

Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework

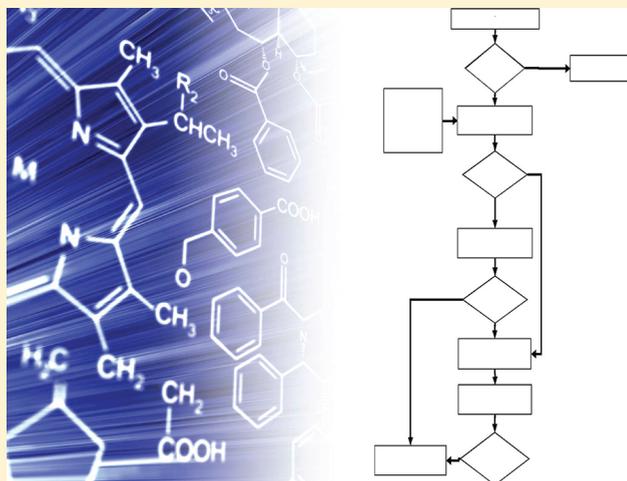
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S Supporting Information

ABSTRACT: Environmental risks of chemicals are still often assessed substance-by-substance, neglecting mixture effects. This may result in risk underestimations, as the typical exposure is toward multicomponent chemical “cocktails”. We use the two well established mixture toxicity concepts (Concentration Addition (CA) and Independent Action (IA)) for providing a tiered outline for environmental hazard and risk assessments of mixtures, focusing on general industrial chemicals and assuming that the “base set” of data (EC50s for algae, crustaceans, fish) is available. As mixture toxicities higher than predicted by CA are rare findings, we suggest applying CA as a precautionary first tier, irrespective of the modes/mechanisms of action of the mixture components. In particular, we prove that summing up PEC/PNEC ratios might serve as a justifiable CA-approximation, in order to estimate in a first tier assessment whether there is a potential risk for an exposed ecosystem if only base-set data are available. This makes optimum use of existing single substance assessments as more demanding mixture investigations are requested only if there are first indications of an environmental risk. Finally we suggest to call for mode-of-action driven analyses only if error estimations indicate the possibility for substantial differences between CA- and IA-based assessments.



INTRODUCTION

Numerous man-made chemicals are concurrently used in any given area and most of these substances and/or their degradation products are finally emitted into the environment. For example, one-third of 139 surveyed streams in the US contained 10 or more different chemicals from a broad range of chemical classes and use groups, such as synthetic hormones, other pharmaceuticals, industrial chemicals, pesticides, biocides and flame retardants.¹ Even if only pesticides are monitored, 20% of surveyed agricultural streams contained 10 or more compounds simultaneously.² Monitoring efforts in Sweden found similarly complex exposures, streams in the vicinity of agricultural activities contained between 10 and 22 pesticides simultaneously.³

Despite this common occurrence of chemical mixtures in the environment, even modern legislations such as REACH focus almost exclusively on the assessment of individual chemicals, which has recently been put forward as a major shortcoming that needs attention by the Council of the European Environmental Ministers.⁴

Chemical mixtures can be assessed by biotesting various samples from an exposed ecosystem (sludge, soil, sediment, water), but such approaches are obviously limited to retrospective

analyses of existing pollution situations. Modeling approaches that make use of the available ecotoxicological information of individual compounds are needed for prospective purposes, such as mixture impact prediction for an exposed ecosystem and the comparison of estimated impacts with environmental quality criteria. Such approaches also lower the need for biotesting, for example, for the classification and labeling of chemical products (CLP). The European CLP Regulation (EC) No 1272/2008 for example uses such approaches for classifying chemical products for their aquatic toxicity.⁵

Ecotoxicological Assessments in a Regulatory Context. Ecotoxicological hazard assessments for individual chemicals are routinely conducted on the basis of laboratory data from standardized tests using organisms from major trophic levels (primary producers, primary and secondary consumers). According to the European chemicals legislation REACH this information is condensed into a Predicted No Effect Concentration (PNEC) for the considered ecosystem. The PNEC is

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derived by selecting the most sensitive biotest (representing the most sensitive trophic level) and applying an appropriate assessment factor (AF), which accounts for intra- and interlaboratory variation of the data, biological variance, short-term to long-term extrapolation and laboratory to field extrapolation.⁶ It is assumed that by protecting the most sensitive trophic level all other organism groups are protected as well and that protecting the structure of an ecosystem also protects ecosystem functions.

The more limited the data set, the higher the inherent uncertainty in the assessment and consequently the higher the applied AF. The minimum set of data (the so-called “base set”) for the calculation of a PNEC consists of short-term toxicity data for algae, crustaceans and fish and is requested by REACH for substances produced or imported into the European Union in quantities of 10 tons per year or more.⁶ An AF of 1000 is then used for the PNEC calculation for the aquatic environment. The quotient of the predicted environmental concentration (PEC) and the PNEC has become a de facto standard for the ecotoxicological risk characterization not only for industrial compounds within the context of REACH, but also, for example, biocides and pharmaceuticals.

