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## Mixture effects in samples of multiple contaminants – An inter-laboratory study with manifold bioassays



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## ABSTRACT

Chemicals in the environment occur in mixtures rather than as individual entities. Environmental quality monitoring thus faces the challenge to comprehensively assess a multitude of contaminants and potential adverse effects. Effect-based methods have been suggested as complements to chemical analytical characterisation of complex pollution patterns. The regularly observed discrepancy between chemical and biological assessments of adverse effects due to contaminants in the field may be either due to unidentified contaminants or result from interactions of compounds in mixtures. Here, we present an interlaboratory study where individual compounds and their mixtures were investigated by extensive concentration-effect analysis using 19 different bioassays. The assay panel consisted of 5 whole organism assays measuring apical effects and 14 cell- and organism-based bioassays with more specific effect observations. Twelve organic water pollutants of diverse structure and unique known modes of action were studied individually and as mixtures mirroring exposure scenarios in freshwaters. We compared the observed mixture effects against component-based mixture effect predictions derived from additivity expectations (assumption of non-interaction). Most of the assays detected the mixture response of the active components as predicted even against a background of other inactive contaminants. When none of the mixture components showed any activity by themselves then the mixture also was without effects. The mixture effects observed using apical endpoints fell in the middle of a prediction window defined by the additivity predictions for concentration addition and independent action, reflecting well the diversity of the anticipated modes of action. In one case, an unexpectedly reduced solubility of one of the mixture components led to mixture responses that fell short of the predictions of both additivity mixture models. The majority of the specific cell- and organism-based endpoints produced mixture responses in agreement with the additivity expectation of concentration addition. Exceptionally, expected (additive) mixture response did not occur due to masking effects such as general toxicity from other compounds. Generally, deviations from an additivity expectation could be explained due to experimental factors, specific limitations of the effect endpoint or masking side effects such as cytotoxicity in *in vitro* assays. The majority of bioassays were able to quantitatively detect the predicted non-interactive, additive combined effect of the specifically bioactive compounds against a background of complex mixture of other chemicals in the sample. This supports the use of a combination of chemical and bioanalytical monitoring tools for the identification of chemicals that drive a specific mixture effect. Furthermore, we

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demonstrated that a panel of bioassays can provide a diverse profile of effect responses to a complex contaminated sample. This could be extended towards mixture adverse outcome pathways. Our findings support the ongoing development of bioanalytical tools for (i) compiling comprehensive effect-based batteries for water quality assessment, (ii) designing tailored surveillance methods to safeguard specific water uses, and (iii) devising strategies for effect-based diagnosis of complex contamination.

## 1. Introduction

The provision of clean water for ecosystems and humans is central for reaching all of the United Nations sustainable development goals (UNEP, <http://web.unep.org/post2015/>). Faced with a rapidly accelerating increase in chemical innovation, production, consumption and emission, and a growing world population with increasing demands, safeguarding the quality of surface waters has become a major challenge (Schwarzenbach et al., 2006). Two complementary approaches have been developed to deal with unwanted chemical contamination. In prospective risk assessment potential environmental risks are assessed by comparing predicted environmental chemical exposure with expected adverse effects based on prior information on compound toxicities and other properties. In monitoring efforts, we seek to screen relevant contaminations in the environment. Both approaches rely strongly on a perspective that focuses on single chemicals, one-by-one, falling short of the reality of contamination of many environmental systems with complex mixtures of chemicals (Loos et al., 2009; Brack et al., 2015; Escher et al., 2013a).

Prospective chemical assessment dealing with mixture exposures and their potential combined effects has progressed considerably (Deneer, 2000; Altenburger and Greco, 2009). A component-based approach which seeks to predict the toxicity of mixtures on the basis of the effects of its components has gained substantial empirical support and is now widely accepted (Kienzler et al., 2016). In routine environmental monitoring, by contrast, exposure-oriented chemical analytical studies and biology-focused investigations are completely separate activities. Under the water framework directive (WFD, 2000) indicators of chemical and ecological quality are regarded as two separate, poorly connected categories. Causal links between chemical exposures and ecological effects are often discussed from a single cause-effect perspective, with a focus on single chemicals, but do not consider the occurrence of multiple chemicals as mixtures, multiple stress factors and their combined effects. The integration of bioassays as effect-based methods in environmental monitoring is intended to bridge this gap, supporting the identification of mixture exposures (Altenburger et al., 2015; Wernersson et al., 2015; Brack et al., 2017).

In a ring trial, Carvalho et al. (2014) investigated two mixtures of substances of concern. Using a panel of 35 different bioassays, mixtures with components at their individual environmental quality standard level (EQS) were shown to elucidate effects in several of the assays. These findings and earlier reviews demonstrated that regulatory single-chemical threshold values may not be fit for purpose to protect against mixture exposure (Carvalho et al., 2014; Kortenkamp et al., 2009). Schoenfuss et al. (2015) studied mixtures of pharmaceuticals at environmentally relevant concentrations together with effluent exposures by using various effect biomarkers in fish. The authors interpreted their observations as interactions between contaminants in the mixture, however, without reference to an expected additive effect of the combination.

Case studies of extracted freshwater samples using chemical and bioanalytical analysis have demonstrated that bioassays can provide complementary information for water monitoring. For instance, the pattern of bioassay responses obtained across 22 sites stretching across a major part of the river Danube resembled well those of chemical analytical concentrations of target chemicals (Neale et al., 2015). Further, a comparison of bioassay effects with samples upstream a effluent outlet, downstream and with the effluent itself with measured

chemicals and their effects consistently showed an increased impact of effluents from wastewater treatment plants at tributaries of the Rhine (Neale et al., 2017a, 2017b) and river Danube (König et al., 2017). When the combined effects are expressed as the sum of bioanalytical equivalent concentrations for quantified chemicals and are then compared to the actually observed effects in environmental samples the findings can be separated into two groups: First, there are assays indicative of highly specific receptor-mediated effects such as algal photosynthesis inhibition, or binding to the estrogen receptor. In these assays, most of the observed bioactivity can be explained in terms of the detected photosystem II inhibiting herbicides or natural estrogens, respectively. Second, with assays sensitive to more general effects triggered by many different chemicals, such as cytotoxicity and induction of oxidative stress response there is an explanation gap of effects that remain unaccounted for. Thus, it is sometimes difficult to explain observed mixture effects using component-based mixture effect prediction. Potential reasons might be due to compounds that were overlooked in the chemical target analyses (Escher et al., 2013a) or to an inaccurate quantification of bioactive concentrations close or below the analytical detection limit, such as for potent xenoestrogens. Furthermore, our current knowledge of the components' bioactivities in specific assays (Neale et al., 2017a, 2017b) and the validity of common mixture effect concepts under conditions of complex exposure need to be scrutinized (Altenburger et al., 2004).

The objective of this study was to verify the ability of a suite of bioanalytical tools to detect bioactivity of specific compounds in a mixture exposure setting against a background of co-occurring water contaminants. We extend previous work (Busch et al., 2016; Neale et al., 2017a, 2017b) by rigorous investigation of the ability of a panel of bioassays to detect joint bioactivities in a mixture of chemicals with diverse modes of actions (MoAs). To achieve our aims, we (i) defined a bioassay panel comprising assays for detection of different key events and apical endpoints (Altenburger et al., 2015; Neale et al., 2017a, 2017b), and (ii) utilised a component-based mixture prediction approach with best-fit modelling of concentration effect relationships (Scholze et al., 2001, 2014). We designed a mixture of twelve compounds with anticipated non-similar modes of actions in two different mixture ratios with the aim of studying (a) the detectability of combined effects against a background of components presumed to be inactive, (b) the ability to capture relevant bioactivities at mixture compositions that may occur in environmental exposures. Results were assessed by comparing predicted and observed combined effects for each assay and through mapping against the expected occurrence of specific biological effects (key events). By testing the same two mixtures in different bioassay we were able to assess the performance of different bioassays for complex exposure analysis and gained an impression of the usefulness of response data for individual compounds for predicting mixture effects in environmental exposure scenarios.

