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Review

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Effect-Directed Analysis: Current Status and Future Challenges

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Abstract – Effect-directed analysis (EDA) has become useful for identification of toxicant(s) that occur in mixtures in the environment, especially those that are causative agents of specific adverse effects. Here, we summarize and review EDA methodology including preparation of samples, biological analyses, fractionations, and instrumental analyses, highlighting key scientific advancements. A total of 63 documents since 1999 (Scopus search) including 46 research articles, 13 review papers, and 4 project descriptions, have been collected and reviewed in this study. At the early stage (1999–2010), most studies that applied EDA focused on organic extracts of freshwater and coastal contaminated sediments and wastewater. Toxic effects were often measured using cell-based bioassays (*in vitro*) and the causative chemicals were identified by use of low resolution gas chromatography with mass selective detector (GC-MSD). More recently (2010–present), EDA has been extended to various matrices such as biota, soil, crude oil, and suspended solids and techniques have been improved to include determination of bioavailability *in vivo*. In particular, methods for non-target screenings of organic chemicals in environmental samples using cutting-edge instrumentation such as time of flight-mass spectrometry (ToF-MS), Fourier transform-ion cyclotron resonance (FT-ICR), and Orbitrap mass spectrometer have been developed. This overview provides descriptions of recent improvements of EDA and suggests future research directions based on current understandings and limitations.

Key words – risk assessment, fractionation, bioassay, non-targeted analysis, sediment

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1. Introduction

The assessment of risks posed by chemicals in the environment based on concentrations is, generally speaking, incomplete and inadequate, because biological effects, bioavailability, and the presence of untargeted toxicants are not fully considered (Brack 2003; Hecker and Hollert 2009; Simon et al. 2015). Novel, but as yet unidentified, chemicals are continually being produced and are entering aquatic environments (Hu et al. 2015). However, newly introduced chemicals and their ecotoxicological properties are largely unknown and analytical methods have been validated for only a few chemicals (Krewski et al. 2010). Environmental media such as water, sediment, and soil are complex mixtures, which include numerous industrial products, unidentified by-products, and transformation products formed in environments and/or deriving from a wide range of sources (Brack 2003). Thus, due to the complexity of environmental samples and site-specific chemical distributions, targeted chemical analysis-driven monitoring strategies are not suitable for identifying key toxic chemicals and assessing risk (Simon et al. 2015).

A number of *in vitro* and *in vivo* bioassays have been developed to screen for potential toxic effects in environmental media. *In vitro* bioassays based on specific mechanisms of actions such as aryl hydrocarbon receptor (AhR), estrogenic receptor (ER), and androgenic receptor (AR)-mediated activities, stereogenecity, and thyroid hormone (TH) disruption are rapid, sensitive, and inexpensive methods, and are complementary

to targeted quantification of known chemicals, including priority pollutants (Windal et al. 2005; Hamers et al. 2010; Li et al. 2014). However, potential toxic effects determined by use of *in vitro* cell bioassays could explain only a small portion of overall toxic potency in extracts of samples (Hollert et al. 2005; Hong et al. 2015). On the other hand, in *in vivo* bioassays, many test organisms are amenable to determining toxicity. However, *in vivo* tests have utilized larger organisms in time- and cost-intensive tests (Hecker and Giesy 2011). More comprehensive strategies that combine chemical and biological analyses are needed for accurate risk assessments to the environment and to understand cause-effect relationships of complex mixtures.

Effect-directed analysis

Integrated strategies such as effect-directed analysis (EDA) that have become useful tools for elucidation of known and unknown toxicant(s) have focused on organic chemicals in environmental samples and mixture effects (Brack 2003; Dévier et al. 2011). EDA is based on a combination of biological analysis (toxicity testing), fractionation procedures, followed by chemical analysis, and facilitates identification of key toxicant(s) (Samoiloff et al. 1983; Schuetzle and Lewtas 1986; Brack 2003). A basic assumption of EDA is that environmental mixtures that create toxic effects chiefly consist of a few active compounds (Brack 2003).

More specifically, biological analyses targeting single or multiple end-points by use of *in vitro* and/or *in vivo* bioassays are first conducted on environmental samples such as sediment, wastewater, biota, or crude oil (Fig. 1). If there is evidence for significant biological responses being induced, the sample is subject to fractionation to reduce its complexity and to separate chemicals (Brack et al. 2008; Hecker and Hollert 2009). The biological effects of fractions are measured by the same testing methods to find the fraction(s) with measurable toxic potencies. Complexities of samples are reduced though fractionation, major toxicants are isolated and finally identified by use of instrumental analysis. Chemical analyses used for EDA can be divided into two basic cases: one determining the contribution of known toxic chemicals (targeted analysis) (Hong et al. 2015) and one identifying unknown toxic chemicals (non-targeted analysis) (Booij et al. 2014; Yue et al. 2015). Finally, during the identification step, key toxicants are chemically and biologically confirmed, if authentic standards are available. In summary, EDA uses biological effects as the basis on which to reduce the huge number of chemicals in samples by use of both targeted and non-targeted identification and quantification of toxicant(s) that significantly contribute to *in vitro* and/or *in vivo* toxic effects (Burgess et al. 2013).

Research efforts and review framework

In the middle of the 1980s, two pioneering studies came up with a new approach called effect- (or bioassay-) directed analysis combining chemical analysis and bioassay (Samoiloff et al. 1983; Schuetzle and Lewtas 1986). They were among the first researchers to apply EDA in a way that was distinct from the US EPA's TIE (toxicity identification evaluation) (Burgess et al. 2013). Since the late 1990s, EDA methods have continuously progressed and have frequently been applied to identify toxicant(s) in environmental samples. In particular, the "Modelkey" project funded by the European Commission within the Sixth Framework Programme from 2005 to 2010

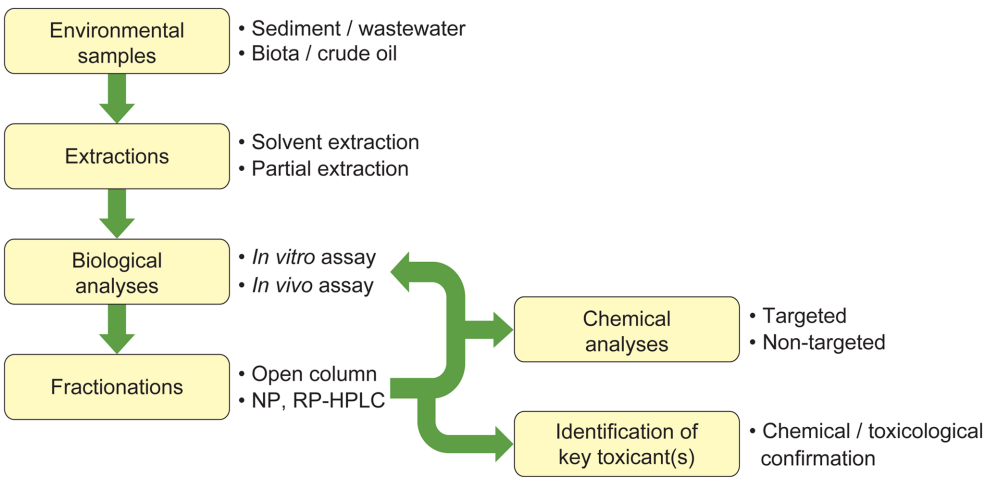

Fig. 1. Scheme of effect-directed analysis (EDA) of environmental samples (modified from Brack 2003)

contributed to development of EDA (Brack et al. 2005a). During that period several papers were published on the development of EDA methods and case studies by researchers from the Helmholtz Centre for Environmental Research. EDA techniques have been driven by the use of cutting-edge instrumentation for non-targeted analysis to identify unknown toxicants (Thomas et al. 2009; Legler et al. 2011; Qu et al. 2011). The history of the applications of EDA can be divided roughly before and after 2010. It was starting in about 2010 that non-targeted screening of unknown chemicals in samples from the environment began being more routinely implemented.

