Exploring the Concepts of Concentration Addition and Independent Action Using a Linear Low-Effect Mixture Model

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Abstract: Chemicals emitted into the environment are typically present at low concentrations but may act together in mixtures. Concentration–response curves of in vitro bioassays were often linear for effect levels <30%, and the predictions for concentration addition (CA) of similarly acting chemicals and for independent action (IA) of dissimilarly acting chemicals overlapped. We derived a joint CA/IA mixture model for the low-effect level portion of concentration–response curves. In a first case study, we evaluated the cytotoxicity of over 200 mixtures of up to 17 components that were mixed in concentration ratios as they occurred in river water. The predictions of the full IA model were indistinguishable from the predictions of the full CA model up to 10% effect, confirming the applicability of the joint CA/IA mixture model at low effect levels. In a second case study, we evaluated if environmental concentrations trigger effects at levels low enough for the joint CA/IA mixture model to apply. The detected concentrations were scaled by their toxic potencies to estimate the mixture effect of the detected chemicals in a complex mixture. In 86% of 156 samples the effects fell in the validity range of the joint CA/IA mixture model (<10% effect level), confirming the CA assumption for toxic unit summation. The joint CA/IA mixture model is not suitable for testing specific mixture hypotheses and interactions of chemicals in mixtures, where more refined models are required; but it is helpful for the interpretation of effects of complex (multicomponent) environmental mixtures, especially for water samples with relatively low effect level. Environ Toxicol Chem 2020;39:2552–2559. © 2020 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Mixture; Water; Toxic unit; Concentration addition; Independent action; In vitro bioassay

INTRODUCTION

In the environment, thousands of chemicals act together in complex mixtures, and there are urgent calls to include mixtures in the risk assessments of the future (Kortenkamp and Faust 2018; Kortenkamp et al. 2019; Drakvik et al. 2020). The 2 main mixture toxicity concepts are concentration addition (CA) and independent action (IA). The former applies for chemicals with the same mode of action (Altenburger et al. 2000) and the latter if chemicals act strictly according to a different mode of action at different target sites (Backhaus et al. 2000). Over the last 20 yr, the concepts of CA and IA have been applied for many different mixtures and biological organisms and tested repeatedly with defined mixtures (Kortenkamp 2007; Kortenkamp et al. 2009; Altenburger et al. 2018). Often (but not always), IA leads to a lower expected mixture effect than CA (Drescher and Boedeker 1995). If chemicals interact in mixtures, this may lead to synergism and antagonism. Such interactions may become important in mixtures with a small number of components and at high concentrations, but interactions are rare and hardly perceptible in complex environmental mixtures with thousands of components, where the majority of components occur at very low concentrations (Cedergreen 2014).

There are numerous studies that have explored effects at low doses (Boobis et al. 2011; Orton et al. 2014) or mixtures of chemicals mixed with constant concentration ratios derived from low effect levels, for example, their no-observed-effect concentration (NOEC) or 1% effect concentration (EC01; Rajapakse et al. 2002; Silva et al. 2002; Walter et al. 2002). In these studies, the resulting experimental mixtures were then typically tested at those fixed concentration ratios up to high effect levels, often up to 100%. There is a lack of experimental studies performed at low effect levels that demonstrate how mixtures behave at true environmental levels. Rider et al. (2018) provided a comprehensive review of additivity models and...
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stated that, at low effect levels, IA is effectively a summation of effects; but they only illustrated this statement with a conceptual consideration, not with experimental data. Kortenkamp (2014) stated that “Dose–response modelling in the range of small effects presents its own challenges—a good regression for large effects does not necessarily mean that the model produces good descriptions in the range of low doses.”