## 2. Materials and methods

### 2.1. Approach

For our round robin mixture effect study we started with single compound testing using 21 different bioassays. The compounds to be characterised by individual concentration-effect relationships were a subset of chemicals of the chemical fingerprinting effort described in Neale et al. (2017a). Components for the mixture testing were selected

such that at least two compounds were active in each assay. Concentration-effect data for the mixture design planning were available from previous studies (Escher et al., 2017; Neale et al., 2017a, 2017b) for all assays (Table 1). Using more sophisticated concentration-effect models (Scholze et al., 2001), we selected twelve compounds for inclusion in the mixture (Table 2). We kept assays with no bioactive compounds (androgen response, glucocorticoid response, and two Ames assays) as negative controls. Two mixtures were generated in different ratios from those twelve compounds (Table 3). The individual concentration-effect relationships for all chemicals and bioassays allowed quantitative mixture predictions according to the mixture additivity concepts (Eqs. (1) and (2)).

## 2.2. Bioassays

A summary of the nineteen bioassays used and references for description of methods and applications is provided in Table 1. Experimental details and standard operating procedures are also provided in the SI of Neale et al. (2017a, 2017b) and in Table S2.

## 2.3. Mixture composition

Individual chemicals were selected from a compilation and hazard ranking of chemicals detected in water contamination monitoring studies and from their known MoA (Busch et al., 2016). The selection, experimental design, and single compound study are further described in the SI. The chemicals selected their identity, use, and MoA group classification are shown in Table 2 (Busch et al., 2016). The suppliers, modelled physicochemical properties, and additional quality information are provided in the SI, Table S1.

## 2.4. Mixture testing

The two mixtures were prepared as stock solutions in methanol (HPLC grade) at UFZ and distributed to all participating laboratories. Dilution series testing was performed with the bioassays based on the expected combined effects. All assays were conducted in at least two

independent repeats against solvent controls and within a period of three months after distribution, with the exception of the AhR- and PPAR $\gamma$ -assays which were completed 6 months after sample distribution.

Several means of quality controlling the nominal concentrations and their stability over the course of the experiments were undertaken, which are described in more detail in the SI.

## 2.5. Concentration-effect data analysis

The selection of concentration-effect model selection for non-linear regression analysis was conducted according to the best-fit regression approach (Scholze et al., 2001), with various regression functions fitted to the same set of the data. As an estimator of the best-fitting model we used the Akaike Information Criterion (AIC). The reader is referred to the SI for further details.

## 2.6. Mixture prediction and assessment

As described by Faust et al. (2001), under the assumption of concentration addition (CA) a mixture concentration producing an effect X can be calculated for a n-component mixture as

$$EC_X(\text{mixture}) = \left( \sum_{i=1}^n \frac{p_i}{EC_{X,i}} \right)^{-1}, \quad (1)$$

where  $EC_X(\text{mixture})$  is the mixture concentration that produces the effect X for a combination of n individual concentrations  $c_i$ ,  $EC_{X,i}$  are the concentrations of the individual components that on their own produce the same effect X as the mixture, and  $p_i$  is the ratio of the  $i^{\text{th}}$  component in the mixture ( $p_i = c_i / (c_1 + \dots + c_n)$ ). The individual effect concentrations are derived from the inverse of the nonlinear regression function which describes best the observed concentration-effect data of the components (as described above).

The basic version of independent action (IA) has been formulated under the simple assumption that the susceptibilities of the individuals of an at-risk-population to different dissimilarly acting mixture

**Table 1**  
Bioassays used in the mixture round robin study.

Biological level	Biosystem	Effect observation	Indication of	Assay name	Method reference
Nuclear receptor	Mammalian and fish cells	Pregnane X receptor activation	Activation of biotransformation	HG5LN-hPXR	Lemaire et al., 2006
		AhR receptor activation	Activation of biotransformation	AhR CALUX	Brennan et al., 2015
		Estrogen receptor activation	Estrogen response	MELN	Balaguer et al., 1999
				ZELH-zfERalpha, ZELH-zfERbeta2	Cosnefroy et al., 2011
		Androgen receptor activation/inhibition	Androgen/anti-androgen	MDA-kb2	Wilson, 2002
		Inhibition of glucocorticoid receptor (GR)	Glucocorticoid response	Anti-MDA-kb2	van der Linden et al., 2008
		PPAR- $\gamma$ nuclear peroxisome proliferator-activated receptor- $\gamma$	Metabolism homeostasis	GR CALUX	Neale et al., 2017b
Cellular	<i>Salmonella typhimurium</i>	Ames test using diagnostic strains	Mutagenicity	Ames microplate agar Ames fluctuation test	Mortelmans and Zeiger, 2000 Reifferscheid et al., 2012
		AREc32 based on MCF7 breast cancer cell line	Nrf2-ARE activation	Adaptive response to oxidative stress	AREc32
Organism receptor	Zebrafish embryo ( <i>Danio rerio</i> )	Estrogenic cyp 19a1b-GFP expression	Estrogen response	Cyp19a1b-GFP or EASZY	Brion et al., 2012
	Medaka embryo ( <i>Oryzias latipes</i> )	Estrogenic choriogenin-GFP activation	Estrogen/anti-estrogen response	ChgH-GFP or REACTIV	Spirhanzlova et al., 2016
Organism apical effects	Algae ( <i>Chlamydomonas reinhardtii</i> )	Growth	Apical effects, multiple MoA	Algal population growth inhibition	de Almeida et al., 2017
	<i>Daphnia magna</i>	Motility	Apical effects, multiple MoA	Daphnia immobilisation	David et al., 2011
	Zebrafish embryo ( <i>Danio rerio</i> )	Development	Apical effects, multiple MoA	zFET - well plate	Seiler et al., 2014
				zFET - glas vials	Schmidt et al., 2016
	<i>Aliivibrio fischeri</i>	Bioluminescence inhibition	Apical effects, cytotoxicity, mainly reactive MoA	Microtox	Escher et al., 2017

**Table 2**  
Chemicals used in the mixture study (information according to Busch et al., 2016).

Compound	CAS RN	Use group	Chemical group	Mode of action group
Diazinon	333-41-5	Pesticide	Organophosphorus	Neuroactive
Diclofenac	15307-86-5	Pharmaceutical	Neutral Organics	Anti-inflammatory
Bisphenol A	80-05-7	Industrial Chemical	Phenols	Endocrine activity
Propiconazole	60207-90-1	Pesticide	Triazole	Sterol biosynthesis inhibition
Triphenylphosphate	115-86-6	Industrial Chemical	Esters	Neuroactive
Diuron	330-54-1	Pesticide	Phenylurea	Photosynthesis inhibition
Chlorophene	120-32-1	Pesticide	Chlorinated phenol	unspecific
Benzo[a]pyrene	50-32-8	Industrial Chemical	PAH	Nucleic acid damage, mutagenicity
Benzo[b]fluoranthene	205-99-2	Industrial Chemical	PAH	Nucleic acid damage mutagenicity
Triclosan	3380-34-5	Biocide	Chlorinated Phenol	Lipid metabolism disturbance
Cyprodinil	121552-61-2	Pesticide	Pyrimidine	Protein biosynthesis inhibition
Genistein	446-72-0	Pharmaceutical	Isoflavone	Mitosis, cell cycle interference, Endocrine activity

For the first mixture (Mix I) we used a mixture composition mimicking the unbalanced nature of 'real' exposure such that a mixture effect was to be expected in most assays (and not only due to a single compound), whereas the second mixture (Mix II) was chosen to represent a realistic ratio of the mixture components as it may occur in freshwaters.