In the present review, a total of 63 documents including 46 research articles, 13 review papers, and 4 project descriptions published since 1999 (Scopus, www.scopus.com) have been collected and reviewed (Fig. 2a). Studies on EDA are increasing in number and are improving techniques for preparing samples, fractionation, and instrumental identification and quantification. Here, we summarize and review the previous research efforts focusing on EDA methodology including techniques for extraction of various matrices (Section 2), biological analyses (Section 3), fractionation techniques (Section 4), and instrumental analyses (Section 5), highlighting key scientific advancements and/or limitations. This paper provides an overview of recent improvements in methods for EDA and suggests future research directions for EDA study based on current understandings and limitations through in-depth review (Section 6).

2. Extraction of Various Matrices

Among various environmental media, approximately 63% of EDA studies have been conducted to determine toxicants in sediments followed by wastewater (17%), biota (6.5%), and crude oil (6.5%) (Fig. 2b). Freshwater and marine sediments and wastewater were focused on during the early stages of EDA (1999–2010). More recently, EDA studies have been extended to the other sample matrices including biotic compartments (e.g., whole organisms, tissue, and blood) (Simon et al. 2015), crude oils (crude and refined oils and oil-contaminated sediments), and suspended solids (Vrabie et al. 2012; Hong et al. 2015). EDA of biological samples can provide useful information on bioavailability, bioaccumulation, and possible metabolization, and this approach seems to be much

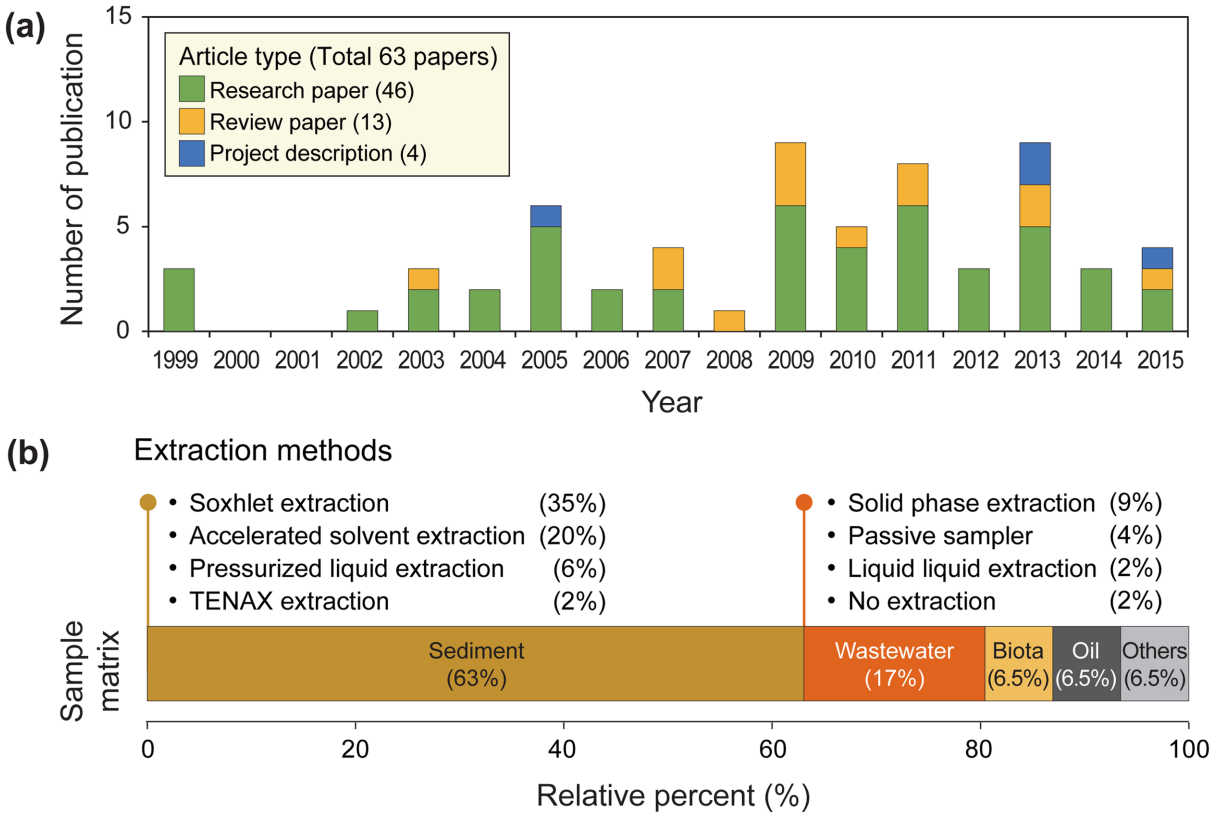

Fig. 2. (a) Number of publications on effect-directed analysis (EDA) from 1999 to present and (b) matrices of environmental samples and extraction methods for EDA studies

more (eco) toxicologically relevant than abiotic compartments (Simon et al. 2015). However, only a few EDA studies have focused on biotic compartments, probably due to difficulties regarding sampling, sample preparation, and instrumental analysis.

Sample preparation such as extraction is the first stage of EDA and is a crucial step for successful detection of biological effects and accurate risk assessment. The extraction step determines concentrations of compounds in the mixture that are subjected to further fractionations (Schwab and Brack 2007). For the extraction of sediments, organic solvents have been employed such as hexane, dichloromethane, acetone, and those mixed solvents in Soxhlet extraction systems, accelerated solvent extraction (ASE), and pressurized liquid extraction (PLE) (Brack et al. 1999; Grote et al. 2005; Weiss et al. 2009; Schmitt et al. 2010). However, solvent extracts could not be used to determine the bioaccessibility of organic chemicals in environmental media, and thus it is limited to precisely detailing the risks for ecotoxicological effects (Cornelissen et al. 2001). To overcome this problem, methods for extraction that assess the rapidly desorbed fraction were developed to improve estimates of bioaccessibility, including Tenax, cyclodextrin, and supercritical fluid extractions (Cornelissen et al. 2001; Schwab and Brack 2007; Schwab et al. 2009). Meanwhile, solid phase extraction (SPE), passive samplers, and liquid-liquid extraction have frequently been used for sample preparations of wastewater samples for EDA (Scheurell et al. 2007; Creusot et al. 2013). Passive sampling techniques could reflect the bioavailable fractions of organic contaminants exposed to living organisms in the field. Finally, similarly to sediments, organic extracts (e.g., eluents of SPE and organic extracts of passive samplers) of wastewater samples were prepared for further biotesting and fractionations.

Many sampling and sample preparation techniques have been introduced and applied in EDA studies. However, there still remain limitations that should be recognized. There is still a lack of standard sample preparation methods of various sample matrices for EDA. Thus, for the sake of consistency, comparing subjects, and to promote scientific progress, it is suggested that sample preparation methods for EDA need to be standardized, as is the case with USEPA's TIE (USEPA 2007).

3. Biological Analyses

Numerous *in vitro* and *in vivo* bioassays have been incorporated into EDA for measuring the integrated effects of mixtures (Larsson et al. 2014a, 2014b) in fractions (Table 1). For EDA to be successful, appropriate measurement end-points and associated bioassays should be selected for each matrix and target toxic effects.

In vitro bioassay

Ames fluctuation assay has been used to assess mutagenic potencies of individual compounds and mixtures. This assay measures the ability of chemicals or mixtures in samples from the environment or their fractions to cause back-mutations in the bacterium *Salmonella typhimurium* (Brack et al. 2005b; Higley et al. 2012; Gallampois et al. 2013). Varying types of mutations caused by different mutagens in fractions can be investigated by use of different strains of Salmonella, such as TA98 or TA100 (Higley et al. 2012). Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene, benzo[a] fluoranthene, and perylene were identified as the major mutagenic chemicals in sediment extracts (Brack et al. 2005b; Higley et al. 2012). In addition, aromatic nitro- and/or amino-compounds in organic extracts of river water seemed to cause mutagenic effects, which have been detected by use of strains YG1024 and YG1041 (Gallampois et al. 2013).

