Mixture Modelling and Effect-Directed Analysis for Identification of Chemicals, Mixtures and Effects of Concern

Peta A. Neale and Beate I. Escher

Abstract

The complex mixtures of organic pollutants detected in environmental matrices means that chemical analysis alone does not provide a full picture of the chemical burden. Instead, bioassays, which detect the effects of all active chemicals in a sample, are proposed as complementary tools for environmental monitoring. This chapter outlines relevant mixture toxicity modelling concepts and demonstrates how the bioanalytical equivalent concentration approach (BEQ) can be used to evaluate the effects of environmental mixtures. Using iceberg modelling, BEQ from bioanalysis and BEQ from chemical analysis can be compared to determine how much of the effect can be explained by detected chemicals, with examples of iceberg modelling in water and sediment discussed. In the case of contamination hotspots, effect-directed analysis can be applied to identify unknown bioactive chemicals using a combination of fractionation, bioanalysis and chemical analysis with structural identification. Finally, effect-based trigger values derived by reading across from existing chemical guideline values were proposed to assess whether the effects of chemical mixtures in water are acceptable or unacceptable. This chapter highlights the importance of using bioassays in parallel to chemical analysis for environmental monitoring to gain a better understanding of the overall chemical burden.

Keywords

Bioassay • Chemical analysis • Concentration addition • Effect-based trigger values • Iceberg modelling

1 Introduction

The aquatic environment contains numerous organic pollutants, such as pesticides, pharmaceuticals and industrial compounds, with chemicals detected in different environmental matrices, including water, sediment and biota (Kadokami et al. 2013; Loos et al. 2013; Scott et al. 2018). Water quality monitoring typically relies on targeted chemical analysis, which provides concentrations of known chemicals in a sample and allows comparison of detected concentrations with available guideline values for priority chemicals. However, chemical analysis alone cannot provide a complete picture of the chemical burden in water as it cannot detect unknown chemicals or account for the mixture effects that occur between the many chemicals in a sample (Wernersson et al. 2015). Chemicals are often present at low concentrations (pg/L to μg/L range) in water and may be below analytical detection limits, but the mixture effects of many chemicals at low concentrations can still result in an observable effect (e.g. "something from nothing" effect) (Silva et al. 2002).

Owing to the limitations associated with chemical analysis, bioanalysis is proposed as a complementary tool for environmental monitoring. Bioassays, which are also known as effect-based methods or bioanalytical tools, provide information about the mixture effects of all chemicals in a sample and are risk-scaled, with potent chemicals having a greater effect in the bioassay (Escher and Leusch 2012). Test batteries of in vitro bioassays indicative of different stages of cellular toxicity pathways, including xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cell viability, as well as early-life stage in vivo assays indicative

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of apical effects, have been proposed for water quality monitoring (Fig. 1) (Neale et al. 2017a). This combination of bioassays is recommended in order to capture modes of action commonly detected in water and to prevent overlooking any unexpected effects. While bioassays detect the mixture effects of chemicals in a sample, they cannot provide information about which chemicals are contributing to the effect, though some assays, such as those indicative of receptor-mediated effects, are highly specific to certain classes of chemicals, for example natural and synthetic estrogens in the activation of the estrogen receptor (ER) assay. Consequently, both bioanalysis and chemical analysis are required to gain a comprehensive understanding of the chemical burden in a sample and subsequent mixture effects.

This chapter will outline relevant mixture toxicity modelling concepts and how chemical analysis and bioanalysis can be applied to evaluate environmental mixtures and identify causative chemicals. It will primarily focus on water samples, reflecting much of the research in this area to date, though some examples for sediment will also be discussed.

2 Mixture Toxicity Modelling

Mixture toxicity concepts can be classified based on chemical mode of action and chemical interaction. Assuming that the chemicals in a mixture do not interact, the mixture toxicity of chemicals that share a common mode of action can be described by the model of concentration addition (CA), while the model of independent action (IA) is used to predict the mixture toxicity of dissimilarly acting chemicals (Backhaus and Faust 2012). The effect concentration predicted by CA ($EC_{y,CA}$) is calculated using Eq. 1, where p_i is the fraction of each chemical i in the mixture and $EC_{y,i}$ is the effect concentration at any effect level y for chemical i. IA predicted effect (E_{IA}) is determined using Eq. 2, where E_i is the effect of each chemical in the mixture.