**Table 3**  
Composition of the mixtures used for the round robin combined effect study across bioassays and environmental concentrations (MEC95, Busch et al., 2016), which defined the Mix II.

Compound	Mixture 1	Mixture 2	MEC 95 <sup>b</sup> [mol/L]	Highest expected sensitivity (based on MoA information and bioactivity cf. Table S3a–j)
	Mix I	Mix II		
	Fraction of total mixture concentration			
	Ratio <sup>a</sup>	Ratio <sup>b</sup>		
Diazinon	4.997E–05	1.303E–03	2.0 E–11	Daphnids
Diclofenac	2.499E–01	1.928E–01	3.0 E–09	Zebrafish
Bisphenol A	5.830E–03	2.768E–01	4.2 E–09	Estrogen activity
Propiconazole	4.997E–01	5.630E–03	8.5 E–11	Anti-androgen activity, biotransformation
Triphenylphosphate	1.249E–01	1.537E–02	2.3 E–10	Daphnids, zebrafish, biotransformation
Diuron	4.997E–03	1.384E–02	2.1 E–10	Algae
Chlorophene	7.496E–02	4.251E–01	6.4 E–09	Anti-androgen activity
Benzo(a)pyrene	4.997E–04	6.289E–04	9.5 E–12	Daphnids, AMES
Benzo(b)fluoranthene	8.329E–04	6.315E–04	9.5 E–12	Oxidative stress
Triclosan	2.915E–02	2.583E–02	3.9 E–10	Algae, zebrafish, anti-androgen activity
Cyprodinil	8.329E–03	1.242E-02	1.9 E–10	Daphnids
Genistein	8.329E–04	2.968E-02	4.5 E–10	Estrogen activity

<sup>a</sup> Given is the fraction of each chemical present in the mixture, i.e. the ratio of its concentration to the total mixture concentration. The number of digits is provided to allow repeatability and does not imply a statement on precision.

<sup>b</sup> 95percentile of measured aquatic concentrations taken from Busch et al., 2016.

components are not correlated with each other (Bliss, 1939). For a n-compound mixture and a relative effect endpoint described by descending concentration response curves (Eq. (1)), IA is defined as

$$E(c_{\text{mixture}}) = \prod_{i=1}^n E(c_i), \quad (2)$$

where  $E(c_i)$  denote the effects produced by the individual compounds  $c_i$ , and  $E(c_{\text{mixture}})$  is the total effect of the mixture. The main assumption is that the effect endpoint is normalised to an effect range 0 to 1, i.e. control and exposure mean estimated outside this range would violate the use of this equation.

As all mixtures were tested according to the fixed-ratio design (i.e. total mixture concentration was varied while the concentration ratio of the components was constant), a huge number of different concentration/effect pairs were generated by using Eqs. (1) and (2) and connected by straight lines, providing a visualization of the predicted concentration–response curve.

To account for the statistical uncertainty in the CA and IA prediction, we used a combination of Monte-Carlo (MC) simulations and bootstrapping regression functions (Efron and Tibshirani, 1993) to produce approximate 95% confidence limits around the predicted mean mixture effect.

### 3. Results

We studied the capability of a panel of 19 bioassays to detect specific combined effects against two background mixtures of 12 organic chemicals, all of which have been identified as relevant water contaminants. The mixture comprised a diverse range of chemical structures and modes of action. The observed bioactivities of the 12 compounds agreed well with available knowledge as discussed in Neale et al. (2017a, 2017b). From these concentration-effect data we estimated a best-fit regression model for each effect endpoint which was then used to calculate the non-interaction additivity expectation of their joint effects. For all assays, these predictions were derived from concentration addition (Eq. (1)). For all apical endpoints we also calculated mixture effects according to independent action (Eq. (2)). These predicted mixture effects were subsequently compared with experimentally observed combined effects.

#### 3.1. Individual compounds

Examples of concentration-effect data from individual compounds are shown in Fig. 1, one for an apical endpoint (mortality in the zebrafish embryo after exposed to diuron, Fig. 1A) and the other for a receptor-mediated response (estrogen receptor activation in a human reporter cell assays exposed to bisphenol A, Fig. 1B). The best-fit regression models for all individual compounds and mixtures are

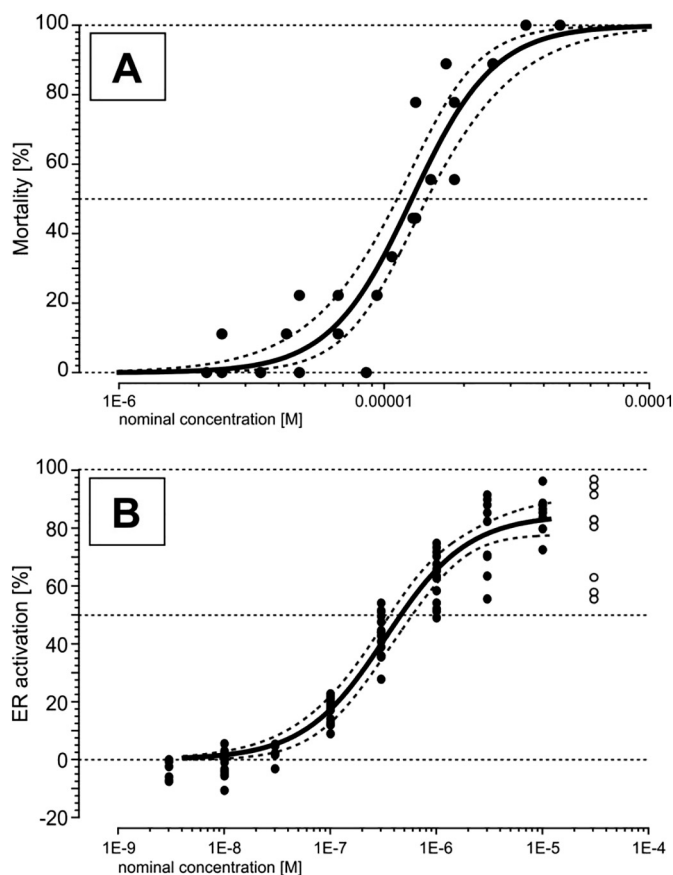


Fig. 1. Examples of experimental concentration-effect data (black dots) and best-fit regression curve for (A) apical effects of diuron on zebrafish embryonic development (zFET, glass vial) and (B) human MCF-7 cell-based estrogen receptor activation (MELN) by bisphenol A. Shown are results from at least three independent experiments, dashed curves are the respective 95% CIs for the regression fits. Responses judged to be influenced by cytotoxicity shown as open circles were not included in data analysis.

provided in the supplement (Tables S3a-S3j). Our aim was to describe the observed concentration-effect data in the best possible way over a large concentration range, and statistical analysis confirmed that this

Table 4

Predicted and observed effect concentrations for mixtures<sup>a</sup> including statistical uncertainty for the apical endpoint bioassays.