Animal testing requires rather high effect levels for visible effects in acute and chronic studies. However, with the advent of cell-based bioassays it has become possible to work reliably at low effect levels. Variability of controls can be reduced to a coefficient of variance of 2 to 10%, making it therefore possible to work at low effect levels and deduced effect concentrations for 10% of effect, EC10. These ECs are higher than concentrations occurring in water, but they come closer (Neale et al. 2020). We can confidently assume that a concentration–response model that explains an effect at approximately the 10% effect level can be used to extrapolate to lower effects, certainly better than a model that focuses on giving a best estimate of EC50.

At low effect levels, typically up to 30% of effect, many concentration–response curves (CRCs) are linear (Escher 2018a). Therefore, we revisited the models of CA and IA at low effect levels in the linear range of the CRC for testing the hypothesis that these 2 mixture models converge to one common model at low effect levels, which we term the “joint CA/IA mixture model.”

We also investigated the significance of these considerations for mixtures of organic chemicals in water. Few studies have addressed the effects of realistic environmental mixtures, combining chemicals in concentration ratios as they occur in water (Junghans et al. 2006; Neale et al. 2020) or sediment (Altenburger et al. 2004). Component-based methods have been recommended to estimate the effect of complex mixtures of chemicals in surface water (Posthuma et al. 2019). The mixture risk of chemicals in water is often estimated by summation of toxic units that are obtained from the measured concentration in water samples and effect concentrations for aquatic organisms from the literature (Sprague 1970). The summation of toxic units implies that mixture components act in a concentration-additive way. The assumption cannot be tested in such simulations, and it is actually a very unlikely assumption because the cocktail of thousands of chemicals in water samples is very likely to also trigger hundreds of different modes of action.

In a first case study, we compared the predictions of CA and IA in over 200 designed mixtures of 2 to 17 chemicals that were mixed in concentration ratios as they had been detected in water samples collected from small rivers during rain events and tested for activation of reporter gene activity and cytotoxicity (Neale et al. 2020). Because reporter gene activation is strictly CA, we used the cytotoxicity for this illustration. In a second case study, we reevaluated the toxic unit of water samples (Kandie et al. 2020) and sediments in a German river (Massei et al. 2018) and tested the applicability of the joint CA/IA mixture model.

THEORY

The basic equation for CA (Equation 1; Altenburger et al. 2000) allows prediction of the effect concentration ECy (mixture) of the mixture at any effect level y for a mixture composed of n components i, present in fractions pi, with pi = Ci/Ctot and ∑pi = 1; Ci is the concentration of the component i and Ctot is the total concentration (Ctot = ∑i=1n Ci).

\[ EC_y (\text{mixture}) = \frac{1}{\sum_{i=1}^{n} p_i} \]  

(1)

Using Equation 2, IA is calculated from the effects (Backhaus et al. 2000).

\[ \text{Effect } y (\text{mixture}) = 1 - \prod_{i=1}^{n} (1 - \text{effect}(C_i)) \]  

(2)

These 2 equations are independent of the model of the CRC, and we will refer to Equations 1 and 2 in the following as “full” CA and IA models.

For effects up to 30% of maximum effect (y < 0.3 expressed in fraction of 1), CRCs from in vitro bioassays are often linear (Escher et al. 2018a) and of the form of Equation 3.

\[ \text{Effect } y = \text{slope } \times C_i \]  

(3)

The effect concentration for y% effect (ECy) and its standard error can then be calculated with Equations 4 and 5 (Escher et al. 2018a).

\[ EC_y = \frac{y}{\text{slope}} \]  

(4)

\[ \text{SE}(EC_y) \approx \frac{y}{\text{slope}^2} \times \text{SE}(\text{slope}) \]  

(5)

The model for CA (Equation 1) can then be simplified for linear CRCs as follows:

\[ EC_y (\text{mixture}) = \frac{1}{\sum_{i=1}^{n} p_i \times \text{slope}_i} = y \times \frac{1}{\sum_{i=1}^{n} p_i \times \text{slope}_i} \]  

(6)

This means the CRC of the CA mixture has the slope mixture given by Equation 7, and Equation 3 applied to mixtures becomes Equation 8.