Transactivation assays such as the chemically activated luciferase expression (CALUX) or chemically activated fluorescence expression (CAFLUX) assays, which are based on recombinant cells, are frequently used to measure biological effects in environmental samples. The cells used in these assays have had constructs transfected into them. The constructs generally include DNA that codes for a receptor associated with the critical pathway of a particular mechanism of toxic action or adverse outcome pathway (AOP). It also includes the DNA that codes for a reporter gene such as luciferase in the CALUX assay or a fluorescent protein such as the fast folding green fluorescence protein. Potential AhR- (Grung et al. 2011; Radović et al. 2014; Hong et al. 2015), ER- (Houtman et al. 2004; Koh et al. 2004), and AR- (Weiss et al. 2009) modulated activities of endocrine disrupting compounds in complex environmental mixtures that act through a specific mode of action can be measured (Giesy et al. 2002). *In vitro* cell bioassays are a rapid, sensitive, and relatively inexpensive tool that enables estimation of total potency of all compounds in a mixture quantitatively. The assay simultaneously corrects for interactions, such as antagonisms or synergisms, between and among constituent chemicals and accounts for all of the active constituents including both those that are known and/or expected to be in the mixture as well as those unknown to

Table 1. A summary of biological analyses (*in vitro* and *in vivo* bioassays) for effect-directed analysis (EDA) studies

Bioassay	Test organism	End-point	Sample	Dosing	Time	Major agonists	References
In vitro assay							
Ames fluctuation assay	Bacteria (*Salmonella typhimurium*)	Mutagenicity	Organic extracts of sediments	DMSO (nd)	48, 72 h	BaP, BaF, and Pery	Brack et al. 2005b
			Organic extracts of sediments	DMSO (2%)	48 h	PAHs, sterols, and naphthoic acids	Higley et al. 2012
			Organic extracts of river water	DMSO (nd)	48 h	Amino- and/or nitro-compounds	Gallampois et al. 2013
CAFLUX assay	Mouse hepatoma cell (H1G1-flu)	AhR-activity	Organic extracts of worm and sediments and crude oils	EtOH (nd)	6, 24 h	Organic compounds (log K_{ow} of 5–8)	Vrabie et al. 2012
CALUX assay	Rat hepatoma cell (H4IIE-luc) and human breast cell (MVLN)	AhR- and ER activities	Organic extracts of sediments	Hex or ACN (1%)	72 h	PAHs for AhR-activity NPs for ER-activity	Khim et al. 1999b, 1999c
	Human breast cancer cell (T47D-luc)	ER-activity	Organic extracts of fish bile	DMSO (0.4%)	24h	E2, estrone, and estriol	Houtman et al. 2004
	Human osteoblast cell	AR-activity	Organic extracts of sediments	DMSO (nd)	24 h	71 unidentified compounds	Weiss et al. 2009
	Mouse hepatoma cell (H1L6.1c3)	AhR-activity	Organic extracts of sediments	DMSO (0.4%)	24 h	N, O containing PAHs	Grung et al. 2011
	H4IIE-luc	AhR-activity	Crude and weathered oils	DMSO (0.8%)	4 h	Alkyl-substituted three and four-ring PAHs	Radović et al. 2014
	H4IIE-luc	AhR-activity	Crude oil	DMSO (0.1%)	72 h	Three to four ring (alkyl)-PAHs	Hong et al. 2015
EROD assay	Rainbow trout cell (RLT-W1)	CYP1A1 enzyme activity (EROD)	Organic extracts of sediments	DMSO (0.5%)	24 h	PCDD/Fs, PCBs, and PCNs	Brack et al. 2002, 2003
	H4IIE	CYP1A1 enzyme activity (EROD)	Organic extracts of sediments	DMSO (nd)	48 h	PAHs, methyl-PAHs, and alkyl-PAHs	Kaisarevic et al. 2009
	RLT-W1	CYP1A1 enzyme activity (EROD)	Organic extracts of suspended solids	DMSO (0.1%)	72 h	Non-prioritized PAHs	Wölz et al. 2010
	Clearfin livebearer cell (PLHC-1)	CYP1A1 enzyme activity (EROD)	Organic extracts of wetland sediment	EtOH (0.4%)	6, 24 h	PAHs, NSAIDs, musk, and pesticides	Regueiro et al. 2013
Microtox assay	Bacteria (*Vibrio fischeri*)	Inhibition of bioluminescence	Organic extracts of sediments	DMSO (0.1%)	30 min.	PAHs	Grote et al. 2005
			Organic extracts of sediments	MeOH (2%)	30 min.	-	Scheurell et al. 2007
			Organic extracts of landfill leachates	MeOH (1%)	15 min.	Organic compounds (log K_{ow} of 3.5–3.7)	Lei and Aoyama 2010
			Organic extracts of oil sands process water	MeOH (nd)	72 h	O2, O3, and O4 C17 to C20 compounds	Yue et al. 2015

Table 1. Continued

Bioassay	Test organism	End-point	Sample	Dosing	Time	Major agonists	References
Primary CGN test	Human cerebellum granule neurons	Neurotoxicity	Organic extracts of sediments	DMSO (0.5%)	24 h	Tetrabromobisphenol A diallyl ether	Qu et al. 2011
Steroidogenesis assay	Human adrenocortical cells (H295R)	Stereogenecity	Organic extracts of sediments	DMSO (0.1%)	48 h	PAHs, sterols, and naphthoic acids	Higley et al. 2012
TTR binding assay	Human TTR (Prealbumin from human plasma)	Thyroid hormone disruption	Organic extracts of polar bear plasma	DMSO (nd)	24 h	OH-PCBs, OH-PBDEs, PCBs, PBDEs, PFASs, and OHPs	Simon et al. 2013
Yeast screening assay	Yeast (*Saccharomyces cerevisiae*)	Estrogenic activity	Organic extracts of produced water	EtOH (nd)	72 h	Short-chain alkylphenols (C1-C5)	Thomas et al. 2009
			Organic extracts of sediments	EtOH (nd)	22 h	E2, estrone, nonylphenols, and chlorophene	Schmitt et al. 2012
			Organic extracts of oil sands process water	MeOH (nd)	72 h	O2, O3, and O4 C17 to C20 compounds	Yue et al. 2015
In vivo assay							
Algal assay	Green algae (*Scenedesmus vacuolatus*)	Inhibition of reproduction (growth)	Organic extracts of sediments	DMSO (0.1%)	24 h	PAHs	Grote et al. 2005
			Organic extracts of sediments	Partition based dosing	24 h	Triclosan	Bandow et al. 2009b
			Tenax extracts of sediments	DMSO (0.1%)	24 h	PAHs	Schwab et al. 2009
Daphnia assay	Daphnia (*Daphnia magna*)	Immobilization	Organic extracts of sediments	Acetone (1%)	24 h	methyl parathion, and tributyltin	Brack et al. 1999
Fish embryo assay	Zebrafish (*Danio rerio*)	Lethality, developmental malformation, and tetratogenicity	Organic extracts of soils	DMSO (0.01%)	6 d	11H-BbF, 9-methylacridine, 4-azapyrene, 2-PhQ, and retene	Legler et al. 2011
		Estrogenic effect (GFP induction)	Organic extracts of sediments	DMSO (0.05%)	5 d	Alkylphenols and estrone	Fetter et al. 2014
PAM assay	Marine microalgae (*Dunaliella tertiolecta*)	Effective photosystem II efficiency	Organic extracts of passive sampled water	MeOH (nd)	4.5 h	Atrazine, diuron, irgarol, isoproturon, terbutryn, and terbutylazine	Booij et al. 2014
Sediment contact test	Mud snail (*Potamopyrgus antipodarum*)	Mortality, growth, and Inhibition of reproduction	Wet field sediments	-	28 and 56 d	NPs and bisphenol-A	Schmitt et al. 2010, 2011

ACN: acetonitrile, AhR: aryl hydrocarbon receptor; AR: androgen receptor; BaF: benzo[a]fluoranthene; BaP: benzo[a]pyrene; BbF: benzo[b]fluorene; CALUX: chemical activated luciferase gene expression; DMSO: dimethyl sulfoxide; E2: 17β-estradiol; ER: estrogen receptor; EROD: ethoxyresorufin-*O*-deethylase; EtOH: ethanol; GFP: green fluorescent protein; MeOH: methanol; NPs: nonylphenols; PAHs: polycyclic aromatic hydrocarbons; PBDEs: polybrominated diphenyl ethers; PCBs: polychlorinated biphenyl; PCDD/Fs: polychlorinated dibenzo-*p*-dioxins and dibenzofurans; PCNs: polychlorinated naphthalene; Pery: perylene; PFASs: polyfluoroalkyl substances; PhQ: phenylquinoline; nd: not described; NSAIDs: non-steroidal anti-inflammatory drugs; OHPs: other halogenated phenols; TTR: transthyretin

be in the sample.

