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Bioanalytical equivalents and relative potencies for predicting the biological effects of mixtures

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[A]bbreviations:2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; EXR, 7-ethoxyresorufin; AhR, aryl hydrocarbon receptor; BEQ, bioanalytical equivalent; Bio-BEQ, bioassay-detected BEQ; Chem-BEQ, chemically analyzed BEQ; CA model, concentration addition model; CALUX, Chemically Activated Luciferase gene eXpression; EROD, 7-ethoxyresorufin-o-deethylase; IA model, independent action model; REP, relative potency; NF-κB, Nuclear Factor Kappa B; MoA, modes of action; PAH, polycyclic aromatic hydrocarbon; TEF, toxicity equivalency factor

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Abstract

Bioanalytical equivalents (BEQs) of mixtures and environmental samples are widely used to reflect the potential threat of pollutants in the environment and can be obtained by bioassays or using chemical analysis combined with relative potencies (REPs). In this study, the relationships between bioassay-detected BEQs (Bio-BEQs) and chemically analyzed BEQs (Chem-BEQs) were studied. BEQs and REPs are correlated with effect level and the concentration-response curves of the reference standard and sample. Thus, effect level (e.g., EC_{10}, EC_{25} and EC_{50}) should be addressed for the BEQ values obtained from bioassays or chemical analyses. The previous prerequisites for REPs application (i.e., curves that are parallel and have the same maximum response) are redundant and the use of REPs for the calculation of BEQs or in risk assessment should instead be based on the same effect level. For a complex mixture with many components, all active components can be regarded as dilutions of a standard compound for inducing a specific effect. Relative toxicity estimates based on EC_{50} ignore the contribution of weak-active components with maximum response below EC_{50} of the reference standard, especially in complex mixtures or environmental samples. REPs based on an effect level EC_{10} that can be clearly discriminated from background response are recommended for BEQ calculation. As an example, the aryl hydrocarbon receptor (AhR)-mediated activity of US EPA priority polycyclic aromatic hydrocarbons (PAHs) in RTL-W1 cells was used to assess the reliability of REPs for mixture toxicity prediction based on the effect level EC_{10}.

Keywords: Standard compound, Concentration-response curve, Effect level, Bioassay

1. Introduction

Bioassays are useful tools to measure the total toxicologically relevant burden of chemicals (Heinrich et al., 2017; Schiwy et al., 2015) and to detect emerging pollutants in the natural environment (Neale et al., 2015). Biological effects of chemical mixtures are usually predicted using different models, including (1) the independent action (IA) model used for chemicals acting with different modes of action (MoAs), (2) the concentration addition (CA) model applying bioanalytical equivalents (BEQs) and relative potencies (REPs) for chemicals with similar MoAs (Altenburger et al., 2004; Larsson et al., 2014a; Neale et al., 2015), and (3) the generalized concentration addition (GCA) model that is a modification of the CA model that considers full agonists, partial agonists, and competitive antagonists (Howard and Webster, 2009; Howard et al., 2010). In risk assessment of chemicals in the environment, CA model is the most frequently used, even in the case of different MoAs, since IA model requires the availability of full concentration-response curves for the mixture and its components, which are rarely available (Belden et al., 2007). Based on CA model but other than REPs considering different endpoints, toxicity equivalency factors (TEFs) have been invented for dioxin-like toxicity in several expert meetings since the early 1990s by the World Health Organization (WHO) to derive consensus TEFs for human and wildlife risk assessment (Van Den Berg et al., 1998; Van den Berg et al., 2006).

Although there is a lack of consistency in the literature, in this paper the term REP is used to describe relative potency of a compound to a reference compound in a

specific test system. REPs are based on concentration–response relationships of individual chemicals (Larsson et al., 2012, 2014a, 2014b; Villeneuve et al., 2000, 2002), and are widely used to evaluate the toxicity of mixtures not only for receptor-mediated effects (e.g. estrogen receptor-mediated endocrine disturbance) (Bonefeld-Jørgensen et al., 2007; Brion et al., 2012; Larsson et al., 2012, 2014a, 2014b; Pillon et al., 2005), but also for non-receptor-mediated effects (e.g. oxidative stress response) (Escher et al., 2020; Neale et al., 2015; Tang and Escher, 2014). Meanwhile, REPs can also be used for bridging chemical analysis and bioassay results by mass balance analysis (Larsson et al., 2014b; Van Den Berg et al., 1998, 2006; Villeneuve et al., 2000, 2002).

Many factors, including interactions between chemicals, differences in the shape of the concentration-response curves, and species responsiveness can cause uncertainties in mass balance analysis and have been discussed previously (Van Den Berg et al., 1998; Villeneuve et al., 2000). For example, Villeneuve et al. (2000) highlighted the possible effects of the response level on REPs and recommended a multiple-point estimate (a range from EC_{20} to EC_{80}) approach to reflect the REPs variations. However, multiple-point REP estimation is a cumbersome and laborious approach and has rarely been applied. At present, the application of REPs in mixture toxicity prediction is still based on several assumptions and limitations. The concentration-response curves for active components should be parallel and exhibit similar efficacy (i.e., maximum response) (Billiard et al., 2008; Payne et al., 2000; Van Den Berg et al., 1998). Actually, these criteria are hardly met or can be met to a

limited degree, and thus are commonly violated or ignored in previous studies (Billiard et al., 2008; Chen et al., 2019; Van den Berg et al., 2006; Villeneuve et al., 2000; Xiao et al., 2016).

Bioanalytical equivalent (BEQ) is the concentration of a reference standard that elicits a response equivalent to the response of the tested sample in a particular assay (Escher et al., 2018a, 2018b; Neale et al., 2015). BEQs can be directly obtained from the application of *in vitro* or *in vivo* bioassays (i.e., Bio-BEQ) or from detected chemical concentrations multiplied by REPs (i.e., Chem-BEQ) (e.g., Chen et al., 2019; Neale et al., 2015; Pan et al., 2010; Richards and Aganovski, 2017). The comparison of Chem-BEQs and Bio-BEQs has been widely applied to quantify the contribution of identified compounds to the bioassay-derived effects (Brack et al., 2000; Hollert et al., 2002; Escher et al., 2018a, 2018b; Larsson et al., 2014b; Neale et al., 2015; Villeneuve et al., 2000; Xiao et al., 2016). Gaps between Bio-BEQs and Chem-BEQs are typically interpreted to be caused by unidentified chemicals (Escher et al., 2020; Giannakis et al., 2020). Interactions between components of the mixtures may also affect the contributions of detected chemicals to biological effects (Billiard et al., 2008; Hong et al., 2015; Larsson et al., 2012). A third reason for gaps apparently suggesting unidentified drivers may be artifacts of data interpretation ignoring the basic requirements of the model on the concentration-response relationships of the mixture components. Although potential deviations of Chem-BEQs calculated on the basis of different effect levels were mentioned in previous studies (Larsson et al.,

2014b; Villeneuve et al., 2000), it remains unclear how effect levels impact Chem-BEQs.

