Effect-Directed Analysis of Ah-Receptor Mediated Toxicants, Mutagens, and Endocrine Disruptors in Sediments and Biota

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Abstract Sediments and associated biota represent important sources for the exposure of aquatic organisms to environmental toxicants including dioxin-like compounds, genotoxic chemicals, and endocrine disruptors. One of the key challenges that environmental toxicologists and risk assessors are facing is the characterization and assessment of toxicological risks associated with such complex matrices such as sediments. Therefore, approaches have been developed supplementing chemical analysis with bioanalytical techniques that make use of the specific properties of certain groups of chemicals to interfere with specific biological processes. This type of analysis has been coined effect-directed analysis (EDA), and is based on a combination of fractionation procedures, biotesting, and subsequent chemical analyses. In this chapter, we review the current state of the art of EDA regarding the assessment of sediment and biota samples for dioxin-like, genotoxic, and endocrine disrupting potentials. We discuss in vivo and in vitro screening concepts that are used in combination with fractionation and chemical analytical techniques to aid in the risk assessment of these chemical groups in sediments and biota. Advantages and disadvantages of current EDA strategies are considered, and recommendations for more realistic and relevant EDA approaches are given. Specifically, these include the use of optimized biotest-batteries covering a broad range of different endpoints as well as the inclusion of in vivo tests, and the parallel assessment of ecologically relevant parameters such

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as benthic community structure. Furthermore, the need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to unknown or emerging chemicals such as endocrine disruptors, perfluorinated compounds, or polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans is discussed.

Keywords Bioassay, Complex mixtures, Dioxin-like chemicals, Endocrine activity, Fractionation, Genotoxicity, In vitro, Toxicity identification and evaluation

Contents

1 Background

Since the middle of the twentieth century there has been increasing concern over the potential adverse effects of xenobiotics present in the environment on human and wildlife populations. Three groups of toxicants that are of particular interest relative to potential environmental health effects are dioxin-like, genotoxic, and endocrine active chemicals. Some of these ubiquitous compounds are hydrophobic, lipophilic, and resistant to biological and chemical degradation. These properties impart persistency and a propensity to bioaccumulate and biomagnify to concentrations that can cause deleterious effects. Sediments, especially, represent long-term sinks for some contaminants [1–8], which can become bioavailable through remobilization events such as floods or dredging, or through benthic or bottom-dwelling organisms [9–13]. Severely contaminated sediments have been reported to occur in rivers and lakes, and have been shown to be acutely and chronically toxic to fish and benthic invertebrate species [8, 14–16]. Under certain conditions, toxicity of sediments has been reported to contribute to decreases in reproductive success, recruitment, and alterations in community structure, effects which could potentially cause changes in population structure. For example, amphipod abundance in San Francisco Bay was found to be inversely proportional to contamination and measured toxicity of sediments [14]. Studies have shown that toxic sediments can affect aquatic species. Zebrafish

embryos exposed to sediments from the Danube River showed impaired development and decreased hatching rates compared to control fish [8]. Sediments have been classified as genotoxic, mutagenic, endocrine disrupting, or recognized for dioxin-like effects [8, 15, 17, 18]. In addition to their acute or chronic toxicity to benthic invertebrates, accumulated residues in sediments can be a pool that can be accumulated through food webs and have subsequent toxic effects on the predators.

Chemicals typically occur as mixtures in environmental matrices such as sediments and biota, and can include different congeners and isomers of both natural and anthropogenic origin. Concentrations and toxic potencies of compounds present in mixtures can range over several orders of magnitude. In addition, interactions among different classes of compounds, such as estrogenic vs. anti-estrogenic, can modulate the toxic potential. Exposure to mixtures and the potential for greater and less than additive effects of mixtures complicates hazard evaluation and risk assessment of complex mixtures of xenobiotics. So, even if complete information is available about the concentrations of all inorganic and organic residues, it is difficult to predict the effects of the mixture. However, this complete knowledge is seldom the case. In fact there might be residues present that have not yet been described in the literature. There are contaminants and/or their degradation products that can cause toxicity for which there are no analytical methods or authentic standards. Furthermore, toxic effects of some contaminants, even those, which are analytically determined, are not well characterized. There are potentially significant classes of contaminants for which little or no information on their effects on organisms is available. In other words, chemical analysis has been used to identify and quantify only those chemicals for which analytical techniques and standards are available. In environmental monitoring, chemists find what they look for. If they do not know to look for a chemical, then it will not be quantified. Finally, instrumental analyses do not account for interactions among the chemicals in mixtures and provide little information on their biological availability and provide no information on their effects. Thus, relying on quantification of individual residues by instrumental analyses, while useful for source identification, can underestimate the potential risks posed by these chemicals and some toxicologically important compounds could be overlooked. In summary, analysis of the vast number of chemicals typically present in an environmental sample would not only be prohibitively expensive but simply impossible due to limits in the available analytical methodologies for many chemicals, especially since often no a priori knowledge of the chemicals present in the sample exists.

1.1 Toxicity Identification and Evaluation vs. Effect-Directed Analysis

To overcome some of the above discussed limitations, bioanalytical approaches have been developed to supplement instrumental chemical analysis. These bioanalytical techniques make use of the specific properties of certain groups of chemicals

to affect biological systems. Such bioassays are often based on in vitro responses of cells or even cell-free responses of biomolecules but can also utilize whole organism in vivo systems. The use of bioassays in chemical identification has several advantages relative to instrumental analyses. First, the assays "read out" directly in terms of a biological response. This could be a molecular change or even something as simple as lethality. The bioassay responds to all of the chemicals in the mixture so even unidentified chemicals that cause a particular endpoint are measured even if they cannot be identified. These assays also respond proportionally to the aggregate effects of mixtures and account for all of the interactions among chemicals. In fact, bioassays can be used in conjunction with instrumental analyses in a "potency balance" approach that helps determine if there are unidentified active compounds. In particular, bioassays can be used with a fractionation scheme to direct instrumental analyses to identify unidentified active chemicals in a mixture.