Potency balance analysis can be used to investigate causative agents, both known and unknown, through targeted and non-targeted instrumental analyses to identify and quantify individual chemicals in mixtures (Khim et al. 1999a, 1999b, 1999c; Villeneuve et al. 2002). In such an analysis, relative potency values (RePs) are applied to the concentration of each chemical. RePs are endpoint and bioassay-specific and their development must meet various assumptions such as equal efficacies with the reference toxicant and parallel dose-response curves (Lee et al. 2013a, 2015). When applying RePs in a potency balance, it is possible to see if the sample and standard cannot reach the same maxima (plateau) which is referred to as the efficacy. However, since the identities or concentrations of individual constituents are unknown, it is impossible to test the assumption of parallelism (Hilscherová et al. 2000a, 2000b). This is because the concentration of the reference toxicant is in units of mass per volume, while those of the mixture are generally given in equivalents of the mass or volume of sample extracted. Thus, since the units on the two axes are not the same, it is impossible to verify whether this critical assumption. For these reasons, to minimize the potential error or systematic bias introduced due to not being able to verify whether the assumption of parallelism, it is suggested that comparisons of EC20 values be made (Villeneuve et al. 2002). Because EC20 is near to the point of departure (POD), which would be the most appropriate threshold but generally greater than the method detection limit, it is a reasonable surrogate for the POD.

In some EDA studies, receptor-mediated effects such as the AhR (Hilscherová et al. 2000b; Eichbaum et al. 2014), ER (Hilscherová et al. 2000a), AR (He et al. 2011), and TH (Shi et al. 2013) agonists and antagonists were successfully identified in sediments, biota, and crude oil samples by use of recombinant cell bioassays combined with instrumental analyses. However, recombinant cell lines are stably transfected with luciferase or fluorescent gene into cells made from humans (MVLN), rats (H4IIE-luc and H1L6.1c3), or mice (H1G1-flu). Thus, the ecotoxicological relevance of biological effects determined using human and mammalian cell bioassays remains an issue with regard to the interpretation of results. To overcome such limitations, wild-type fish cell lines such as RLT-W1 (rainbow trout) (Villeneuve et al. 1999) and PLHC-1 (clearfin livebearer) (Villeneuve et al. 2001) have also been used to determine CYP1A1 enzyme activity by use of ethoxyresorufin *O*-deethylase (EROD) assay. In several EDA studies, the EROD assay was applied for detection of dioxin-like chemicals in freshwater or marine sediments and suspended solids (Brack et al. 2002, 2003; Wölz et al. 2010; Regueiro et al. 2013). However, *in vitro* bioassay based on PLHC-1 cells was 4- to 6-fold less sensitive than H4IIE-based cells for detecting AhR-mediated (dioxin-like) activity in complex mixtures (Villeneuve et al. 2001). One possible explanation for differences in sensitivity observed between fish and mammalian cells is differences in membrane permeability and metabolic capacity and structural differences of the AhR (Villeneuve et al. 2001; Farmahin et al. 2014; Doering et al. 2015).

Inhibition of luminescence by marine bacteria (*Vibrio fischeri*), called the Microtox toxicity test, has been used for the measurement of baseline toxicity in complex mixtures as part of the EDA (Grote et al. 2005; Scheurell et al. 2007; Lei and Aoyama 2010). This testing method is relatively cheap, quick, and convenient. However, due to their less aqueous solubility, toxic effects could not be observed for very hydrophobic chemicals ($\log K_{ow} > 5$) (Lee et al. 2013b). Primary testing for neurotoxicity using human cerebellum granule neurons (CGN) (Qu et al. 2011) and steroidogenesis assay to determine effects in steroidogenic enzymes (Hilscherova et al. 2004; Zhang et al. 2005) and production of hormones (Hecker et al. 2006) using H295R cells (human adrenocortical cell) has been conducted (Higley et al. 2012). TTR binding assay for thyroid hormone disruption using prealbumin from human plasma (Simon et al. 2013) and yeast screening assay for estrogenic activity using yeast *Saccharomyces cerevisiae* (Thomas et al. 2009; Schmitt et al. 2012; Yue et al. 2015) have also been applied in the EDA studies.

Each of these *in vitro* bioassays for EDA has advantages and disadvantages, and thus suitable bioassays should be selected for specific purposes. However, there are a few matters such as dosing techniques and duration of exposure that EDA researchers need to consider regarding exposure methods. Most of *in vitro* bioassays conducted in EDA studies have used solvent dosing techniques (Table 1). Organic extracts of environmental matrices or their sub-fractions are first dissolved or exchanged to organic solvents such as DMSO, ethanol, methanol, or acetone as a carrier solvent and the solution directly dosed into assay medium, which minimizes concentrations of solvent applied to 0.01 to 2%. Solvents such as nonane or isooctane can be also used. These volatilize quickly leaving the dosed materials in the medium, but without the confounding effects of the solvent. According to physicochemical properties

and partitioning behaviors of chemicals in samples, volatile compounds can be evaporated during incubation and hydrophobic chemicals can adsorbed onto plastic surfaces of well plates and into cellular matrices (Kwon et al. 2009). Although partition-based dosing techniques have been developed for more accurate estimates of exposure using polydimethylsiloxane (PDMS) (Kwon et al. 2009), only a few studies have applied this technique in the EDA (Bandow et al. 2009a, 2009b). In these systems, toxicants in extracts are loaded into the PDMS, which has properties similar to membranes, and are slowly released into the medium so as to avoid super-saturation in the medium. Durations of exposure of *in vitro* bioassay varied on a case-by-case basis even when the same bioassay was used (Table 1). Stability, metabolic rate, and binding affinity of chemicals in environmental mixtures vary among chemicals based on their individual properties (Villeneuve et al. 2001). For example, PAHs are thought to be rapidly transformed within a few hours through *in vitro* metabolism, whereas 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyl (PCBs) were not easily metabolized (Larsson et al. 2012; Pieterse et al. 2013). Duration of exposure for bioassay is therefore chosen according to the specific objective of the measurement and potential for biotransformation (Windal et al. 2005).

In vivo bioassay

In vivo bioassays are increasingly being utilized in EDA studies due to their more accurate estimations of (eco) toxicologically relevant effects than *in vitro* measurements. Algal assay (Grote et al. 2005; Bandow et al. 2009b; Schwab et al. 2009), daphnia assay (Brack et al. 1999), zebrafish embryo larval assay (Legler et al. 2011; Fetter et al. 2014), PAM assay (Booij et al. 2014), and sediment contact test (Schmitt et al. 2010, 2011) have been used as a tool for measurement of biological effects in environmental samples (details in Table 1). In algal bioassays used in EDA studies, growth inhibition of green algae, *Scenedesmus vacuolatus,* is applied (Grote et al. 2005; Bandow et al. 2009b; Schwab et al. 2009). PAHs have been identified a major toxicant for growth inhibition of green algae, both in organic extracts (Grote et al. 2005) and Tenax extracts (bioaccessibility-directed extraction) (Schwab et al. 2009) of sediment samples. However, more polar compounds such as triclosan have been identified as key toxicants by use of partition-based dosing techniques, while fractions including PAHs did not exhibit significant effects (Bandow et al. 2009b). Thus, more uses of bioaccessibility-directed extraction and partition-based dosing techniques are recommended to prioritize and identify major toxic fractions to approximate more closely to real environmental conditions.