The first aim of this study was to theoretically unravel the impact of slopes, maximum effects and selected effect levels on REPs and BEQs, and to establish criteria for the scientifically sound application of the REPs in the calculation of Chem-BEQs by mass balance approaches. The second aim was to use an experimental study to compare Chem-BEQs with Bio-BEQs in order to validate the theoretical concept. Specifically, a bioassay measuring 7-ethoxyresorufin-o-deethylase (EROD) activity was used to measure the aryl hydrocarbon receptor (AhR)-mediated response of mixtures of polycyclic aromatic hydrocarbons (PAHs) based on compositions detected in sediment extracts from the River Danube. This experimental study helped explain how the effect level under consideration affects the Chem-BEQ, evaluate the explanatory power of Chem-BEQ to Bio-BEQ, and verify the feasibility of optimized REPs for mass balance analysis.

2. Theoretical analysis of BEQs and REPs

2.1 CA model and calculation of mixture toxicity

When the composition of mixture is known, CA model can be expressed using the following equation (1):

$$EC_{F,\mathrm{mix}} = \left(\sum_{i=0}^{n} \frac{R_i}{EC_{\mathrm{F},i}}\right)^{-1} \quad (1)$$

Where: $EC_{F,\ mix}$ is the predicted concentration of the mixture provoking F% response between the maximum response to the reference compound and the blank response; $EC_{F,i}$ is the concentration of the *i*th mixture component provoking F%

response between the minimum and the maximum induction by the reference compound when applied individually; n is the number of components of mixtures; R*i* is the ratio of the *i*th component in the mixture (Berenbaum, 1985; Larsson et al., 2014a).

2.2 The calculation of REPs

REPs were used to calculate the concentration of a reference standard that is equivalent to a given concentration of the sample inducing an absolute response level (Safe, 1998). There are different equations modeling concentration-response curves of the reference, the most widely accepted one is the four-parameter logistic equation, which means that there is a plateau and reduction in response probably caused by toxic effects and which cannot be considered. For the reference this reads as given in equation (2):

$$I_{F,ref.} = I_{blank} + \frac{I_{max,ref.} - I_{blank}}{1+10^{\left(LogEC_{50,ref.} - LogEC_{F,ref.}\right)*H_{ref.}}} \quad (2)$$

Where: $I_{F,ref.}$ is the biological response to the reference compound with a concentration $EC_{F,ref.}$ (0< F<100, expressed as a percentage); I_{blank} is the blank response to the solvent; $I_{max,ref.}$ is the maximum response to the reference compound in the solvent; $EC_{50,ref.}$ is the reference concentration that causes 50% response between $I_{max,ref.}$ and I_{blank}; $H_{ref.}$ represents the hill slope of the curve of the reference compound.

Similarly, the concentration-response curves of the samples (i.e., compounds, mixtures and complex environmental samples) can be expressed as the following equation (3):

$$I_{sample} = I_{blank} + \frac{I_{max,sample} - I_{blank}}{1+10^{(LogEC_{50,sample} - Log\ C_{sample})*H_{sample}}} \quad (3)$$

Where: I_{sample} is the response of the sample with a concentration C_{sample}; I_{blank} is the blank response to the solvent; $I_{max,sample}$ is the maximum response to the sample in the solvent; $EC_{50,sample}$ is the sample concentration that causes 50% response between $I_{max,sample}$ and I_{blank}; H_{sample} represents the hill slope of the curve.

REPs can be calculated according to the equation (4) being aware that $I_{max,sample}$ and $I_{max,ref.}$ may differ significantly and F always represents an effect level relative to $I_{max,ref.}$.

$$REP_{EC_F} = \frac{EC_{F,ref.}}{EC_{F,sample}} \quad (4)$$

$EC_{F,sample}$ can be converted from $EC_{50,sample}$ using the following equation (5) that is converted from the equation (3)

$$LogEC_{F,sample} = LogEC_{50,sample} - Log(\frac{I_{max,sample} - I_{blank}}{I_{F,ref.} - I_{blank}} - 1)/H_{sample} \quad (5)$$

When the maximum response of a compound is greater than half of the maximum response of the reference standard, $REP_{EC_{50}}$ was widely used in previous publications (Bols et al., 1999; Villeneuve et al., 2000). The relationship between $REP_{EC_{50}}$ and REP_{EC_F} can be established using the equation (6) derived from equations (2), (4) and (5).

$$REP_{EC_F} = \left(\frac{I_{F,ref.} - I_{blank}}{I_{max,ref.} - I_{F,ref.}}\right)^{\frac{1}{H_{ref.}}} \cdot \left(\frac{(I_{EC_{50},ref.} - I_{blank}) \cdot (I_{max,sample} - I_{F,ref.})}{(I_{F,ref.} - I_{blank}) \cdot (I_{max,sample} - I_{EC_{50},ref.})}\right)^{\frac{1}{H_{sample}}} \cdot REP_{EC_{50}} \quad (6)$$

Where: REP_{EC_F} is a relative potency on the basis of $EC_{F,ref.}$; $REP_{EC_{50}}$ is a relative potency on the basis of $EC_{50,ref.}$; $I_{EC_{50},ref.}$ is the half response between the

maximum response of the reference compound and the blank response.

Simply, the maximum responses of the sample can be expressed as the relative percentage of the maximum response of the reference standard ($I_{max,ratio}$ = $I_{max,sample}/I_{max,ref.}\times100\%$), and the response $I_{F,ref.}$ can be expressed as F% of $I_{max,ref.}$ with a concentration $EC_{F,ref.}$ ($I_{F,ref.}$ = F×100%). Thus, the relationship between $REP_{EC_{50}}$ and REP_{EC_F} can be expressed using a simplified equation (7):

$$REP_{EC_F} = \left(\frac{F}{100-F}\right)^{\frac{1}{H_{ref.}}} \cdot \left(\frac{50\cdot(I_{max,ratio}-F)}{(I_{max,ratio}-50)\cdot F}\right)^{\frac{1}{H_{sample}}} \cdot REP_{EC_{50}} \quad (7)$$

Where:$I_{max,ratio}$> 50×100%; 0<F<$I_{max,ratio}$ and F<100.

