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Improving the accuracy of effect-directed analysis: the role of bioavailability

Jing You and Huizhen Li*

Aquatic ecosystems have been suffering from contamination by multiple stressors. Traditional chemical-based risk assessment usually fails to explain the toxicity contributions from contaminants that are not regularly monitored or that have an unknown identity. Diagnosing the causes of noted adverse outcomes in the environment is of great importance in ecological risk assessment and in this regard effect-directed analysis (EDA) has been designed to fulfill this purpose. The EDA approach is now increasingly used in aquatic risk assessment owing to its specialty in achieving effect-directed nontarget analysis; however, a lack of environmental relevance makes conventional EDA less favorable. In particular, ignoring the bioavailability in EDA may cause a biased and even erroneous identification of causative toxicants in a mixture. Taking bioavailability into consideration is therefore of great importance to improve the accuracy of EDA diagnosis. The present article reviews the current status and applications of EDA practices that incorporate bioavailability. The use of biological samples is the most obvious way to include bioavailability into EDA applications, but its development is limited due to the small sample size and lack of evidence for metabolizable compounds. Bioavailability/bioaccessibility-based extraction (bioaccessibility-directed and partitioning-based extraction) and passive-dosing techniques are recommended to be used to integrate bioavailability into EDA diagnosis in abiotic samples. Lastly, the future perspectives of expanding and standardizing the use of biological samples and bioavailability-based techniques in EDA are discussed.

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Environmental significance

The risk of multiple stressors in aquatic ecosystems has attracted increasing attention and become a great challenge to be overcome in the current framework of ecological risk assessment. Diagnosing the key toxicity contributors is an indispensable step in prioritizing the toxicants concerning environmental risk assessment, management and policy decision. Effect-directed analysis (EDA) is an efficient tool to address this challenge, yet suffers from the lack of environmental relevance. In particular, the ignorance of bioavailability in EDA may cause a biased identification of the key toxicants in a mixture. Based on the current understanding and limitations, the current article reviewed the methods and applications of incorporating bioavailability into EDA approaches to improve the accuracy of cause diagnosis.

Introduction

Along with the global population increase and economic booms, a vast number of chemicals have been used and released into the environment. As a consequence, complex mixtures of chemical residues and their transformation products co-occur in different environmental compartments and pose significant threats to the ecosystem and human health. To evaluate and mitigate ecological risk, it is necessary to find out the main toxicants responsible for any observed adverse outcome. Chemical-based risk assessment is often used to assess the occurrence, fate, and toxicity of environmental contaminants. A combination of quantitative analysis of target analytes and tiered biological assessments (*e.g.*, *in silico*, *in vitro*, and *in vivo* bioassays and trait-based effect assessment at population and community levels) can provide multiple lines of evidence for elucidating potential risks related to contaminants with a known identity.[1,2] These conventional approaches have proven effective in regional risk assessment, particularly in areas where key toxicants are included in the lists of target analytes. For example, the toxicity of sediments to benthic invertebrates in urban streams in some regions was well explained by the occurrence of pyrethroid insecticides.[3–5]

In most cases, however, the link between chemical exposure and an adverse outcome may not be so obvious. To date, more than 100 million chemicals have been indexed in the American Chemical Society's database and over 100 thousand of these are currently in use (https://www.cas.org/content/counter). However, only a very small portion (several thousands) of this

School of Environment, Guangzhou Key Laboratory of Environmental Exposure and Health, Guangdong Key Laboratory of Environmental Pollution and Health, Jinan University, Guangzhou 510632, China. E-mail: lihuizhen@jnu.edu.cn; Tel: +86-20-8522-6326