$$EC_{y,CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{y,i}}} \quad (1)$$

$$E_{IA} = 1 - \prod_{i=1}^{n} (1 - E_i) \quad (2)$$

Chemicals that interact at the toxicokinetic or toxicodynamic level can be described by synergism, where the mixture components have a greater effect than expected based on CA, or antagonism, where the mixture components show less effect than IA. While synergism and antagonism are observed in some binary mixtures, these interactions are not likely to be relevant in environmental samples containing many chemicals at low concentrations (Cedergreen 2014). Instead, CA is considered a conservative model to evaluate the mixture toxicity of dissimilarly acting compounds (Backhaus et al. 2000) and is suitable to predict the toxicity of chemical mixtures in assays indicative of receptor-mediated effects (Kortenkamp 2007; Tang and Escher 2014), adaptive stress responses (Escher et al. 2013) and apical effects (Tang et al. 2013b; Altenburger et al. 2018). The effect of chemical mixtures that act in a concentration additive manner can be translated to bioanalytical equivalent concentrations (BEQ) (Sect. 2.1) or toxic units (TU) (Sect. 2.2).

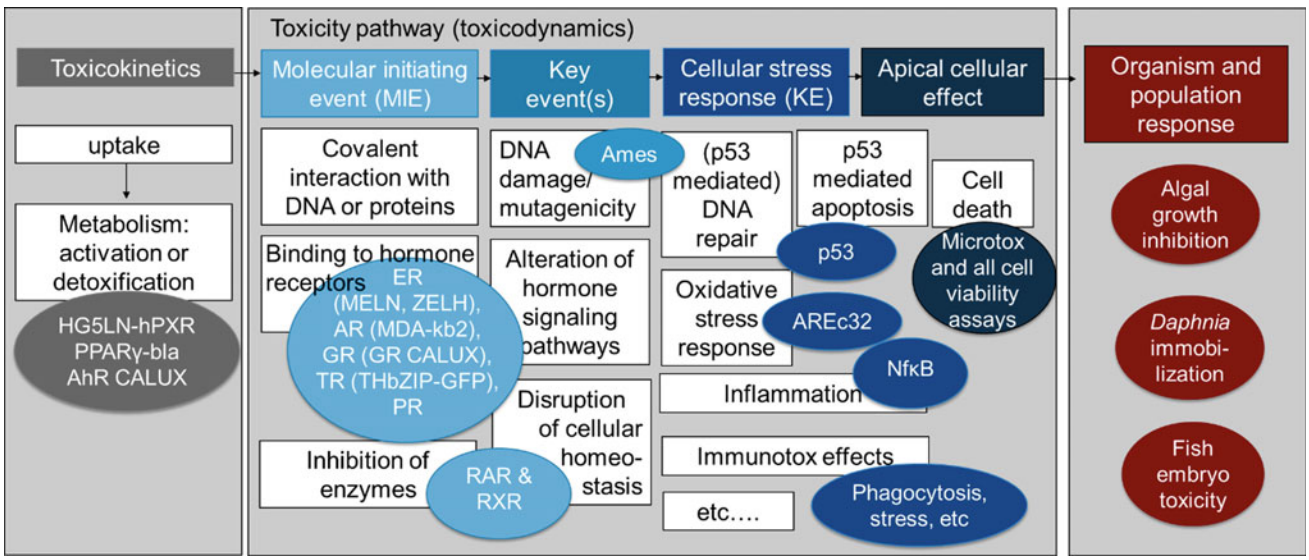

Fig. 1 Proposed bioassay test battery covering assays indicative of different stages of the cellular toxicity pathway and apical effects in whole organisms. Reprinted from Neale et al. (2017a). Copyright 2017, with permission from Elsevier

2.1 Bioanalytical Equivalent Concentrations

The BEQ from bioanalysis (BEQ_{bio}) relates the effect of a complex chemical mixture (EC_y (sample)) to the effect elicited by the assay reference compound (EC_y (ref)) (Fig. 2). This approach was initially applied to receptor-mediated effects, such as estradiol equivalent concentrations (EEQ) for assays indicative of estrogenic activity (Murk et al. 2002; Rutishauser et al. 2004), but is now applied more widely to assays indicative of xenobiotic metabolism, adaptive stress responses and non-specific toxicity (Escher et al. 2008, 2012; Neale et al. 2015). The BEQ from chemical analysis (BEQ_{chem}) is calculated using the molar concentration of each detected chemical i and its relative effect potency (REP_i) in the assay (Fig. 2). REP_i is determined based on the effect of the reference compound and the effect of the detected chemical i, with only experimental EC_y (i) data used (Eq. 3).

