% response), to a particular amount of component 1, $\frac{1}{3}EC_{x1}$, must elicit the same additional response as would be the result of adding component 1 whose amount is equal to $\frac{2}{3}EC_{x1}$ to component 1 itself, whose amount is $\frac{1}{3}EC_{x1}$. There is no need to know the effect of $\frac{1}{3}EC_{x1}$ or $\frac{1}{3}EC_{x2}$ ($Q\%$). Thus, if the amount of component 1 from the first is $\frac{1}{3}EC_{x1}$, the mixture effect must be X%, because $\frac{1}{3} + \frac{2}{3} = 1$, regardless of the response shape. The irregular shape of the response curve emphasizes that the concept of CA holds with an arbitrary toxic interaction rule (TIR) as long as it is common among all components and the mixture (see text for explanation of TIR).

Table 1. Functions and model parameters used in the present study[a]

Symbol	Term: explanation
c	Concentration of a component
EC_X	The concentration that induces X% effect
C	Concentration of the mixture: $\sum_{i=1}^{n} c_i$ (n: the number of components)
$R(c)$	Response function
θ	Scale of concentration: the concentration corresponding to a certain response
β	Response shape: an index for nonlinearity of responses
TIR	Toxicant interaction rule
$F(Z)$	Response mother function: the functional form of concentration–response function
Z	Standardized concentration: the unique argument of the response mother function
$R_M(\mathbf{c})$	Mixture response function: the response function for the mixture whose component concentrations are $\mathbf{c} = (c_1 \quad \cdots \quad c_n)$
β_M	Response shape for component addition: an index for nonlinearity between components
u	Transformed concentration: the concentration of a component transformed into the scale of the mixture response function

[a]The parameters, variables, or functions c, EC_X, $R(c)$, θ, β, and u append subscripts for specifying particular components.

$F(Z) = 1 - \exp(-Z)$ (the Weibull model), $F(Z) = \min[1, \max(0, \log_{10}Z)]$ (the threshold model), and $F(Z) = \int_0^Z \frac{1}{\sqrt{2\pi}x}\exp\left\{-(\log_{10}x)^2/2\right\}dx$ (the probit model). The basic concept of the GCA approach does not depend on the particular response mother functions.

In the above modeling framework, the TIR of a toxicant is indicated by the response shape β in Equation 2. In general, nonlinearity of responses to toxicant concentrations increases as β deviates from 1. However, $\beta = 1$ does not necessarily mean that the responses are linear, because the response mother function in general does not linearly relate standardized concentrations to responses (see *Comparison with the IA model* section). If the response shape is identical among all components and there are no synergistic or compensatory effects among components, the concept of CA in the strict sense holds; in that case, the response to the mixture $R_M(\mathbf{c})$ as a function of concentrations of all components $\mathbf{c} = (c_1 \quad \cdots \quad c_n)$ can be estimated from $R_M(\mathbf{c}) = F\left[\left(\sum_{i=1}^{n}\frac{c_i}{\theta_i}\right)^{\beta}\right]$, where c_i and θ_i are the concentration and the scaling factor, respectively, of the ith component. Similar methods of accumulating scaled component concentrations with identical response shapes have been used by Olmstead and LeBlanc [33]. However, for the case in which the components have different response shapes or nonzero interactions, simple mathematical approaches relevant to the description and prediction of mixture effects by the summation of concentrations of components have not yet been presented (see Hewlett and Plackett [34,35]).

The GCA approach relies on 2 heuristic assumptions for formulating the effect of mixtures whose components have different response shapes and arbitrary interacting effects (Figure 3). The first assumption is that adding together different components to make a mixture follows a mixture-specific TIR that is independent of the TIRs for the individual components in eliciting mixture effects. A corollary of this assumption is that a new concentration–response function, the response function for component addition, is introduced to describe the effect of adding together different components in causing responses. The general form of the response function for component addition is $R_M(\mathbf{c}) = F\left[\left(\sum_{i=1}^{n} u_i\right)^{\beta_M}\right]$, in which β_M is the mixture-specific response shape for component addition, and u_i is the transformed concentration of the ith component on the scale of the response function for component addition (see below; Figure 3).