Assays based on embryos and larvae of zebrafish (*Danio rerio*) have advantages including small size, ease of culture, high fecundity, rapid development, external fertilization and development, and transparency of the embryo. Also their small size makes it feasible to do assays with individual larvae in well of 96-well plates. This makes it possible to have large numbers of true replications and avoid pseudoreplication and also to use automated techniques to monitor the characteristics of embryos, including behavior, which is well suited to EDA (Di Paolo et al. 2015). Numerous endpoints have been developed including lethality (coagulation of the embryo and/or undetected heartbeat) and teratogenicity (malformation of the head, tail, or heart, scoliosis, deformity of yolk, and growth retardation). 11H-benzo[b]fluorene, 9-methylacridine, 4-azapyrene, 2-phenylquinoline, and retene were successfully identified as major developmental toxicants in soils by use of zebrafish embryo larval assay combined with liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry (Legler et al. 2011).

Sediment contact tests with the mudsnail *Potamopyrgus antipodarum* is the only method for assessment of endocrine effects on organisms after exposure via spiked sediments in EDA (Schmitt et al. 2010, 2011). *P. antipodarum* is a very sensitive organism with regard to endocrine disrupting chemicals, and their reproduction appeared to be more sensitive than the induction of estrogenic gene expression in the YES assay (Schmitt et al. 2010). *P. antipodarum* was only exposed to bioavailable compounds in sediments, and thus the sediment contact test led to more realistic exposure scenarios.

Most *in vivo* assays conducted in EDA have common features: small scale (24- or 96-well plate scale) and dosing with organic extracts of environmental samples, except for sediment contact tests. The reason for the use of small-scale *in vivo* testing that is mainly employed in EDA studies seems to be due to the limited quantities of samples and the large numbers of fractions to be tested (Di Paolo et al. 2015). Thus, solvent dosing methods in *in vivo* assays are still limited. In addition, linkages between effect endpoints in *in vitro* and *in vivo* tests are needed to advance the concept of AOP (Henneberg et al. 2014).

4. Fractionations

After *in vitro* and/or *in vivo* screening of raw extracts of environmental samples, samples exhibiting significant toxicity

are subject to fractionation for separating and subsequent identification and quantification of putative toxic agents. Fractionation of mixtures is conducted by use of column chromatography, based on various physical and chemical properties, but one of the major properties is hydrophobicity (e.g., log K_{ow}), which is affected by molecular mass and number of aromatic rings (Brack et al. 2003). Specific techniques for separation in EDA have included SPE cartridge (Qu et al. 2011), normal-phase (NP)-HPLC (Lübcke-von Varel et al. 2008), and/or reverse-phase (RP)-HPLC (Houtman et al. 2004). Most previous studies have carried out rough fractionation by use of open column chromatography, and biotesting, and then fine fractionation for major toxic samples by use of NP- or RP-HPLC (Snyder et al. 2001). Some of the more important elements associated with fractionation in EDA are: 1) recovery of parent sample; 2) sufficient volume of fraction for biotesting; 3) reproducibility; and 4) precision.

Compounds in organic extracts of environmental samples are fractionated into three to five fractions according primarily to polarity (Table 2). Solid packing materials used to separate compounds include: Alumina (Brack et al. 2003; Brack and Schirmer 2003; Grote et al. 2005; Wölz et al. 2010; Vrabie et al. 2012), Florisil (Khim et al. 1999a, 1999c; Koh et al. 2004), silica gel (Schmitt et al. 2011; Hong et al. 2015), and two phases of silica gel and alumina (Schmitt et al. 2012; Radović et al. 2014) column chromatography, which are most widely used to fractionate law extracts into aliphatic (F1, non-polar or saturates), aromatic (F2, mid-polar), and polar compounds (F3). Residues that remain on the solid phase, often considered one of the fractions (F4), are collected for further analysis (Brack and Schirmer 2003). During open column chromatography, despite the same principle, the amount of absorbent, internal diameter of column, elution solvent, volume of eluting solvent, and flow rates are applied differently among researchers. Each method is optimized, thus fractionation conditions are suitable according to potential chemicals of interest and the bioassay system to be applied. Gel permeation chromatography (GPC) (Weiss et al. 2009) and SPE cartridge (Qu et al. 2011; Simon et al. 2011; Yue et al. 2015) have also been used to separate fractions of organic extracts prior to separation by use of HPLC.

NP-HPLC columns (Lübcke-von Varel et al. 2008; Thomas et al. 2009; Regueiro et al. 2013; Fetter et al. 2014; Hong et al. 2015) and RP-HPLC (Houtman et al. 2004; Grung et al. 2007; Legler et al. 2011; Vrabie et al. 2012) have been used for fine fractionations in EDA studies (Snyder et al. 2001). Various NP-HPLC columns have been used to fractionate organic extracts into 3 to 31 sub-fractions according to polarity, aromatic ring number, planarity, or molecular size. NP-HPLC has a definite advantage for fractionation of hexane- or dichloromethane-based organic extracts without solvent exchange, but the sub-fractions are needed to substitute water-based assay medium for biotesting. Automated fractionation of organic extracts has been developed by use of three connected columns including cyanopropyl- and nitrophenylpropyl-bonded silica and porous graphitized carbon stationary phases (Lübcke-von Varel et al. 2008). The fractionation produces 18 fractions, and is thus very useful for primary screening, and has been successfully applied in EDA studies (Bandow et al. 2009a, 2009b; Kaisarevic et al. 2009; Schwab et al. 2009; Grung et al. 2011).

The C18 column eluted with a gradient mobile phase of water and methanol is the most commonly used to fractionate samples into from 9 to 30 fractions based on hydrophobicity (as measured by log K_{ow}) in RP-HPLC system (Houtman et al. 2004; Grung et al. 2007; Legler et al. 2011; Vrabie et al. 2012). Gradient conditions for fractionation on the C18 column have been optimized in each case study using calibration curves between log K_{ow} values of known chemicals and HPLC retention times. For example, we have optimized gradient conditions for HPLC when fractionating by use of the calibration curve using 34 PCBs, 16 PAHs, 7 alkylphenols, and 5 phthalates compounds (Fig. 3 and Table S1 of the Appendix). Based on this calibration curve, fine fractions can be collected at intervals of 0.5 or 1 of log K_{ow} values of compounds in organic extracts of environmental samples. Larger volumes of fraction samples are needed for *in vivo* testing, thus more use of semi-preparative or preparative scale HPLC are suitable for fractionation in EDA.

Initially, fractionation in EDA tended to include multistep fractionation procedures, while recently simpler fractionation techniques are being applied to get larger volumes of each fraction and then further fractionations are applied. This approach is more efficient in use of the available materials and the number of tests is reduced. It seems to be associated with development of instrumental analysis techniques and its application in EDA. It has taken considerable effort to reduce the complexity and to isolate the key toxicants in samples using multistep fractionation techniques due to past limitations of low resolution GC-MSD in instrumental analysis (Lübcke-von Varel et al. 2008). More recently, simple fractionation techniques such as C18-based RP-HPLC have been shown to be appropriate for use in EDA. Causative

Table 2. A summary of fractionation techniques for effect-directed analysis (EDA) studies