Strategies for the environmental risk assessment of chemical mixtures have been proposed especially by the group of Leo Posthuma and Dick de Zwart^{7–10} and have recently also been discussed in the context of the tissue residue approach in environmental risk assessment.¹¹ The more advanced approaches, based on species sensitivity distributions (SSDs) or detailed toxicokinetic and/or -dynamic modeling develop their full potential mainly in data-rich situations, where a substantial amount of ecotoxicological knowledge is at hand for each and every component in the mixture. However, REACH requests only three ecotoxicological data sets (base set) for most compounds, which is insufficient for the estimation of SSDs or more elaborate modeling approaches.

The aim of the present paper is therefore to introduce, rationalize and discuss a general tiered approach for the predictive environmental risk assessment of chemical mixtures in a regulatory context, assuming that only the base set of ecotoxicological data is available for each mixture component. The suggested approach is developed to make optimum use of the information that is generated during the current practice of chemical risk assessment and only calls for specific information if there are indications for environmental risks. It can hence serve as a first tier, before more elaborate mixture assessments are implemented.

■ THE FUNDAMENTAL MIXTURE TOXICITY CONCEPTS

Concentration Addition (CA) and Independent Action (IA) are the two classical concepts that allow calculating the expected mixture toxicity, based on the toxicities of the individual compounds and their concentrations in the mixture. Both concepts are hence applicable only for well-defined mixtures. The concepts have been introduced in the scientific literature under a range of different names, see compilation and references in ref 12 and are implemented in a diverse set of models for predicting or assessing mixture toxicities, see compilations, for example, in refs 13–17.

Concentration Addition. Concentration Addition (CA, also termed Dose Addition) was first formulated in a publication by the German pharmacologist Loewe in 1926.¹⁸ For a mixture of n components, the concept can be mathematically

expressed as

$$\sum_{i=1}^n \frac{c_i^*}{ECx_i} = 1 \quad (1)$$

where c_i^* gives the concentration (or dose) of the i th component in an n -compound mixture which elicits $x\%$ total effect and ECx_i denotes the concentration of that substance which provokes $x\%$ effect if applied singly. Every fraction c_i/ECx_i —also termed a “toxic unit”—gives the concentration of a compound in the mixture scaled for its relative potency. If the sum of the toxic units equals 1 at a mixture concentration that provokes $x\%$ effect, the mixture behaves according to CA. Under these circumstances any mixture component can be exchanged by another chemical without changing the overall mixture toxicity, as long as the size of the concerned toxic unit is constant. Such interchangeability is generally assumed to result from the compounds binding to the same receptor, that is, substances that have a similar mechanism of action, and that do neither interact on a physicochemical level nor in their toxicokinetics and toxicodynamics.

Independent Action. The alternative concept of Independent Action (IA, also called Response Addition) also assumes that all mixture components affect the same end point. But in contrast to CA, Independent Action assumes that the mixture components act on different subsystems (tissues, cells, molecular receptors) of an exposed organism and that impaired subsystems affect the end point under observation independently of each other.¹⁹ IA, as well as CA, assumes that there are no interactions between the components in a mixture, that is, that they do not influence each other’s uptake, distribution or metabolism. The expected mixture effect can hence be calculated according to the joint probability of statistically independent events as

$$E(c_{\text{mix}}) = E(c_1 + \dots + c_n) = \prod_{i=1}^n E(c_i) \quad (2a)$$

if the value of the response parameter (biological end point) decreases with increasing concentrations (e.g., when survival rates are recorded), or

$$E(c_{\text{mix}}) = E(c_1 + \dots + c_n) = 1 - \prod_{i=1}^n [1 - E(c_i)] \quad (2b)$$

when a response parameter (such as e.g. mortality) increases with increasing concentrations. $E(c_{\text{mix}})$ is the IA-expected overall effect (scaled to the range 0–1) of a mixture composed of n chemicals at a total concentration c_{mix} . $E(c_i)$ gives the effect of chemical i if applied singly in a concentration c_i which corresponds to the concentration of that component in the mixture.

Independent action of the individual compounds in a mixture is commonly interpreted as the compounds having dissimilar mechanisms of action, for example, refs 20,21.

Calculating the IA-expected mixture effect for the classic ecotoxicological end points that make up the base-set of data (e.g., mortality, growth, reproduction) relies on the knowledge of the individual effect, $E(c_i)$, that each compound would provoke if applied singly at the concentration at which it is present in the mixture (eqs 2a and 2b). This usually requires that the concentration–response curves of all individual toxicants are known. Moreover, the IA-predicted effect of a mixture,

$E(c_{\text{mix}})$, is always higher than the effect of each individual component in the mixture, $E(c_i)$. This implies that with an increasing number of compounds lower and lower effects have to be described for each component in order to predict a certain mixture effect. For example: if two compounds provoke an individual effect of 29.3% at certain concentrations, their combination causes 50% effect. However, if 10 or 50 compounds are present, already 6.7%, respectively 1.4% individual effect combine to a mixture effect of 50%. Hence, huge amounts of reliable ecotoxicological data that cover the region of low effects are needed for the application of IA to multi-component mixtures. Such data are usually not available and substantial experimental efforts are needed for generating them. Hence, the application of IA for the prediction of the joint toxicities of multicomponent mixtures has so far been largely limited to experimental mixture studies in which the necessary data were specifically recorded. It should be pointed out, however, that the mixture toxicity assessment strategy based on species sensitivity distributions (SSDs), as put forward by Posthuma and colleagues,^{7–10} allows to calculate the IA-expected species sensitivity distribution using standard EC50- and/or NOEC-values, assuming that data for a sufficient number of taxa is at hand for each mixture component.