	Effect Concentration EC50 <sub>mix</sub> [M]						EC50: predicted/observed	
	Observed		Predicted by CA		Predicted by IA		CA	IA
	Mean	95% CI	Mean	95% CI	Mean	95% CI		
zFET – glass vial, fish mortality (48 h exposure)								
Mix I	2.03E-05	[1.89E-05–2.13E-05]	<b>1.48E-05</b>	[1.33E-05–1.60E-05]	<b>3.85E-05</b>	[3.24E-05–4.37E-05]	<b>0.73</b>	<b>1.90</b>
Mix II	1.59E-05	[1.42E-05–1.77E-05]	1.30E-05	[1.11E-05–1.47E-05]	<b>2.32E-05</b>	[1.82E-05–2.95E-05]	0.82	<b>1.46</b>
zFET-96 well plate, fish mortality (48 h exposure)								
Mix I	5.50E-05	[4.66E-05–6.43E-05]	<b>3.19E-05</b>	[2.84E-05–3.60E-05]	6.32E-05	[5.08E-05–7.68E-05]	<b>0.58</b>	1.15
Mix II	3.55E-05	[2.99E-05–4.28E-05]	<b>2.27E-05</b>	[2.00E-05–2.58E-05]	4.08E-05	[3.39E-05–5.01E-05]	<b>0.64</b>	1.15
Algae population growth inhibition (72 h exposure)								
Mix I	2.52E-05	[2.41E-05–2.63E-05]	<b>1.58E-05</b>	[1.33E-05–1.81E-05]	2.70E-05	[2.14E-05–3.24E-05]	<b>0.63</b>	1.07
Mix II	9.20E-06	[8.98E-06–9.45E-06]	1.02E-05	[8.03E-06–1.20E-05]	<b>1.65E-05</b>	[1.23E-05–2.04E-05]	1.11	<b>1.79</b>
Daphnia immobilisation test (48 h exposure)								
Mix I	5.78E-06	[5.20E-06–6.32E-06]	<b>2.53E-06</b>	[2.11E-06–2.84E-06]	4.08E-06	[2.82E-06–5.23E-06]	<b>0.44</b>	0.71
Mix II	6.19E-07	[5.67E-07–6.67E-07]	5.66E-07	[4.84E-07–6.30E-07]	6.97E-07	[5.93E-07–8.01E-07]	0.91	1.13
Microtox (30 min exposure)								
Mix I	7.68E-04	[5.91E-04–1.01E-03]	<b>2.04E-04</b>	[1.47E-04–2.54E-04]	<b>3.58E-04</b>	[2.24E-04–4.53E-04]	<b>0.27</b>	<b>0.47</b>
Mix II	9.18E-05	[7.59E-05–1.11E-04]	5.64E-05	[3.84E-05–7.87E-05]	7.75E-05	[4.85E-05–1.24E-04]	0.61	0.84

CA – Concentration Addition, IA – Independent Action, CI – Confidence Interval; numbers in bold indicate statistical significance between predicted and observed mean.

<sup>a</sup> Mixture ratios as defined in Table 3.

cannot be achieved by using a pre-defined single nonlinear regression function for all endpoints and compounds. For cell-based continuous responses the logit function was typically selected as the best-fitting model: logit is a reparameterization of the Hill equation (on log-transformed concentrations) which is commonly used in biochemistry and pharmacology to analyse the binding equilibria in ligand-receptor interactions. Its selection as best-fitting regression model was therefore not surprising, as most cell-based effect endpoints are expected to reflect closer proximity to specific pharmacological activities, i.e. adhere to the law of mass action (Kenakin, 2015). For apical endpoints, the selected best-fitting models varied, with the Weibull model often providing the best data description.

The biological activity expressed as EC for the 12 compounds spanned up to six orders of magnitude (Tables S3a-l). If these values are compared with concentrations estimated for their baseline toxicity (apical endpoints) or their cytotoxicity (cell-based endpoints), the differences suggest biological effects from close to baseline activity up to highly specific responses. Compounds were not reported if they showed responses only at cytotoxic or water non-soluble concentrations.

### 3.2. Mixtures

The observed combined effects and the corresponding CA and IA mixture effect predictions for mixtures 1 and 2 are provided in Tables 4 and 5 for bioassays where statistically significant mixture effects were recorded. For two mixtures, Fig. 2 provides exemplarily their predicted and observed concentration-response data as two illustrations of data situations and modelling. Fig. 3 than displays the resulting concentration-response relationships for expected and observed mixture effects from Mix I exposure for all assays that showed statistically significant mixture responses.

The assessment of the observed mixture effects was subsequently performed in a two-step procedure. First, we analysed whether a combined effect can be detected by comparing against the components' individual activities. Secondly, we compared the observed with the predicted combination effects.

For the five bioassays with apical effect observations we found in 9 of the 10 cases that the combined effects were clearly higher than the effects of any of the individual components (Fig. S1). Expected combined effects based on the mixture modelling for all apical bioassays showed that concentration addition always provided the more

**Table 5**

Predicted and observed effect concentrations for mixtures including statistical uncertainty for receptor-based bioassays.

	Effect concentration EC10 <sub>mix</sub> [M]				Predicted/observed
	Observed		CA		
	Mean	95% CI	Mean	95% CI	
AhR CALUX - Aryl hydrocarbon receptor activation (24 h exposure)					
Mix I	8.79E-07	[2.54E-07–2.41E-06]	1.81E-07	[6.76E-08–3.13E-07]	0.21
Mix II	1.52E-06	[5.21E-07–3.43E-06]	2.25E-07	[7.80E-08–3.93E-07]	<b>0.15</b>
HG5LN-hPXR activation (16 h exposure)					
Mix I	1.35E-06	[9.97E-07–1.69E-06]	<b>2.58E-06</b>	[1.74E-06–3.41E-06]	<b>1.91</b>
Mix II	1.21E-05	[9.37E-06–2.05E-05]	5.52E-06	[3.90E-06–6.02E-06]	0.46
PPAR $\gamma$ UAS-293H - PPAR gamma activation (24 h exposure)					
Mix I	1.07E-06	[5.35E-07–1.67E-06]	9.74E-07	[4.64E-07–1.79E-06]	0.91
Mix II	–	–	1.30E-06	[6.11E-07–2.46E-06]	–
MELN - Estrogen receptor activation (24 h exposure)					
Mix I	2.98E-06	[1.83E-06–4.70E-06]	2.91E-06	[1.87E-06–4.24E-06]	0.98
Mix II	7.30E-08	[3.21E-08–1.42E-07]	9.48E-08	[5.92E-08–1.51E-07]	1.30
ChgH-GFP – Estrogen response (24 h exposure)					
Mix I	5.59E-06	[4.39E-06–7.21E-06]	1.10E-05	[6.08E-06–1.52E-05]	1.97
Mix II	4.55E-06	[1.88E-06–6.17E-06]	4.87E-06	[2.36E-06–8.05E-06]	1.07
MDA-kb2 - Anti-androgenicity (24 h exposure)					
Mix I	2.96E-06	[8.61E-07–6.26E-06]	1.16E-06	[8.90E-07–1.45E-06]	0.39
Mix II	6.95E-07	[3.49E-07–1.17E-06]	1.14E-06	[8.68E-07–1.42E-06]	1.64
AREc32 - oxidative stress (24 h exposure)					
Mix I	3.93E-05	[3.21E-05–4.66E-05]	<b>1.15E-04</b>	[9.72E-05–1.29E-04]	<b>2.93</b>
Mix II	4.52E-05	[4.21E-05–4.79E-05]	4.94E-05	[3.88E-05–5.67E-05]	1.09

CA – Concentration Addition, CI – Confidence Interval; bold indicates statistical significance between predicted and observed mean;