\[ \text{Slope}_\text{mixture} = \sum_{i=1}^{n} p_i \times \text{slope}_i \]  

(7)

\[ \text{Effect } y (\text{mixture}) = \sum_{i=1}^{n} p_i \times \text{slope}_i \times C_{\text{tot}} = \left( \sum_{i=1}^{n} p_i \times \text{slope}_i \right) C_{\text{tot}} = \text{slope}_\text{mixture} \times C_{\text{tot}} \]  

(8)

The standard error \( \text{SE}_{\text{slope mixture}} \) of \( \text{slope}_\text{mixture} \) was calculated by propagation of error through partial derivatives of...
Equation 9 and the standard errors of slopes $SE_{\text{slope}_i}$ (Equation 9).

$$SE_{\text{slope mixture}} = \sqrt{\sum_{i=1}^{n} p_i^2 \times SE_{\text{slope}_i}^2}$$  \hspace{1cm} (9)

Starting from IA (Equation 2) and by inserting Equation 4, we obtain the full IA model (Equation 10):

$$\text{Effect y(mixture)} = 1 - \prod_{i=1}^{n} (1 - \text{slope}_i \times p_i \times C_{\text{tot}})$$

$$= 1 - \prod_{i=1}^{n} (1 - \text{slope}_i \times p_i \times C_{\text{tot}})$$  \hspace{1cm} (10)

At low effect levels when CRCs are linear, the equation for IA can be simplified to Equation 11, which is equivalent to Equation 8 that was derived for CA. This assumption is only valid under certain circumstances, as will be discussed and illustrated in the Results and Discussion section.

$$\text{Effect y(mixture)} \approx 1 - \left[ 1 - \sum_{i=1}^{n} p_i \times \text{slope}_i \times C_{\text{tot}} \right]$$

$$= \left( \sum_{i=1}^{n} p_i \times \text{slope}_i \right) \times C_{\text{tot}} = \text{slope mixture} \times C_{\text{tot}}$$  \hspace{1cm} (11)

The approximation in Equation 11 can be derived from the development of Equation 10 as follows:

$$\text{Effect y(mixture)} = 1 - \prod_{i=1}^{n} (1 - \text{slope}_i \times p_i \times C_{\text{tot}})$$

$$= 1 - \left[ 1 - \sum_{i=1}^{n} p_i \times \text{slope}_i \times C_{\text{tot}} \right]$$

$$= \left( \sum_{i=1}^{n} p_i \times \text{slope}_i \right) \times C_{\text{tot}} = \text{slope mixture} \times C_{\text{tot}}$$  \hspace{1cm} (12)

Transformation by stepwise multiplication of all factors delivers Equation 13, which, in the end, consists of $n$ terms with increasing power numbers from 1, 2, 3, and so on up to $n$, which is why the first 3 terms are given “only” to illustrate the pattern of the general development of the equation.

Effect y(mixtures) = 1 - \[ (1 - \text{slope}_1 \times p_1 \times C_{\text{tot}} - \text{slope}_2 \times p_2 \times C_{\text{tot}} + \text{slope}_3 \times p_3 \times C_{\text{tot}} + \cdots ) \times (1 - \text{slope}_3 \times p_3 \times C_{\text{tot}} + \cdots ) \times (1 - \text{slope}_n \times p_n \times C_{\text{tot}}) \]

$$= 1 - \left[ 1 - \sum_{i=1}^{n} p_i \times \text{slope}_i \times C_{\text{tot}} \right]$$

$$= 1 - \sum_{i=1}^{n} p_i \times \text{slope}_i \times C_{\text{tot}} + \sum_{i=1}^{n} p_i \times \text{slope}_i \times p_i \times C_{\text{tot}}$$

$$+ \sum_{i=1}^{n} p_i \times \text{slope}_i \times p_i \times C_{\text{tot}} - \cdots$$  \hspace{1cm} (13)

### MATERIALS AND METHODS

All simulations for the conceptual part and the case studies were prepared with Microsoft Excel and all figures with GraphPad Prism.