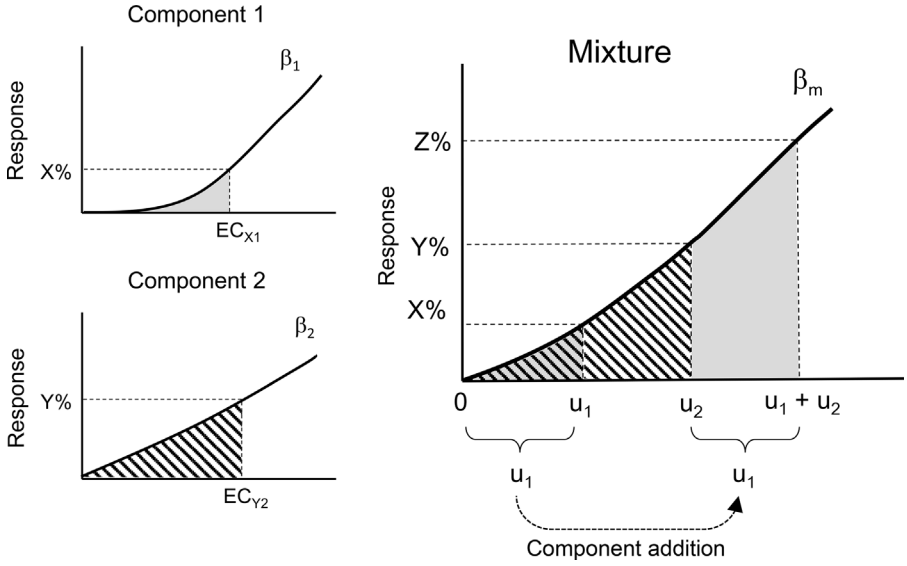

Figure 3. Schematic explanation of the generalized concentration addition (GCA) approach applied for components with different response shapes to give the mixture effect. The GCA method allows addition of 2 component substances (components 1 and 2) with different response shapes, characterized by response shapes β_1 and β_2 (on the left), respectively. Each component has the single effect of X% and Y% responses at concentrations EC_{X1} and EC_{Y2}, respectively. To add these component concentrations with the purpose of estimating the mixture effect, a new concentration–response function with a definite response shape β_M is introduced (on the right). The component concentration EC_{X1} and EC_{Y2} are transformed into the corresponding concentrations u_1 and u_2 in the concentration–response function for component addition eliciting the same effects as the single component effects. The mixture effect Z% is predicted from the summation of component concentrations $u_1 + u_2$ taken on the concentration axis of the concentration–response function for component addition.

The second assumption is that the concentration of each component can be transformed to an equitoxic value (a scaled dimensionless concentration) in the response function for component addition (EC_{X1} to u_1 and EC_{Y2} to u_2 in Figure 3) such that each component individually elicits the same response in the response function for component addition as it does in the response function for each component (u_1 for X% and u_2 for Y% in Figure 3). In our notation, the transformed concentration is $u_i = \left(\frac{c_i}{\theta_i}\right)^{\beta_i/\beta_M}$ because the transformation must satisfy the equation $F\left[\left(\frac{c_i}{\theta_i}\right)^{\beta_i}\right] = F[u_i{}^{\beta_M}]$. If these 2 assumptions are met, the transformed values of component concentrations (referred to as transformed concentrations) can be summed over components to generate the mixture effect according to the TIR for component addition (the right panel in Figure 3).

Three lines of reasoning may justify the second assumption. The first is that a concentration of a substance that induces a particular response can be associated with the equitoxic concentrations of other substances that induce the same response (Figure 3). Hence, the concentration–response function in general is mathematically transformable across substances, whereas it is usually impossible to share the TIR among substances with different response shapes. Likewise, concentrations of any components can be converted into transformed concentrations in the response function for component addition. The second line of reasoning is that the above-mentioned transformation is regarded as a part of the assumed formulation of the response function and the response shape for component addition, both of which cannot be defined until component concentrations are transformed onto the scale of the response function for component addition. Lastly, for mixtures consisting of 1 major component and other components at extremely low concentrations (all fractions except for the dominant component are close to 0), the compound effect is likely to be dominated by the effect of the major component. The equitoxic transformation of component concentrations into transformed concentrations is 1 of the simplest assumptions that are compatible with this situation.

Provided these assumptions are met, the GCA method gives a general equation for the mixture effect with particular component concentrations as follows,

$$R_M(\mathbf{c}) = F\left[\left(\sum_{i=1}^{n}\left(\frac{c_i}{\theta_i}\right)^{\beta_i/\beta_M}\right)^{\beta_M}\right] \quad (3)$$

For the special case of the logit model, we have

$$R_M(\mathbf{c}) = 1 - \frac{1}{1+\exp\left(\beta_M \log_{10}\sum_{i=1}^{n}\left(\frac{c_i}{\theta_i}\right)^{\beta_i/\beta_M}\right)} \quad (4)$$

Estimation of the model parameters θ_i, β_i, and β_M requires concentration–response data for each component and at least 1 response datum for a complete mixture with known component concentrations. Given estimates of these parameters, responses to mixtures of arbitrary concentrations and fractions can be predicted as long as the mixtures have nearly the same value of β_M as determined in the mixture experiment. The effect of interactions among components in the mixture can be examined by comparing the response shape for component addition β_M and the response shape for individual components β_i.

The degree of demands on response data for the whole mixture depends on how broadly the parameter β_M can be defined, as well as on the extent to which precision of β_M is required. If β_M is regarded specific for a certain composition of the mixture or certain fractions of the single substances, β_M need to be determined experimentally for each composition to get the fraction-specific values of β_M. In contrast, an estimate of β_M may be shared by mixtures having different fractions within a range of fractions in which β_M is regarded as approximately constant.