In the late 1980s, one of the first standardized effect-directed analysis (EDA) procedures, the toxicity identification and evaluation (TIE) approach, had been established by the US-EPA. This approach focuses primarily on the identification and evaluation of organic or inorganic contaminants in aqueous samples using a combination of in vivo tests, fractionation, and chemical analysis, and is characterized by the following three steps (reviewed in [19]):

1. Toxicity characterization by assignment of toxicity to general groups of toxicants (typically bioassay directed analysis)
2. Identification of suspected toxicants (chemical analytical determination)
3. Confirmation of the suspected cause of toxicity

The basic concept of the TIE approach is the removal of compound groups from the tested matrix until the toxicity of the sample disappears. Suspected chemicals are then identified by analytical chemistry, and their toxicity is confirmed by means of the same bioassay used in the initial characterization phase. Approaches like TIE have been an important step towards improving environmental risk assessment (ERA) focusing on surface waters. It has been increasingly recognized, however, that particle-bound contaminants such as suspended matter or sediments, or those accumulating through the food chain might be of greater ecotoxicological relevance with respect to moderately or strongly lipophilic compounds. While there have been some recent developments of standardized EPA techniques and procedures for sediment TIE [20, 21], there are still a number of uncertainties associated with this approach. For example, it is often difficult to construct an artificial mixture of the suspect compounds identified in the characterization phase, because these may not always be commercially available. Furthermore, exposure of tests organisms as part of the confirmation step is problematic since it requires spiking of clean or artificial sediments with the suspected chemicals, and which often alters the toxicological properties of the tests matrix because of differing adsorption and/or bioavailability properties. Finally, TIE primarily relies on whole organism tests that often do not provide information on the specific mode of toxicity of a sample, and which can be helpful in identifying the compound groups responsible for the measured effect.

A recent approach that aims to overcome some of the shortcomings of TIE is the EDA procedure as reviewed by Brack [19, 22]. Like TIE, the principle of EDA is to use a biological response to direct the identification of causative agents in a complex matrix such as sediment. During the conduct of an EDA, however, the causal substances are identified by analyzing different extracts that were prepared from the original sample, e.g., by separation based on lipophilicity, polarity, size, etc., and not by a retrospective step-by-step exclusion of certain compound groups as done in TIE. Furthermore, during EDA the biological analysis phase is typically dominated by in vitro or in vivo mechanistic assays that enable identification of the properties of a sample to interact with specific biological pathways or targets, and thus, can narrow the group of suspected chemicals (e.g., dioxin-like chemical that can be detected by means of an AhR assay as described in Sect. 2 in [23]). Thus, EDA has some distinct advantages over sediment TIE by better intertwining the specific biological (toxicity) and analytical pathways. However, the TIE approach is considered to be of greater ecological relevance because it does not require alteration of the sample through extraction, and utilizes whole organisms that are tested in direct contact with the matrix of concern.

1.2 Dioxin-Like Activities

Chemicals that elicit toxic effects similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), known as dioxin-like chemicals, are of concern due to their ability to cause hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, carcinogenesis, wasting syndrome, and tumor promotion in many different species at relatively small concentrations [24, 25]. A number of studies have demonstrated that several toxic and biochemical effects caused by dioxin-like chemicals are mediated through the aryl hydrocarbon receptor (AhR) [26, 27] and associated dioxin responsive elements (DREs) on DNA. The AhR, which belongs to the basic helix-loop-helix protein family [28], is a ligand-dependent transcription factor, complexed with heat shock proteins located in the cytosol. The strength with which congeners bind to the AhR is directly proportional to the toxicity, enhanced gene transcription, and enzyme activities mediated by the AhR mechanism [29]. The role of the AhR in mediating toxic and biological effects of dioxin-like chemicals has been well documented in a number of studies, even though the exact biochemical mechanism leading to the pleotrophic toxic responses is yet to be elucidated [22]. After binding of ligands to cytosolic AhR, heat shock proteins dissociate from the complex, the receptor ligand complex is activated and translocated to the nucleus, where it forms a dimer with the Ah receptor nuclear translocator (ARNT) protein and possibly other factors. The heteromeric ligand: AhR:ARNT complex binds with high affinity to specific DREs on DNA. Binding of the transformed ligand-AhR-ARNT complex to DREs results in DNA bending, disruption of chromatin and nucleosome, and thus increased promoter accessibility and transcriptional activation of adjacent responsive genes (Fig. 1) [31, 32].

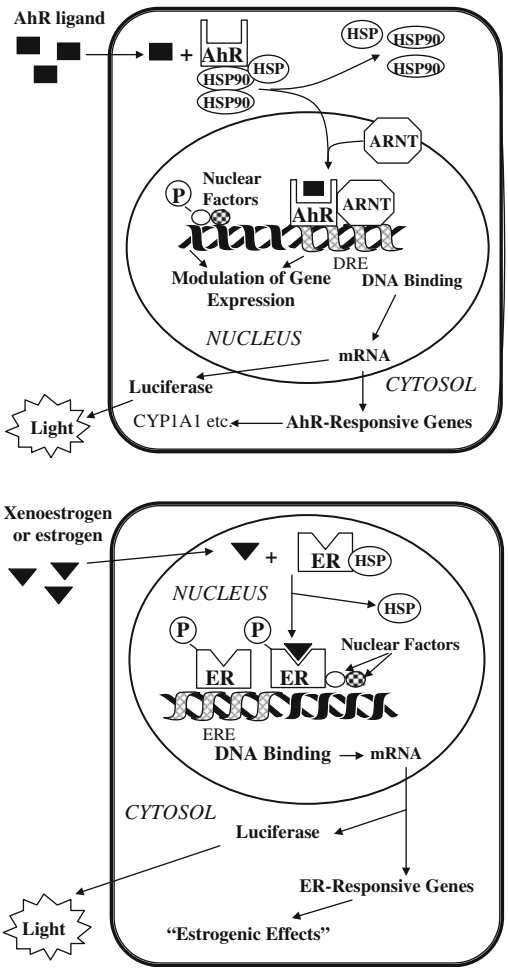

Fig. 1 Mechanism of AhR- or ER-receptor-mediated response in cells (adapted from [30]). For description see text. *HSP* heat shock proteins, *P* = phosphates: phosphorylation is an important regulatory factor for receptor function

The traditional, well-known ligands for AhR have been described as hydrophobic aromatic compounds with planar structure of a particular size of the molecule or a part of the molecule, which fits the binding sites [33, 34]. Thus, the ability of these ligands to bind to the AhR and to cause toxic effects greatly depends on their structure and substitution pattern. These include planar congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDDs and PCDFs), chlorinated azobenzenes and azoxybenzenes, polychlorinated biphenyls (PCBs), several polycyclicaromatic hydrocarbons (PAHs) and polychlorinated napthalenes (PCNs) [35]. Other chemicals that have been suggested as potential AhR agonists due to their stereochemical configuration, but not yet experimentally confirmed, include polybrominated and chloro-/bromo- analogs of the previously listed classes of compounds [36], alkylated-chlorinated dioxins and furans, chlorinated

dibenzothiophenes, chlorinated xanthenes and xanthones [37], polychlorinated diphenylthiophenes (PCDTs), anisols (PCAs), anthracenes (PCAN), fluorenes (PCFL), and others [38]. Recently, new types of relatively weak AhR ligands or inducers (compared to TCDD) have been identified, which include both natural and synthetic compounds [31]. These compounds deviate from the traditional criteria of planarity, aromaticity, and hydrophobicity. The natural compounds that bind to the AhR include, among others, indoles, tryptophan-derived products, oxidized carotinoids, and heterocyclic amines. Some pesticides or drugs with various structures, such as imidazoles and pyridines, also possess the AhR binding ability. These ligands act as transient inducers and bind to the AhR with weak affinity and are rapidly degraded by the induced detoxification enzymes.