EMPIRICAL EVIDENCE ON THE PERFORMANCE OF CA AND IA IN ECOTOXICOLOGICAL ASSESSMENTS

Neither CA nor IA makes any assumption on the biology of the exposed organisms. That is, the presence or absence of certain uptake or clearance routes and mechanisms (e.g., MXR pumps), catabolic enzymes (e.g., cytochrome P450) or ecological traits (reproductive behavior, generation times, etc.) is not taken into consideration. Nor does either concept consider specific chemical characteristics of the components ($\log K_{\text{OW}}$, $\text{p}K_{\text{A}}$, etc.). This simplicity allows establishing general guidelines for mixture toxicity assessment. But it also has to be assumed that such simple concepts do not fully describe biological reality, except perhaps in very simple systems. A multitude of studies has therefore been carried out in which CA- and/or IA-predicted mixture toxicities were compared to experimental observations, in order to characterize the predictive power of the concepts.

The available empirical evidence on the ecotoxicology of chemical mixtures has been compiled and reviewed for several environmental chemicals such as pesticides,^{22–24} heavy metals,²⁵ endocrine disrupters,²⁶ pharmaceuticals,²⁷ and for narcotic, industrial chemicals.^{28,29} The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) released a report on the aquatic toxicity of chemical mixtures in 2001.³⁰ The current evidence with respect to the performance of CA and IA in an ecotoxicological context can be briefly summarized as follows:

- 1 A majority of the published experimental studies investigated the applicability of CA, while only a comparably small fraction applied IA or even compared the predictive power of both concepts for the investigated mixture.
- 2 In those cases in which the performance of CA and IA has been comparatively assessed, CA usually predicts either a slightly higher mixture toxicity (see examples and discussion in ref 31) or CA and IA predict virtually identical mixture toxicities.^{32–34}
- 3 Most studies were conducted with selected species of freshwater organisms. Mixture studies in marine and terrestrial systems are still rare and so are studies on a higher, ecological, level of biological complexity.
- 4 Most studies investigated mixtures of 2–3 compounds,^{23,25,27,28,31} Experimental studies with multi-component mixtures are largely limited to specifically designed, so-called “reference mixtures”, that is, mixtures whose composition and concentration ratios do not reflect any real environmental situation, but which were specifically designed for the exploration of conceptual mixture toxicity questions. Examples include mixtures of only similarly acting chemicals (e.g., ref 12), of only dissimilarly acting chemicals (e.g., refs 20,21), of compounds that all belong to the same chemical class (e.g., ref 25), or use class (e.g., refs 12,27,33,35).
- 5 CA usually provides good to excellent predictions of the observed mixture toxicities. Belden and co-workers concluded in their review that for 88% of the considered pesticide mixtures the prediction by CA falls within a factor of 2 from the observation, independent of the similarity or dissimilarity of the mechanisms of action of the mixture components.²³ This agrees well with the observations by Cedergreen and her co-workers, who also observed substantial deviations from CA (more than a factor of 2 between predictions and observations) only for 6% of the investigated 158 data sets.³⁴ The information on metal mixtures that was evaluated by Norwood and his colleagues does not provide an equally clear picture, as most studies in the field do not provide enough information in order to assess the quantity of deviations.²⁵ The assessment of metal mixtures is furthermore complicated by the fact that many metals are essential elements and organisms hence have a well-developed active system for metal uptake, internal storage and sequestration. Furthermore, metal interactions may already occur at the level of bioavailability and uptake.
- 6 For those situations in which CA and IA predict different toxicities, the similarity or dissimilarity of the molecular mechanisms of action was a valid criterion for selecting the most appropriate concept. As environmentally realistic mixtures might usually be neither composed entirely of strictly similarly acting nor entirely of strictly dissimilarly acting substances, the issue of how to apply CA and IA for such mixtures has recently gained substantial attention in the scientific literature (see below).

In view of the rather consistent body of evidence on the performance of CA and IA, Altenburger and Greco concluded that “*Scientific concepts for risk assessment of [...] mixtures of chemicals are reasonably validated and ready to use*”.³⁶

It should be pointed out that advanced CA- and IA-based approaches have been put forward, based on species-sensitivity distributions (SSDs),^{7–10,37} using mechanistic modeling based on the DEB-theory³⁸ or employing tissue-residue approaches.¹¹ Despite their appealing features, such approaches are currently not applicable in many situations, especially when industrial chemicals are considered, simply because the necessary single substance toxicity data for the required range of different species and taxa are often not available (in the case of SSD approaches), the information on the relation between aqueous and internal body concentrations is not at hand (which is a

prerequisite for the tissue residue approach), or the available biological knowledge (toxicokinetics and -dynamics) is insufficient (in the case of mechanistic models and tissue-residue approaches).

ENVIRONMENTALLY REALISTIC MIXTURES

In order to account for the fact that environmentally realistic mixtures do neither obey to the simple case of a mixture composed entirely of similarly acting compounds nor are composed entirely of dissimilarly acting substances, it has been suggested to group the compounds of a mixture into subgroups of similar mechanism of action, predict the toxicity for each subgroup using CA and then predict the toxicity of the complete mixture by applying IA to the CA-predicted concentration–response curves of the subgroups.^{39–44} The power of this two-stage prediction has been demonstrated in a range of experimental studies, for example, refs 41–44,46.