$$REP_i = \frac{EC_y(ref)}{EC_y(i)} \tag{3}$$

BEQ_{bio} and BEQ_{chem} can be compared to determine the contribution of detected chemicals to the observed effect using an approach termed iceberg modelling (Eq. 4). Iceberg modelling has been applied to different environmental samples, including water, sediment and biota, with examples provided in Sect. 3. BEQ_{bio} and BEQ_{chem} can be used in mass balance models to understand how much of the effect is explained by detected chemicals (e.g. how much of the iceberg is visible) and how much of the effect is due to undetected chemicals ($BEQ_{unknown}$) (Fig. 2). The BEQ approach was also used to differentiate the effect of micropollutants and disinfection by-products in drinking water by comparing BEQ_{bio} before and after chlorination (Hebert et al. 2018).

$$\%\,\text{effect explained} = \frac{BEQ_{chem}}{BEQ_{bio}} \cdot 100\% \tag{4}$$

Iceberg modelling relies on experimental effect data for each detected chemical in order to calculate REP_i and the lack of available effect data is a limitation of this approach. However, US federal research collaborations Toxicity Forecaster (ToxCast) and Toxicology in the 21st Century (Tox21) have greatly increased the amount of available data, with effect data for over 9000 chemicals in up to 1192 assays in the iCSS ToxCast Dashboard (https://actor.epa.gov/dashboard). Several studies have also fingerprinted environmentally relevant chemicals in a range of bioassays (Leusch et al. 2014; Neale et al. 2017a).

2.2 Toxic Units

Effect data is also expressed in TU, particularly for in vivo assays. TU from chemical analysis (TU_{chem}) are often used for chemical risk assessment (Kuzmanovic et al. 2015;

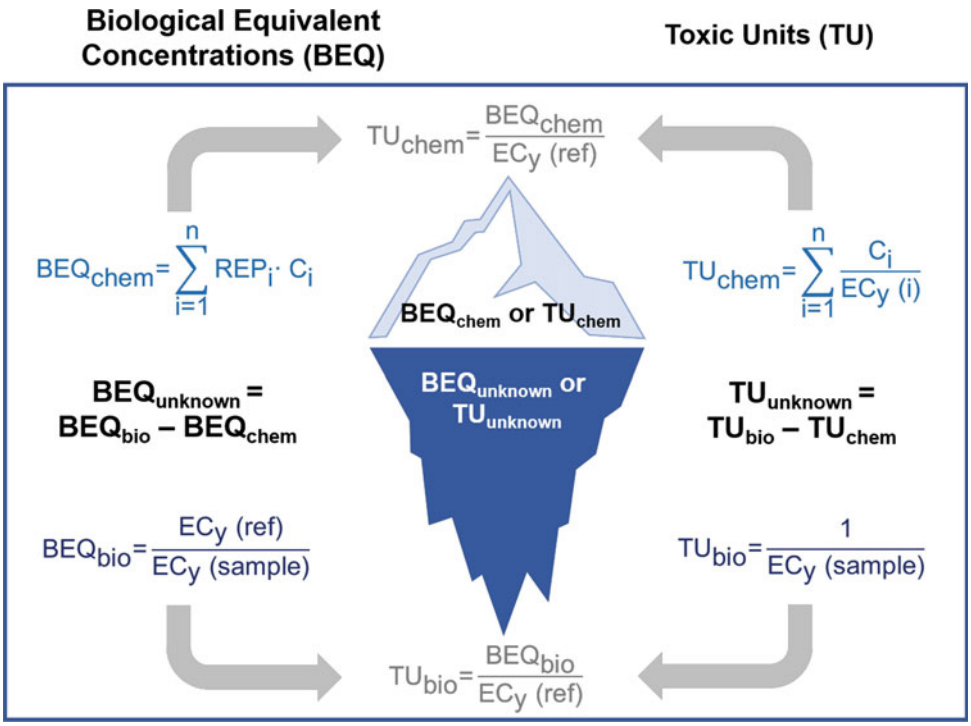

Fig. 2 Bioanalytical equivalent concentration (BEQ) and toxic unit (TU) approaches for evaluating mixture toxicity, with equations linking BEQ and TU shown in grey. NB: C_i: concentration of chemical i; EC_y: effect concentration; ref: reference compound; REP_i: relative effect potency

Beckers et al. 2018) and are calculated based on the detected chemical concentration and effect in a target organism (e.g. algae, daphnids, fish), with both experimental and Ecological Structure Activity Relationships (ECOSAR) predicted effect data used (Fig. 2). The TU are summed for each detected chemical to determine TU_{chem} and, like BEQ, CA is assumed. TU can also be calculated based on bioanalysis (TU_{bio}) (Fig. 2) and several studies have compared TU_{chem} and TU_{bio} to determine which chemicals are contributing to the observed effect (Booij et al. 2014; Tousova et al. 2017; Guo et al. 2019).