For the first case study on cytotoxicity in “simulated environmental mixtures” we used the experimental data of 227 mixtures of 2 to 17 chemicals mixed in realistic environmental concentrations that were recently presented in a study on water quality in small agriculture-impacted streams during rain events (Neale et al. 2020). Mixture risk drivers for the specific modes of action detected with reporter gene assays were identified by a combination of chemical analyses and bioassays of the water samples (Neale et al. 2020). The chemicals identified as toxicologically most important (frequent occurrence or high potency) in the water samples were mixed in the concentration ratios at which they were detected and tested for mixture effects in the 3 reporter gene assays for activation of the arylhydrocarbon receptor (AhR CALUX), oxidative stress response (AREc32), and activation of the peroxisome proliferator activated receptor (PPARy GeneBLAzer) (Neale et al. 2020). For the purpose of the present study, we used the cytotoxicity data of these cell lines because only specifically acting chemicals will activate the reporter genes (and hence reporter gene activation is CA per definition [Escher et al. 2013]), but all chemicals in a sample will contribute to cytotoxicity. The inhibitory concentrations causing 10% cytotoxicity (IC10) were not directly reported, but instead cytotoxicity was reported in the form of toxic units, $TU_{\text{bio}} = 1/IC10$, in Tables S7 to S9 of the supplementary information of Neale et al. (2020). For the present study, the $TU_{\text{bio}}$ values were converted back into $IC10 = 1/TU_{\text{bio}}$. The CA predictions were made with Equation 1, and the IA predictions were made with Equation 2. The IC10 had been derived for 106 of 107 designed mixtures in AhR CALUX, 38 of 43 mixtures in AREc32, and 17 of the 29 mixtures in PPARy GeneBLAzer.
76 mixtures in PPARy GeneBLAzer (Neale et al. 2020). Because the focus of the present study was the activation of reporter genes, concentrations were not always high enough to derive IC10 values. The number of 161 mixture IC10 values is still high enough to illustrate the similarity between CA and IA.

The second case study evaluated the \( T\text{U}_{\text{chem}} \) concept using analytical water data from Kandie et al. (2020) and sediment data from Massei et al. (2018). We first converted the reported EC50 data toward algae, and 50% lethal concentration (LC50) data toward Daphnia and fish for the individual chemicals (see supplementary Table S10 of Massei et al. 2018) to EC10 and LC10 assuming a log-sigmoidal CRC with slope 1 (Escher et al. 2018b). In Massei et al. (2018) the reported concentrations of chemicals in sediment (their supplementary Table S11) were normalized to organic carbon and converted to water concentrations with the partition constant between organic carbon and water. From the EC/LC10 and the resulting water concentration (\( C_i \)) we recalculated the \( T\text{U}_{\text{chem}} \) on the 10% effect level with Equation 14.

\[
T\text{U}_{\text{chem}} = \sum_{i=1}^{n} \frac{C_i}{\text{LC10}_i} \tag{14}
\]

In the study from Kandie et al. (2020), only toxic units were reported; and we assumed that the \( T\text{U}_{\text{chem}} \) values based on LC10 were 9 times higher than the \( T\text{U}_{\text{chem}} \) values based on LC50 data because the conversion factor between LC50 and LC10 was 9, provided that the slope of the log-logistic curve was 1.

In a linear CRC, the slope is the ratio of \( y_i / C_i \) (Equation 3), which holds for any effect level up to 0.3 (30%), such as for 10%/LC10. Hence, the effect contribution of each mixture component \( i \) can be deduced by Equation 15 for a linear CRC (Escher et al. 2018a).

\[
\text{Effect } y_i = \frac{10\%}{\text{LC10}_i} \times C_i \tag{15}
\]

Then, the mixture models were applied in the same way as for the first case study.