However, there are at least three major hurdles when applying this approach in an environmental risk assessment that uses existing single substance data. First of all, a good deal of knowledge about the mechanisms of action of the components is needed for a sound classification into groups of similar action. Second, as the modes of action of a given set of compounds might depend strongly on the considered (groups of) species a separate clustering of the mixture components would be needed for each (group of) species. And finally, when applied to classical population-level end points, the two-stage methodology also has considerable demands in terms of input data, which is due to the inherent data demands of IA (see above).

CA and IA can be regarded as the two extreme cases of a noninteractive mixture toxicity, providing a so-called “prediction window”^{21,47} into which the toxicity of mixtures that are not entirely composed of similarly respectively dissimilarly acting substances can be expected to fall. Again, the data demands of IA set the limits for the determination of the actual width of the prediction window for a given mixture.

ELEMENTS FOR A TIERED APPROACH 1: INTEGRATING THE BASE SET OF TOXICITY DATA FOR ESTIMATING RISK QUOTIENTS FOR CHEMICAL MIXTURES

The calculation of an “ecosystem risk quotient” for mixtures (ratio between the expected exposure and the hazard of the mixture) which is based on a set of single substance ecotoxicity data from different trophic levels (groups of species) involves two extrapolation steps: (a) the extrapolation from the experimental toxicity data for the tested species after a certain exposure duration to the whole ecological community after infinite exposure, a step that is implemented for single substances by calculating the PNEC using assessment factors (see above); and (b) the extrapolation from single substances to chemical mixtures, which can be achieved by the application of CA and/or IA (Figure 1).

Already in 1992 an approach for the development of water quality standards using the base set of ecotoxicological data was suggested by Calamari and Vighi,⁴⁸ which was taken up again in subsequent publications.^{49,50} In the suggested methodology the PNECs of the individual compounds are calculated first. A second step then extrapolates from single substances to the mixture by summing up the PEC/PNEC ratios (i.e., risk quotients for the individual substances) in order to yield the

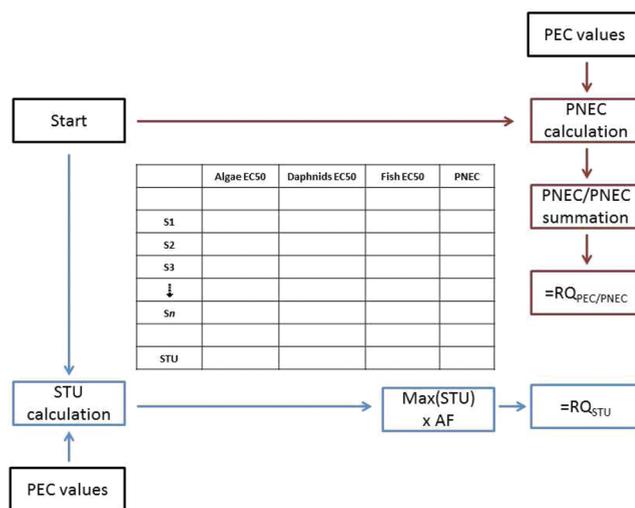


Figure 1. Two approaches for calculating mixture risk quotients (RQs) Blue: calculation of RQ_{STU} , based on the sum of toxic units for the most sensitive trophic level. Red: calculation of $RQ_{PEC/PNEC}$, based on the sum of PEC/PNEC values. It is assumed, that the base set is available for each substance. In this case, a constant Assessment Factor (AF) of 1000 is applied to the lowest EC50 to calculate the PNEC and after the calculation of the STU for each trophic level. The ratio between $RQ_{PEC/PNEC}$ and RQ_{STU} is then confined to the range of 1–3 (see text and Supporting Information for the mathematical proof). S1, S2, S3,...Sn: Compounds in the mixture; TU = toxic unit (PEC/EC50), STU = sum of toxic units, PEC = predicted environmental concentration, PNEC = predicted no effect concentration, RQ = risk quotient.

final risk quotient for the mixture, $RQ_{PEC/PNEC}$:

$$RQ_{PEC/PNEC} = \sum_{i=1}^n \frac{PEC_i}{PNEC_i} = \sum_{i=1}^n \frac{PEC_i}{\min(EC50_{algae}, EC50_{daphnids}, EC50_{fish})_i \times (1/AF)_i} \quad (3)$$

According to this approach the environmental quality standard is exceeded if $RQ_{PEC/PNEC}$ is above 1. However, although the summation of PEC/PNEC ratios might closely resemble the CA-equation (eq 1), it is conceptually different from CA because the involved PNECs might be based on different (groups of) species. This implies that the final risk quotient might result from summing up toxicity estimates for different species. This certainly violates a fundamental assumption of CA, that is, that all individual toxicity data refer to the same biological end point and organism. Therefore it has recently been argued that PEC/PNECs sums should not be used in mixture toxicity assessments.⁵¹ Additionally, the PNECs of the mixture components can be derived by using different individual assessment factors (AF_i), which would make the resulting sum hard to interpret directly.