The basic procedure to estimate β_M consists of the following 2 steps: 1) estimation of θ_i and β_i for each component from concentration–response data of individual components; and 2) determination of β_M from observed effects of mixtures with specific component concentrations using Equation 3. Numerical solutions using solver or finder functions, for example, *uniroot* in R and *Solver* in Excel, must be used to determine β_M, because we do not have any explicit solutions for β_M in Equation 3.

A single observed response to a mixture can result in an estimate of β_M. Thus, if there are 10 measurements of responses to a mixture with the same fractions (e.g., 5 concentrations with 2 replicates), there will be 10 independent estimates of β_M. On the assumption that β_M is specific to fractions, the mean β_M for mixtures with the same component fractions may provide fraction-specific estimates of β_M. If β_M is specific to neither fractions nor concentrations, the maximum likelihood method based on the entire dataset may give the best estimate of β_M.

Synergistic and antagonistic interaction

Following the standard definition [1,10], the synergistic (or the antagonistic) interaction refers to the case in which more (or less) responses are elicited than expected by 1 of the reference models (the CA or IA model) in the present study.

Comparison with the CA model

In cases in which the response shapes are similar among all components, it is easy to infer if the interaction among components is synergistic, antagonistic, or noninteractive. The term noninteractive is different from the strict additivity, which means no interaction between substances, and denotes that the interaction between substances is equal to that within components.

When the CA (the Loewe additivity) is held among all components, the response shape for component addition β_M is equivalent to the response shape for individual components β. Defining the response shape for component addition when the Loewe additivity holds as β_{CA}, the response shape for CA (β_{CA} is equal to β if all components have the same response shape), we can infer the strength of interaction between substances by comparing estimates of β_M and β_{CA}, which works as the standard of CA. We regard the case of $\beta_M > \beta_{CA}$ as more interactive than CA, the case of $\beta_M < \beta_{CA}$ as less interactive than CA, and the case of $\beta_M = \beta_{CA}$ as noninteractive. The case of more interactive than CA is illustrated in Figure 4.

Determination of synergism or antagonism based on the comparison between β_M and β_{CA} depends on whether the sum of transformed concentrations over all components $\sum_{i=1}^{n} u_i$ is larger than or less than unity. Because $Z = \left(\sum_{i=1}^{n} u_i\right)^{\beta_M}$ is an

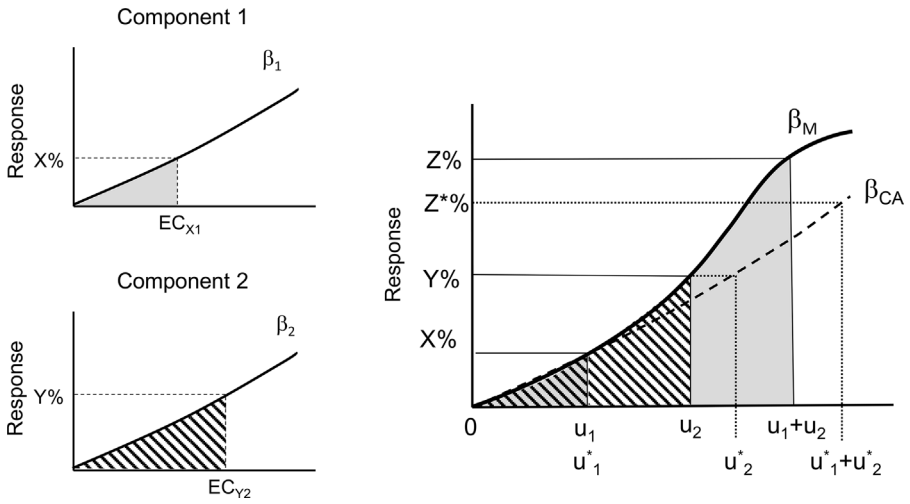

Figure 4. Schematic explanation for the case in which the response slope for component addition β_M is estimated as more interactive than the CA model. For simplicity, the response slopes of the 2 components are assumed to be identical; thus the response slope for CA is equivalent to that for both components, that is, $\beta_{CA} = \beta_1 = \beta_2$. The component concentrations EC_{X1} and EC_{Y2} are transformed into the corresponding concentrations u_1 and u_2 according to the GCA model, and u^*_1 and u^*_2 according to the CA model. In this example, the observed mixture effect Z% deviated from the effect Z*% predicted by the CA model. The GCA method adjusts the response slope for component addition β_M such that the predicted and the observed mixture effects are in accord.

increasing function of β_M if $\sum_{i=1}^{n} u_i$ is larger than unity, and vice versa, the mixture effect is judged as synergistic if $\beta_M > \beta_{CA}$ or antagonistic if $\beta_M < \beta_{CA}$ when $\sum_{i=1}^{n} u_i > 1$. The judgement is reversed when $\sum_{i=1}^{n} u_i < 1$.