<sup>1)</sup> mixture ratios as defined in Table 3.

conservative mixture prediction, i.e. concentration addition calculated combined effects at lower concentrations compared to the concept of independent action. Comparing the predicted with observed combined effects for the two mixtures in these bioassays (Table 4) at the estimated median effect levels and including statistical uncertainty revealed the following picture. Qualitatively, i.e. looking for a statistical significant difference between expected and observed median effect level, six out of the ten mixture-bioassay combinations studied were less active than anticipated on the basis of a concentration additive response, while three of these ten were more active than independent action would predict (Table 4). The mixture observations for Mix I in the Microtox assay suggest less than expected combined effects compared against both concepts, while for the Mix II this was not observed. The quantitative differences ranged from a maximum of a 3.7-fold overestimation of the combined effect at median effect concentration to a 1.9-fold underestimation of the actual effect compared to independent action. Concentration addition in none of the cases provided an underestimation of the observed combined effect. The quantitative differences to either model were clearly less than a factor of two despite being significant in several cases, except for the Mix I in the Microtox assay, where the mixture effect was much lower than expected. Here, we suspect that some compounds precipitated invisibly in solution because the Microtox assay is run in artificial seawater that is more likely to precipitate organic chemicals due to relatively high ionic strength compared with freshwater buffers or tissue culture media.

From the 14 bioassays which detected specific responses, seven recorded a response with the 12-component mixture (Fig. S2). The individual components in the cell-based assays showed higher variability between independent experimental repeats (Tables S3a–j), and baseline responses were sometimes different from the control. Moreover, cytotoxicity was a potential confounder at higher concentrations, e.g. for the hPXR response. The effect concentrations of the mixture corresponding to median effects varied by four orders of magnitude across all bioassays, from  $7.3 \cdot 10^{-8}$  to  $7.7 \cdot 10^{-4}$  mol/L regarding the total mixture concentration (=sum of individual components). At the highest usable mixture concentrations, no mixture effect were found for

the AMES assays, the androgenic response in MDA-kb2 in agonistic response mode and the GR CALUX response assays, which is in line with the expectations from the 12 studied components with no to very low individual bioactivities in these assays. Thus, most of the seven assays reported here successfully discriminated combined effects from those of the most active components in the mixtures over a wide range of concentrations (Fig. S2a–e). Remarkable exceptions were found for the ZELH-alpha and -beta2 cells, and the Cyp19a1b, where no agonistic effect could be detected for mixtures (not illustrated) although this was predicted (Tables S4 and S5). In the PPAR $\gamma$ -assay, neither of the two mixtures reached a 20% response level, which is consistent with the very low effect expected by the CA prediction (Fig. S3).

The comparison of predicted with observed combined effects for the two mixtures in the bioassays using receptor-based endpoints at the estimated low effect levels (EC<sub>10</sub>, IC<sub>20</sub>, or EC<sub>IR1.5</sub>) and including statistical uncertainty is displayed in Table 5. For these responses only concentration addition was used as a reference. Ten out of the 13 observed combined responses were within the uncertainty range of the expected concentration additive response and could thus be assessed to be in agreement with the expected combined effect. For two assays the observed mixture response of Mix I was statistically significantly more active than expected. For the hPXR activation Mix I was 1.9-fold more active than expected and just outside the confidence interval for the expected response, and for the oxidative stress response detected using the AREc32 assay Mix I showed a 2.9-fold significantly higher mixture activity than expected. By contrast, the Mix II showed a 6.7-fold lower activity in the AhR-assay compared to a concentration additive response. For the PPAR $\gamma$ -assay, Mix II showed no measurable effect (Fig. S3) whereas the Mix I was in line with the expected concentration additive response, however this response needs to be interpreted with caution as effects did not reach 20% before cytotoxicity started to compromise the cells.

In summary, a combined effect in response to exposure to a mixture of 12 organic compounds with different modes of action could clearly be detected in 12 of the 14 bioassays where it was expected. Those 5 bioassays where any individual compound showed no bioactivity by

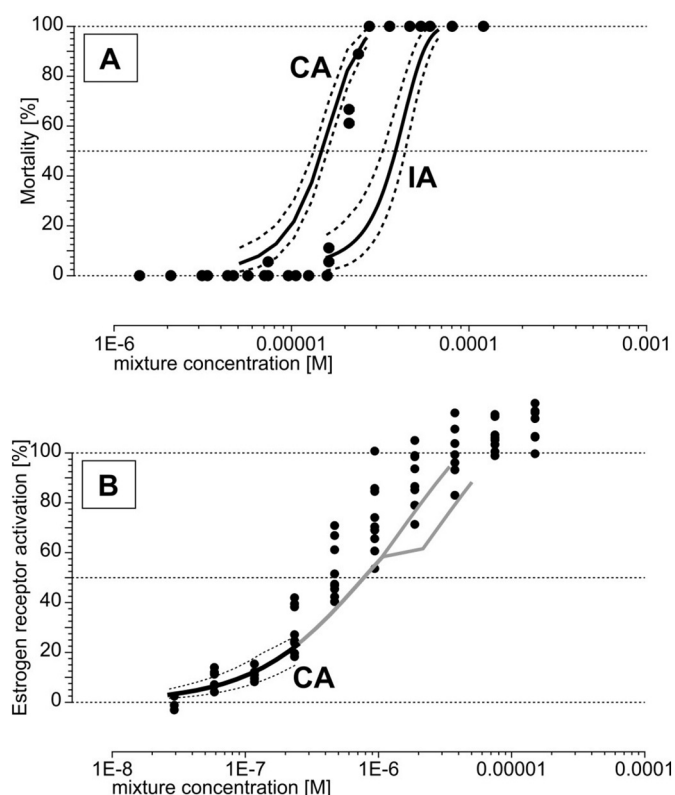


Fig. 2. Examples of observed and predicted mixture responses for (A) apical effect on zebrafish embryonic development (zFET - glass vial, Mix I) and (B) human MCF-7 cell based estrogen receptor activation reporter (MELN, Mix II). Mixture effects were predicted according to CA and IA (only for apical endpoints) (solid line) and are shown with their respective 95% CIs (dashed line), observed mixture responses are from at least three independent experiments (dots). CA mixture prediction in (B) was only possible at low concentrations, here a range of most likely CA mixture responses at higher concentrations was calculated according to the toxic unit extrapolation method by Scholze et al. (2014) (grey solid line).

itself also showed no effects with the mixture. The three assays, which consistently failed to detect expected mixture effects, showed different directions of effects (stimulation/inhibition) for different mixture components. Finally, most assays detecting specific responses showed combined effects that were quantitatively close to component-based mixture effect expectations based on concentration addition. The independent action concept, however, was the better predictor of the observed mixture effects for assays detecting apical effects, whereas in one of the four assays the combined effect observation fell in the range spanned between concentration addition and independent action predictions.

#### 4. Discussion

The results of the presented interlaboratory study for two mixtures are discussed with regard to the data quality, the detectability and predictability of combined effects and the consequences for the composition of panels of bioassays for effect-based monitoring and its interpretation.