1.3 Genotoxicity

Chemicals in the environment can cause overt toxicity, but they can also cause subtle changes that may not result in immediate toxicity. One such effect is genotoxicity. Chemicals, such as biotransformation products of some polycyclic aromatic hydrocarbons (PAH), which are common contaminants in sediments, can bind to DNA where they cause a number of types of damage. The resulting DNA adducts can result in point mutations or strand breaks or other types of reorganization of the DNA that can result in adverse effects in germ cells and can result in decreased fitness of individuals in subsequent generations [39].

A number of in vitro and in vivo techniques have been developed to screen for these effects. Here we provide three examples of tests that have been found to be useful in screening of sediments for genotoxic potentials. The first measures point mutations or mutagenicity in vitro, while the second is an in vivo test that measures the occurrence of DNA strand breaks. The Ames assay uses the TA 98 *Salmonella typhimurium* bacteria strain to measure frame-shift mutations and the TA 100 *S. typhimurium* bacteria strain to measure base pair substitutions [4]. These strains are mutants that cannot produce histidine. A colorimetric measure of the number of back-mutated cells that are able to produce histidine is used as a measure of the mutagenic potency of a pure chemical or sediment extract.

Some chemicals need to be metabolically activated to cause mutagenicity. Because *Salmonella* do not possess the metabolic machinery to bioactivate molecules such as certain PAHs, S9 microsomal preparation can be added to samples. The Ames assay can be conducted using different bacteria strains, the most used of which are the mutated strains (TA 98 and TA 100) and with and without preincubation of the extract with the S9 microsomes to enable a comprehensive assessment of the mutagenic potential of a sample.

Another method to determine genotoxic effects measure DNA fragmentation [40]. This assay, which is variously called the alkaline DNA unwinding assay or the comet assay, measures small fragments of DNA that occur due to breaks in the DNA. Under alkaline conditions double-stranded DNA will unwind, such that when

separated by polyacrylamide gel electrophoresis (PAGE), the smaller fragments of DNA migrate more quickly than the larger strands of DNA. The more fragmentation, the more bands that can be identified. Because of the pattern formed on a two-dimensional PAGE that represents a comet, this assay is often referred to as the "Comet" assay [41]. DNA strand breaks can be studied in either in vitro or in vivo assays.

A third type of genotoxicity assay that is commonly used to assess genotoxicity of chemicals or complex matrices such as sediments is the micronucleus test. The assay measures the formation of a micronucleus during the metaphase/anaphase transition of mitosis, e.g., as the result of an acentric chromosome fragment detaching from a chromosome after breakage, and which coalesce into bodies of chromatin material referred to as micronuclei. The number of micronuclei present in cells is directly proportional to DNA damage [42].

1.4 Endocrine Disruption

Over the past 2 decades, there has been increasing concern about the possible effects of chemicals in the environment on the endocrine and reproductive systems in humans and wildlife [43, 44]. Such compounds have the potential to disrupt normal reproduction or developmental processes which can lead to adverse health effects such as compromised reproductive capacity, breast and testicular cancer, reproductive dysfunction such as feminization or demasculinization of males, and other adverse effects. A range of classes of compounds including natural products, pharmaceuticals, pesticides, and other industrial chemicals have been shown to affect endocrine systems of wildlife and humans. The manner by which these chemicals can interact with the endocrine system is manifold, and in general it is distinguished between compounds that elicit their response through binding to hormone receptors, and those that act through other mechanisms such as interference with steroidogenesis. While any chemical that causes an organism to be unable to maintain homeostatic regulation could be classified as an endocrine disrupting chemical (EDC), chemicals classified as EDCs have typically been those that are either able to bind to hormone receptors or can modulate the expression of steroid hormones such as estrogens or androgens or thyroid hormones. There are both natural and artificial chemicals that can modulate the endocrine system. These chemicals can be direct-acting and cause effects as receptor agonists or antagonists or can have indirect effects that ultimately modulate expression of genes that lead to effects that are similar to those caused by direct-acting effects. For example, a chemical that can induce the activity of CYP19 (aromatase) can result in the production of more endogenous estradiol (E2), and subsequently would cause an estrogenic effect even though it might not bind to the estrogen receptor (ER).

Much of the research in the area of environmental endocrine disruption has been focused on chemicals that can bind to hormone receptors including the estrogen receptor (ER) and androgen receptor (AR) as either agonists or antagonists.

However, various modes of actions have been reported, which include binding of chemical to other nuclear receptors, which then interact with an estrogen responsive element; acting through other receptors and/or signal transduction pathways; modulations of steroidogenesis and catabolism of active steroid hormones [45–48]. Estrogenic compounds are characterized by their ability to bind to and activate the estrogen receptor, which is a transcription factor belonging to the steroid receptor family. While there are structural similarities among some compounds that are ER agonists, other ER-active compounds do not share similar structures. Upon binding of an estrogenic compound to the ligand binding domain of the ER (located predominantly in the nucleus), the associated heat shock protein complex, which masks the DNA binding domain, dissociates and subsequently the ligand occupied receptor dimerizes. The homodimer complex interacts with specific DNA sequences referred to as estrogen response elements (EREs) located in the regulatory regions of estrogen-inducible genes. ER complexes bound to an ERE recruit additional transcription factors, leading to increased gene transcription and synthesis of proteins required for expression of hormonal action (Fig. 1) [49]. A series of natural and synthetic endocrine disrupting compounds have been identified by different in vivo and/or in vitro methods. Unlike chemicals that can directly interact with the nuclear hormone receptors, there is a multitude of different ways by which chemicals can interact with other endocrine processes such as steroidogenesis. For instance, substances such as some imidazole-like fungicides and phyto-flavonoids have been shown to modulate hormone production by affecting activities of the steroidogenic enzymes aromatase (CYP19) and 17β-hydroxysteroid-degydrogenase (17β-HSD), respectively [45, 46, 50]. Other chemicals such as naphthenic acids can inhibit estradiol metabolism, and thereby increase estradiol concentrations in vitro [51].