In order to follow the conceptual foundation of CA the order of the two extrapolation steps needs to be reversed: First the sum of toxic units (STU) is calculated for each trophic level/organism group (extrapolation from the single chemicals to the mixture). Afterward, the STU can be treated in a second step as if it were an estimate for an individual chemical (Figure 1). Hence the organism group that is most sensitive to the mixture (i.e., for which the highest STU was calculated) is selected and by applying the corresponding assessment factor a risk quotient

for the mixture is then calculated (subsequently termed RQ_{STU}):

$$\begin{aligned}
 RQ_{STU} &= \max(STU_{\text{algae}}, STU_{\text{daphnid}}, STU_{\text{fish}}) \times AF \\
 &= \max\left(\sum_{i=1}^n \frac{PEC_i}{EC50_{i,\text{algae}}}, \sum_{i=1}^n \frac{PEC_i}{EC50_{i,\text{daphnids}}}, \sum_{i=1}^n \frac{PEC_i}{EC50_{i,\text{fish}}}\right) \times AF \tag{4}
 \end{aligned}$$

AF depicts a common assessment factor used for the extrapolation from the short-term laboratory studies to the chronic exposure under field situations. The AF equals 1000 for the limnic aquatic environment under REACH, if base-set data are at hand only. A comparison of the two approaches for the assessment of a simple two compound mixture of benzene and $K_2Cr_2O_7$ is given in Table 1. Benzene has a high toxicity toward fish, while being only moderately toxic to daphnids. In contrast, $K_2Cr_2O_7$ is predominately toxic to daphnids.

The calculation shows that the resulting $RQ_{PEC/PNEC}$ (0.43) is higher than the RQ_{STU} (0.24), which reflects the fundamental relationship between the two risk quotients. It can be proven (see Supporting Information), that the $RQ_{PEC/PNEC}$ will always be higher than the RQ_{STU} and that the ratio $RQ_{PEC/PNEC}$ to RQ_{STU} will always be equal or smaller than the number of considered organism groups (trophic levels). If the mixture assessment is using the base set of data the ratio of $RQ_{PEC/PNEC}$ to RQ_{STU} is hence equal or smaller than 3. The difference between the risk quotients increases if the components in the mixture have a species-specific mechanism of action that leads to large differences in the sensitivities of the different species, as in the provided example. If the toxicity profiles of the mixture compounds are similar, or if even all species have on average similar sensitivities to the mixture components both methods

yield similar risk estimates (see example of a 2-compound mixture of benzene and chlorbenzene in the Supporting Information, Table S1). The $RQ_{PEC/PNEC}$ is conceptually analogous to the hazard index of a mixture, which is applied for the human health assessment of chemical mixtures using so-called reference doses (RfD).¹⁷

Due to its more solid conceptual basis a mixture risk assessment should in principle be based on RQ_{STU} . However, its calculation does not only assume that the individual EC50 values are actually at hand, it also assumes a homogeneous data situation for all components. As can be seen from eq 4, only end points for which data are available for *all* compounds in the mixtures can be considered for calculating the corresponding (sums of) toxic units. If, for example, additional chronic toxicity data or SSDs are available for a few, but not all, mixture components, this information cannot be easily incorporated for the calculation of an RQ_{STU} . Currently, there is no agreed methodology at hand how an overall AF could be calculated in this situation.

However, such unbalanced data situations are inherently considered in the initial PNEC calculation by applying a specific AF to each individual component. The $RQ_{PEC/PNEC}$ can hence be calculated without problems for imbalanced data situations. This feature of the $RQ_{PEC/PNEC}$ has to be considered a major advantage in a regulatory context. It is hence hardly surprising, that the $RQ_{PEC/PNEC}$ is already applied in practice, for example, ref 52.

ELEMENTS FOR A TIERED APPROACH 2: ESTIMATING THE ERROR THAT RESULTS FROM THE SOLE CONSIDERATION OF CA

The ecotoxicological data for the single substances that can be compiled from literature references and databases (EC50 values, NOECs) typically do not allow to calculate the IA-expected mixture toxicity for a particular species, while CA

Table 1. Example of the Relationship Between RQ_{STU} and $RQ_{PEC/PNEC}$ Using a Simple 2-Compound Mixture of Benzene and $K_2Cr_2O_7$ ^a

	benzene	$K_2Cr_2O_7$
EC50 (algae)	3202 $\mu\text{mol/L}$	24.44 $\mu\text{mol/L}$
EC50 (daphnids, acute)	4117 $\mu\text{mol/L}$	5.22 $\mu\text{mol/L}$
EC50 (fish, acute)	0.85 $\mu\text{mol/L}$	14 560 $\mu\text{mol/L}$
resulting PNEC (AF=1000)	0.85 nmol/L	5.22 nmol/L
PEC	0.2 nmol/L	1 nmol/L
$RQ_{PEC/PNEC}$ (based on sum of PEC/PNECs)	$RQ_{PEC/PNEC} = \frac{PEC_{\text{Benzene}}}{PNEC_{\text{Benzene}}} + \frac{PEC_{K_2Cr_2O_7}}{PNEC_{K_2Cr_2O_7}} = 0.43$	
RQ_{STU} (based on sum of Toxic Units)		
algae	$STU_{\text{algae}} = \frac{0.2 \text{ nmol/L}}{3202 \mu\text{mol/L}} + \frac{1 \text{ nmol/L}}{24.44 \mu\text{mol/L}} = 0.04 \times 10^{-3}$	
daphnids	$STU_{\text{daphnids}} = \frac{0.2 \text{ nmol/L}}{4117 \mu\text{mol/L}} + \frac{1 \text{ nmol/L}}{5.22 \mu\text{mol/L}} = 0.19 \times 10^{-3}$	
fish	$STU_{\text{fish}} = \frac{0.2 \text{ nmol/L}}{0.85 \mu\text{mol/L}} + \frac{1 \text{ nmol/L}}{14560 \mu\text{mol/L}} = 0.24 \times 10^{-3}$	
final RQ_{STU}	$RQ_{STU} = \max(STU_{\text{algae}}, STU_{\text{crustaceans}}, STU_{\text{fish}}) \times AF = 0.24$	