##### 4.1. Data quality considerations

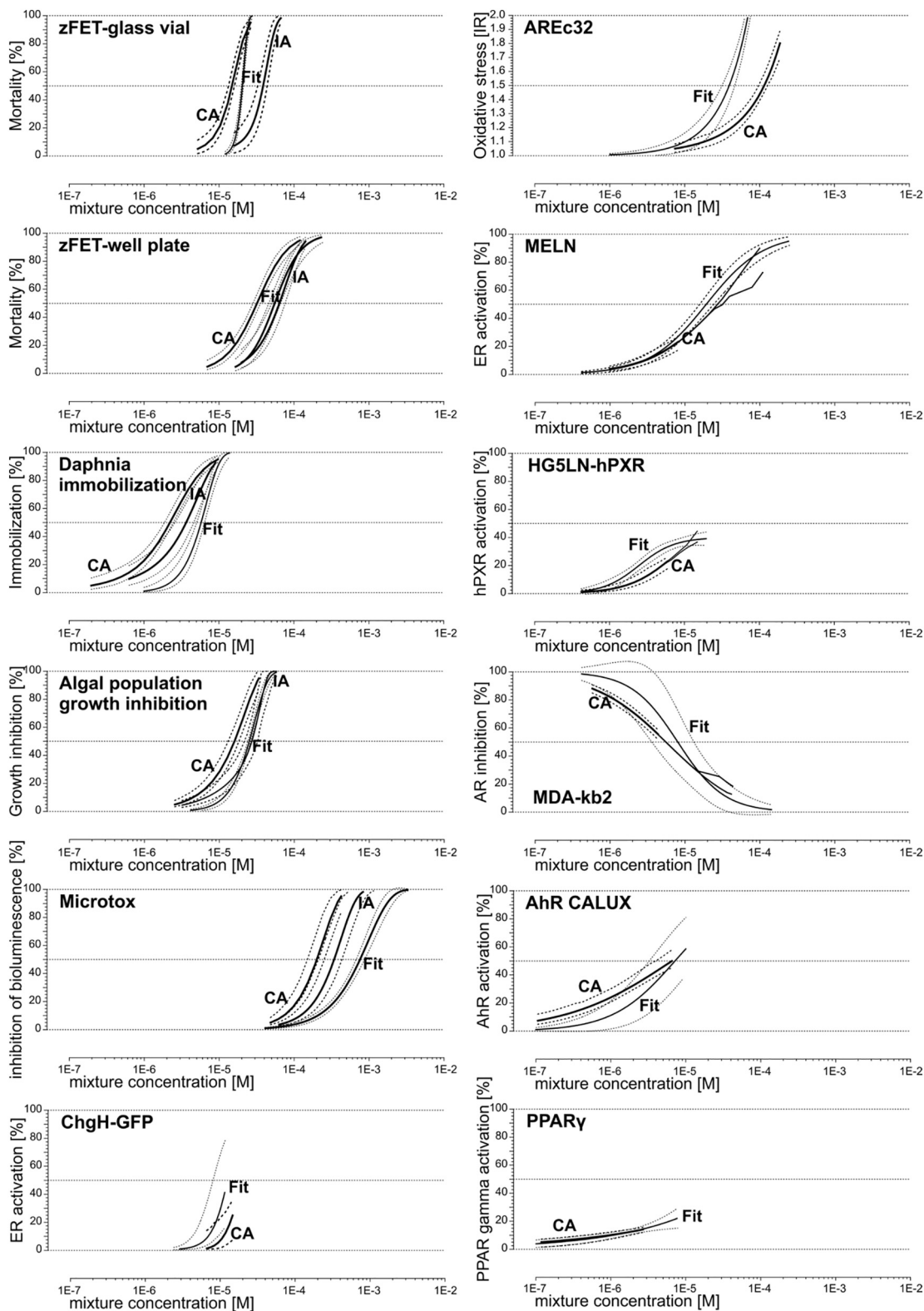
A mixture round robin study by Carvalho et al. (2014) showed the occurrence of effects in various bioassays at concentrations of the individual components at EQS levels, i.e. concentrations where no adverse biological effects should be expected. To assess whether such effects are due to predictable combined effects or whether they instead derive from unpredictable mixture interactions, explicit knowledge of

the components concentration-dependent bioactivity is required. Moreover, for discriminating the effects of single compounds from those of a mixture and deciding on possible deviation from prediction, the experimental design and the response variability play a crucial role and need to be scrutinized.

Based on these considerations, we included several measures to ensure that the characterisations of the compound's effects were accurate. Thus, we used independent experimental repeats, adaptive spacing of concentrations to capture the dynamic effect range and to derive robust EC estimates, considered physicochemical properties to achieve soluble concentrations, provided identical stock solutions of the two mixtures for biotesting across the different laboratories, and checked the stability of stock solutions. Using data from earlier work reported by Neale et al. (2017b), we used a best fit approach for the concentration-effect relationship modelling and accounted for inter-experimental variation and overdispersion. The finding that the logit model (which is equivalent to the Hill function) proved to be the best fitting model for assay data that representative of specific responses is in line with an understanding that receptor-binding as a limiting process should adhere to the law of mass action (Kenakin, 2015), while for organismic assays other processes such as kinetics or effect chains may become determining and may thus modify the concentration-effect relationship which, for example, is then better captured by the asymmetrical Weibull model.

As we had two assays using zebrafish early development observations (zFET), one that used a microtiter plate format, the other using glass vials, we compared the findings for the individual compounds and the mixtures. For three compounds (bisphenol A, diclofenac, and propiconazole) the EC<sub>50</sub> were not statistically different (Table S3). Eight of the other nine compounds and the two mixtures were consistently shown to be of higher bioactivity in the assay using glass vials, by a factor ranging between 1.5 and 5.6. As the assay showing higher sensitivity was performed in glass vials as opposed to a plastic microtiter plate, it seems reasonable to consider systematic differences in the exposure regime as causative, rather than biological variability. Schreiber et al. (2008) and Riedl and Altenburger (2007) have provided evidence of systematic differences in EC estimates in systems using different materials for exposure vessels and it is assumed that sorption to plastic microtiter plates may constitute a major loss process altering nominal concentrations for compounds with a log K<sub>ow</sub> of 3 and higher. Kramer et al. (2012) have further provided analytical access to quantitatively determine the different underlying processes. For the selected substances substantial loss processes could be assumed for all of the compounds except genistein (log K<sub>ow</sub> 2.3). In our experiments, this was the only compound showing a lower effect concentration in the microtiter plate assay. As this study did not target the absolute effect concentrations but rather investigated the assumption of additive mixture effects in a complex mixture, these differences are of no major concern but have to be kept in mind, when concluding on environmentally relevant effect concentrations. For high-throughput biotesting in the future, either experimental solutions, or modelling loss processes from compound properties, could be ways to reduce the existing error sources.

A second prominent feature in this study derives from the use of a uniform mixture stock solution for both mixtures across all bioassays. This, on the one hand, guaranteed that exactly the same mixture compositions were tested in each lab. On the other hand, we had to accept the inaccuracies arising from the use of different lots of chemicals during individual compound and mixture testing or using different co-solvents. The latter could be excluded by comparing the influence of DMSO and methanol as co-solvents in their influence on the individual components effects, e.g. for bisphenol A and genistein in the MELN assay (data not shown). Solubility issues due to the highly concentrated stock solutions were not apparent up front for any of the bioassays, though in hindsight, regarding the specific case of the high salt medium it seemed plausible for the Microtox assay.



**Fig. 3.** Predicted and observed combined effect responses of Mix I in various bioassays. Mixture effects were predicted according to CA and IA (only for apical endpoints), observed mixture responses are from at least three independent experiments and indicated by their common best-fit regression model (Fit). Dashed curves show the respective 95% CIs for the predictions and regression fit. In three cases CA mixture predictions were only possible at low concentrations, here we calculated a range of most likely CA mixture responses at higher concentrations according to the toxic unit extrapolation method by Scholze et al. (2014). For positive reference compounds please refer to SI, Table S2.