2.3 The calculation of BEQs

The bioassay-derived BEQ based on a fixed effect level EC_F (i.e., $Bio-BEQ_{EC_F}$) was calculated by dividing $EC_{F,ref.}$ for the reference compound by the $EC_{F,sample}$ (equation (8)) (Giannakis et al., 2020; Neale et al., 2017).

$$Bio-BEQ_{EC_F} = \frac{EC_{F,ref.}}{EC_{F,sample}} \quad (8)$$

For a single chemical, chemically derived BEQ based on EC_F (i.e., $Chem-BEQ_{EC_F}$) can be calculated by multiplying the concentration with related REP (equation (9)).

$$Chem-BEQ_{EC_F} = Conc. \times REP_{EC_F} \quad (9)$$

For a sample containing only one active component, Bio-BEQ is equal to Chem-BEQ. For a complex sample with more than one active component,

Chem-BEQ of the mixture can be expressed as the sum of individual Chem-BEQs of these active components on the basis of CA model (Larsson et al., 2012).

Based on the equations (2), (5) and (8), a relationship between $\mathrm{Bio-BEQ_{EC_F}}$ and $\mathrm{Bio-BEQ_{EC_{50}}}$ can be expressed using the simplified equation (10):

$$\mathrm{Bio-BEQ_{EC_F}} = \left(\frac{F}{100-F}\right)^{\frac{1}{H_{ref.}}} \cdot \left(\frac{50\cdot(I_{max,ratio}-F)}{(I_{max,ratio}-50)\cdot F}\right)^{\frac{1}{H_{sample}}} \cdot \mathrm{Bio-BEQ_{EC_{50}}} \quad (10)$$

Where: $I_{max,ratio} > 50\times100\%$; $0<F<I_{max,ratio}$ and $F<100$

Based on equations (6) and (10), it is clear that the variations of REPs and BEQs of the samples are correlated with the maximum responses and hill slopes of concentration-response curves of the compounds and reference standard. The effect level should be addressed when using REPs and BEQs for comparative, risk assessment or mass balance analysis.

When the concentration-response curves of the sample and the reference standard are parallel (i.e. $H_{ref.}=H_{sample}$) and have a same efficacy (i.e., $I_{max,ref.}=T_{max,sample}$), $\mathrm{REP_{EC_F}}$ and $\mathrm{REP_{EC_F}}$ can be regarded as the values independent from the effect levels. Nevertheless, the previous REP estimates (Billiard et al., 2008; Villeneuve et al., 2000) were calculated on the basis of these unproven or rarely existing prerequisites. Actually, the previous prerequisites for REP application are redundant and unnecessary, and the use of REPs for the calculation of BEQs should be based on the same effect level (equation (7)). Based on mass balance analysis, there is no doubt that the contribution of known components to the toxic potency of a complex mixture is a constant value, regardless of which effect level the contribution

is analyzed. Environmental risk assessment of the samples using the different BEQs (Bio-TEQ and Chem-TEQ) should be based on the same effect level to ensure data comparability. For REP and TEQ based on an effect level EC_{50} a great challenge is that these values cannot be obtained when the maximum response of the sample fails to reach half maximum response of the reference standard.

2.4 Bio-BEQs and Chem-BEQs obtained by using REPs

For a given mixture containing only one active component and no interactions between the components, the concentration-response curve of the mixture is determined by this active component ignoring the potential disturbance of cytotoxicity. Equation (11) converted from the equations (7) (9) and (10) shows that the ratio between Chem-BEQ and Bio-BEQ should be a constant value.

$$\frac{\text{Chem}-\text{BEQ}_{\text{EC}_F}}{\text{Bio}-\text{BEQ}_{\text{EC}_F}} = \frac{\text{Chem}-\text{BEQ}_{\text{EC}_{50}}}{\text{Bio}-\text{BEQ}_{\text{EC}_{50}}} \quad (11)$$

For complex mixtures with more than one active component, the basic assumptions of the BEQ calculation are that the combined effects of the components are dose additive and act in a similar manner without interaction (Larsson et al., 2014a; Payne et al., 2000; Van Den Berg et al., 1998). However, many other assumptions and limitations (i.e., a similar slope and efficacy) are not always met and based on an understanding of the REP concept that the components are supposed to behave as the dilutions of the standard compound (Billiard et al., 2008; Villeneuve et al., 2000). Actually, the REP concept was designed based on a specific effect level $EC_{F,ref}$ (equation (4)) and all active components of the mixture are supposed to behave as the dilutions of the standard compound for inducing this effect. Therefore, weak inducers

with efficacies below the response level EC_F cannot be regarded as the standard dilutions. For a given mixture, although bioassay-derived and chemically estimated BEQs vary with the effect level selected, the ability of Chem-BEQ to interpret Bio-BEQ at the same effect level will be stable theoretically. To correctly calculate BEQs of the complex samples, a reasonable approach is that the BEQ calculations of the complex mixtures should be based on a lower effect level recommended by Belden et al. (2007) and Escher et al. (2018b).

In previous studies (Neale et al., 2015), the sigmoidal concentration-response curve was used for BEQ calculation at an effect level higher than 30% of the maximum effect. However, a linear concentration-response curve was used at an effect level lower than 30%. Bio-BEQ calculations based on different curves are laborious and the comparability of Bio-BEQs would be affected.

In this study, REPs based on an effect level EC_{10} that can be significantly distinguished from solvent response are recommended for obtaining Chem-TEQs. If the maximum response of *i*th component in a mixture containing *n* components ($n \geq i > 0$) is less than 10% of the reference standard, the *i*th component can be regarded as a non-active component and be excluded from Chem-BEQ calculation. $\mathrm{Chem-BEQ_{EC_F}}$ of a mixture can be calculated by the sum of $\mathrm{Chem-BEQ_{EC_F}s}$ of its active components ($10 \leq F \leq \min(I_1, I_2, I_3 \ldots I_i)$ and $F<100\times100\%$). Since environmental samples collected from the air, soil, and water typically elicit weak responses, high enrichment is required to induce a response equal or greater than 50% of the response of the reference standard, but lower enrichment is required to induce a response equal

or greater than 10%. The contribution of the detected chemicals to bioassay-derived BEQ can be determined by the ratio of $\mathrm{Chem-BEQ_{EC_{10}}}$ to $\mathrm{Bio-BEQ_{EC_{10}}}$. EC_{10}-based REPs can be used to calculate the Chem-BEQs of the mixtures with specific MoA (e.g., *in vivo* or *in vitro* inductions of endocrine disturbance and AhR activity) and other mixtures that can be simulated by the CA model, such as oxidative stress response, daphnia acute immobilization test and fish embryo toxicity.