EDCs such as pesticides, plasticizers, plant sterols, PAHs, etc., have all been measured in sediments and have been shown to disrupt the endocrine system in in vitro and in vivo assays [46, 52, 53]. For example, known estrogen receptor agonists, such as 17α-ethinylestradiol (EE2), 17β-estradiol (E2), and bisphenol A, have been measured in sediments in several ecosystems [54–56]. Sediment extracts from the Upper Danube River produced estrogenic-like responses in a transcriptional ER assay [8]. It has also been reported that the same sediments caused embryo toxicity, disruptions in hatching rates and time to hatch in *Danio rerio* embryos [8]. Other endocrine effects that were caused by sediment-associated contaminants were changes in the expression of key genes involved in steroidogenesis [57], and alteration in the production of the sex steroids testosterone (T) and E2 [18] using the H295R cell line.

2 Effect-Directed Analysis in Sediments and Biota

Specific testing systems have been developed for the detection of dioxin-like, genotoxic, and endocrine active potential in environmental samples. These systems can be separated into two general categories: (1) in vivo assays using whole

organisms, or (2) in vitro tests utilizing cellular or sub-cellular systems that detect interactions with specific biological functions. These bioassays are used to assess the net effects of a complex sample to an animal or in vitro system. Organisms are predominantly used to identify effects of sediment-bound pollutants in direct contact assays with the unaltered sample on apical endpoints such as growth, reproduction, and survival. They are typically utilized to assess the biological risk of a given exposure but often do not allow pinpointing the effect to specific contaminants in a sample. Therefore, whole organism assays are often paired with a parallel exposure assessment by means of a combination of in vitro assays and analytical chemistry. In vitro bioassays are based on the responses of either wild type or genetically altered eukaryotic or prokaryotic cells that enable the assessment of potencies of individual chemicals or complex mixtures of environmental contaminants in extracts to cause effects specific to the exposure with certain chemical groups. Specifically, either endogenous responses or specific exogenous induced alterations incorporated into a cell are used for the measurements. The induction of specific responses following the exposure of cells to specific chemicals or mixtures of compounds can be assessed by measuring endogenous or engineered responses such as mutations, DNA strand breaks, protein expression, enzyme activity, etc., depending on the test system and endpoint.

2.1 In Vivo Bioassays

There are a number of organisms that are amenable to determine the toxicity of sediments [58]. Here we will focus on those that are most useful for studying the three classes of chemicals discussed in this chapter. While there are a number of hypotheses and recommendations for the use of invertebrate systems to assess the endocrine-disrupting potential of sediments to date, only very few studies have used this approach relative to EDA [59, 60]. One of the key issues associated with the use of different species is uncertainties regarding the predictability of endocrine effects to vertebrates because they have different hormone systems. Also, since most invertebrates do not express the AhR, they are essentially unresponsive to dioxin-like compounds. Finally, while benthic invertebrates might represent useful sentinels for the assessment of genotoxic potentials of sediments [61], they are rarely used in this context.

For application in the EDA process described here, therefore, we will focus on vertebrate assays because they have been successfully used in EDA of sediments. There have been numerous efforts to use fish species such as sanddab (*Citharichthys stigmaeus*), California halibut (*Paralichthys californicus*), flounder (*Platichthys flesus*), or trout (*Oncorhynchus mykiss*) to assess exposure to dioxin like or EDCs in sediments [54, 62–64]. However, most of these studies utilized large organisms in time- and cost-intensive experiments. One promising test is the zebrafish (*D. rerio*) embryo sediment contact test [15, 65].

The zebrafish is a small, easily cultured freshwater fish that reaches sexual maturity in approximately 3 months. They produce between 50 and 200 eggs every 2–3 days and the embryos develop rapidly. The eggs have a transparent chorion, which makes it relatively easy to monitor development of the embryo. The size of the zebrafish allows exposures to be performed in 24-, 48-, or 96-well culture plates. The protocol for using these fish is fairly simple. Zebrafish embryos 1–2 h postfertilization are exposed directly to sediments or to diluted sediment extracts in 96-well plates in 100 µL ISO water containing 20 mM $CaCl_2 2H_2O$, 5 mM $MgSO_4 7H_2O$, 7.5 mM $NaHCO_3$, and 0.037 mM KCl. Embryos are covered with an oxygen permeable cover and incubated at 27°C for 48 h. The embryos can be monitored for various endpoints, including lethality and deformities, but can also be used in subsequent measures of gene expression and enzyme activities such as CYP1A, which is under the control of the DRE, and genotoxicity, e.g., by means of the comet assay [15, 66]. The only shortcomings of embryo-based assays, such as the zebrafish egg contact test, are its limitation to measure effects on the endocrine system due to the lack of sexual differentiation at this life stage.

2.2 In Vitro Bioassays

In vitro bioassays have been used to assess dioxin-like, genotoxic, and endocrine activities in a variety of environmental matrices, including sediments and biota. Various environmental samples, such as sediments [4, 18, 52, 53, 67, 68] or particulate matter [69, 70], sludge [71, 72], and animal tissues, have been assessed regarding their potential to cause toxicity in vivo or in vitro. Significant dioxin-like activity has been observed in egg extracts of birds such as herring gull, cormorant, and great blue heron [73, 74] as well as in birds at different stages of development [75]. Among other animals, extracts of fish (white sucker, juvenile whitefish) [37, 72], bivalves [76], and otter [77] have also been tested. Different than in tests with live organisms, in vitro assays typically require clean up and extraction of the original sediment or tissue samples prior to testing. This is usually done through extraction by organic solvents. The solvent of choice needs to be compatible with the cell system, not causing any effect by itself, but enabling distribution of the extracted material to the cells. Extracts can be cytotoxic, which is caused by some compounds present in complex mixtures. For example, sulfur is a major cytotoxic constituent in sediment extracts, which should be eliminated prior to performing dioxin-like or estrogenic activities. The measurement of cell viability/cytotoxicity is essential in all bioassays dealing with complex mixtures as well as single compounds. Cell bioassays with multi-well plate formats enable the measurement of several samples at the same time. In addition, current procedures allow subsequent measurement of viability index, enzyme activity, and protein content in the same multi-well plates [35].