enormous number of chemicals have ever been analyzed and/or regulated in the environment, and even less (tens to hundreds) have been enrolled in the priority lists for regular monitoring.[6] It is possible that the thousands of target analytes assessed could only explain a small fraction of the observed adverse outcomes.[6–8] Recently, a nationwide survey of organic contaminants in stream water was carried out by the U. S. Geological Survey.[7] A total of 719 bioactive anthropogenic contaminants were analyzed in surface water samples collected from 38 streams across the U.S. Within the target compounds, 406 were detectable in at least one of the samples. Complementarily, *in vitro* bioassays were conducted as effect-based assessments, and estrogen (ER), androgen (AR), and glucocorticoid receptor (GR) activities in part of the water samples were significantly different from the controls. Although the ER activity was well correlated to the measured concentrations of estrogen in water, the detected AR and GR activities could not be explained by the detected target chemicals in the extended list of 719 analytes.[8] Similar inconsistency between the chemical and biological analyses of the same samples has also been reported in other regions. In a European-wide water monitoring program, Brack *et al.*[9] showed that the target analytes (151 organic pollutants) were not capable of explaining the major portions of the observed biological effects, particularly the AR and GR activity and fish embryo toxicity. Therefore, it is imperative to develop alternative nontarget approaches for diagnosing the causes of noted toxicity in the cases where target chemical-based risk assessment cannot match the results of effect-based assessments.

Cause diagnosis: toxicity identification evaluation and effect-directed analysis

Two approaches, namely toxicity identification evaluation (TIE) and effect-directed analysis (EDA), have been developed to fulfill the purpose of identifying key toxicants in environmental samples under the guidance of biological analysis.[10–12] In general, TIE procedures comprise three phases: characterization, identification, and confirmation.[13] In phase I characterization, the toxicants are classified into different groups, such as nonionic organic contaminants, cationic metals, ammonia, and sulfides, by comparing toxicity with and without sample manipulation. In phase II identification, possible toxicants belonging to the toxic classes characterized in phase I are analyzed for their identities, environmental concentrations, and toxicity contributions. In phase III confirmation, the toxicity contribution of the identified toxicants in phase II is confirmed using independent lines of evidence. On the other hand, EDA combines bioassays, fractionation, and chemical analysis to unravel the key organic toxicants in complex mixtures.[14] For solid samples, an extraction step is mandated to transfer the mixture of contaminants into a solution for bioassays and fractionation. Under the guidance of biological effects, EDA reduces the sample complexity for merely identifying the suspect toxicants in the fractions with high biological activity after cycles of fractionation and bioassays. Confirmation of the candidate toxicants is also required to warrant EDA identification.

Both approaches have been successfully used for aquatic samples, including water, sediments, and biota.[6,14–17] TIE and EDA share the same goal of tracing the causative agents for the observed toxicity by screening, identifying, and confirming the suspect toxicants in certain chemical classes and fractions exhibiting high biological activity. Nevertheless, TIE and EDA differ a lot in the methodologies used to reduce the complexity of the original samples and to narrow down the lists of pollutants to be analyzed.[15] For sediment samples, TIE characterizes the possible toxicants into different classes, *e.g.*, nonpolar organics, cation metals, and ammonia, using respective manipulations to alter the bioavailability of certain classes of chemicals, such as handling the sediments with coconut charcoal, cation resin, and zeolite.[13,18] Instead, EDA focuses on identifying the main organic toxicants causing toxicity in sediment extracts by performing fractionation and a bioassay iteratively.[6,14]

The two approaches have their own merits and weaknesses in diagnosing causative chemicals in complex environmental

Dr Jing You is currently a professor in the School of Environment, Jinan University. Her research is focused on addressing research questions about the environmental fate and adverse effects of organic contaminants, with a particular focus on the bioavailability and toxicity of current-use pesticides in sediments. Currently, her special research interest is to identify the principal cause of toxicity in complex mixtures of contaminants using toxicity identification evaluation and effect-directed analysis techniques.

Dr Huizhen Li is currently an associate professor in the School of Environment, Jinan University. Her general research interests are aquatic ecotoxicology and the risk assessment of organic contaminants, with a special interest in studying the toxicokinetics and toxicodynamics of metabolizable pesticides in aquatic species. She is also interested in evaluating the behavior, bioavailability, and toxicity of multiple contaminants in water-sediment systems.