Equipment & column	Mobile phases (elution solvents)	No. of fractions	Identified chemicals in fractions	Further fractionation	References
Open column chromatography					
Alumina	F1: Hexane F2: Hexane:DCM (95:5, v/v) F3: DCM F4: MeOH:acetic acid (99:1, v/v)	4	F1: Non-polar aliphatics F2: Non-polar aromatics F3, F4: Polar fractions	NP-HPLC	Brack et al. 2003
	F1: n-Hexane F2: n-Hexane:DCM (90:10, v/v) F3: DCM F4: MeOH:acetic acid (99:1, v/v) treated with 0.01 M HCl and extracted by hexane F5: The pH adjusted to 12 and extracted by hexane	5	F2: PAHs	NP-HPLC	Grote et al. 2005
	F1: Hexane F2: 10% DCM in hexane F3: DCM	3	F1: Non-polar aliphatics F2: Non-polar PAHs F3: Polar substances	NP-HPLC	Wölz et al. 2010
	F1: Hexane F2: DCM F3: Methanol	4	F1: Saturates F2: Aromatics F3: Resins	RP-HPLC	Vrabie et al. 2012
Florisil	F1: Hexane F2: 20% DCM in hexane F3: 50% DCM in MeOH	3	F1: Non-polar (PCBs) F2: Mid-polar (PAHs) F3: Polar (APs)	No	Khim et al. 1999a, 1999c
Gel permeation chromatography (GPC)	F1: 16:3–24.0 min. fraction using DCM F2: 29.0–36.0 min. fraction using DCM	2	F2: Androgenic compounds	RP-HPLC	Weiss et al. 2009
Silica gel	F1: Hexane F2: 50% DCM in hexane F3: DCM F4: Methanol	4	F1: Aliphatic hydrocarbons F2: PAHs, PCBs, and dioxins F3: Nitro-PAHs F4: Polar compounds	No	Schmitt et al. 2011
	F1: Hexane F2: 20% DCM in hexane F3: 40% Acetone in DCM	3	F1: Aliphatic hydrocarbons F2: PAHs and alkyl-PAHs F3: Polar and resins	NP-HPLC	Hong et al. 2015
Silica gel + Alumina	F1: n-Pentane F2: n-Heptane F3: n-Heptane/ethylacetate (7:3) F4: Ethylacetate F5: MeOH	5	F5: Estrogenic compounds (alkylphenols, chlorophene, bisphenol A, cholesterol, estrone, 17β-estradiol, etc.)	No	Schmitt et al. 2012
	F1: Hexane F2: DCM F3: Methanol	3	F1: Aliphatics F2: Aromatics F3: Polar and resins	NP-HPLC	Radović et al. 2014
	A: Water B: MeOH	9	Extracts fractionated into 9 fractions based on log K_{ow}	No	Houtman et al. 2004

Table 2. Continued

Equipment & column	Mobile phases (elution solvents)	No. of fractions	Identified chemicals in fractions	Further fractionation	References
NP-HPLC					
Nucleosil 100-5 NO_2, Nucleosil 100-5 CN, Cosmosil PYE and Hypersil PGC	A: Hexane B: DCM	18	Extracts fractionated automatically into 18 fractions based on polarity, number of aromatic carbons, and planarity	No	Lübcke-von Varel et al. 2008
PAC (cyano-amino bonded silica)	A: Hexane B: DCM C: Iso-propanol	31	Water extracts fractionated into 31 fractions based on HPLC retention time	No	Thomas et al. 2009
Hypersil APS-2	A: Hexane B: DCM C: Ethyl acetate	3-4	PLE 1 extract fractionated into 3 fractions using solvents A and B and PLE 2 extract fractionated using solvents B and C	No	Regueiro et al. 2013
Nucleosil 100-5 CN	A: n-Hexane B: DCM C: Acetonitrile	10	Organic extracts fractionated into 10 sub-fractions based on HPLC retention time	No	Fetter et al. 2014
Nucleosil 100-5 NO_2	A: Hexane:DCM (95:5, v/v), Isocratic	6	PAHs and alkyl-PAHs fractionated into 6 sub-fractions based on aromatic ring number	No	Hong et al. 2015
RP-HPLC					
C18 column	A: Water B: MeOH	9	Aromatics and resins fractionated into 9 fractions based on log K_{ow}	No	Vrabie et al. 2012
	A: Water B: MeOH	20	Extracts fractionated into 20 fractions based on HPLC retention times (log K_{ow})	No	Legler et al. 2011
	A: Water B: MeOH	30	Extracts fractionated into 30 fractions based on HPLC retention times (log K_{ow})	No	Grung et al. 2007
	A: Water B: MeOH	9	Extracts fractionated into 9 fractions based on log K_{ow}	No	Houtman et al. 2004
SPE cartridge					
Bakerbond (silica gel, SiOH)	F1: DCM:hexane (1:9, v/v) F2: DCM:hexane (2:8, v/v)	2	F1: PBDEs F2: HBCDs and TBBPA	RP-HPLC	Qu et al. 2011
Oasis MCX (polymeric sorbent)	F1: MeOH F2: 5% NH_4OH in MeOH	2	F1: OHPs, OH-PCBs, OH-PBDEs, and PFASs	NP-HPLC	Simon et al. 2011
C18 (octadecylsilane) and PSDVB	F1-F4: 20, 60, 80, and 100% MeOH for C18 F5-F8: 20, 60, 80, and 100% MeOH for PSDVB	8	F2: Estrogenic compounds	RP-HPLC	Yue et al. 2015

APs: alkylphenols; DCM: dichloromethane; GPC: gel permeation chromatography; HBCDs: hexabromocyclododecanes; NP: normal phase; PBDEs: polybrominated diphenyl ethers; PCBs: polychlorinated biphenyl; PSDVB: polystyrene divinylbenzene; RP: reverse phase; SARA: saturates, aromatics, resins, and asphaltenes; SPE: solid phase extraction; TBBPA: tetrabromobiphenol A; TLC: thin layer chromatography; UPLC: ultra performance liquid chromatography

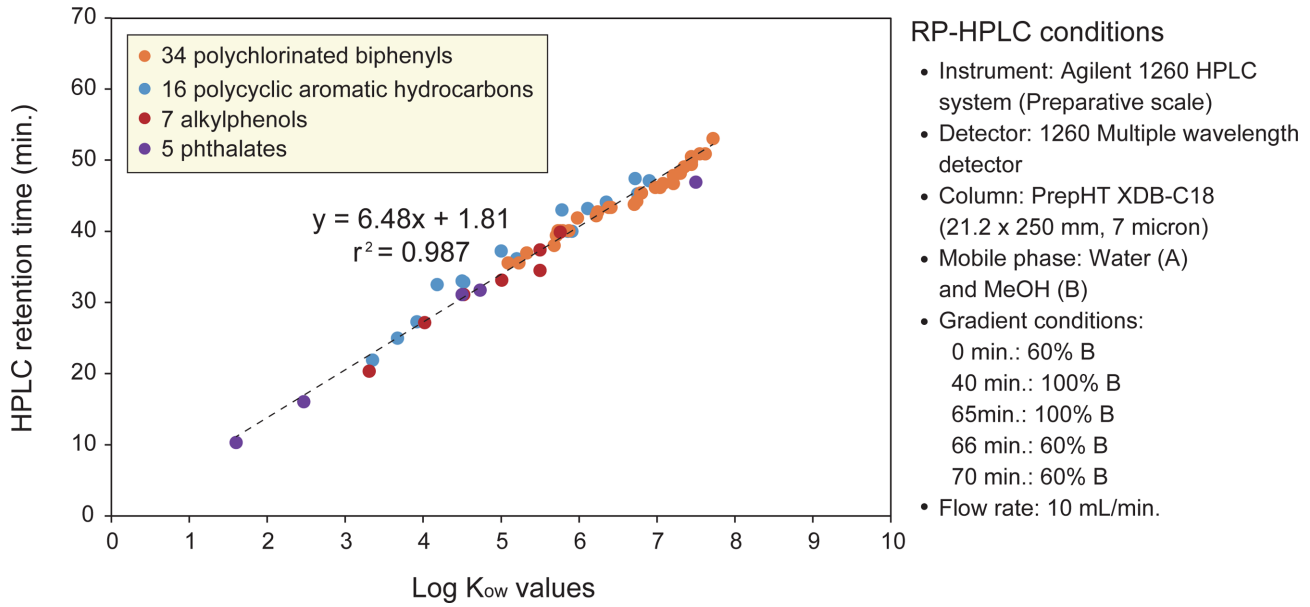

Fig. 3. Retention times of various organic chemicals (n = 62) as a function of the chemical's log K_{ow} values by use of reverse phase-HPLC (XDB-C18 column) (details in Table S1 in Appendix)

chemicals can be identified through non-targeted analysis of great toxic fractions by use of cutting-edge instrumentation such as time of flight mass spectrometry (ToF-MS), Fourier transform-ion cyclotron resonance (FT-ICR), and Orbitrap MS. Instead of using physical separation to help identify causative agents, the instrumental element of the analysis is used. Thus, instrumental analyses are more weighted in EDA studies than sample fractionation in more recent years.