**RESULTS AND DISCUSSION**

**Concentration addition**

Predictions of CRCs for CA were only possible up to now if the equations of the sigmoidal CRC were known. There was no simple function to predict the CRCs of the mixtures, but a CA prediction had to be made for every effect level of interest, which allowed the derivation of a CRC of the mixture. Another problem arising from this approach was that the maximum effect of the mixture could not exceed the maximum effect of the least potent mixture component unless very complex models were applied (Scholze et al. 2014).

By making use of the low-level linear CRCs, we circumvented the problem of maximum effect. The mixture prediction for CA can be solved analytically and yields a CRC function of the form of Equation 8, which greatly simplifies mixture modeling. The remaining limitation is that all CRCs of all components and the mixture must be in the linear range (\( y_i < 0.3 \)). For environmentally realistic mixtures and water samples the assumption of linearity held in practice, as numerous previous studies have shown (Escher et al. 2014b; Hebert et al. 2018; Neale et al. 2020).

**Independent action**

The simplification of Equation 10 to Equation 11 requires some justification. Basically, the quadratic term \( \sum_{i=1}^{n} \sum_{j=1}^{n} \text{slope }_i \times p_i \times \text{slope }_j \times p_j \times C_{\text{tot}} \) and any higher term need to be \(<1\); then, all nonlinear terms would approach 0, and Equation 10 would simplify to Equation 11. In addition, \( \Sigma p_i = 1 \), which means that the factors composed of the compound fractions (\( p_i \times p_j \times p_k \times p_i \times \text{etc.} \)) are becoming smaller with an increasing number of components because all \( p_i < 1 \) and at least one \( p_i \leq 0.5 \). For example, in binary equipotent mixtures the quadratic term \( p_i \times p_j \) remains, leading to a maximum of \( 0.5 \times 0.5 = 0.25 \). We also must remember that \( y_i = \text{slope }_i \times p_i \times C_{\text{tot}} < 0.3 \) is the initial condition for linearity of the single compound’s CRCs. Thus, the quadratic term would still be significant with \( 0.3 \times 0.3 = 0.09 \), exceeding 0.3 in sum with the other term factors. Of course, not each of the \( n \) components \( i \) of a mixture would have \( y_i \) close to 0.3, but each component’s effect contribution would decrease with increasing number of components. If \( y_i = 0.2 \), then the quadratic term is 0.04 and the deviation from linearity will become almost insignificant. Figure 1 demonstrates this phenomenon on the example of equipotent mixtures with 2, 3, 4, and 10 components that were calculated with the full IA (Equation 10) using arbitrary EC10s of 2, 4, 8, and 16 for illustration purposes. The black line for CA is equivalent to the joint CA/IA model (Equation 11).

These illustrations (Figure 1) clearly show that up to an effect level of 0.1 (10%), the prediction of the CA and IA models are overlapping, which means that the quadratic and higher terms are indeed negligible. With increasing number of components (Figure 1A–D), the deviation between the 2 models is becoming larger at the same effect level \( y \) (when \( 0.3 > y > 0.1 \)), but the overall deviation is small. Even an equipotent mixture with 10 components with different EC10 yields the same predictions as one with 10 components with the same EC10 (Figure 1D vs E). Even up to an effect level of 0.3 (30%) there is only a small difference between CA and IA, which in most cases is likely to be lower than the variability of the experimental data, making it impossible to decide which model would be better. Given the perfect overlap between CA and IA for \( y \leq 0.1 \), the EC10 of any mixture can be predicted with high certainty using the joint CA/IA model. Even if the joint CA/IA model is strictly applicable only up to 0.1 (10%), it appears pragmatic to apply the joint CA/IA model up to 30%. For in vitro bioassays, 10 to 30% is within a measurable range, but this will not be feasible for many in vivo bioassays, whose controls vary too much to allow the derivation of EC10 values.