^aToxicity data were collected from ECETOC's EAT-5 database.⁶⁶ Exposure data are arbitrary figures. RQ = risk quotient, STU = sum of toxic units, PNEC = predicted no effect concentration, PEC = predicted environmental concentration (arbitrary values), TU = PEC/EC50, AF = Assessment Factor (1000, according to ref 6).

can be applied (see above). CA as well as IA are fundamentally wrong for the majority of environmentally realistic mixtures, which are usually not composed entirely of only strictly similarly or of only strictly dissimilarly acting substances. The linchpin for selecting between CA and IA (or a two-stage combination of both) is the mechanism of action of the mixture components, which is often unknown and varies between the different considered groups of species (trophic levels) in an ecosystem.

These data gaps are a severe impediment for the application of CA and IA. However, filling in those data gaps might require the investment of substantial time and resources, which is not warranted if already simple first-tier estimations can prove that such an investment would not change the regulatory outcome. The classical tiered approach usually starts with an initial, rough risk estimate and the assessment whether a given exposure situation provides potential reason for concern using cautious, conservative assumptions. The additional investment of resources is then justified if such an initial tier indicates potential reason for concern.

As CA provides the more cautious risk estimate and is applicable using standard ecotoxicity data, we suggest to apply this concept in a precautious first step to any type of mixture, ignoring the similarity or dissimilarity of the mechanisms of action of the involved compounds. This step should be complemented with an estimation of the maximum possible error that may result from such a simplification. An appropriate approach has been put forward by Junghans and co-workers for mixtures of pesticides,⁴⁵ but can be applied to chemical mixtures in general. The maximum possible ratio between the mixture EC50s predicted by both concepts is given by

$$\frac{EC50^{IA}}{EC50^{CA}} \leq \frac{\sum_{i=1}^n \frac{c_i}{EC50_i}}{\max_{i \in (1..n)} \left(\frac{c_i}{EC50_i} \right)} \quad (5)$$

That is, the ratio between the IA- and CA-predicted EC50s is equal or smaller than the sum of all toxic units ($c_i/EC50_i$) divided by the highest individual toxic unit of the components that make up the mixture. Accordingly, the maximum possible ratio between the predicted EC50s occurs in a mixture in which all components contribute with an equal toxic unit ($c_1/EC50_1 = c_2/EC50_2 = \dots = c_n/EC50_n$, a so-called “equitoxic” mixture). Under these circumstances a maximum possible ratio of n (=number of mixture components) results, see also ref 53. For 2 compound mixtures eq 5 gives a theoretical maximum ratio of 2 between $EC50^{IA}$ and $EC50^{CA}$, which explains why for such mixtures both concepts often predict virtually identical mixture toxicities.^{32,34} If a mixture is dominated by certain compounds in terms of toxic units, the maximum possible ratio between $EC50^{IA}$ and $EC50^{CA}$ decreases accordingly.

Environmentally realistic mixtures might easily contain tens or even hundreds of compounds. Under these conditions, and depending on the specific toxic unit distribution and on the (dis)similarity of the components modes/mechanisms of action, CA might lead to a substantial overestimation of the actual mixture risk. Equation 5 allows to estimate this maximum error that might result from ignoring IA for a particular mixture and the ratio $EC50^{IA}/EC50^{CA}$ can hence serve as guidance on whether evaluations that consider the data-demanding concept of IA are worth the effort. It should finally be pointed out that

an error estimation using eq 5 is easily applicable and does not require additional information beyond those that are needed for calculating CA in the first place.

■ PROPOSAL FOR A TIERED APPROACH FOR THE PREDICTIVE ECOTOXICOLOGICAL RISK ASSESSMENT OF CHEMICAL MIXTURES

Taking Concentration Addition and Independent Action as two fundamental cornerstones of predictive mixture ecotoxicology and acknowledging their fundamental relationship (eq 5) as well as the need to extrapolate from laboratory conditions to real life and from single substance data to chemical mixtures we suggest a two-tiered approach for predictive environmental risk assessment of mixtures (Figure 2).

The proposed approach hinges on the initial scenario definition. This step follows the “problem formulation phase” of the EPA-guidelines for ecological risk assessment⁵⁴ in principle, amended by mixture specific issues. In particular a list of the compounds that are to be included in the assessment needs to be compiled in this initial step. The aim is to be as complete as possible while at the same time keeping the scope of the analysis manageable. The substance compilation can be based on fate and exposure modeling, on monitoring data from the environmental compartments of concern, on biomonitoring data, on the identification of relevant emission sources in a local or regional scenario, and/or on observations of biological effects that can be traced back to particular (groups of) compounds. Only compounds that are either proven or expected to co-occur in sufficiently close proximity in space and time (so that their ecotoxicological effects can overlap) need to be subjected to a mixture risk assessment.