2.5 Concentration-response curves of the mixture and its components

The relationships of concentration-response curves between the mixture and its multiple components are quite complex and cannot be clearly characterized mathematically. However, the possible relationships and interactions of components are shown in Fig. 1. Based on the concept of additive behavior and non-interactions between components, the concentration-response curve of the mixture is directly related to the concentration-response curve and the ratio of each active component in the mixture (Fig. 1A). The mixture curve is steeper than the component curve with the minimum slope (component II), but flatter than the component curve with the maximum slope (component III). The efficacy of the mixture should be between the minimum (component II) and maximum efficacies (component I) of its components. When the concentration of the mixture is lower than the lowest concentration of its components alone to induce a specific effect level (Fig. 1B), the synergistic interactions between components definitely exist. Conversely, when the concentration of the mixture is higher than the highest concentration of its components alone to produce a specific effect level without cytotoxicity (Fig. 1C), the antagonistic

interactions between components must be present. For any more specific interpretation the observed effect concentrations have to be compared to the predicted effect concentrations.

It should be noted that the response of a mixture higher than the maximum response of the weakest component (i.e., component II) cannot be predicted by the CA model (Larsson et al., 2012; Rajapakse et al., 2001). Therefore, it is unreasonable to obtain BEQ based on high effect levels and even to extrapolate beyond the maximum response of the weakest component to get a CA-converted REP for toxicity estimates.

The concentrations of the mixture inducing a specific response (e.g., EC_{10}) below the maximum response of the weakest component vary in a limited range (e.g., "c") due to variations in the slopes and efficacies of the components. However, the concentrations of a mixture inducing a response (e.g., EC_{50}) above the maximum response of its weakest component vary in an infinite range (e.g., "d") without cytotoxicity. A wider range means a more ambiguous linkage between the results of chemical analysis and the potential effects of the mixtures. Environmental samples may be weak inducers (Neale et al., 2015), thus the linkage between the results of chemical analysis and the environmental risks of samples will be more ambiguous at a higher effect level. Therefore, it appears reasonable to calculate BEQ at a lower effect level.

3. Materials and Methods

3.1 PAHs and mixtures preparation

AhR-mediated activities of seven US EPA priority PAHs (i.e., benzo[*k*]fluoranthene, dibenzo[*a,h*]anthracene, indeno[*1,2,3-cd*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]pyrene, benzo[*a*]anthracene, chrysene) were evaluated using the EROD assay in RTL-W1 cells. The remaining nine US EPA priority PAHs (i.e., benzo[*g,h,i*]perylene, naphthalene, phenanthrene, anthracene, pyrene, acenaphthylene, acenaphthene, fluorene, fluoranthene) were not evaluated individually since no EROD induction of these PAHs was observed in a previous study using similar conditions (Bols et al., 1999). Benzo[*k*]fluoranthene (≥98%), dibenzo[*a,h*]anthracene (≥98%), benzo[*b*]fluoranthene (≥98%), benzo[*a*]pyrene (≥97%), benzo[*a*]anthracene (≥98%) and chrysene (≥97%) were purchased from Sigma-Aldrich GmbH, and indeno[*1,2,3-cd*]pyrene (≥99.5%) was purchased from Dr. Ehrenstorfer GmbH. Meanwhile, six synthetic mixtures with 2 to 6 PAHs were prepared according to the detected concentrations in multilayer fractions of sediment samples from three sites at Sigmaringen (MS), Lauchert (ML) and Oepfingen (MO) in Upper Danube River (Grund, 2010). The concentrations of individual components in the mixtures are given in Table 1. One widely detected non-AhR-active PAH benzo[*g,h,i*]perylene (≥98%, Sigma) was also included in five of the synthetic mixtures to determine whether the presence of non-AhR-active PAHs affect the activities of the mixtures. Benzo[*b*]fluoranthene, benzo[*k*]fluoranthene and benzo[*j*]fluoranthene could not be chromatographically separated and thus their

individual concentrations in the sediment samples were not quantifiable. Thus, two mixtures MS1 and MS2 were prepared containing benzo[*k*]fluoranthene and benzo[*b*]fluoranthene, respectively. Benzo[*j*]fluoranthene was not included in any mixture because it did not elicit any AhR activity in the RTL-W1 cells (Bols et al., 1999). MS3 was prepared based on the concentration of another fraction of sediment sample from the site at Sigmaringen.

3.2 EROD assay

EROD induction was measured in RTL-W1 cells according to methods described by Heimann et al. (2011) and Wölz et al. (2008) with slight modifications. Briefly, RTL-W1 cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and allowed to grow to 90% confluence for 72 h. Following incubation, the medium was removed and the cells were exposed to samples diluted in medium using eight dilutions. The dimethyl sulfoxide (DMSO) (Promochem, Wesel, Germany) content in the wells was less than 1%. The compound 2,3,7,8-tetrachlordibenzo-p-dioxin (2,3,7,8-TCDD) (Promochem, Wesel, Germany) was used as a positive control and serially diluted to a final concentration range of 3.13-100 pM. After 72 h exposure, induction was stopped by removing the medium and freezing for at least 1 h at -70 °C to lyse the cells. Deethylation of exogenous 7-ethoxyresorufin (EXR) (Sigma-Aldrich, Deisenhofen, Germany) was initiated by adding EXR to each well and incubating the cells in the dark at room temperature for 10 min, followed by addition of NADPH solution (Sigma-Aldrich, Deisenhofen, Germany) and incubating for a further 10 min. The reaction was terminated by adding

fluorescamine dissolved in acetonitrile and incubating for 15 min. EROD activity was measured fluorometrically using a multiwell plate reader (Tecan, Crailsheim, Germany) with an excitation/emission wavelength of 544/590 nm. Protein was determined fluorometrically using the fluorescamine method with an excitation/emission wavelength of 355/390 nm.

The AhR-mediated activities of the chemicals and mixtures were converted to Bio-BEQ by relating the $EC_{F\text{-}sample}$ of sample to the mean EC_F of *2,3,7,8*-TCDD using the equation (8) based on fixed effect level EC_F.