As shown in Fig. 2, the BEQ and TU approaches are essentially equivalent, so both approaches can be used for in vitro and in vivo assays. The main difference is that only experimental EC values are used for BEQ_{chem}, while both experimental and predicted EC values are used to calculate TU_{chem}. Thus, TU_{chem} cannot be converted to BEQ_{chem} if predicted EC values are used. Owing to the inclusion of predicted data, a larger fraction of the effect may be explained using the TU approach compared to the BEQ approach. For example, between 0.84 and 20.6% of the effect in the 96 h fish embryo toxicity (FET) assay could be explained by detected chemicals in European surface waters using the TU approach with effect data for 90 chemicals predicted using the ECOSAR program (Tousova et al. 2017). In contrast, no more than 0.33% of the effect in the 48 h FET assay could be explained in the Danube River using the BEQ approach with experimental EC values for 19 detected chemicals (Neale et al. 2017a). These examples are not directly comparable as they represent different sampling sites, but help to illustrate the potential differences between BEQ and TU. It should be noted that the lower reliability of predicted data compared to experimental data is a limitation of the TU approach. Consequently, this chapter will primarily focus on the BEQ approach. It would also be possible to close data gaps in the BEQ approach using QSAR predictions, but to our knowledge there are no studies where predictive methods for effects were combined with a BEQ approach. The typically lower number of chemicals that are characterized for their effects is a limitation of the present application of the BEQ approach.

3 Iceberg Modelling Examples

Iceberg modelling using the BEQ approach has been applied to different environmental matrices to determine the contribution of detected chemicals to the observed effect. This section will focus on examples from the literature for water and sediment.

3.1 Water

Iceberg modelling has been applied to drinking water (Hebert et al. 2018; Shi et al. 2018), surface water (Neale et al. 2015; Conley et al. 2017), storm water (Tang et al. 2013a), wastewater and recycled water (Murk et al. 2002; Mehinto et al. 2015; Jia et al. 2016) and swimming pool water (Yeh et al. 2014). These studies have focused on a range of endpoints from different stages of cellular toxicity pathways (Fig. 3), though estrogenic activity is by far the most studied endpoint. In most cases, the effect in assays indicative of molecular initiating events, such as hormone receptor-mediated effects and photosystem II (PSII) inhibition, can be explained by the detected chemicals (e.g. Bengtson Nash et al. 2006; Jia et al. 2016; Conley et al. 2017). For example, over 100% of PSII inhibition in the Brisbane River can be explained by three herbicides, diuron, simazine and atrazine (Bengtson Nash et al. 2006), while simazine and atrazine also explain most of the PSII inhibition in samples from an advanced water treatment plant (Escher et al. 2011). Similarly, potent estrogenic hormones, such as estrone and 17β-estradiol, typically explain most of the estrogenic activity in wastewater and surface water, with other weakly estrogenic compounds (e.g. bisphenol A, nonylphenol) only having a minor contribution to the effect (Rutishauser et al. 2004; Neale et al. 2015; Conley et al. 2017; Konig et al. 2017). In some studies only a small fraction of estrogenic activity could be explained (e.g. <5%), but this can be attributed to the presence of active compounds that are below the instrument limit of quantification but still contributed to the mixture effects in the sample (Escher et al. 2011).

Detected chemicals often only explain a small fraction of the effect in assays indicative of xenobiotic metabolism, adaptive stress responses and whole organism effects (Fig. 3). In many cases, <1% of the effect could be explained in the pregnane X receptor (PXR) (Creusot et al. 2010; Neale et al. 2017a), peroxisome proliferator-activated receptor (PPARγ) (Konig et al. 2017), oxidative stress response (Escher et al. 2013; Yeh et al. 2014; Neale et al. 2017b), p53 response (Neale et al. 2015) and FET assays (Neale et al. 2015). Unlike assays indicative of molecular initiating events, a wide variety of different chemicals can contribute to the effect in these assays.

Owing to low environmental concentrations, water samples need to be enriched prior to chemical analysis and bioanalysis, with solid-phase extraction (SPE) often applied to enrich water samples. Low recovery of some chemicals could mean that BEQ_{chem} underestimates the effect. However, recent studies have shown similar recovery of both individual