A number of different measurement endpoints are used to assess the exposure to dioxin-like, genotoxic, and endocrine active chemicals. Exposure to chemicals that exhibit dioxin-like properties can be measured by increased expression and induced

activity of cytochrome P4501A1 and its monooxygenase activities, such as 7-ethoxyresorufin *O*-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) [63, 78]. Genotoxicity can be assessed by measuring a variety of endpoints, including DNA strand breaks using, e.g., the comet assay, micronucleus formation, and mutations [40, 79–81]. The potential of chemicals or environmental samples to interact with the endocrine system is typically assessed by means of three endpoints: (1) binding to the estrogen receptor (ER), (2) binding to the AR, and (3) alteration of sex steroid production through interaction with steroidogenic pathways. Determination of the potential of a chemical to interact with the ER or AR is either conducted by means of direct receptor binding assays or by transcriptional assays using genetically modified cells [49]. The latter are typically obtained by transfection of the so-called wild type cells with recombinant expression vectors, which contain selective responsive elements upstream of a reporter gene. The most common reporter genes are firefly luciferase (luc), alkaline phosphatase (PAP), chloramphenicol acetyl transferase (CAT), or β galactosidase (LacZ) [82, 83]. Effects on steroidogenesis can be measured at the gene, protein, or end-product level. Common assays include the quantification of changes in steroidogenic or metabolic genes [84], CYP19 aromatase enzyme activity (transforms androgens into estrogens) [46], or quantification of steroid hormones [47, 85]. A list of some bacterial, yeast, animal, and human cell lines used for the detection of in vitro TCDD-like, genotoxic, or endocrine activity is shown (Table 1).

2.3 Bioassay-Directed Fractionation

As discussed previously, in vitro bioassays as well as certain in vivo tests such as the *D. rerio* embryo sediment toxicity assay can be used in support of the characterization of complex mixtures in sediments or biota. Specifically, bioassays can be used in combination with specific analytical techniques in a tiered approach, which is termed bioassay-directed fractionation methodology. This approach provides information needed for monitoring and risk assessment of the compounds with specific modes of action and may lead to identification of novel classes of environmental toxicants [70]. Specifically, a complex sample (e.g., sediment or tissue extract) is first analyzed using one (for specific questions such as the characterization of dioxin-like chemicals) or a combination of multiple (nontarget analysis) bioassays representing different modes of biological action. Parallel to the assessment of effects on specific biological processes, the general toxicity of a sample (e.g., cytotoxicity for a cell-based bioassay) that could mask a specific response is evaluated. If a sample has been identified as having a significantly altered biological activity it is then subjected to fractionation, separating the chemicals contained in it by chemical–physical properties, such as polarity, molecular weight, or any other physical–chemical properties or combinations thereof [19]. These fractions are then again analyzed for their potential to interfere with biological processes using both acute and mechanism specific bioassays. This procedure is repeated until the activity can be pinpointed to one or

Table 1 Examples of cell lines used in effect directed analyses of sediments and biota

Effect type	Cell line	Source	Toxicity endpoints	References
AhR receptor ligands	H4IIE-Luc	Rat hepatoma cell line	Ah receptor-mediated luciferase reporter gene assay	[30, 86]
	RTL-W1	Rainbow trout liver cell line	7-ethoxyresorufin O-deethylase (EROD) activity (CYP1A1)	[68, 87]
Genotoxic chemicals	DNA-repair-deficient DT40	Chicken DT40 B-lymphocyte line	Screening and characterizing the genotoxicity	[88]
	Ames test	Salmonella TA98 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause point mutations	[80]
		Salmonella TA100 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause frame shift mutations	[80]
Endocrine disrupting chemicals	H295R	Human adrenal cancer cells	Endocrine disrupting activities: modulation of steroidogenesis	[84, 85, 89]
	MVLN-assay	Transformed MCF-7 human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay	[90]
	T47D-KBluc	Transformed T-47D human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay	[91]
	MDA-KB	Transformed MDA human breast cancer cell line	Androgen receptor-mediated luciferase reporter gene assay	[92]
	YES	Transformed yeast cell	Estrogen Lac-Z reporter gene assay	[93]
	YAS	Transformed yeast cell	Androgen Lac-Z reporter gene assay	[94]

multiple specific fractions that contain chemicals of certain properties. These fractions are then subjected to chemical analysis for suspected compounds. One possible strategy for toxicants identification and evaluation (TIE) in complex mixtures is shown (Fig. 2). The general steps are as follows: (1) Screening of the whole extract – to determine the samples containing significant toxic potencies, which require further chemical analysis. If no significant response is observed, there is no need to conduct expensive, time- and material-consuming chemical analysis. Since the method detection limit is known for the bioassay, an upper limit of concentration of toxic equivalents in the sample can be defined. (2) Fractionation of the samples that were active in bioassays and chromatographic analysis can be used to determine the most