Whether or not compounds that are present at concentrations below their individual quality criteria need to be included in the assessment depends on the final scenario in terms of total number of components, their concentrations, potency and mechanisms of action. A compliance of compounds with their individual environmental quality criteria might provide guidance in the later stages of the assessment, but does not allow to exclude the corresponding compounds in the initial stages of the assessment. All substances that are present above the analytical limit of quantification or that are expected to be present due to an exposure modeling need to be initially included, in order to describe their potential contribution to the expected mixture toxicity (i.e., their toxic unit). However, they may be ignored in subsequent higher tier assessments or risk management steps, if their toxic units are considered sufficiently small in comparison to the overall sum of toxic units. Compounds that might potentially be present in a given environmental compartment but for which available monitoring data give negative findings may initially be assumed to be present at their analytical limit of detection. This would provide a worst-case scenario, which allows to analyze whether it could be principally possible that those compounds contribute substantially to the mixture toxicity.

The mere presence of a compound is obviously insufficient as a sole decision criterion on whether a compound needs consideration during a mixture toxicity assessment. This is a major limitation for the practical applicability of the so-called “top n ” approach, in which it has been suggested to focus the investigation on the n most important compounds.⁵⁵ However, ecotoxicological ranking of compounds in a scenario and the determination of the “top n ” that are expected to dominate the overall mixture effect can only be achieved if the toxic units of

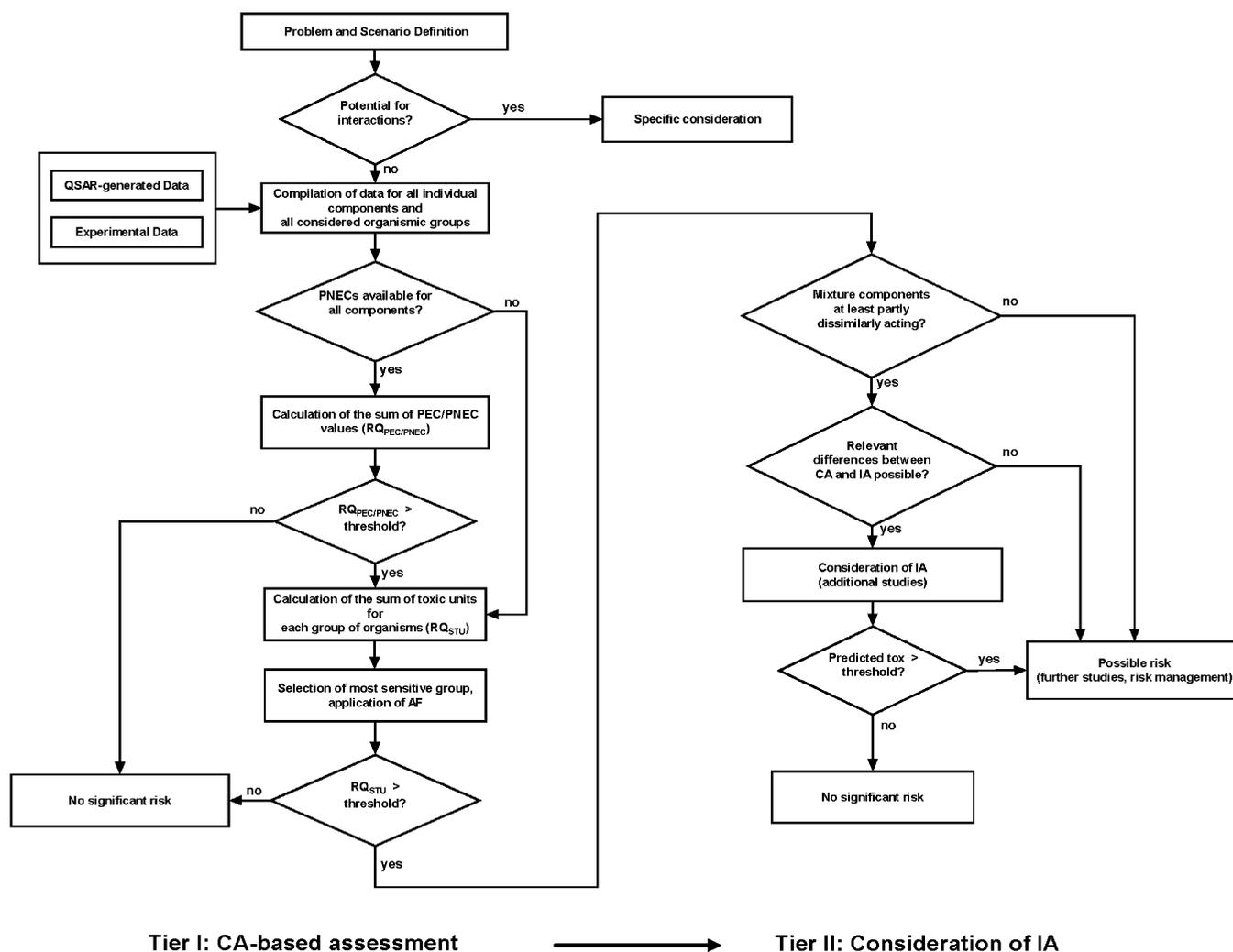


Figure 2. Outline for the predictive ecotoxicological risk assessment of chemical mixtures.

all compounds are known in the first place. Thus, the information that is needed for focusing on only a subset of all the compounds present is the outcome of a first tier assessment but not its starting point. The “top n ” approach might hence be most useful for subsequent steps of the assessment process, especially if appropriate risk management or mitigation measures need to be identified.