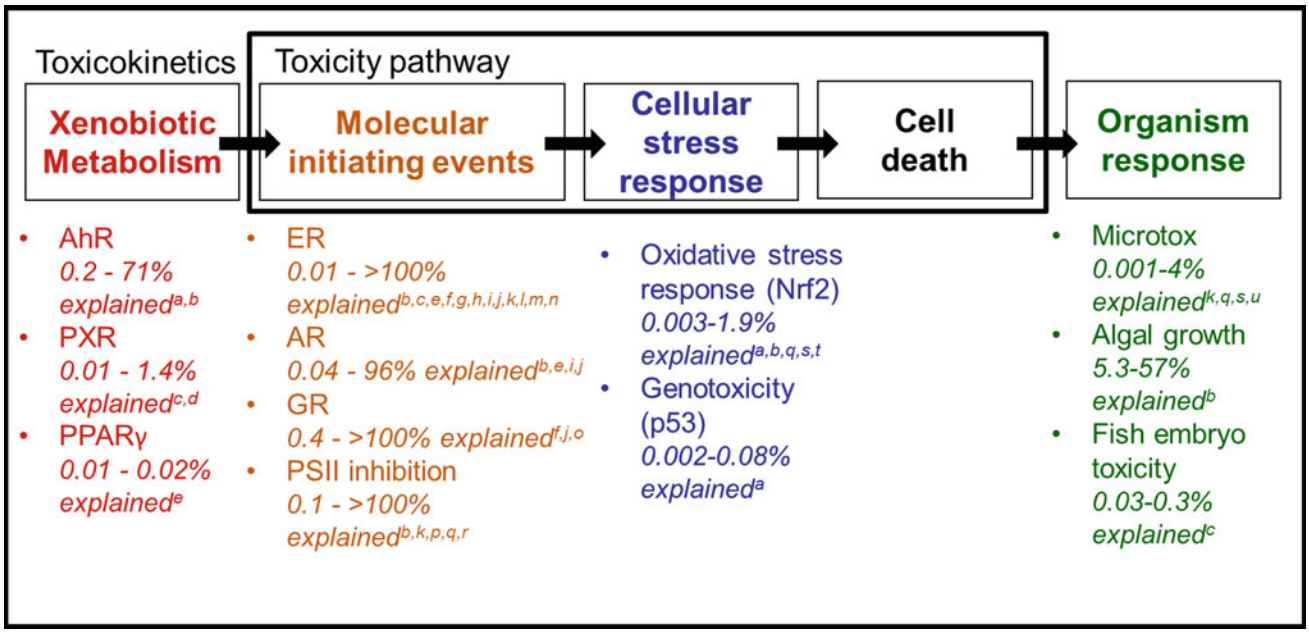

Fig. 3 Examples of assays from the literature where iceberg modelling using the BEQ approach has been applied to determine the fraction of effect explained by detected chemicals. [a]Neale et al. (2015); [b]Neale et al. (2017b); [c]Neale et al. (2017a); [d]Creusot et al. (2010); [e]Konig et al. (2017); [f]Mehinto et al. (2015); [g]Fang et al. (2012); [h]Chou et al. (2015); [i]Conley et al. (2017); [j]Tousova et al. (2017); [k]Escher et al. (2011); [l]Murk et al. (2002); [m]Rutishauser et al. (2004); [n]Shi et al. (2018); [o]Jia et al. (2016); [p]Bengtson Nash et al. (2006); [q]Tang et al. (2014); [r]Tang and Escher (2014); [s]Yeh et al. (2014); [t]Escher et al. (2013); [u]Tang et al. (2013b). NB: AhR: aryl hydrocarbon receptor (AhR); PXR: pregnane X receptor; PPARγ: peroxisome proliferator-activated receptor; ER: estrogen receptor; AR: androgen receptor: GR: glucocorticoid receptor; PSII: photosystem II

chemicals and biological effect by common SPE sorbents (Neale et al. 2018; Simon et al. 2019). Further, reverse recovery modelling was used to calculate BEQ_{chem} assuming 100% chemical recovery and showed good agreement with BEQ_{chem} from the literature, supporting the application of iceberg modelling for water extracts (Neale et al. 2018).

In addition to SPE, several studies have applied iceberg modelling to passive sampler extracts from surface water and wastewater (Vermeirssen et al. 2010; Creusot et al. 2014; Hamers et al. 2018; Novak et al. 2018; Tousova et al. 2019). Passive samplers absorb chemicals from the water phase over the deployment period and represent a time-integrated picture of the chemical mixture in water. Passive sampling showed similar trends to SPE extracts, with detected chemicals explaining the majority of estrogenic activity and PSII inhibition in wastewater (Vermeirssen et al. 2010; Escher et al. 2011), but <1% of PXR and oxidative stress response in river water (Creusot et al. 2014; Novak et al. 2018). Up to 10% of the aryl hydrocarbon receptor (AhR) response could be explained in wastewater effluent (Hamers et al. 2018). The fraction of effect explained is also affected by the type of passive sampler deployed, with polar samplers, such as polar organic chemical integrative sampler (POCIS) or Empore disks, more suitable for estrogenic compounds and non-polar samplers, such as semi-permeable membrane device (SPMD) or silicone rubber, more appropriate for capturing hydrophobic contaminants. For example, a greater fraction of the effect in the activation of AhR assay could be explained by detected chemicals in SPMD extracts compared to POCIS extracts (Tousova et al. 2019). In contrast, a larger fraction of the estrogenic activity could be explained in Empore disk extracts compared to silicone rubber extracts (Novak et al. 2018). Therefore, the type of passive sampler will affect the chemical mixture extracted.