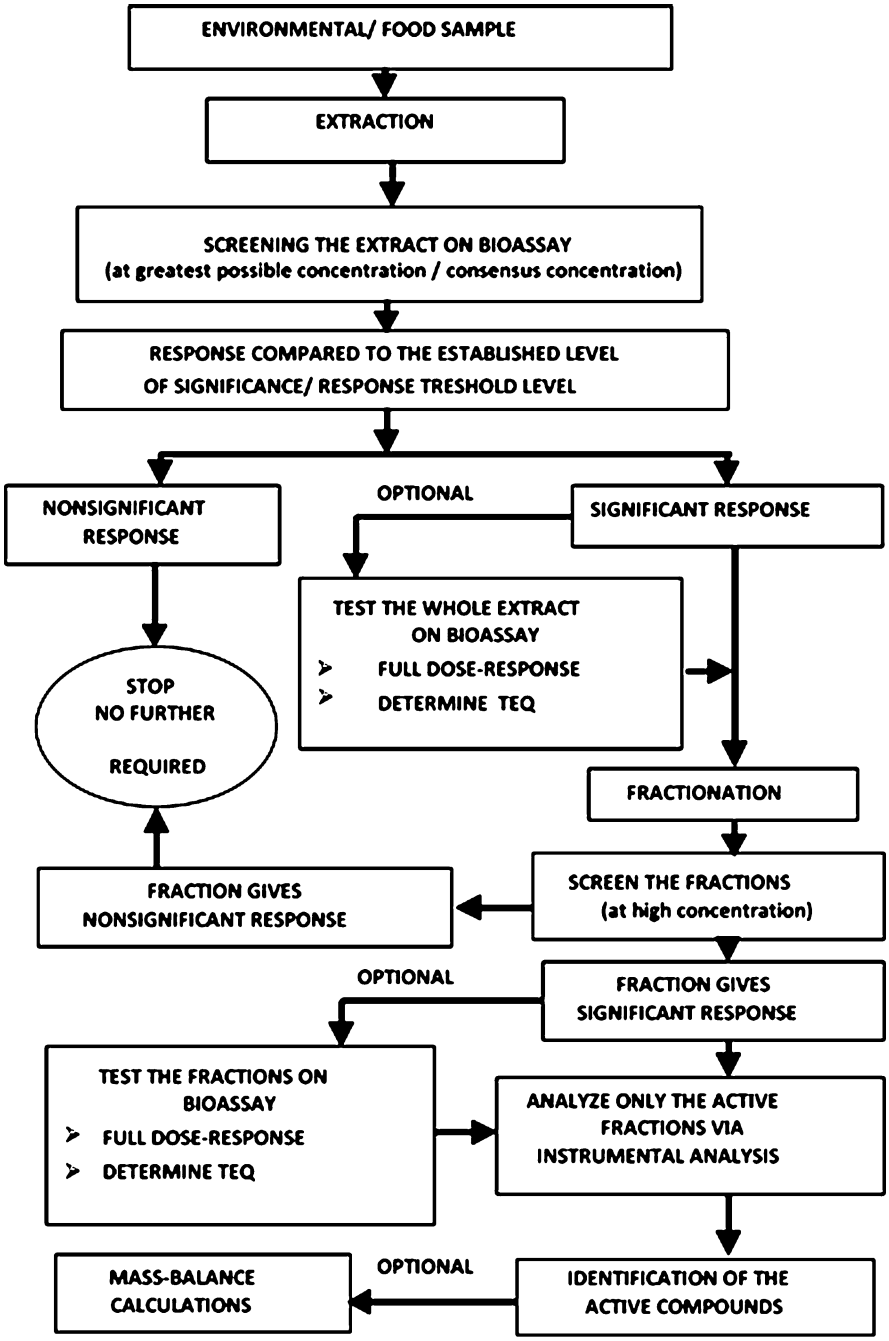

Fig. 2 Screening system: Toxicant identification and evaluation strategy (adapted with permission from [30])

probable contributors to the total activity. (3) Generating the full dose–response relationship of the unfractionated sample or fractions thereof, so that the total activity of the sample can be determined as response equivalents. It should be noted, however, that such a mass-balance approach is only feasible for chemicals for which potencies relative to a positive control, such as 2,3,7,8-TCDD for AhR-mediated responses or E2 for ER binding assays, can be calculated. For more complex assays such as the H295R Steroidogenesis assay that enables the determination of multiple parallel endpoints such as effects on androgen and estrogen production as well as changes in enzyme activity, relative potency approaches have yet to be developed. In such cases, data is simply expressed in terms of changes relative to the applicable solvent control [85].

Where applicable, calculation of the potency balance is accomplished by comparing the potency of a mixture observed in a bioassay with the potency calculated as the sum of the products of concentrations of individual, compounds quantified by instrumental analyses and their RP vales. If the values derived and fractionation does not indicate that there were antagonistic effects in the whole extract, it can be concluded that all of the significant contributors to the total complex mixture have been identified. However, if the total activity determined from the bioassay is significantly greater than those predicted from the instrumental analysis it can be inferred that there are unidentified compounds or that there is synergism. By comparing the activity of the whole extract to that of the various fractions, it is possible to determine if the difference is due to the presence of unidentified compounds or synergism. Also, depending on the fraction where an unknown compound occurs gives insight into the most appropriate instrumental analysis to apply for identification and quantification of active chemicals in a fraction. Antagonisms can occur, particularly between non-AhR-active and AhR-active PCB congeners [38]. These antagonisms will affect the potency balance and need to be considered.

Finally, some nonactive parent compounds in complex mixtures contained in environmental matrices can be metabolically activated to potent inducers of specific biological responses; alternatively the active compound can be biotransformed to nonactive metabolites. This phenomenon can also influence mass-potency balance analysis, metabolic activation or deactivation of chemicals would result in an apparent synergism or antagonism of the chemicals detected in the mixture. For most compounds, the activity of their metabolites is unknown. Some of the cell lines possess a number of metabolic capabilities and upon prolonged duration of exposure they can partly simulate in vivo biotransformation of some compounds. This fact can be used analytically by use of selective inhibitors.

2.4 Relative Potency and Potency Balance Analysis

To apply the potency balance approach with complex mixtures, species- and endpoint-specific relative potencies (RPs) or toxicity equivalency factors (TEFs)

need to be determined relative to validated standard chemicals previously shown to exert the desired effect.

2.4.1 Estimation of Relative Potencies of Complex Mixtures in Sediments and Biota

The RPs of samples are usually calculated as the amount of standard (reference toxicant) giving the same response as the sample, commonly based on the amount of sample needed to produce 50% of the maximal standard response (EC_{50}). The exogenous compound with the greatest known affinity as well as toxicity, 2,3,7,8-tetrachlodibenzo-*p*-dioxin, is used as a standard for AhR-mediated responses. The endogenous substrate E_2 serves as a standard for ER-mediated activity, and the endogenous nonaromatizable androgen dihydrotestosterone (DHT) is used for determining RPs in AR assays. Activities of samples are then expressed as bioassay-derived equivalents: dioxin equivalents (TCDD-EQ), estradiol equivalents (E_2–EQ), or DHT equivalents (DHT-EQ) per specified amount of sample. For calculating and comparing the equivalents, complete dose–response curves from step-wise diluted extracts and standards should be obtained. This is rather difficult with mixture extracts obtained from environmental matrices such as sediments or biota. Common problems encountered when determining the relative potencies of complex mixtures include different efficacy (maximal induced response), nonparallel slopes, cytotoxicity at greater concentrations or insufficient mass of agonists to reach full efficacy or the occurrence of partial agonists that do not attain the maximum possible response. These limitations must be taken into account when calculating the relative potency of the sample. There is always variation in the EC_{50} in replicates measured on different days due to differences in plating density. For some cell lines, the normalization for protein content can solve this problem. For endogenous enzyme activities, the normalization to protein content is necessary. In some transgenic cell lines, normalization to the amount of protein present has been inadvisable because of increased variability of the normalized results. Protein normalization is not recommended in cell lines used for estrogen-receptor mediated activity, where response induction correlates with estrogen-induced protein synthesis [95].