The parallel determination is applicable to cases in which components in the mixture have dissimilar response shapes. The only difference is that the value of β_{CA} cannot be equated to the response shape for 1 of the components, because they have different response shapes. Thus, β_{CA} is calculated on the assumption that responses to the mixture follow the basic equation for concentration addition (Supplemental Data, Equation S1). This equation provides an operational definition of additivity in the GCA framework. Supplemental Data S1 shows that β_{CA} is approximately equivalent to a weighted average of the response shapes of all components, $\beta_{CA} \cong \frac{\sum_{i=1}^{n} w_i \beta_i}{\sum_{i=1}^{n} w_i}$ in which $w_i = -\frac{c_i}{\theta_i} \log_e \left(\frac{\frac{c_i}{\theta_i}}{\sum_{i=1}^{n} \frac{c_i}{\theta_i}} \right)$ (see Supplemental Data, Equation S1).

Comparison with the IA model

The IA model is based on probabilistic independence (Bliss independence [1,13]) between actions of multiple substances in a mixture: $R_M(\mathbf{c}) = 1 - \prod_{i=1}^{n} \{1 - R_i(c_i)\}$. If the sum of responses across all components is much smaller than 1, the right-hand side of the above equation is approximated by $\sum_{i=1}^{n} R_i(c_i)$, which is the response addition (RA) model.

For the special case in which the response mother function is linear, that is, $F(Z_1 + Z_2 + \cdots) = F(Z_1) + F(Z_2) + \cdots$, the GCA method is identical to the RA model if $\beta_M = 1$. In other cases, the response shape for RA is defined as β_{RA} such that it gives mixture effects approximately equivalent to those given by the RA model. For nonlinear response mother functions, β_{RA} depends on the response mother function (Supplemental Data, S2). For the particular case of the logit function, it is indicated to be $2.5 \leq \beta_{RA} \leq 3$ (Supplemental Data, S2).

Expression of mixture concentration–response function

For the purpose of risk assessment for a mixture of chemicals, we need a concentration–response function for the mixture. In the present study we follow the standard definition of the concentration C of a mixture, which has a specific array of fractions of components. The mixture concentration C and the component fractions f_i are defined as $C = \sum_{i=1}^{n} c_i$ and $f_i = c_i/C$, where c_i is the concentration of the ith component [1]. When applying the GCA model (Equation 3), the concentration–response function for a mixture is described as $R_M(\mathbf{c}) = F(Z_M)$, where Z_M is the standardized concentration of the whole mixture, $Z_M = \left(\sum_{i=1}^{n} \left(\frac{f_i}{\theta_i} C \right)^{\beta_i/\beta_M} \right)^{\beta_M}$, and $\mathbf{c}$ denotes the array of all component concentrations. Supplemental Data S3 shows that, starting from these definitions, the following expression is derived for concentration–response relations for mixtures with specific fractions:

$$R_M(\mathbf{c}) = F\left[\left(\frac{C}{\Theta} \right)^{\hat{\beta}(\mathbf{c})} \right] \quad (5)$$

in which $\hat{\beta}(\mathbf{c})$ represents the response shape for the mixture (not the response shape for component addition β_M), and Θ is the scaling factor for the mixture concentration, specified as $\Theta = \left\{ \sum_{i=1}^{n} \left(\frac{f_i}{\theta_i} \right)^{\beta_i/\beta_M} \right\}^{-1}$. The response shape for the mixture $\hat{\beta}(\mathbf{c})$ is the weighted mean of the response shapes among components, weighted by the transformed concentration of each component: $\hat{\beta}(\mathbf{c}) = \frac{\sum_{i=1}^{n} \beta_i u_i}{\sum_{i=1}^{n} u_i}$ (Supplemental Data, S3), in which u_i is the transformed concentration, $\left(\frac{c_i}{\theta_i} \right)^{\beta_i/\beta_M}$. Thus, the response shape for a mixture $\hat{\beta}(\mathbf{c})$ depends on the transformed concentrations rather than simply on fractions of components. The implication is that components with steeper

response shapes (larger values of β_i) have a smaller influence on the shape of the response in the mixture than other components with flatter response shapes when the concentrations of these components are very low (if $\frac{c_i}{\theta_i} \ll 1$ for all components, the components that have larger β_i have a smaller contribution to $\hat{\beta}(\mathbf{c})$ than other components). Inversely, when $\frac{c_i}{\theta_i}$ is equal to or more than 1 for most components, components with steeper response shapes (larger β_i) have a more predominant influence on the response shape in the mixture. Therefore, the mixture effect tends to be explained by components with flat response shapes when all components have very low concentrations, whereas it tends to be explained by components with steep response shapes when such components have concentrations comparable to or higher than the median effect concentration of single substances.

APPLICATIONS TO ECOTOXICOLOGICAL DATA

In this section we attempt to apply the proposed approach to a mixture of toxicity data reported in 2 previous studies, 1 of which examined the effect of mixtures of similarly acting substances and the other of dissimilarly acting substances. The purpose of the present reanalyses is to illustrate the utility of the proposed approach and the kind of issues for which it is relevant; the purpose is not to give an alternative interpretation to the original reports. In addition, we conducted an experiment for the mixture effect of copper and zinc in *Daphnia magna* acute toxicities as a case study of synergistic interaction between components.