Finally, we found that the concentration extrapolation approach for CA predictions for compounds with incomplete concentration-response curves developed by Scholze et al. (2014) helped to describe the observed mixture effects. Incomplete regression curves pose problems

with the applicability of CA, as the maximally predictable combined effect level is determined by the lowest maximal effect of a component in the mixture. The extrapolation approach thus provided confidence regarding the predictability of combined effects (e.g. Fig. 2b).



The plausibility of the derived effect concentrations for the individual components (Table S3) was in line with expectations of reported data using ToxCast bioassays (comptox.epa.gov; August 2017). More detailed considerations are provided in the SI.

#### 4.2. Detectability and predictability of combined effects

The comparative assessment between an observed and predicted mixture response can only provide reliable judgements when both sides of the comparison as well as the comparison itself are valid and free of any bias. For the predictions it meant that we repeated the single substance experiments several times in order to rule out any “day-specific” outcomes and thus consolidated robust concentration-response pattern (“averaging over experiments and time”), with the same rationale the mixture experiments were repeated and the overall mean used for the comparison, and technical factors that could have led to a biased comparison were minimised in the best possible way (e.g. avoiding the testing of the wrong mixture composition by identical master solutions). The comparison itself was performed only between effect (or effect concentrations) that were supported by data (i.e. no data extrapolations) and quantitative differences confirmed statistically by considering the uncertainty on both sides of the comparison, i.e. predictions and observed mixture response. Here we strongly recommend to base not only the prediction on repeated experimental data, but also to repeat the final mixture experiment at least twice to achieve robust effect estimations on both sides of the comparative assessment.

The mixture effect observations and the modelled concentration-response functions were compared with the contributions from the individual components in the mixtures. For the majority of the assay/mixture combinations these comparisons showed that the effects of the mixture were indeed larger than any of the mixture components alone. Thus, we detected true combined effects different from cases where an individual substance dominates the observed effect, despite the presence of several other compounds.

The only exception was the Microtox assay, where the concentration response relationship for chlorophene alone could explain the observed mixture effect in Mix II while in Mix I the mixture effect appeared lower than for the chlorophene exposure alone. Multi-component mixtures of bioactive components have been studied previously using this assay and have shown, in contrast to the findings here, that combined effects can be detected and that results were in line with the mixture model expectations both for similarly and dissimilarly acting components (e.g. Altenburger et al., 2000; Backhaus et al., 2000). The lower than expected mixture toxicity found in the Microtox assay for the Mix I is thought to result from a limited solubility of components as the predictivity for a mixture effect improved when using a solubility cut-off corrected for the salting out effect with the Setchenow equation for the 3 M salt concentration in the saltwater medium used in the Microtox assay with the marine bacteria *A. fischeri* (Escher et al., 2017). The modelled solubility data are for freshwater and may not be sufficient in this specific case. As the Microtox assay sensitivity is lower by an order of magnitude than all other bioassays, this potential problem does not have to be considered for the other assays.

Observations for seven receptor/cell-based assays, namely AR activation, GR activation, Ames, Cyp19a1b activation, zfERalpha, and zfERbeta2 activation detected no or very little combined effects with the mixtures. For AR activation, GR activation, and the Ames assays these findings are consistent with the observations that none of the individual components of the 12 compound mixture would provoke any substantial effect in the plausible concentration ranges and, furthermore, that the explicit mixture prediction modelling did not calculate any observable effect for the mixture. For the zf estrogen receptor alpha and beta2 activity in zebrafish cells, and for the cyp19a1b activation assays in the transgenic zebrafish the situation was different. For the zfER responsive in vitro assays, we found deviation from expected

additivity, in particular for the zfERbeta2 assay. Co-occurrence of activities heading in opposite directions, i.e. stimulatory and inhibitory, was suspected. The mixture effect of estrogen receptor activation may thus be confounded by this phenomenon. A separate in depth investigation is underway and will be reported separately (Serra et al., in prep.). The cyp19a1b transgenic assay has been shown to be useful to assess additive effects of binary (Brion et al., 2012) and multi-component mixtures (Petersen et al., 2013) of ER agonists. Overall, a good correspondence has been reported between the cellular responses measured by zfER responsive in vitro assays and the embryonic cyp19a1b for single compounds and mixtures (Sonavane et al., 2016; Le Fol et al., 2017). The lack of estrogenic response observed at the non-lethal concentrations of the mixtures could also have resulted from the co-occurrence of activating and inhibiting activities on ER-signalling pathways, while at higher concentrations the general toxicity observed on cyp19a1b zebrafish embryos as in the FET assay, expected from the mixture components diclofenac, propiconazole, TPP, chlorophene, and triclosan, may have excluded the observation of an estrogenic response. This argument is supported by the known effect concentration - response function from the zFET assays (Table S3c,e). These findings support including both mechanism-based and apical endpoints for monitoring multicomponent mixtures.

The oxidative stress response assay for Mix I was greater than the response predicted by the concentration addition model and correspond with findings in an earlier mixture study which also showed higher than expected mixture effects (Escher et al., 2013a). Two interpretations are possible in this case: The variance in the mixture predictivity for defined mixtures was higher than estimated here and falls into the range of a factor of 2–3 as previously described, or chemicals whose activity is masked by cytotoxicity when they are tested alone are contributing to the mixture effect (Escher et al., 2013a, 2013b).

The question whether the combined effects in a multiple mixture composed of components with various modes of action and at heterogeneous concentrations are still predictable using component-based mixture models will be discussed first by considering the apical responses (Table 4). Since Walter et al. (2002) first described a case where the observed combined effect of a multiple mixture of heterogeneous chemicals fell between the mixture effect predictions derived from CA and IA an assessment dilemma became apparent: The observed mixture responses can be interpreted as antagonism (effects smaller than expected) in relation to CA and as synergisms (effects larger than expected) in relation to IA, a rather unsatisfactory situation. If additional knowledge is available on the similarity or dissimilarity of modes of action of the mixture components, a stepwise modelling can improve the accuracy and precision of the combined effect prediction (Altenburger et al., 2004, 2005, Ermiler et al., 2013). This stepwise approach involves modelling, first by concentration addition for the similarly acting components, and subsequently, of the dissimilarly acting compound groups using independent action (Altenburger et al., 2004). In environmental mixtures the information on modes of action for all compounds is, however, not easily available. In this study the observed mixture responses for the two fish embryo assays and the algal growth assay in this study fell into this ‘prediction window’ spanned by concentration addition and independent action, and thus can be interpreted as in line with responses expected for a mixture of similarly and dissimilarly acting compounds. In all cases studied here concentration addition predicted an effect concentration lower than for independent action, i.e. the higher combined effect. For the apical assays this also in all cases accommodated for the experimentally observed worst case combined effect. This also means, that if one intends to identify mixture drivers in environmental samples based on available concentrations-response relationships for components, e.g. through toxic unit summation, the CA assumption may underestimate the number of relevant contributions.

For the assays sensitive to more specific responses (Table 5), we evaluated mixture responses only in relation to CA and not to IA. The

effects in these assays are receptor-driven, and at the level of receptors only competition for binding and differences in intrinsic activity are of importance. For such effect concentration addition provides a reasonable reference. This idea is supported by our observation that the combined effects were quantitatively well predicted by concentration addition. It has to be acknowledged, however, that for higher response levels, the concentration addition model cannot always be used in a straightforward manner, due to its limitation to model the concentration relationship only up to the effect level of the component with the lowest effect maximum. The advanced extrapolation approach by Scholze et al. (2014) for such cases was used here and provided evidence that the observed responses were in good agreement with expected CA (Fig. 3b). As a novel finding of this study we conclude that seven of the bioassays used here, representing different receptor-mediated responses, were able to capture expected combined effects against a background of multiple other compounds with known bioactivities. We also deduce that confounding factors such as cytotoxicity or other interfering biological activities (e.g. overlap between competitive agonists and antagonists) should be recorded to avoid mislead.