4. Results and discussion

4.1 REPs of PAHs for the AhR-mediated activities

Taking the AhR-mediated activities of PAHs as an example, REPs were obtained based on different effect levels EC_5, EC_{10}, EC_{25} and EC_{50}. Biphasic concentration-response curves were found in earlier studies with the EROD assay (Brack et al., 2000) and the decreasing trend in activity at the greatest concentrations was excluded because it is probably caused by cytotoxicity (Altenburger et al., 2018). In the present study, full concentration-response curves were acquired for individual PAHs with no decreasing trend. These curves varied in hill slopes and the maximum responses after 72 h exposure using RTL-W1 cells. Most of the US EPA priority PAHs are relatively weak inducers, the derived EC_{50} values for US EPA priority PAHs were three orders of magnitude higher than the EC_{50} values for *2,3,7,8*-TCDD standard (7.16 pM). The maximum EROD inductions of US EPA priority PAHs, except

benzo[*k*]fluoranthene and indeno[*1,2,3-cd*]pyrene, were lower than the maximum induction of the reference standard (Table 2).

For a given PAH, REP is not a constant value and varies greatly, such as at different effect levels (Table 3). Furthermore, great variations in REPs for different PAHs were observed. The REPs of benzo[*k*]fluoranthene were two orders of magnitude higher than that of the benzo[*a*]anthracene at a same effect level. Generally, REPs of the PAHs at an effective level EC_{50} were lower than those at lower effective levels (i.e., EC_5, EC_{10} and EC_{25}). The width of variation between REP_{EC_5} and $REP_{EC_{50}}$ was correlated with the hill slopes and the maximum EROD inductions of individual PAHs. Besides the determination of the effect levels on REP values, earlier studies reported that REPs were different when the compound was tested using different methods and cell lines at different exposure times (Bols et al., 1999; Larsson et al., 2012, 2014b; Villeneuve et al., 2002). When the Bio-BEQ of a sample was obtained from one bioassay, Chem-BEQ calculation should use REPs obtained from the same assay using the same cell lines at the same exposure time to analyze the contribution of detected chemicals. It is worth mentioning that regardless of which reference standard (e.g., *2,3,7,8*-TCDD, benzo [*a*] pyrene, 17- *β*- estradiol), or cell line (e.g., H4IIE rat hepatoma, RTL-W1, U2OS cell line), or species (zebrafish, water flea) is used to assess the mixture activity with a specific MoA or the activity that can be simulated by the CA model, REPs at different effect levels can be expressed by equation (6) under the same conditions.

4.2 Predicted BEQs based on CA model

The predictability of the CA model for the activities of the mixtures of chemicals acting with similar MoAs has been demonstrated in other bioassays (Larsson et al., 2012, 2014a; Payne et al., 2000; Zhang et al., 2008). The relationship between the bioassay-derived BEQs and REP-predicted BEQs can be reflected by the status of experimental and CA-predicted concentration–response curves since REPs were designed based on the CA model. Regardless of whether Chem-BEQs were determined based on REPs or the CA model, the prediction of mixture activity was based on the additive interactions of components, and non-additive behavior of the components was not considered. Thus, the effects of non-additive behavior could be reflected by comparing the concentration–response curve of the tested with that of the CA-model predicted (Fig. 2).

The CA model tends to overestimate the toxicity of the mixtures (Kakaley et al., 2019; Olmstead and LeBlanc 2003). the predicted effects of the mixtures (except MS2) were slightly higher than those observed effects in this study. Underestimated toxicities were also observed when REP and CA model were used to predict the toxicities of the mixtures containing two to four PAHs (Larsson et al., 2012). It is possible that the differences between predicted and observed results were caused by non-additive interactions. It is also possible that the differences are caused by the bioassay (Larsson et al., 2012), taking into account the deviations of the EC_{50}s of PAHs. The concentrations of chemicals in environmental samples are quite low and synergism is rare (Payne et al., 2000; Neale et al., 2015). Generally, CA model and CA-converted REPs can be used to confirm the contribution of detected compounds

to the effects observed in a complex sample. In recent years, it has been recommended in publications to use the GCA model based on CA for mixture activity prediction (Howard et al., 2010; Hadrup et al. 2013; Kakaley et al., 2019). The application of the GCA model requires the maximum efficacy and the EC50 value of each component, while the application of the REP is relatively simple, whether REP or GCA model is more suitable for mixture prediction needs further study.

4.3 Comparison of Bio-BEQs and Chem-BEQs at different effect levels

The effectiveness of the REPs at different effect levels for the evaluation of EROD activities was assessed using the RTL-W1 cell line (Fig. 3). For the synthetic mixtures MS1 and MS2, there was no significant difference between the detected Bio-BEQs and Chem-BEQs. For the mixtures MS3, ML2 and MO1, there were significant differences between the calculated Chem-BEQs and the measured Bio-BEQs at an EC50 effect level, which may be caused by the potential non-additive behavior of the components with high concentrations (Larsson et al., 2014a). $\mathrm{Bio-BEQ_{EC_{50}}s}$ of the ML2 (14.55 ±1.67 ng/g SEQ) and MO1 (7.05 ± 2.84 ng/g SEQ) were lower than $\mathrm{Chem-BEQ_{EC_{50}}s}$ of their respective weakest components alone (25.64 ± 3.20 and 13.45 ±1.67 ng/g SEQ) with the same mixture concentration, indicating antagonistic interactions of the components. The maximum deviation between Chem-BEQs and Bio-BEQs was observed for the two-component mixture ML1 in which Chem-BEQs were 1.5-3 times higher than Bio-BEQs, depending on the different effect levels. The gaps between Chem-BEQs and Bio-BEQs were smaller at lower effect levels. At the effect levels EC5 and EC10,

only one mixture ML1 showed significant differences between Chem-BEQs and Bio-BEQs, which might be due to metabolism. It was reported that PAHs and their derivatives could be metabolized in cells (Larsson et al., 2014b), however whether the deviation is more pronounced at high concentrations or at low concentrations remains unclear.