Besides the question on which compounds are to be included in the assessment, the definition of the assessment end point warrants particular attention. The application of CA as well as IA builds on the notion that only those compounds that are also toxic alone (although perhaps only at higher concentrations) contribute to the toxicity of a mixture. Inert compounds that do not affect the species, population or system of concern individually can also be ignored for the mixture toxicity assessment, unless there are indications that they confound the toxicity of other mixture components, see example in ref 56.

The first tier of the suggested approach uses exclusively CA as a basis for the preliminary risk assessment of the mixture(s) of concern. The necessary input data for the mixture toxicity calculation can be derived either from experimental data, or from QSAR estimates (quantitative structure activity relationships). This tier itself contains two consecutive steps, based on $RQ_{PEC/PNEC}$ and RQ_{STU} . The former provides the more conservative approach, is often easier to apply (assuming

PNECs are at hand for the mixture components), but might violate the assumption of a common biological end point (see discussion above). Hence, if $RQ_{PEC/PNEC}$ is above 1, that is, indicates the potential for reason for concern, RQ_{STU} can be calculated in a next step. Only if there are still indications for a potential risk (i.e., when $RQ_{STU} >$ threshold) tier II commences, which takes IA into consideration (either alone, or in the form of the aforementioned two-stage prediction). This tier will, due to the data demands of IA, often require additional experimental studies.

Such a sequential application of CA and IA in two tiers relieves the need for an immediate classification of the mixture components into groups of similar and dissimilar modes and mechanisms of action already at the very beginning of the assessment, which is often central to published approaches⁵⁷ and guidance documents on the human health risk assessment of chemical mixtures.^{10,17,58,59} Especially in an ecotoxicological setting, with its multitude of potentially affected species with often fundamentally different but largely unknown physiology and biochemical pathways, the demand to group compounds into compounds with similar modes and mechanisms of action might otherwise be a major hurdle for the assessment. This initial application of CA under conditions of uncertainty in order to provide a preliminary, cautious assessment is in concordance with recently published strategies for environ-

mental^{7–10,60} and human health risk assessment of chemical mixtures.^{61,62}

In case the first tier exceeds the critical value of 1 for the mixture risk quotient, exposure and/or hazard estimates of the mixture can be refined. Depending on the rational for the initial exposure estimates, these can be refined by additional or improved analytical surveys and/or advanced exposure modeling (e.g., using probabilistic approaches). The critical decision on whether to proceed to a tier 2 hazard assessment is driven by the possible ratio of the CA- and IA-expected mixture toxicities, calculated by eq 5. A tier 2 assessment only makes sense when the number of compounds present in the scenario and their specific toxic unit distribution indicate that the sole application of CA might lead to a substantial risk overestimation. The question on what a “substantial overestimation” might constitute can only be discussed in the specific context of the actual assessment, especially in view of the uncertainties of the toxicity and exposure estimates.

The strategy that is proposed in Figure 2 provides details on how the comparatively data-poor situations (only three acute data for each mixture component) that will result from the current phase-in of chemicals into the REACH system can be handled in a manner that is consistent to the current practice of single-substance assessments. It is completely compatible to the broader mixture assessment outlines that have been put forward earlier.^{8,10} However, we now provide clear decision criteria for moving to a higher tier, and what might (or might not) be gained by investing additional time and effort. This will facilitate the incorporation of sound mixture assessments into regulatory practice.

As discussed earlier, both, CA as well as IA assume a noninteraction between the mixture components. Such interactions can lead to either antagonistic (less toxic than expected) or synergistic (more toxic than expected) toxicities. Although such situations seem to be rare, they do occur, especially in binary mixtures. For example, Laetz and his co-workers recently demonstrated that certain binary pesticide mixtures had a clearly more than additive toxicity toward salmon.⁶³ Synergistic mixture effects have also been demonstrated for a mixture of the fungicide prochloraz and the insecticide esfenvalerate.⁶⁴ Such cases of more than additive mixture toxicity are specific for a certain mixture (compound types, their concentrations and mixture ratios), a particular biological system and end point. Hence, such exceptional cases cannot be properly handled in a general risk assessment scheme, but where the available knowledge allows their identification, they must be treated on a case-by-case basis. To this end, any regulatory strategy must include a corresponding element of flexibility.

Comparable to the ecotoxicological hazard and risk assessment of individual chemicals as outlined in the guidance documents of the REACH regulation,⁶⁵ the outlined scheme does not consider local conditions and confounding factors, but instead provides an approach for a general “screening level” risk assessment of chemical mixtures than can be flexibly adapted and extended as needed.

■ ASSOCIATED CONTENT

Supporting Information

The mathematical proof of the quantitative relation between $RQ_{PEC/PNEC}$ and RQ_{STU} is provided together with a second example on their application to a 2-compound mixture

(benzene and chlorbenzene) in Table S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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