2.4.2 Potency Balance Calculations

In the potency-balance approach, the potency of a mixture to elicit a particular measurement endpoint determined by a bioassay is compared with the sum of potencies of individual compounds determined by instrumental analysis and corrected by application of a RP. This strategy has been widely used for dioxin-like [24, 96] and estrogenic compounds [29]. Toxic equivalents (TEQs) are calculated by multiplying the relative potency (RP) for the specific assay (if available) or the international toxic equivalency factor (TEF) by concentration of the specific

congener giving total sum toxic equivalents per mass unit. For calculating the TEQs from chemical data, effects are assumed to be additive (Equation 1).

$$\mathrm{TEQ} = \sum_{I=1}^{N} \mathrm{CONC.\,OF\ COMPOUND}_i \times \mathrm{TEF}_i \tag{1}$$

TEFs are species-, endpoint-, and assay-specific determination of potency expressed relative to the standard, they can vary widely depending on the species and endpoint. The relative potencies (RPs) should be used for bioassay-directed potency-balance calculation for complex mixtures that are specific for studied endpoint and assay [96]. The international TEFs are consensus values, based on many different types of assays [97, 98] including multiple in vitro and in vivo endpoints for multiple species. TEF values are order-of-magnitude estimates suitable for risk assessment purposes. Because of the differences in RPs among species, specific sets of international TEFs have been established for mammals, fish, and birds [97, 98]. Currently, TEFs and RPs are available for dioxins, furans, some PCBs and PAHs from a number of assays. There are many compounds with potential AhR-mediated activity for which RPs are unavailable and TEFs have not been established. Therefore, those compounds cannot be included in the mass-balance calculations.

Limited RP values are available for estrogenic compounds. RPs have been established only by use of in vitro bioassays for a few alkylphenolic compounds, phytoestrogens, some pesticides, PCBs, and PAHs [95, 99]. In this case, by calculating the E_2-EQs based on analytical results, one can estimate the proportion of the total activity determined by bioassay that is represented by the compounds which have been quantified and have known relative potencies. There are several limitations of calculating TEQs from analytical results: (1) RPs or TEFs are available for only a limited number of chemicals; for some compounds there are no endpoint-specific nor consensus values for TEFs available; (2) TEFs derived for other species, usually from mammals, where the most research has been conducted, may not be suitable for use with nonmammalian species due to the interspecies differences in sensitivity; (3) there may be some compounds not routinely detected whose contribution to the activity would be overlooked; (4) application of the additive approach is routinely used in the total activity calculation; potential interactions among compounds in a mixture, such as synergism or antagonism are neglected; and (5) detailed analysis of trace contaminants require specialized equipment such as HRGC/HRMS (high resolution gas chromatograph/mass spectrophotometry), which is not available in all laboratories and may be prohibitively expensive. Toxic equivalents estimated based on analytical data are correlated with the bioassay results in some situations, depending on the composition of the complex mixture of compounds in the samples. For biota, highly significant correlations have been found between bioassay derived EROD activity and instrumentally measured TEQs in extracts of fish or bird samples [37, 73]. However, toxic activities determined in the bioassays and concentrations of known dioxin-like or

xenoestrogenic compounds are sometimes not correlated. For instance, data obtained from bioassays may be an independent parameter that is predictive of ecotoxicological effects. Besides nonadditive (synergistic or antagonistic) interactions among individual ligands, differences between TEQs derived in bioassays and those calculated from concentrations of individual compounds may be caused by the following events: (1) there are some other active compounds present, which were not identified by the chemical analysis [100]; (2) noncomplete dose responses or cytotoxicity disabling accurate estimations of toxic equivalents; (3) the RPs or TEFs used may not be appropriate. Potencies of mixtures determined by use of bioassays have ecotoxicological relevance because they represent an integrated biological response, but are limited in that the TEQ concentration cannot be "moved" among trophic positions. While the REP can correct for the relative potency of a chemical it does not embody any information on partitioning behavior or degradation or rates dissipation, which are factors that influence disposition of chemicals in the environment.

3 Limitations and Perspectives of Bioassay-Directed EDA

It is necessary to point out disadvantages and limitations of bioassays to be used in support of EDA of sediments and biota. While they provide a greater degree of realism and enable assessment of biological relevant exposures, the main limitations associated with benthic in vivo tests are the lack of specificity of the response and the often resource and time intensive nature of studies including whole organisms, especially vertebrates. The former is particularly of concern in the context of EDA because it limits the characterization of specific types of exposure, which is the core component of bioassay-directed EDA. However, there are currently efforts under way such as the DanTox project that aims to overcome these limitations by utilizing high throughput and mechanism-specific in vitro bioassay formats, including exposure of eggs or embryos [66]. In contrast, in vitro bioassays do not account for the pharmacokinetics, tissue distribution, and biotransformation that may occur in vivo. If cell lines possess only limited metabolic activities, substances active after bioactivation may not be detected by in vitro system [101]. Bioassays do not identify the individual compounds causing the response and are often limited in scope because they focus on specific compounds, e.g. those who act through a specific receptor-mediated mechanism of action. This is especially true in context with the assessment of endocrine disrupting potentials of environmental samples. The majority of studies that were conducted to characterize exposure with endocrine-active chemicals in sediments, effluents, and surface waters focus on the presence of compounds that can interact with the nuclear hormone receptors, namely the ER or AR. Recent studies have demonstrated that relying on a few "popular" endpoints such as ER-mediated effects can provide a false assessment of the actual exposure and are prone to miss relevant effects [18, 102]. In this context, it seems to be commonly misunderstood that the phenomenon of endocrine

disruption is not limited to chemicals that can mimic hormones such as estrogens or androgens. Endocrine disruption appears to be a much broader issue that cannot be seen separated from other classic toxicological issues. This is especially true with regard to impacts on the synthesis or metabolization of sex steroids, processes that primarily depend on P450 enzymes and can be affected by many different chemicals including PCBs, pesticides, etc. [46, 103, 104]. In fact, induction or inhibition of the production of estradiol or testosterone can have much greater impacts than exposure to estrogen or androgen mimics because they are among the most potent receptor agonists. This indicates the need for more comprehensive and integrative approaches in support of EDA of environmental samples such as sediments or biota. Potentials of residues in sediments from the Danube River, Germany would not have been identified if the sediments had been examined solely for ER-modulating potential [18]. To address these issues it is becoming increasingly popular to include a variety of biossays detecting multiple different types of effects in EDA of environmental samples [18, 102]. Furthermore, incorporation of high throughput microwell format, in vivo tests, such as the *D. rerio* embryo assay into such test batteries has the potential to overcome the above discussed concerns regarding the lack of realism of in vitro systems [66, 102]. We strongly encourage expansion of on these recent trends by further broadening the spectrum of endpoints utilized in biotest batteries as well as to integrate in vivo tests such the above discussed *D. rerio* embryo assay used in support of EDA of sediment or biota samples. Similarly, it was recently suggested that EDA could be used as an additional line of evidence in comprehensive weight-of-evidence studies [105], aiming at the identification of the unknown substances responsible for the biological effects in the bioassay paired with parallel assessment of the ecological relevant endpoints such as benthic invertebrate community structure (Fig. 3).