The ratio between $\mathrm{Chem-BEQ_{EC_F}}$ / $\mathrm{Bio-BEQ_{EC_F}}$ and $\mathrm{Chem-BEQ_{EC_{50}}}$/$\mathrm{Bio-BEQ_{EC_{50}}}$ should theoretically be a constant value (i.e., one), but the ratios for the synthetic mixtures varied between 0.6 and 1.9. These deviation ratios should be common since approximately 90% deviation ratios between the tested and CA-predicted toxicity varied in a similar range (0.5-2.0) in previous studies (Belden et al., 2007). Overall, the comparison of Bio-BEQs with Chem-BEQs of the synthetic mixtures revealed that the REPs at a certain effect level can be used to analyze the contribution of detected compounds to mixture toxicity. The explanatory power of Bio-BEQ by Chem-BEQ will be covered by the artifact when both BEQs are obtained at different effect levels. For example, Bio-BEQ of the MS2 could be completely explained by Chem-BEQ at the same effect level while $\mathrm{Chem-BEQ_{EC_{50}}}$ could only account for approximately 15% of Bio-BEQ at an effect level EC_5, resulting in an underestimated contribution. In earlier studies, the prediction of the effects of mixtures with many weak inducers, especially those with maximum response below half that of the reference standard, proved to be a challenge (Payne et al., 2000). Actually, this issue can be solved by REPs at a lower effect level.

5. Conclusions

The results of the theoretical analysis revealed the impact of slopes, efficacy and selected effect levels on REPs and BEQs. BEQs and REPs varied at different effect levels and these differences directly related to the slopes of the concentration-response curves of the sample and standard and to the efficacy of the sample relative to the standard. The previous prerequisites for REP application are redundant, and the effect level should be addressed when using REPs and BEQs for comparative, risk assessment or mass balance analysis. Although bioassay-derived and chemically estimated BEQs vary with the effect level selected, the ability of Chem-BEQ to interpret Bio-BEQ at the same effect level will be stable theoretically. Therefore, we recommended that Bio-BEQs should be calculated based on a lower effect level EC_{10} that can be significantly distinguished from the solvent response, and Chem-BEQs calculated from $REP_{EC_{10}}$ should be used to analyze the contributions of chemically analyzed compounds. Thus, the presence of weak agonists can be considered. Finally, we used experimental studies to help explain how the effect level affects REPs and Chem-BEQs, evaluate the explanatory power of Chem-BEQ to Bio-BEQ, and verify the feasibility of optimized REPs for mass balance analysis.

Declaration of competing interest

The authors declare no competing financial interest.

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Fig. 1. The possible relationships between the concentration-response curve of the mixture and the curves of its components. The concentration-response curves of the mixtures in, above and below the range consisting of the curves of its components are shown in Fig. 1A, 1B and 1C, respectively. Components I, II and III represent the components of the mixture with the highest response, the weakest response and the steepest curve, respectively. The concentration of the mixture represents the sum of concentrations of all active components. In Fig. 1A, the deviations of the environmental concentration away from the concentrations of the mixture for inducing EC_{10} and EC_{50} are indicated by the letters "a" and "b", respectively. The concentrations of the mixture inducing the effect levels EC_{10} and EC_{50} vary in the ranges of "c" and "d", respectively. In Fig. 1B, the deviations between the concentration of the mixture and the lowest concentration of its components alone for inducing EC_{10} and EC_{50} are represented in the ranges of "e" and "f", respectively. In Fig. 1C, the deviation between the concentration of the mixture and the highest concentration of its components alone for producing an effect level EC_{10} is represented in the range of "g".

Fig. 2. Observed (solid lines) and predicted (dashed lines) concentration-response curves of the synthetic mixtures in the EROD assays. Observed data was calculated based on the average of three repetitions. Error bars represent the standard deviations. EROD activities of the synthetic mixtures were normalized against the maximum response observed for their corresponding standard. The unit mg/ml means mg sediment equivalent (SEQ) in 1 ml medium.

Fig. 3. Comparison of measured Bio-BEQs (lighter grey) and calculated Chem-BEQs (dark grey) of the mixtures at different effect levels (EC_5, EC_{10}, EC_{25} and EC_{50}). Chem-BEQs were calculated using the relative potencies (REPs) based on mass concentrations. Error bars represent the

standard deviations (n=3). Asterisk (*) represents a significant difference between the measured Bio-BEQ and the calculated Chem-BEQ ($p<0.05$). The unit ng/g SEQ means ng TCDD equivalent detected in g sediment equivalent (SEQ).

Table 1 The concentrations (ng/mL) of PAHs in synthetic mixtures.

Compounds	Sigmaringen			Lauchert		Oepfingen
	MS1	MS2	MS3	ML1	ML2	MO1
Benzo[*b*]fluoranthene	2216	0	0	0	0	0
Benzo[*k*]fluoranthene	0	2216	0	2902	0	0
Benzo[*a*]pyrene	1104	1104	476	1422	0	0
Dibenzo[*a,h*]anthracene	196	196	166	0	326	106
Indeno[*1,2,3-cd*]pyrene	384	384	420	0	902	488
Benzo[*g,h,i*]perylene	476	476	476	0	1046	454
Sum of active components	3900	3900	1062	4324	1228	594
Sum of all components	4376	4376	1538	4324	2274	1048

The dose of a single PAH in 1 ml solvent is equal to that detected in 20 g sediment equivalent (SEQ).

Table 2 US EPA priority PAHs that induced EROD activities in RTL-W1 cells after 72 h exposure.

PAHs	EC_{50} (nM) ± SD	Maximum EROD activity (pmol/mgP/min) ± SD	$I_{max,ratio}$[a](%)	Hill slopes (H_{sample})
Benzo[*k*]fluoranthene	9.98 ± 1.45	3.92 ± 0.2[illegible]	103.52	0.81
Dibenzo[*a,h*]anthracene	12.13 ± 2.01	3.68 ± 1.28	92.49	1.07
Indeno[1,2,3-cd]pyrene	15.41 ± 1.65	4.45 ± 0.87	107.82	1.12
Benzo[*b*]fluoranthene	112.07 ± 31.55	[illegible].46 ± 0.64	87.02	0.89
Benzo[*a*]pyrene	230 ± 61.53	2.94 ± 0.25	73.74	0.89
Benzo[*a*]anthracene	1097.51 ± 2[illegible].57	2.72 ± 0.21	68.26	0.90
Chrysene	1312.5[illegible] ± 140.47	3.03 ± 0.26	76.26	0.99

[a] $I_{max,ratio}$ was the maximum EROD induction of PAHs ($I_{max,sample}$) relative to the maximum induction induced by the reference *2,3,7,8*-TCDD. The maximum EROD induction of the reference was 3.98 ± 0.73 pM/mgP/min. The hill slope of the reference was 1.64.

Table 3 The REPs based on molar concentrations for US EPA priority PAHs derived from EROD induction using RTL-W1 cells.