Similarly acting substances

Altenburger et al. [29] examined the combined effects of 2 herbicides, atrazine (2-chloro-4-ethylamino-isopropylamino-1, 3,5-triazine), and metribuzin (4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4-triazin-5-(4H)-one), on the photosynthetic activity of a green algae, *Chlorella fusca*. Data on the responses to specific concentrations of each component and their mixture are not provided in the original report. However, Drescher and Boedeker [12] presented some of the raw data in their reanalysis (their Table 3 [12]).

We used the logit model as the response mother function because the goodness of fit to the response data did not differ between the probit and the logit models, and the latter was more analytically tractable. Use of the least-squares method, which was based on the predicted and the observed responses, resulted in estimated parameter values of $\theta_1 = 0.65$ and $\beta_1 = 2.86$ for exposures to only atrazine, and $\theta_2 = 0.202$ and $\beta_2 = 3.65$ for exposures to only metribuzin. The response shape was considerably larger and the response curve was more convex upward for metribuzin than for atrazine (Figure 5). The mixture response was then $R_M = 1 - \frac{1}{1+\exp(\log_{10} Z_M)}$, where $Z_M = \left\{ \left(\frac{c_1}{\theta_1}\right)^{\beta_1/\beta_M} + \left(\frac{c_2}{\theta_2}\right)^{\beta_2/\beta_M} \right\}^{\beta_M}$, and c_1 and c_2 are the concentrations of atrazine and metribuzin. Here, β_M was treated as a constant, identical for all exposure treatments, because the component fraction was constant (0.75:0.25).

The least-squares estimate of β_M for mixture effects was 2.99 based on the entire dataset. The standard response shape for CA was calculated to be $\beta_{CA} = 3.25$, the indication being that the observed combined effects were almost noninteractive. A simple statistical test based on the resampling method (the Jackknife test [36]) was attempted to decide whether the discrepancy between β_M and β_{CA} was significant. The resampling unit was set as the experimental treatment with particular concentrations either of the 2 components in the single substance tests. The resampling generated distributions of θ and β for both substances across resampled sets of data. Also, by applying the entire sets of θ and β to the equation for β_{CA} (see *Comparison with the CA model* section), we obtained the distribution of β_{CA} around the Jackknife estimate produced by the resampling, which was used as the null distribution of β_M for the case where it did not differ from β_{CA}. The resultant 95% confidence interval for β_{CA} was between 2.035 (the lower limit) and 5.478 (the upper limit). The estimate of β_M was within this interval, which indicated that β_M did not differ from β_{CA}.

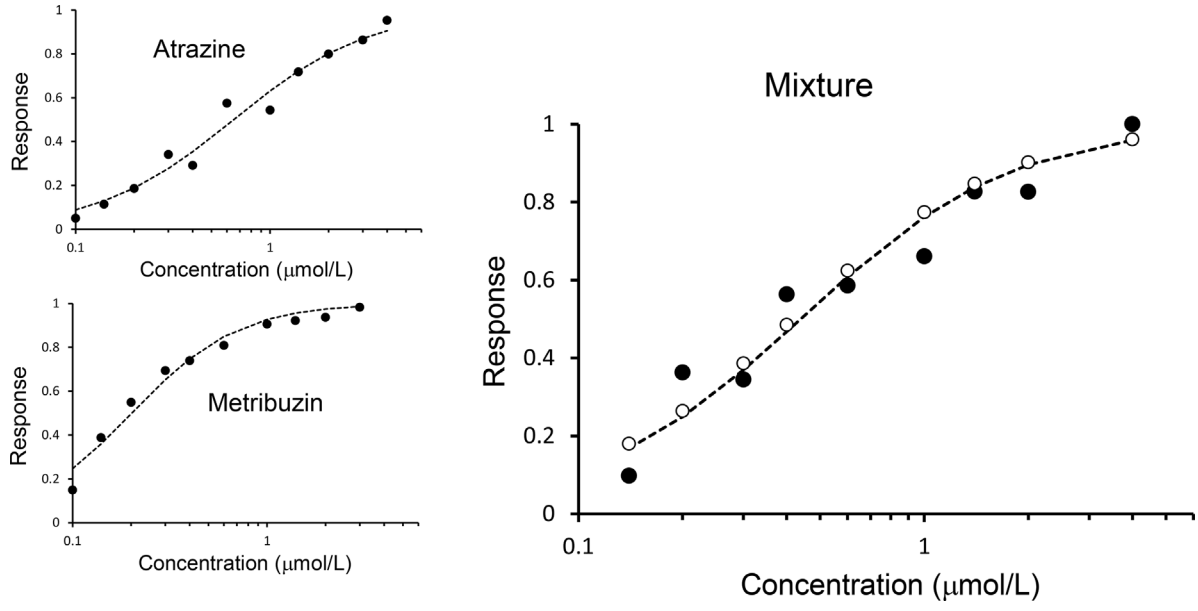

Figure 5. Concentration–response curves of photosynthetic activity of *Chlorella fusca* in response to 2 herbicides, atrazine (upper left) and metribuzin (lower left), and the mixture (on the right). The closed circles in all figures represent the observed responses (data from Altenburger et al. [29]), whereas the open circles represent responses predicted from the CA model. The broken lines in the left figures denote the best-fit concentration–response model (the logit model), and the broken line in the right figure denotes the best-fit mixture model based on the GCA method.