Sometimes, solvent exchange is needed for bioassay because some organic extracts dissolved in hexane and dichloromethane are immiscible with water and assay medium. Compounds in environmental complex mixtures have different solubilities and polarities and thus precipitates can be formed in the new solvent during this step. Thus, some chemicals are necessarily excluded in the bioassay that can result in losses for toxic potencies of parent fractions (Khim et al. 1999a, 1999b). In addition, some volatile compounds in samples can be lost during evaporation of the solvent, leading to reduced availability of the compounds in the bioassay. Thus, validation and optimization of sample preparation for additional bioassay after fractionation including solvent exchange and concentration steps are necessary.

5. Instrumental Analyses

Toxicant(s) in active fractions are identified by use of instrumental analyses. Chemical analysis during EDA can be divided into two cases: one determining contribution of known toxic chemicals (targeted analysis) and the other identifying unknown toxic chemicals (non-targeted analysis) in environmental complex mixtures.

Targeted analysis

Early in the development of EDA, fractionation of samples considering the characteristics of target chemicals was conducted prior to confirmation of toxicity in bioassays, and then toxicity contributions of targeted chemicals were determined. Traditional toxic chemicals such as PCBs, organochlorine pesticides (OCPs), PAHs, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), and polychlorinated naphthalene (PCNs) in sediment samples were isolated and analyzed (Brack et al. 1999, 2002; Khim et al. 1999c; Koh et al. 2004). For isolation of key toxicants and reducing complexities in mixtures, multistep fractionations were performed (Brack et al. 2003). Then, unknown chemicals with greater peak heights in chromatograms of the more toxic fractions were identified by use of low-resolution GC-MSD (Brack and Schirmer 2003; Weiss et al. 2009). Confirmation of putative causative chemicals was also carried out, if standard materials were available (Brack et al. 2005b). However, because of several limitations of GC/MSD analysis, such as ambiguous structural elucidation of unknown chemicals, only a few studies were successful.

Contributions of toxic effects by known chemicals can be

characterized by use of potency (or mass) balance analysis (Villeneuve et al. 2000, 2002; Hong et al. 2015). This approach is conducted through a direct comparison between bioassay-directed toxic potency and instrument-derived toxicity equivalents (TEQs). Assay-specific ReP values of known chemicals in relation to the positive control (e.g., TCDD) are needed to convert concentrations being expressed in TEQs. Mixture effects are not to be considered in this approach, assuming additivity of responses. Finally, contributions of each known toxic chemicals can be compared to the overall induced toxic effects of extracts of environmental samples in bioassays. RePs of individual toxic chemicals including PCDD/Fs, PCBs, PCNs, and PAHs for certain toxicity endpoints are available (Villeneuve et al. 2000, 2002; Behnisch et al. 2003; Van Wouwe et al. 2004; Lee et al. 2013a; Hong et al. 2015). However, there were a few cases where key toxicants have been successfully identified in environmental samples by use of potency balance analysis (Koh et al. 2004; Hong et al. 2014). Most of the studies showed that contributions of known toxic chemicals could explain only a small portion of total induced toxicity (Brack and Schirmer 2003; Brack et al. 2005b; Bandow et al. 2009b; Weiss et al. 2009; Wölz et al. 2010; Hong et al. 2012, 2015). Overall, in EDA, the TEQ approach based on targeted chemicals is useful for the determination of the toxicity contribution of individual compounds. However, ReP values are available for only a limited number of endpoints and then for each endpoint only a few chemicals. Thus, potency balance could not deal with non-targeted chemicals in environmental samples.

Non-targeted analysis

Non-target screening of organic chemicals in environmental samples has been progressively developed by use of cutting-edge instrumentation and applied to identify certain toxicant(s) in recent years. Strategies for identifications and confirmation of active compounds in fractions were previously described (Kind and Fiehn 2007; Krauss et al. 2010; Weiss et al. 2011; Simon et al. 2015). Briefly, during the process of identification of unknown chemicals, high resolution (e.g., ToF-MS) or ultra-high resolution (e.g., Orbitrap-MS) analyses are used for non-targeted identification of putative active chemicals in the more potent fractions of environmental samples (Fig. 4). For data analysis, mass data purification is conducted including: 1) internal calibration; 2) subtraction of active fraction and non-active fraction chromatogram; and 3) generation of accurate masses to charge ratios from which it is possible to discern molecular formulas using various software programs (Booij et al. 2014; Simon et al. 2015). In this step, mass data is cleaned-up by subtraction of chromatograms of procedural blank and/or non-toxic fractions by use of software tools, such as DataAnalysis (Bruker Daltonics, Bremen, Germany), Bruker Daltonics (Bruker Daltonics), and/or MetaboliteDetect (Bruker Daltonics) (Simon et al. 2015). Irrelevant masses were excluded by further analysis based on the "Seven Golden Rules" such as: 1) element number restrictions; 2) Lewis and Senior chemicals rules; 3) isotopic patterns; 4) element ratio of hydrogen versus carbon; 5) element ratios of nitrogen, oxygen, phosphorus, and sulfur versus carbon; 6) element ratio probabilities; and 7) presence of trimethylsilyl-compounds (Kind and Fiehn 2007). Chemical formulas of candidates for causative chemicals are extracted from the accurate mass via elemental composition software tools, such as Xcalibur (Thermo Fisher, Bremen, Germany) or CompoundCrawler (Bruker Daltonics) (Weiss et al. 2011; Simon et al. 2015). Next, tentative toxic chemicals are evaluated for physico-chemical and toxicological properties through database search (e.g., ChemSpider, Royal Society of Chemistry). Finally, chemical (e.g., retention time and mass spectra) and biological (e.g., dose-response characterization) confirmations are performed, if standard materials are available.

Non-targeted screening combined with EDA has been applied to various abiotic (e.g., sediment, soil, water, crude oil, and oil sands process water) and biotic environmental compartments (e.g., blood plasma of polar bear) using cutting-

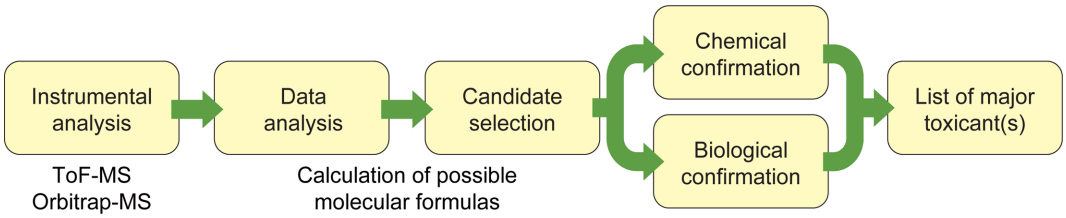

Fig. 4. Non-targeted chemical analysis strategy for identification of major toxicant(s) in environmental samples in effect-directed analysis (EDA) (modified from Booij et al. 2014 and Simon et al. 2015)

edge instrumentation in recent years (Legler et al. 2011; Qu et al. 2011; Gallampois et al. 2013; Simon et al. 2013; Booij et al. 2014; Radović et al. 2014; Yue et al. 2015). For examples of abiotic environmental compartments, tetrabromobisphenol A diallyl ether was identified as an emerging neurotoxicant in sediment samples collected from near a brominated flame retardant manufacturing plant by use of LC-Q-ToF-MS applied in EDA (Qu et al. 2011). In addition, 9-methylacridine, 4-azapyrene, and 2-phenylquinoline were newly introduced as developmental toxicants in soil samples from a former municipal landfill site by use of LC-hybrid linear ion trap Orbitrap MS (Legler et al. 2011). Non-targeted screening of more mutagenic fractions of water extracts using LC-linear trap quadrupole (LTQ)-Orbitrap MS successfully provided a list of mutagens of molecular formulas including amino- and nitro-compounds (Gallampois et al. 2013). Also, major AhR binding chemicals were identified as alkyl-substituted three to four-ring aromatic compounds in active fractions of fresh and artificially weathered crude oil by use of GC×GC-ToF-MS (Radović et al. 2014). Finally, Yue et al. (2015) reported that O2, O3 and O4 C17 to C20 compounds (6–20 double bond equivalents) were identified as major estrogenic compounds in oil sands process water (OSPW) by use of HPLC-LTQ-Orbitrap MS. In biotic environmental compartments of EDA, linear and branched nonylphenol, 4′-OH-CB201 (octachlorinated biphenyls), and 4,4′-OH-CB202 in blood plasma of polar bear were found to be thyroid disrupting chemicals by use of LC-ToF-MS (Simon et al. 2013). However, identification of causative compounds in environmental complex mixtures still remains challenging due to the limited number of chemical standards, presence of lessor concentrations and more potent chemicals, mixture toxic effects, and difficulties of data processing.