Effectively, the overlapping of CRCs for CA and IA in the linear range of the CRCs means that effect summation applies in this range. Rider et al. (2018) also mentioned this special case in their recent review and noted that effect summation is applicable below 15% effect of the mixture. Whereas Rider et al. (2018) just gave one intuitive example, our general deviation supports this statement in a more general manner.
Case study 1 on cytotoxicity in simulated environmental mixtures

We compared the predictions for the CA model (Equation 8) with the IA model (Equation 10) and the predictions to experimental mixture data to test the hypothesis that, if these predictions overlap and the effects in the original water sample are <10%, the joint CA/IA model is applicable (Equation 11).

The data for the case study stemmed from a recent experimental study where 2 to 17 chemicals were mixed in the concentration ratios detected in river samples during rain events and run in 3 reporter gene assays (Neale et al. 2020). We used only the reported cytotoxicity data of water samples and designed mixtures for testing the applicability of the joint CA/IA model. The measured cytotoxicity of the designed mixtures agreed within a factor of 10 with the prediction for CA (Figure 2A). This is a poorer agreement than for the specific effects in the same experiments (Neale et al. 2020), where agreement was typically within a factor of 2; but this is because the cytotoxicity measurements were performed using imaging methods that have a larger variability than the quantification of the reporter gene product. In addition, the solvent dimethylsulfoxide, which was required to prepare such multi-component mixtures, might have contributed to the cytotoxicity, in particular for AhR-CALUX.

Most important for the message of the present study is that the agreement between the prediction of CA and IA was next to perfect (Figure 2B). These predictions were done with the original models in Equations 8 and 10, not with the joint CA/IA model (Equation 11). In all cases, the mixture effects in the experiments that were used to derive the IC10 were <30%, and the effects calculated back to the native water sample were in all cases <0.01% (i.e. <1% within the linear range of the joint CA/IA mixture model).

Case study 2 on application of the toxic unit concept to analytical data

We converted the LC50 of the original data sets on sediment samples by Massei et al. (2018) to LC10 to assure that the calculations were in the linear range of the CRC. Note that in vivo assays typically do not allow a robust derivation of LC10, but they can be interpolated from the dose–response model. As customary, only the LC50 values, but not the equations of the sigmoidal CRC models, were reported in Massei et al. (2018) and only TUchem in Kandie et al. (2020); hence, we had to make this pragmatic assumption.

A TUchem of 1 for TUchems calculated from LC10 means that the mixture triggers a 10% effect. From the reported toxic unit and detected concentrations, we calculated the expected effect of the original water or sediment sample (converted to water concentrations by the organic carbon–water partition constant; for more details, see Massei et al. 2018). The algal toxicity predicted by CA agreed well with that anticipated by IA (Figure 3A). The expected mixture effect of 8 of 78 samples exceeded 30% algal toxicity; that is, 10% of the data were outside the applicability domain of the model, but even in that range the correspondence of CA and IA was very good. For Daphnia magna the majority of samples fell on the one-to-one line, with 14 out of the 48 samples exceeding the linearity criterion of 30% (Figure 3B), mainly in the water samples, due to the presence of high concentrations of insecticides, in particular diazinon. For fish, all predicted lethality stayed

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<10%, and there was a good agreement between CA and IA (Figure 3C). This exercise demonstrates that at environmentally realistic concentrations, the toxic unit concept is applicable for mixtures of chemicals irrespective of the mode of action, provided they do not interact.

CONCLUSION

Chemicals occur in the environment in low concentrations—in most cases, too low to show any direct effects. Water samples with high concentrations and many different chemicals, like those from wastewater-treatment plant effluent and storm water, might show direct effects. In contrast, surface water needs to be enriched typically by more than a factor of 10 to show an effect and recycled and drinking water even further (Escher et al. 2014a). Effects in the original environmental samples are then calculated back to often far less than 10%. Thus, we can invoke low-effect models when developing mixture models of chemicals from complex water samples.