Table 2. The benchmark concentrations and the estimated model parameters (θ: scale of concentration, β: response shape) of the logit model for bioluminescence inhibition in the marine bacteria *Vibrio fischeri*[a]

Component	EC1(μmol/L)	EC50(μmol/L)	θ	β
8-Azaguanine	0.0013	0.07	0.07	2.654
Chloramphenicol	0.0066	0.23	0.23	2.98
Azaserine	0.0251	0.83	0.83	3.024
Nalidixic acid	0.1621	0.86	0.86	6.341
5-Fluorouracil	0.0205	0.92	0.92	2.781
Actinomycin	0.2965	1.68	1.68	6.1
Ethacrynic acid	0.1035	1.79	1.79	3.712
Fusidic acid	0.0453	3.66	3.66	2.409
Streptomycin	0.1734	6.76	6.76	2.888
Dibutyl phthalate (DPB)	5.252	7.99	7.99	25.217
Diltiazem	11.09	375	375	3.005
Ampicillin	225.92	450	450	15.355
Metalaxyl	1.0305	586	586	1.668
Cycloserine	22.17	670	670	3.104

[a]Data from Backhaus et al. [9].
EC1, EC50 = concentration that induces 1% and 50% effect respectively.

Dissimilarly acting substances

Backhaus et al. [9] examined the effects of 14 substances with dissimilar modes of action on the long-term inhibition of bioluminescence of the marine bacterium *Vibrio fischeri*. Their major focus was an experimental test of the IA model as the reference model for dissimilarly acting substances. Although these investigators did not report response data for each substance, they did report numerical endpoints, EC50 and EC1 values, which were calculated from the best-fit response model for each substance chosen from 6 various functions. As an alternative, we used a common response model, the logit model, for all components to determine the best-fit model parameters, θ and β, that were consistent with the reported 2 endpoints (Table 2). Thus, the response function we used may not have precisely represented the observed pattern of responses for each component, but was expected to reflect the variability in the response shapes across components.

Toxicity tests for mixtures were also reported by Backhaus et al. [9] for 2 component fractions, each proportional to the EC50 and EC1 values of all components. These 2 fractions are referred to as the EC50 fraction and the EC1 fraction, respectively. We used the concentration–response data, which were derived from graphical scanning of the reported figures.

The mixture effect can be predicted by using the GCA method with $Z_M = \left\{ \sum_i \left(\frac{q\mathrm{EC}_{50i}}{\theta_i} \right)^{\beta_i/\beta_M} \right\}^{\beta_M}$, in which $\mathrm{EC50}_i$ is the EC50 for the *i*th component, and q is the ratio of the component concentrations to EC50 for the case of the EC50 fraction (EC50 is replaced by EC1 for the case of the EC1 fraction). We again used the logit model for the mixture effect. Each mixture concentration C was used to determine a q value from $q = C/\sum_i \mathrm{EC}_{50i}$ and provided an estimate of β_M by comparison with the observed response and the predicted response to the mixture. The estimated values of β_M are plotted against q for each fraction (Supplemental Data, S4). For both fractions, β_M tended to increase with mixture concentration, an indication of concentration–dependent interaction. The fact that the relation of β_M to the mixture concentration exhibited a much clearer pattern with the EC50 fraction than with the EC1 fraction implied a fraction dependence of interacting effects among components. The similarity in values of β_M between the 2 fractions suggested that the gross pattern of interactions among the components was not substantially affected by fractions.

These estimates of β_M were summarized by expressing β_M as a fourth-order polynomial of the EC50 fraction ($\beta_M = -1082.6q^4 + 1101.6q^3 - 379.2q^2 + 54.2q + 0.21$) and a linear function of the EC1 fraction ($\beta_M = 1.14q + 1.20$). We examined the fit of the linear, the quadratic, the third-order, and the fourth-order polynomial functions to the β_M values on q, and have chosen the above functions as the best model by visual inspection. The concentration–response curves for the mixture calculated from these expressions for β_M and Z_M were compared with the observed mixture effects and the 2 reference models (Figure 6). The mixture effects were better approximated by the IA model than by the CA model. The GCA approach tracked the observed responses even better than the IA model did, even though the GCA approach is conceptually based on CA.