PAHs	REP_{EC_5} $\times10^{-3}$	$REP_{EC_{10}}$ $\times10^{-3}$	$REP_{EC_{25}}$ $\times10^{-3}$	$REP_{EC_{50}}$ $\times10^{-3}$	$REP_{EC_{50}}$ [a] $\times10^{-3}$
Benzo[*k*]fluoranthene	3.700 ±0.397	2.896 ±0.439	1.563 ±0.372	0.717 ±0.104	1.040
Indeno[*1,2,3-cd*]pyrene	0.661 ±0.366	0.657 ±0.313	0.558 ±0.155	0.453 ±0.045	0.278
Dibenzo[*a,h*]anthracene	0.839 ±0.014	0.655 ±0.020	0.484 ±0.046	0.361 ±0.06	0.350
Benzo[*b*]fluoranthene	0.183 ±0.014	0.175 ±0.030	0.131 ±0.046	0.069 ±0.028	0.193
Benzo[*a*]pyrene	0.114 ±0.031	0.109 ±0.041	0.077 ±0.030	0.035 ±0.009	0.300
Chrysene	0.044 ±0.004	0.035 ±0.004	0.022 ±0.002	0.011 ±0.002	0.047
Benzo[*a*]anthracene	0.035 ±0.006	0.022 ±0.008	0.014 ±0.005	0.006 ±0.001	0.043

The units of EC_5, EC_{10}, EC_{25} and EC_{50} values were pM, and the mean EC_5, EC_{10}, EC_{25} and EC_{50} values for *2,3,7,8*-TCDD standard were 1.19, 1.86, 3.67 and 7.16 pM, respectively; [a] The values from Bols et al. (1999).

Credit Author Statement

Shangbo Zhou, Shuchan Peng, Thomas-Benjamin Seiler and Henner Hollert designed the study. Shangbo Zhou conducted the experiments. Shangbo Zhou and Shuchan Peng evaluated the data and wrote the paper. Werner Brack and Jon A Doering made substantial contributions to the interpretation of data and to the revision of the paper.

Declaration of competing interest

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Effect level should be addressed for bioanalytical equivalents.
- Components can be viewed as dilutions of a compound for inducing a specific effect.
- Toxicity estimates at a high effect level ignore weak-active components.
- Relative potencies at EC_{10} can be used to calculate bioanalytical equivalents.

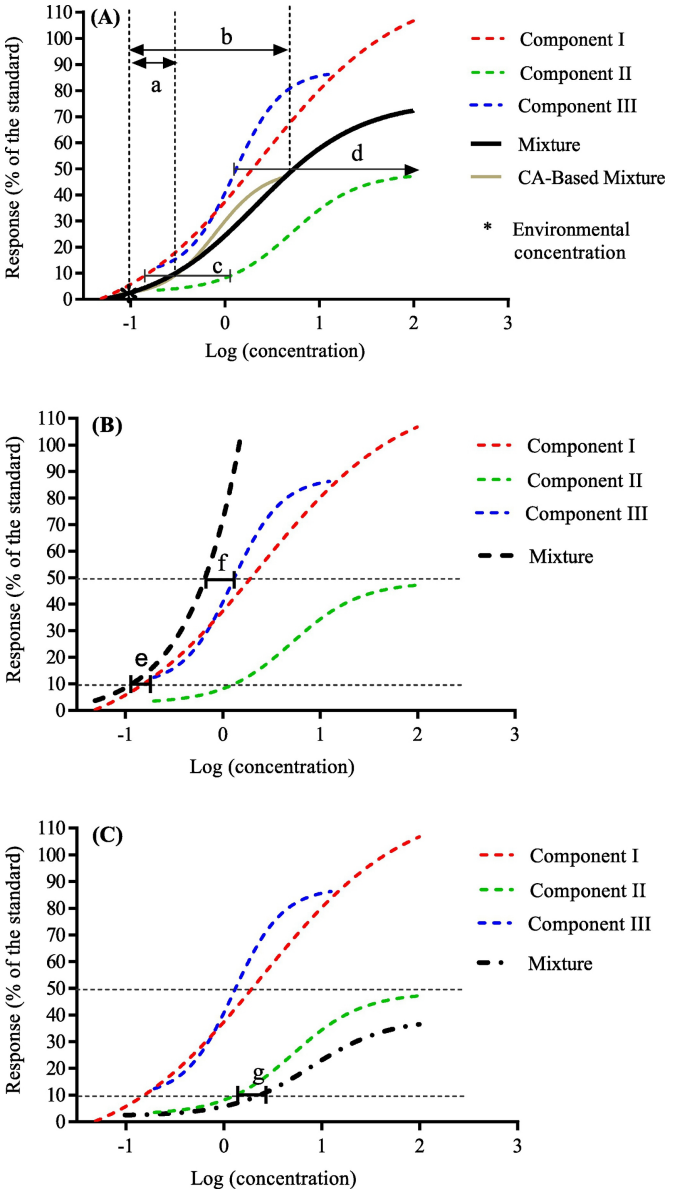
(A)
Response (% of the standard)
Log (concentration)
a
b
c
d
Component I
Component II
Component III
Mixture
CA-Based Mixture
* Environmental concentration
(B)
e
f
Component I
Component II
Component III
Mixture
(C)
g
Component I
Component II
Component III
Mixture