3.2 Sediment

Compared to water extracts, fewer studies have applied iceberg modelling to sediment extracts, with most focusing on AhR and estrogenic activity endpoints. For example, David et al. (2010) found that detected polycyclic aromatic hydrocarbons (PAH) explained between 63 and 121% of the effect in river and coastal sediment in the ethoxyresorufin-O- deethylase (EROD) assay after 4 h, but only 4–19% of the effect in the same assay after 24 h, suggesting undetected persistent contaminants were contributing to the effect. Further, up to 55% of the estrogenic activity in sediment from the Upper Danube River could be explained by detected chemicals (Grund et al. 2011), though $BEQ_{unknown}$ was >94% for most of the sediment extracts. While most studies apply iceberg modelling to in vitro assays, Hu et al.

(2015) found that 54–125% of the effect in the 48 h *Daphnia magna* immobilization test could be explained by detected chemicals in sediment from an agriculturally impacted lake, with insecticides chlorpyrifos and cyfluthrin explaining most of the effect.

Exhaustive solvent extraction is commonly used to extract the total chemical mixture prior to bioanalysis. Creusot et al. (2016) used the iceberg modelling approach to assess effect recovery of spiked endocrine disrupting chemicals in artificial sediment using a range of solvents with pressurized liquid extraction. Extraction with 50:50 dichloromethane/methanol was optimal, with over 90% recovery of effects in assays indicative of activation of ER and activation of PXR. In addition to solvent extraction, the bioavailable chemical mixture in sediment can be analysed using passive sampling with polydimethylsiloxane (PDMS). Using an assay indicative of activation of AhR, Li et al. (2016) used iceberg modelling to evaluate the contribution of total and bioavailable PAHs to the effect in exhaustive solvent and PDMS extracts, respectively. Up to 41% of the effect could be explained by PAHs in the exhaustive solvent extract, while up to 71% could be explained in the PDMS extract, though the fraction explained varied greatly with different sampling sites.

4 Iceberg Modelling Reveals Different Bioassay Categories

From the literature reviewed in Sect. 3, iceberg modelling indicates that bioassays generally fall into one of two categories: category 1 and category 2 bioassays (Escher et al. 2018) (Fig. 4). Category 1 bioassays detect the effect of defined mixtures where a small number of highly potent chemicals explain up to 100% of the effect. Category 1 bioassays are indicative of receptor-mediated effects, such as activation of hormonal receptors and PSII inhibition in green algae. In contrast, category 2 bioassays detect more integrative effects and include assays indicative of adaptive stress responses, most notably the oxidative stress response, and apical effects in whole organisms. These assays detect the mixture effects of mainly low potency chemicals, so the detected chemicals will only explain a small fraction of the effect. For example, <2% of the oxidative stress response was explained in wastewater effluent and surface water from small streams in Switzerland despite effect data available for 26 detected chemicals (Neale et al. 2017b). A wide range of chemicals can induce a response in category 2 bioassays and analysing more chemicals will not close the gap between the measured and predicted effect. This highlights the importance of using bioanalysis as well as chemical analysis for environmental monitoring.

It should be noted that not all assays will fit neatly into the two bioassay categories. For example, while detected chemicals typically explained <1% of the effect in some xenobiotic metabolism assays (e.g. PXR and PPARγ), a greater fraction of the effect could be explained in some cases for activation of AhR assays, particularly in sediment (David et al. 2010; Li et al. 2016) and biota (Jin et al. 2013, 2015a, b). Similarly, some compound classes will have a specific effect in whole organism assays indicative of apical effects, such as PSII inhibiting herbicides in algal growth assays and acetylcholinesterase (AChE) inhibitors in *D. magna* (Neale et al. 2017a).