The standard response shape for CA was calculated to be $\beta_{CA} = 5.80$ for the EC50 fraction and $\beta_{CA} = 10.91$ for the EC1 fraction. These values must be interpreted with caution because they were derived on the assumption of low variability of the response shapes among components (Supplemental Data, S1), an assumption that was hardly met in the present case. Nonetheless, much less interaction than expected from the

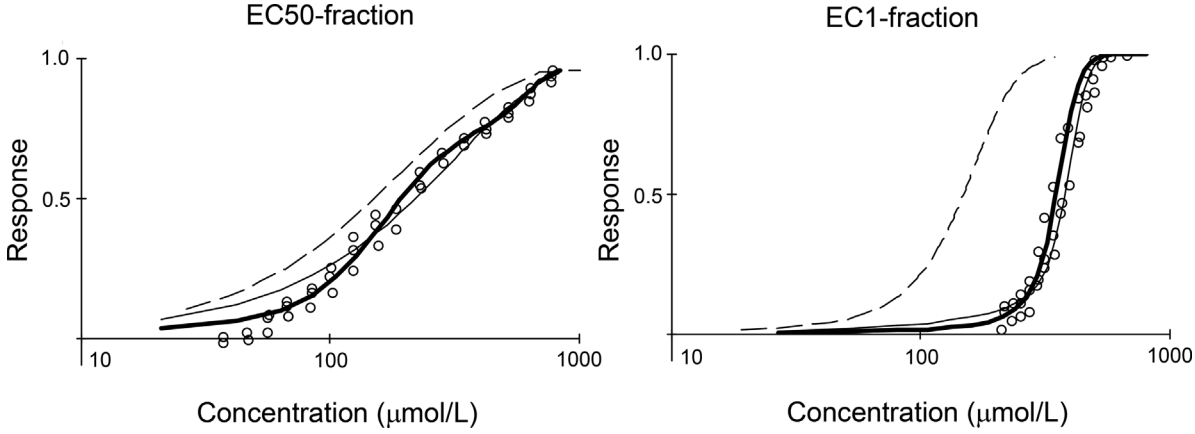

Figure 6. Concentration–response curves of the bioluminescence inhibition of *Vibrio fischeri* responding to a mixture of 14 herbicides. The open circles denote the observed responses (data from Backhaus et al. [9]). The bold solid lines represent the predicted mixture effects based on the GCA model. The plain solid line and the broken line represent the independent action model and the CA model, respectively.

CA model was indicated for both fractions, unlike the previous example, because values of β_M were much less than β_{CA} for all q regardless of the fractions (Supplemental Data, S4).

The standard response shape for RA, β_{RA}, was specified to be $\beta_{RA} = 2.5 \sim 3.0$ and was compatible with the observed value of β_M in the range of mixture concentrations $0.1 \leq q \leq 0.3$ for the EC50 fraction and $1 \leq q \leq 1.5$ for the EC1 fraction (Supplemental Data, S4).

Nonadditive interaction

For demonstrating the applicability of the present study's approach to nonadditive interaction, we conducted acute immobility tests (48 h) of copper, zinc, and their mixtures for *D. magna* (see Supplemental Data, S5 for descriptions of materials, methods, and the data). The concentration at which 50% of individuals were immobilized after 48 h (48-h EC50) were estimated as 11 μg/L and 889 μg/L for Cu and Zn, respectively. The experimental design for detecting mixture effects followed the isobole design, in which the fraction of copper and zinc (Cu/Zn) was set as 1/0, 0.8/0.2, 0.6/0.4, 0.4/0.6, 0.2/0.8, and 0/1, based on the relative scale to the acute EC50 of each substance (Supplemental Data, S5).

We used the log–logistic model as the response mother function. The least-square method gave the parameter values as $\theta_1 = 10.1$ (μg/L) and $\beta_1 = 9.50$ for the single exposure by Cu, and $\theta_2 = 630$ (μg/L) and $\beta_2 = 3.32$ for the single exposure by Zn, which indicated a more upward convexed response curve for Cu than Zn (Supplemental Data, S5).

Analyses of the mixture effects were separately practiced to estimate the response shape for component addition β_M for each exposure treatment, because the nonadditive interaction between Cu and Zn appeared to be dependent on fractions of components. As for the fifth treatment ([Cu/Zn] = 0.2/0.8), the GCA approach was not applicable because the observed mixture effect was less than the response predicted from the single effect of Zn.

The results indicated that strong synergistic interaction existed between Cu and Zn in all combinations, because β_M much exceeded β_{CA} (β_M and β_{CA} are, respectively, 13.7 and 5.68 for 0.8/0.2, 11.5 and 6.32 for 0.6/0.4, and 11.0 and 6.86 for 0.4/0.6). For an index, $I_M = \beta_M/\beta_{CA}$, which represents the degree of synergistic interaction among components compared with that under the additivity, we have $I_M = 2.41$, 1.83, and 1.60 for 0.8/0.2, 0.6/0.4, and 0.4/0.6, respectively. Thus, it was implied that Cu and Zn are more interactive with higher fractions of Cu.

According to the statistical method used in the *Similarly acting substances* section, the 95% confidence intervals [the lower limit–the upper limit] for β_{CA} were calculated as [3.70–7.24] for 0.8/0.2 (Cu/Zn), [4.32–7.97] for 0.6/0.4 (Cu/Zn), and [4.80–8.65] for 0.4/0.6 (Cu/Zn). All estimates of β_M were outside these intervals, and were larger than the upper limits, which indicated that Cu and Zn were more interactive to each other than within each substances.