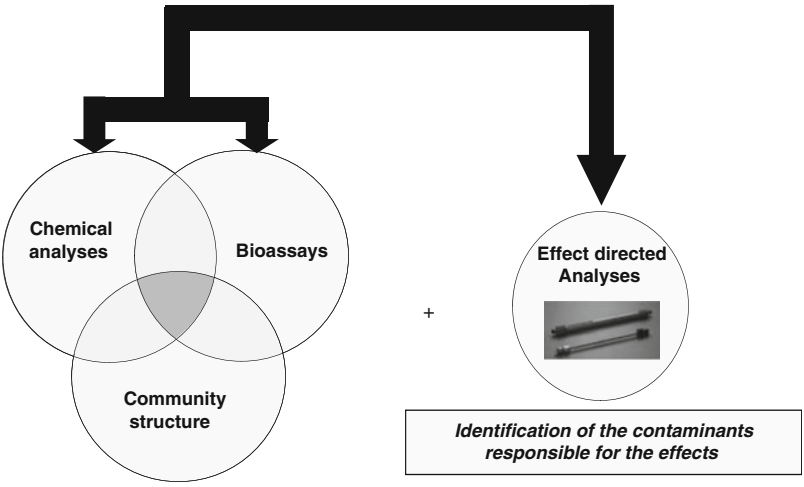

Fig. 3 Recommended combination of the triad approach with EDA (adapted from [105])

There are increasing concerns about emerging contaminants including perfluorinated compound [106, 107] as well as polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans [108]. For these compounds, no, or only a limited number of sufficiently specific bioassays are available, and thus, such exposures often cannot be appropriately addressed. As a consequence, there is still a great need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to these chemicals. Furthermore, while well established for its use in ERA of contaminants such as dioxin-like, genotoxic/mutagenic, or estrogenic substances, the continuing discovery of new contaminant groups of concern in the environment or new effect types pose new challenges to classical EDA approaches. These challenges include establishing bioassays that are specific to the biological activity of chemical groups of concern, as well as the identification and description of relative potencies to model compounds characteristic for these types of effects to enable the utilization of mass-balance approaches.

Another issue is the lack of correlation between the chemical analytical data and the biological response measured by the bioassay. For example, as discussed in one of the previous sections, the chemically detected TEQs can often not explain the biological activity measured with the bioassay (bio-TEQ) (e.g., [109, 110]). Similarly, attempts to correlate genotoxicity of complex environmental samples measured by tests such as the Ames, the micronucleus, or the comet assay with PAH concentrations often fail [111–115], suggesting a contribution of other non-regulated mutagens to the observed biological effect. In a number of studies assessing sediments by EDA it could be demonstrated, for example, that in addition to priority pollutants several nonregulated PAHs, including perylene and benzo[a] fluoranthene, 11H-indeno[2,1,7-cde]pyrene, a methylbenzo[e]pyrene and a methyl perylene, were present at significant concentrations in the analyzed samples [116]. Furthermore, Fernandez et al. [117] showed that more polar compounds, including several polycyclic quinones and nitroquinones, as well as nitro-PAHs, contributed significantly to the mutagenic effects of marine sediments. Furthermore, one of the key challenges for the assessment of the contribution of individual chemicals to the bioassay derived estrogenic potential (estradiol equivalents; EEQs) of a sample is the sensitivity of the utilized analytical method, as has been demonstrated by a study assessing estrogenic compounds in complex environmental samples in the catchment area of the River Neckar, Germany [118]. Those estrogenic chemicals that were detected at concentrations above the method detection limits, including nonyl- and octylphenol, phthalates, PCBs, bisphenol A, and DDT, were only able to explain 9–14% of the total Bio-EEQs. In contrast, when the method detection limits of chemicals that could not be detected by the utilized analytical methods, specifically 17β-estradiol and ethinylestradiol, were taken as a basis of estimation for the Bio-EEQs, 95% of the Chem-EEQ could be explained. Advanced analytical methods for natural and synthetic hormones with lower detection limits such as HRLC/HRMS (high resolution liquid chromatography/high resolution mass spectroscopy) are one way to reduce this problem [119–121].

EDA has been shown to have the potential as a powerful tool in support of ERA, and already is routinely utilized in environmental monitoring programs

[13, 109, 120, 122–125]. However, to date EDA is almost exclusively based on measurable effects in in vitro and in vivo biotests. To address current needs of regulators and risk assessors, therefore, an increasing focus should be on the integration of EDA into ERA. Specifically, there is need for the development of tools to confirm EDA-determined key toxicants as stressors in populations, communities, and ecosystems [120].

4 Conclusions

In summary, there have been increasing efforts over the past decade to improve current strategies in the assessment of ecological risks associated with the exposure to environmental contaminants of concern through sediments or biota. It has been recognized that classic approaches relying on a few endpoints or analysis types (e.g., analytical chemistry) alone are not only impractical but also tend to wrongly or incompletely assess a given exposure. This is particularly true with respect to exposure to complex mixtures of different types of chemicals that are typical for many aquatic environments. One example is the focus on chemicals that interact with the estrogen receptor (ER) as a representative of endocrine effects. As discussed in the previous section, identification of ER binding potentials represents only one facet of a much more complex issue and can lead to a wrong assessment of the true endocrine disrupting potential of a sample. To address these shortcomings, therefore, studies increasingly rely on advanced EDA concepts by using multiple-endpoint bioassay batteries in combination with fractionation techniques followed by confirmatory targeted chemical analysis.

Also, there are increasing concerns about emerging contaminants including EDCs, perfluorinated compounds, as well as polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans, for which no or only a limited number of sufficiently specific bioassays are available. As a consequence, such exposures often cannot be appropriately addressed, and there is still a great need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to these chemicals. Furthermore, while well established for its use in ERA of contaminants such as dioxin-like, genotoxic/mutagenic, or estrogenic substances, the continuing discovery of new contaminant groups of concern in the environment or new effect types pose new challenges to classical EDA approaches. These challenges include establishing bioassays that are specific to the biological activity of chemical groups of concern, as well as the identification and description of relative potencies of model compounds characteristic for these types of effects to enable the utilization of mass-balance approaches.

There is need to increase the realism of environmental effect analysis to enable linking exposure to biological relevant outcomes that can ultimately predict effects at the population and/or community level. As discussed in this chapter, it is therefore recommended to integrate standard EDA practices based on mechanism-specific in vitro bioassays, high throughput in vivo tests, and analytical

chemistry into more comprehensive assessments of ecologically relevant endpoints such as benthic invertebrate community structure, e.g., as part of weight-of-evidence studies.

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