5 Identifying the Drivers of Toxicity Using Effect-Directed Analysis

Effect-directed analysis (EDA) is a tool that can be used to identify unknown chemicals that are driving toxicity in a contaminated site. After identification of a biologically active environmental extract, sample complexity is reduced by chromatographic fractionation, then additional bioanalysis is conducted to identify active fractions (Brack et al. 2016). These active fractions undergo chemical analysis and structural identification to identify the contributing chemicals, with the effect of the identified causative chemicals confirmed in the studied bioassay. As EDA aims to identify the chemicals that are driving the observed effect, it has primarily been applied to category 1 bioassays (e.g. assays indicative of receptor-mediated effects) in matrices including water, sediment and biota (Houtman et al. 2004; Weiss et al. 2009; Creusot et al. 2013; Sonavane et al. 2018). While iceberg modelling using the BEQ approach applies targeted chemical analysis to identify chemicals contributing to the effect, structural identification of unknown chemicals in active fractions in EDA can result in the discovery of new potent causative chemicals. For example, Muschket et al. (2018) found that 4-methyl-7-diethylaminocoumarin (C47), a compound used in consumer products, could explain the anti-androgenic activity in a polluted river. Further, two aromatic amines, 2,3- and 2,8-phenazinediamine, were found to explain up to 86% of the mutagenic activity in surface water receiving industrial wastewater effluent (Muz et al. 2017). EDA can also reveal the effects of contaminants masked by antagonists or non-specific toxicity (Brack et al. 2016).

EDA is not suitable for category 2 bioassays where a large number of chemicals contribute to the mixture effects. This was demonstrated by Hashmi et al. (2018) for an oxidative stress response assay, with the combined effect of the fractionated samples only able to explain 16% of the oxidative stress response detected in the unfractionated sample. While whole organism assays indicative of apical effects are often considered as category 2 bioassays, some assays sensitive to specific chemical modes of action are suitable for EDA. For example, insecticide methyl parathion