Figure 1

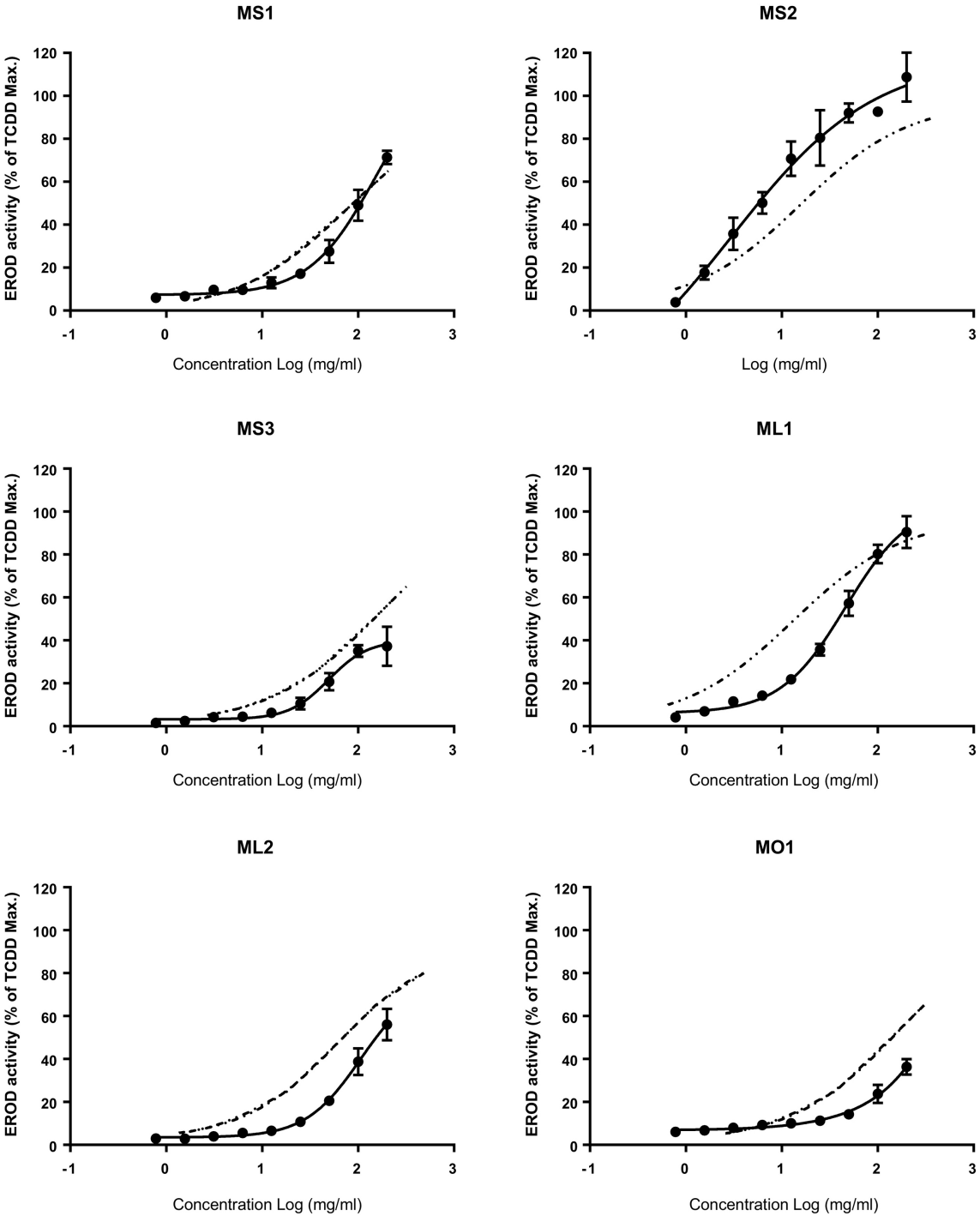
MS1
EROD activity (% of TCDD Max.)
Concentration Log (mg/ml)
MS2
EROD activity (% of TCDD Max.)
Log (mg/ml)
MS3
EROD activity (% of TCDD Max.)
Concentration Log (mg/ml)
ML1
EROD activity (% of TCDD Max.)
Concentration Log (mg/ml)
ML2
EROD activity (% of TCDD Max.)
Concentration Log (mg/ml)
MO1
EROD activity (% of TCDD Max.)
Concentration Log (mg/ml)

Figure 2

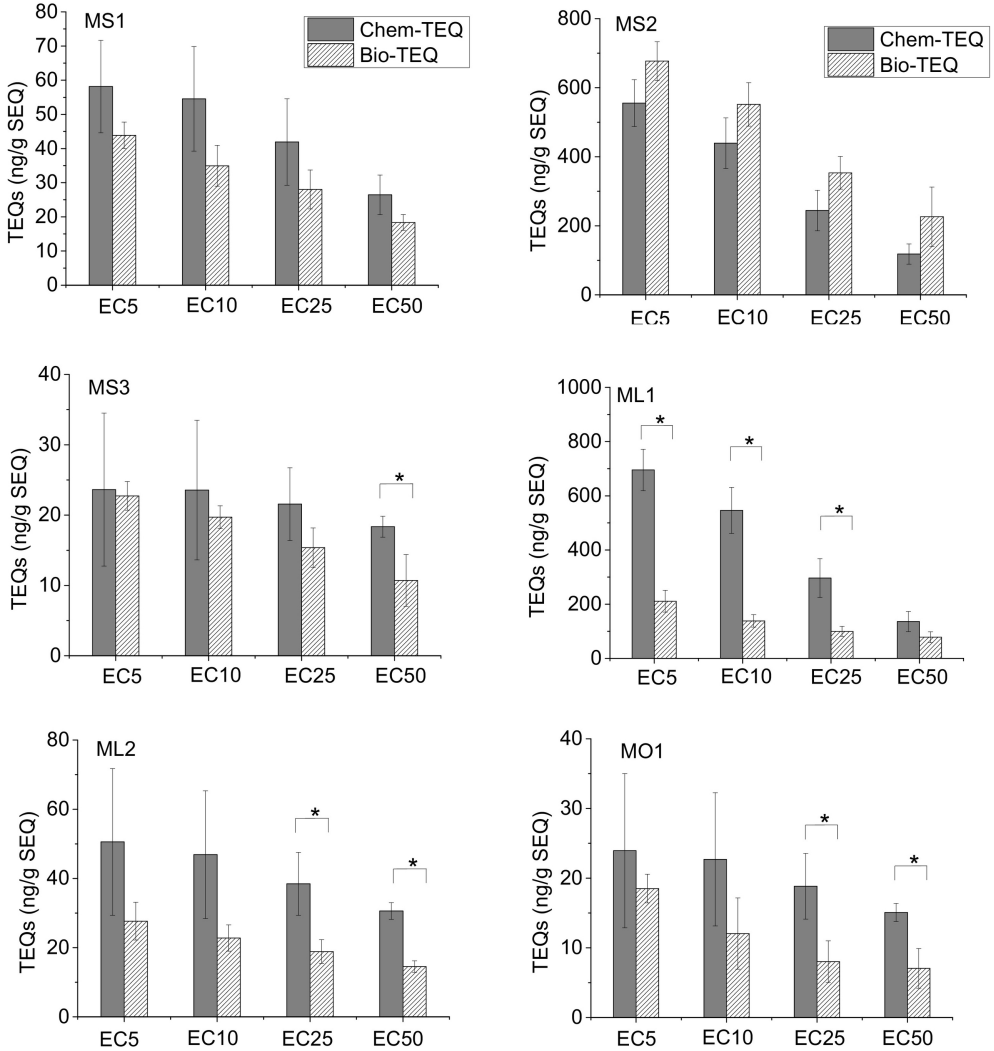
MS1
Chem-TEQ
Bio-TEQ
TEQs (ng/g SEQ)
EC5
EC10
EC25
EC50
MS2
MS3
ML1
ML2
MO1
*

Figure 3