The limiting conditions outlined for the joint CA/IA model were met in the real-world scenarios of the case studies presented. Hence, it appears possible to predict the mixture effects of complex mixtures in environmental samples by applying the joint CA/IA model. The joint CA/IA model also circumvents various problems encountered when using reporter gene assays with variable maxima in mixture studies (Scholze et al. 2014; Watt et al. 2016). It must be noted that the limit of linear low effect levels in CRCs is strict and that

FIGURE 2: (A) Comparison between the concentration addition (CA) prediction (Equation 8) and the experimental cytotoxicity of 106 designed mixtures in AhR CALUX, 38 mixtures in AREc32, and 17 mixtures in PPARγ GeneBLAzer. (B) Comparison between CA (Equation 8) and independent action (Equation 10) predictions for the designed mixtures.

FIGURE 3: Comparison between the acute toxicity toward (A) algae, (B) Daphnia, and (C) fish in sediment and water samples predicted with the mixture models of concentration addition and independent action. Data recalculated from Kandie (2020) and Massei (2018). The dotted line shows the 30% toxicity/lethality, above which concentration–response curves become typically nonlinear.
extrapolations to higher effect levels are not recommended even if it appears that CRCs extend in practice the linear range to higher effect levels. Prior to application of the model to predict CRCs, one must test the applicability of the model by ascertaining that experimental data are within the linear range of the CRC.

As a rule of thumb, any complex environmental sample that needs to be enriched 10-fold before effects become detectable will fall within the applicability domain of the joint CA/IA model. When testing such samples, one must assure that the samples are not enriched too much to avoid the experiment exceeding the applicability domain of the linear model. Robotic tools allow the pipetting of linear dilution series to fit the dosed concentration range to expectations.

This also means that the concept of bioanalytical equivalent concentrations is valid without any additional constraint, as has been discussed for CA (Escher et al. 2018a); but now we can also apply this concept for chemicals that act together via IA, provided the constraint of low effect levels is fulfilled, which is likely to be in surface water samples.

If the toxic unit concept is used to interpret chemical analytical data, we must note that many assumptions are made that we have not explicitly investigated in the present study. The only assumption we have evaluated is the validity of the CA assumption for all chemicals, irrespective of their mode of action and expected joint action; and we could clearly demonstrate that this assumption is valid at low effect levels. Often, the predicted-no-effect concentrations of single compounds are applied to calculate toxic units. In these cases, it should be even safer to apply the summation method because the effect level is certainly lower than 10%. In our case study, despite a minority of predicted effects exceeding 10%, the agreement between CA and IA was still very good. However, there remain limitations when the surface water sample is highly toxic, as exemplified by Nowell et al. (2014), who proposed a pesticide toxicity index (PTI) that is mathematically equivalent to toxic units. They demonstrated that if the PTI starts to exceed 0.1, the survival of associated biological species is compromised for these waters. Munz et al. (2017) used multispecies potentially affected fractions (msPAFs) to estimate the risk from organic micropollutants in wastewater-impacted streams. They combined the msPAF of mixtures of chemicals in 2 steps, first accounting for CA of chemicals with the same mode of action and second combining the groups with different modes of action via IA. This is a commendable endeavor, but considering that the cumulative msPAF was low in all cases, an alternative data treatment could have been the joint CA/IA model presented in the present study. Overall, the proposed linear low-level mixture model for CA and IA will be especially useful for future water quality studies with in vitro bioassays but also justifies the application of the toxic unit concept for complex mixtures of chemicals.

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Data Availability Statement—All toxic unit data of the first case study are available in Neale (2020) or from the corresponding author (beate.escher@ufz.de). The effect data and concentrations of the second case study are available in the Supplemental Data files of Massei (2018) and Kandie (2020). Data, associated metadata, and calculation tools are available from the corresponding author (beate.escher@ufz.de).

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