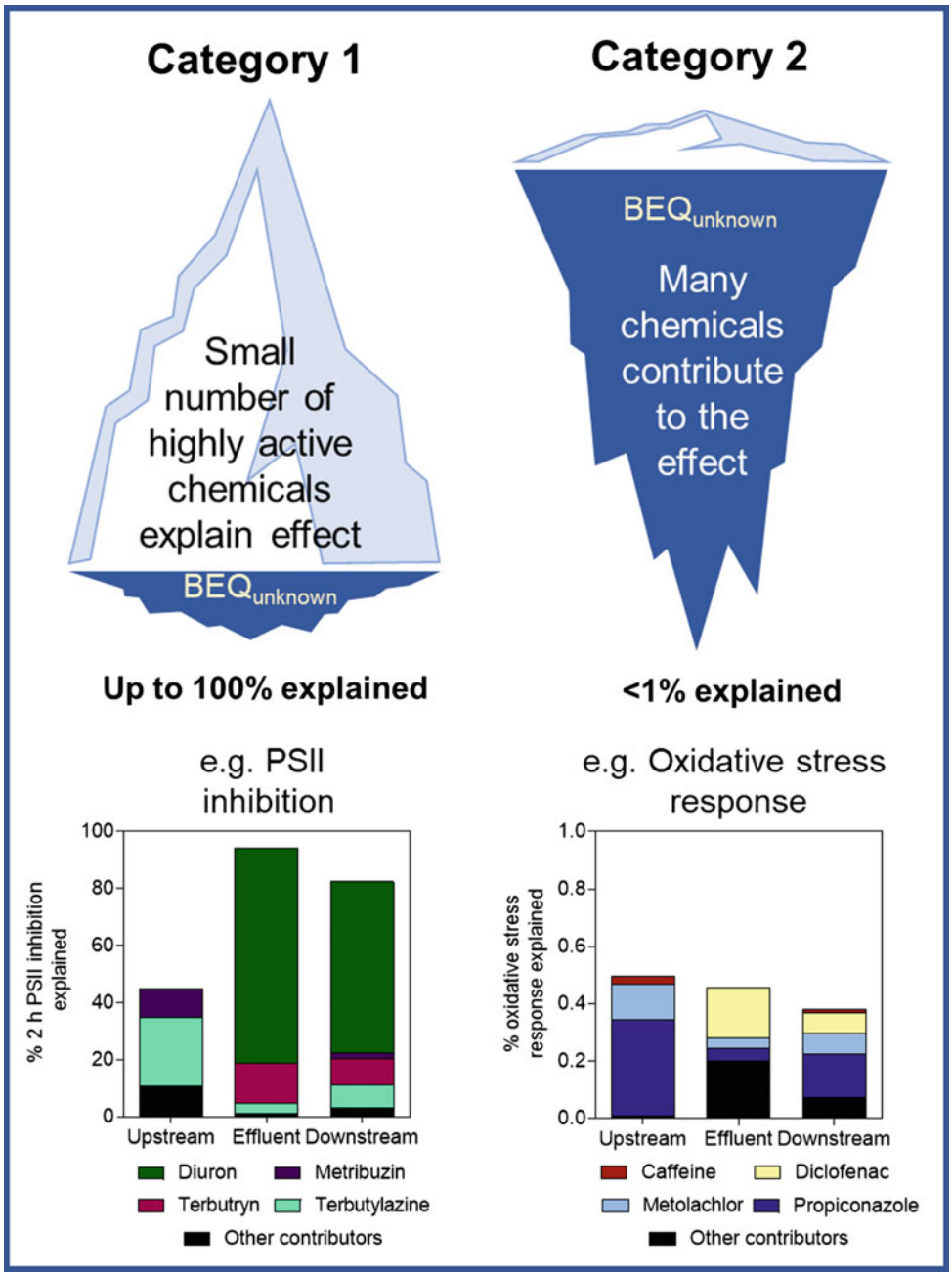

Fig. 4 Category 1 bioassays include assays indicative of receptor-mediated effects and most of the effect is explained by known highly active chemicals. Category 2 bioassays include assays indicative of apical effects and adaptive stress responses and typically only a very small fraction of the effect can be explained. Photosystem II (PSII) inhibition and oxidative stress response data adapted from Neale et al. (2017b)

could explain 97% of the effect in *D. magna* in one fraction of river sediment, though the observed effect in other fractions could not be fully explained by detected chemicals (Brack et al. 1999).

6 Effect-Based Trigger Values for Environmental Mixtures

The chemical status of water is typically evaluated based on chemical guideline values for priority chemicals, but, as mentioned in Sect. 1, this approach does not consider chemical mixture effects. While bioassays can detect the effects of complex chemical mixtures, the combination of high sample enrichment and increasingly sensitive bioassays means that effects can be detected in clean samples. For bioassays to be used for water quality monitoring, it is important to be able to differentiate between an acceptable effect in water and an unacceptable effect. Consequently, effect-based trigger values (EBTs) have been developed for drinking water (Brand et al. 2013; Escher et al. 2015), surface water (van der Oost et al. 2017; Escher et al. 2018) and wastewater effluent (Jarosova et al. 2014). EBTs are assay-specific and the comparison of an EBT with an effect

detected in a water sample can indicate whether the sample is compliant or not, with an exceedance of an EBT indicating further investigation, such as chemical analysis, is warranted.

Most studies initially focused on hormone-receptor mediated effects (e.g. category 1 bioassays), but more recently EBTs have been derived for assays indicative of xenobiotic metabolism and adaptive stress responses and apical effects in whole organisms (e.g. category 2 bioassays). Several approaches have been applied to derive EBTs, but one simple method to calculate EBTs is to convert existing guideline values to BEQ using the chemical concentration from the guidelines and its REP_i. This approach was applied to derive EBTs for drinking water using the Australian Guidelines for Water Recycling (Escher et al. 2015) and for surface water using average annual Environmental Quality Standards (AA-EQS) from the European Union Water Framework Directive (Escher et al. 2018), with 32 preliminary EBTs derived for surface water. In the case of category 2 assays, where a large fraction of the effect is not explained by known chemicals, an additional mixture factor was included. Reading across from existing guideline values does have some limitations, including the lack of effect data for some guideline chemicals and the lack of guideline values for potent chemicals in some important environmental endpoints such as glucocorticoid activity. However, the approach can be applied to any bioassay and represents an important step forward towards regulatory acceptance of bioassays.

7 Conclusions

The complementary use of bioassays and chemical analysis represents a valuable approach to evaluate the effects of environmental mixtures and determine the contribution of detected chemicals to the observed effect. Both BEQ and TU approaches have been applied to describe the mixture effects in environmental samples, but the two approaches are interchangeable, provided only experimental effect data is used to derive TU_{chem}. Iceberg modelling has shown that most of the mixture effect can be explained by detected chemicals for category 1 bioassays (e.g. assays indicative hormone receptor-mediated effects), with EDA a suitable approach to detect unknown active chemicals in these assays. While the lack of single chemical effect data is a limitation for some less-studied bioassays, detecting more chemicals will not close the BEQ mass balance for category 2 bioassays as a wide range of low potency chemicals contribute to the effect. This highlights the importance of a combined chemical analysis and bioanalysis approach as it provides a better picture of the chemical burden. Chemical mixtures are currently not considered when evaluating the chemical status of water in a regulatory context, but EBTs that read across from existing chemical guideline values represent away forward. EBTs for some category 1 bioassays are already quite advanced, though further work is still required to develop robust EBTs for category 2 bioassays.

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