6. Future Research Directions

The developmental history of EDA methodology can be divided into two phases, before and after 2010 whether or not the non-targeted screening of unknown chemicals in the environmental samples was conducted (Fig. 5). Earlier (1999–2010), most of EDAs focused on organic extracts of sediments and wastewater. Cell-based bioassays (*in vitro*) were mainly used to measure biological effects in multistep fractionation samples, and toxicity contributions of targeted chemicals were determined by use of low resolution GC-MSD. More recently, the EDAs have been extended to the various sample matrices such as biota, soil, crude oil, and suspended solids. *In vivo* bioassay tools are more frequently applied to improve environmental realism. Non-targeted screening techniques by use of cutting-edge instrumentation have been applied to identify certain unknown toxic chemicals in EDA studies. Non-targeted analysis seems to be useful and much more powerful for prioritizing chemicals with regard to environmental management strategies. Overall, we suggest future research directions for EDA studies based on the current understandings and limitations (details in Table

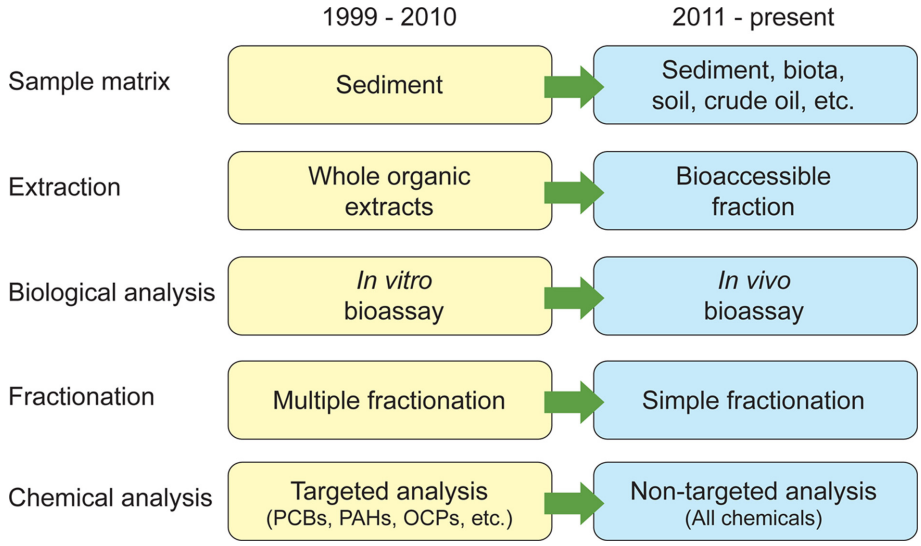

Fig. 5. Key scientific advancements focusing on effect-directed analysis (EDA), including sample matrix, extraction, biological analysis, fractionation, and chemical analysis

Table 3. Suggestions for future research directions of EDA studies based on current understandings and limitations

Future research directions	Viewpoints	Advantages	Remarks
(1) Extension of sample matrix	• Organismal tissues and biological fluids	Useful information on bioavailability, bioaccumulation, and possible metabolization	Section 2
(2) Standardization of EDA procedure	• Sample preparation (e.g., extraction) • Fractionation procedures	Consistency and comparing subjects	Sections 2 & 4
(3) Consideration of ecotoxicological relevance	• Bioaccessibility-directed extraction • Partition-based dosing • Selection of suitable biological analysis • Linkage between *in vitro* and *in vivo* effects • Community-level responses related with major toxic substances	Accurate ecotoxicological risk assessment and understanding of source to outcome pathways	Sections 2 & 3
(4) Identification of unknown toxic chemicals	• Non-targeted analysis using cutting-edge instrumentations	Prioritizing toxic chemicals (chemicals of concerns)	Section 5
(5) Application of EDA results	• Multiple lines of evidence risk assessment • Region-specific chemicals of concerns	Providing scientific knowledge to environmental decision and policy makers	

3), including: 1) extension of sample matrix (e.g., organismal tissues and biological fluids); 2) standardization of EDA procedure; 3) consideration of ecotoxicological relevance; 4) identification of unknown toxic chemicals; and 5) application of EDA results in multiple lines of evidence risk assessment.

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Appendix

Table S1. Log K_{ow} values and HPLC retention times of 34 PCBs, 16 PAHs, 7 alkylphenols, and 5 phthalates

Chemicals	Log K_{ow}	HPLC RT (Min.)	References
Polychlorinated biphenyls (PCBs)			Han et al. 2006[a]
8	5.09	35.563	
15	5.23	35.563	
18	5.33	36.991	
31	5.68	38.048	
28	5.71	39.448	
37	5.71	39.448	
44	5.73	40.101	
77	5.76	40.101	
52	5.79	40.101	
49	5.87	40.101	
81	5.98	41.881	
87	5.98	41.881	
70	6.22	42.197	
60	6.24	42.739	
101	6.38	43.381	
149	6.41	43.381	
114	6.71	43.824	
118	6.74	44.22	
105	6.79	45.411	
153	6.8	45.411	
126	6.98	46.178	
123	7.04	46.178	
170	7.08	46.726	
180	7.21	46.726	
187	7.21	47.85	
167	7.29	48.193	
128	7.3	48.193	
195	7.35	49.083	
138	7.44	49.426	
156	7.44	49.808	
157	7.44	50.536	
169	7.55	50.92	
194	7.62	50.922	
189	7.72	53.07	
Polycyclic aromatic hydrocarbons (PAHs)			Kang et al. 2014[b]
Naphthalene	3.35	21.927	
Acenaphthylene	3.67	25.026	
Acenaphthene	3.92	27.315	
Fluorene	4.18	32.518	
Phenanthrene	4.52	32.906	
Anthracene	4.50	33.054	
Fluoranthene	5.20	36.173	
Pyrene	5.00	37.237	
Benz[*a*]anthracene	5.91	40.017	

Table S1. Continued

Chemicals	Log K_{ow}	HPLC RT (Min.)	References
Chrysene	5.86	40.017	
Benzo[*b*]fluoranthene	5.78	43.018	
Benzo[*k*]fluoranthene	6.11	43.217	
Benzo[*a*]pyrene	6.35	44.104	
Indeno[*1,2,3-cd*]pyrene	6.72	47.423	
Dibenz[*a,h*]anthracene	6.75	45.281	
Benzo[*ghi*]perylene	6.90	47.119	
Alkylphenols			ChemSpider 2015[c]
p-t-Butylphenol	3.31	20.364	
p-n-Pentylphenol	4.02	27.202	
p-n-Hexylphenol	4.52	31.144	
p-n-Heptylphenol	5.01	33.168	
p-n-Octylphenol	5.5	34.522	
p-t-Octylphenol	5.5	37.427	
p-n-Nonylphenol	5.76	39.907	
Phthalates			PubChem 2015[d]
Dimethyl phthalate	1.6	10.323	
Diethyl phthalate	2.47	16.073	
Di-n-butyl phthalate	4.5	31.123	
Butyl benzyl phthalate	4.73	31.738	
Bis(2-ethylhexyl) phthalate	7.5	46.920	

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