DISCUSSION

There are 2 major approaches for treating chemical mixtures in a regulatory context: the single compounds (or the component-based) approach and the mixture of concern approach or the similar-mixture (the whole-mixture) approach [3,37,38]. The component-based approach assumes no interaction between components, whereas the whole-mixture approach allows interactions between components but needs additional data for the effects of particular mixtures or a particular range of mixtures. The GCA approach is an attempt to make 1 of the simplest and most parsimonious links between the 2 contrasting approaches in mixture toxicology.

Successful application of the component-based approach requires appropriate selection between the 2 major reference models based on the similarity of the mode of action among components. However, knowledge of the mode of action is lacking for many chemicals [3]. Uncertainties have also been pointed out regarding the connection between the similarity or dissimilarity of the mode of action and the appropriate reference model [14].

Chemical mixtures in the environment may include substances having either similar or dissimilar modes of action [39]. For analyzing such complex mixtures, an integrative approach, which combines the CA model and the IA model in a nested frame, has been suggested [33,40]. This method also requires identification of the appropriate reference model to define classes of components in a mixture.

The GCA approach presented in the present study does not require identification of the right reference model, because the approach is based exclusively on CA. At the same time, the GCA model can precisely reconstruct even a mixture effect that follows the IA model but does not follow the CA model. Also, the estimated model parameter (the response shape for component addition, β_M) in the GCA approach is expected to indicate which reference model has closer affinity to the observed mixture responses.

Strictly speaking, we have not found any theoretical basis that would permit the use of the CA model for substances with heterogeneous response shapes. Berenbaum [26] has stated "it is often assumed that zero-interactive combinations of agents with dissimilar dose-effect curves should also have linear isoboles, but no proof of this appears to have been suggested." Components with much steeper response slopes than other components appear to have response thresholds with concentrations under which other components still have adverse effects. Thus accumulation of concentrations lower than the apparent threshold across such components according to the prescribed response functions may give overestimates for mixture responses. This issue is addressed by the GCA approach, which does not assume homogeneity of response shapes among components.

There are very few general models in mixture toxicology that allow interactions between compounds. Hewlett and Plackett [34] proposed 1 of the most general mathematical methods, which predicted the effect of binary mixtures with arbitrary interactions based on the bivariate distribution of tolerances within populations of the target species. This approach is mathematically refined and self-consistent. However, the model demands correlation estimates between tolerances to different compounds, and these estimates are rarely available in real test systems.

Most other analytical approaches to the toxicity of mixtures of chemicals are also based on the 2 reference models: the CA and the IA models. One of the most general mathematical methods, the concentration–response surface model [15–18,41], involves formulation of biases of mixture responses from predictions by 1 of the reference models. This model allows straightforward prediction of mixture toxicity with arbitrary strengths of interactions among components.

What distinguishes the GCA approach from the concentration–response surface approach is the former's description of the consequence of component addition by introduction of a new response function, which is specialized for addition of concentrations across components and is characterized by a new

response shape, β_M, rather than direct utilization of 1 of the reference models. The GCA approach only summarizes in a statistical way the cumulative effect of all components by an additional function and parameter. It is possible, based on this model, to infer the pattern of interactions among components by comparing the estimated response shape β_M with the predicted value (β_{CA} or β_{RA}) that follows from 1 of the reference models as the null model for nonzero interaction.

Owing to the holistic nature of the GCA approach, the response shape β_M can be flexibly defined to take into account different ranges of fractions and concentrations of components as well as which components are included in the mixtures. For example, if mixtures of very similar but partly different components are regarded as mixtures from the same group, the β_M value may be defined for the entire group and can be extrapolated between similar mixtures within the same group. In contrast, mixtures consisting of the same components but with different fractions of the components may have different values of β_M, in which case β_M should be defined according to the fractions. It is even possible, as the example in the *Dissimilarly acting substances* section implied, to determine the functional relationship of β_M to concentrations or fractions if detailed response data for the mixture are available. Thus, the response shape for component addition can be used as a statistical measure to characterize component interactions and thereby define similar mixtures that have overlapping components and similar values of β_M within a limited range.

The extent to which the GCA approach requires response data for a mixture depends on the required statistical precision and how strictly the response shape for component addition is defined regarding fractions and concentrations of components. As an extreme case in which interactions between components are assumed to be independent of fractions and concentrations, 1 mixture response datum in addition to the concentration–response data for each component is sufficient for a generic estimate of β_M. This is the minimum requirement for data in addition to the data required for the component-based approach. In contrast, for mixtures whose effects are largely dependent on fractions and concentrations, rigorous factorial data are needed to endow the GCA model with good predictive ability.

Because of the heuristic assumption of the GCA approach that the cumulative effect of all components in a particular mixture is subject to a particular TIR regardless of the number of components, the performance of the GCA model is limited to a holistic or statistical description of the mixture effect. This property makes the GCA approach unable to directly include any definite mechanisms of interactions, but this property may allow the flexible nature to function as a data analytical tool. No theory has yet been presented to link binary interactions and ternary or higher order interactions in this framework. In the future such interactions may accommodate the present holistic approach to reductionistic understanding of component interactions in the mixture.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3503.

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Data Availability—Data are available on request from the corresponding author (y_tanaka@genv.sophia.ac.jp).

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