#### 4.3. Effect detection for mixture comprising multiple modes of action

Looking at the coverage (Fig. 4, Table SI 4) of biological response across various bioassays, the following picture emerges. For 7 of the 12 compounds a response-specific assay would detect a component of the mixture at the lowest concentration. For 9 of the 12 components, however, apical assays, in particular the daphnia assay, are among the two most sensitive to detect a component from the mixture studies. The explanation is straightforward, while the assays designed to detect specific biological effects are expected to capture few components from the studied mixture (here typically two), the bioassays using apical effect observation show combined effects from three (Microtox) to seven (daphnids) components (Table SI 4), thus explaining their apparent sensitivity.

The selection of compounds in this mixture study reflected the heterogeneity of water contaminants and the diversity of their anticipated modes of action (Busch et al., 2016). More detailed reflection of the plausibility of mode of action specific responses are given in the SI.

While the experimental mixture results cannot proof the occurrence of a specific mode of action, we confirmed that a compound's specific MoA can be mirrored by using an adequate receptor-based assay in case of receptor-transmitted endocrine effects. However, neither do we have bioassays that capture all known modes of action ready for contaminant monitoring, nor do compounds always adhere to one specific or even receptor-related mechanism of action, e.g. Bisphenol A is known to act via several pathways until leading to an adverse outcome (Goodson et al., 2015). We also demonstrated that apical effect detection captures more comprehensively the complexity of mixture contamination through joint effect description. Therefore, the specific bioassays discussed here as potentially suitable for monitoring are rather diagnostic indicator systems for groups of compounds, whereas interpretations with respect to biological adverse effects need separate research in the frame of e.g. the AOP concept.

Several suggestions and considerations on the assembly of bioassay panels for water monitoring purposes have been made in the literature (e.g. Diamond et al., 2011, Escher and Leusch, 2012, Wernersson et al., 2015, Di Paolo et al., 2016, Neale et al., 2017a, 2017b, van der Oost et al., 2017). Mostly, they comprise compilations of available assays filtered by criteria concerning their practicability (e.g. Kienle et al., 2015; Schmidt et al., 2017). Given that all mentioned technical requirements can be adequately accounted for as laid out above, the first question for a panel definition should be whether the monitoring is targeted at exposure diagnosis or towards ecological effect assessment. For example, in drinking water assessment the detection of potential chronic effects on human health such as endocrine, mutagenic, carcinogenic or reproductive effects should be emphasised. Effect-based monitoring would therefore focus on proxies for these, such as receptor-mediated or adaptive stress response assays. If the monitoring purpose is to assess complex chemical contamination with regard to protecting aquatic ecosystems and its services for humans, given the lack of comprehensive coverage of the relevant modes of action using only effect-specific bioassays (Busch et al., 2016), bioassays detecting apical endpoints would be a priority choice.

In summary, we conclude that (i) a modular bioassay panel can accommodate for different application purposes, and (ii) apical bioassays currently continue to have their virtue for panels where comprehensive coverage of contaminants and effect qualities is the goal.

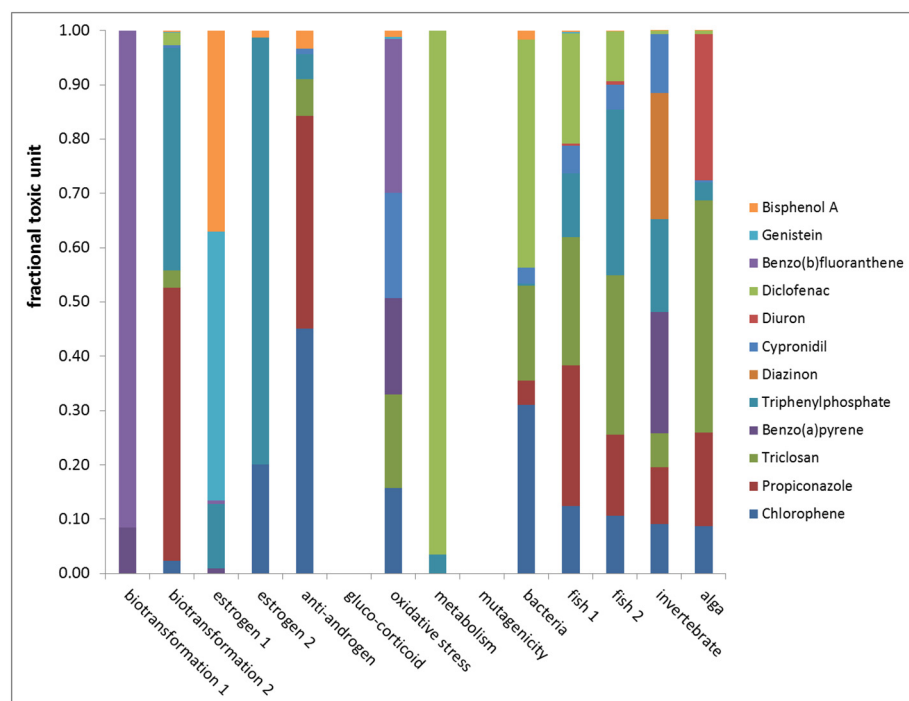


Fig. 4. Scaled toxic unit contribution to the concentration additive mixture effect in the various bioassays displayed by the indicated effect type (see Table 1) for Mix I; effect indication - assay assignment from left to right: biotransformation 1 - AhR CALUX, biotransformation 2 - HGN5LN-hPXR, estrogen 1 - MELN; estrogen 2 - ChgH-GFP; anti-androgen - Anti-MDA-kb2; glucocorticoid - GR CALUX; oxidative stress - AREc32; metabolism - PPAR- $\gamma$ UAS-293H; mutagenicity - AMES, bacteria - Microtox; fish 1 - zFET-well plate; fish 2 - zFET-glas vials; invertebrate - daphnia immobilisation, alga - algal population growth.

Although apical assays respond to many chemicals present, they give only limited information on the mode of action. (iii) Reporter gene assays that target exclusively one specific molecular initiating event or one key event (e.g. adaptive stress response) will only be responsive to a limited number of chemicals and therefore cytotoxicity may be a severe problem as it can mask any specific effects. Therefore, those assays are only valid if it is assured that they are run at non-cytotoxic concentrations. These conclusions are in line with literature suggestions derived from complex environmental samples testing including wastewater and surface water (Diamond et al., 2018; Neale et al., 2017a, 2017b).

## 5. Conclusions

We conclude from this interlaboratory mixture study that it is possible to decipher combined effects from multiple mixture exposure as they might occur in water monitoring. Concentration addition provides a worst case component-based prediction for combined effects on apical effect endpoints and serves as a reasonable model for receptor-based responses. Thus, component-based predictions and mass balance comparison of chemically determined contaminants and bioanalytical effects are possible. Such studies require adequate quality controls for confounding factors such as concomitant cytotoxic effects that may mask specific effect potencies. Also, exposure regimes in high-throughput bioassay protocols need to be designed to account for processes that could lead to loss of bioavailable concentrations. For a comprehensive effect monitoring of chemicals that are known to occur in freshwaters, apical effect assays are essential, as we do not yet have all the methods to specifically account for all or even only the most relevant modes of action. Assays detecting specific effects lend themselves to diagnostic monitoring.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2018.02.013>.

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