mixtures. TIE considers toxicity contributions from organics, ammonia, and metals and has the advantage of environmental relevance by using whole organism toxicity testing with a consideration of the bioavailability of the contaminants. However, TIE suffers from the limitation that it only considers target analysis in phase II when identifying the principal toxicants in complex mixtures. Alternatively, EDA is a promising tool to use in diagnosing organic toxicants with the aid of sophisticated fractionation procedures and advanced analytical methods for target, suspect, and nontarget analyses. However, conventional EDA is short of environmental relevance because *in vitro* bioassays with exhaustive solvent extracts are commonly used in EDA rather than *in vivo* bioassays with original environmental samples as in TIE, mainly as a sacrifice to achieve high-throughput and high-sensitivity biological analysis. Therefore, it would be profitable to establish an integrated method by complementing the environmentally relevant TIE and the toxicant specific EDA.[15,19]

Taking bioavailability into consideration is vital to improve the environmental relevance of cause-diagnosing approaches. A comparison of whole sediment and porewater TIE techniques showed that the testing matrix significantly impacted the characterization of the causes of toxicity. While ammonia was identified as the major toxicant by porewater TIE, whole-sediment TIE concluded that polycyclic aromatic hydrocarbons (PAHs) were the primary reason for the toxicity to benthic invertebrates in sediment samples from the Illinois River in the U.S.[20] This suggests that it is preferable to conduct phase I TIE characterization under environmentally realistic exposure conditions, which can well address the bioavailability issue. Meanwhile, the use of bioavailability/bioaccessibility-based extraction (*e.g.*, Tenax extraction for nonpolar organics and the Bureau Commune de Reference speciation for metals) in phase II TIE could improve the accuracy in identifying major toxicants by eliminating the contaminants with low bioavailability from the list of putative toxicants.[21] On the contrary, conventional EDA methods generally ignore the bioavailability of the contaminants, which may bias the toxicant diagnosis by overestimating the toxicity of the highly toxic but less bioavailable contaminants and by underestimating the toxicity contribution from the polar toxicants.[14,15]

The majority of synthetic chemicals ever produced have been organic chemicals.[22] Upon reviewing the whole-sediment TIE practices in the last 20 years, Ho and Burgess[13] concluded that 90% of the TIE tests revealed organic pollutants, either singly (70%) or in combination with metals (10%) or ammonia (10%), were responsible for the observed sediment toxicity. This conclusion was also supported by Malaj *et al.*,[23] who found that organic contaminants were the major toxicants jeopardizing biodiversity and the ecological functions of freshwater ecosystems in Europe. Therefore, accurately elucidating the toxicity contribution from organic pollutants that have an ever-expanding chemical space is of great importance in diagnosing the causes of noted toxicity in the environment. This promotes the use of the EDA approach, which is superior to TIE in recognizing toxicity contributions from suspected organic toxicants with an unknown identity.[6] To take bioavailability into consideration is of importance for improving the accuracy of EDA diagnosis.

The objective of this review article is to summarize the current status and applications of EDA, with a special focus on discussing the role of bioavailability in cause diagnosis for biological and abiotic samples in aquatic environments. To incorporate bioavailability into EDA practices for abiotic samples, bioavailability-based techniques are integrated in the sampling (extraction) and/or dosing steps. Here, the future perspectives of applying and standardizing the bioavailability/bioaccessibility-based techniques in EDA are also discussed.

The concept and measurements of bioavailability

Bioavailability is a measure of the quantity of a chemical that can be utilized by an organism.[24,25] Bioavailability is controlled by an array of variables related to the exposure medium, chemical properties, organism traits, and environmental parameters, thus it is highly variable.[26,27] The influence of bioavailability on the bioaccumulation and toxicity of organic contaminants has been well documented and bioavailability-based measures have been recommended as more accurate dose metrics for predicting toxicity than the total chemical concentrations in an environmental matrix, particularly in sediment and soil.[4,26,28–32] Extensive studies have been conducted to better understand the impact of various factors on bioavailability, to develop more accurate methods for estimating bioavailability and to incorporate bioavailability into ecological risk assessment.[26,33]

Despite acknowledgment of the importance of including bioavailability in toxicity assessments, the practice is lagging behind due to the lack of a universally accepted definition of bioavailability.[27] The amount of chemicals accumulated in organisms well represents the bioavailability, but it is species-dependent, difficult to quantify, and unsuitable for easily metabolizable chemicals.[34] Alternatively, chemical techniques have been developed to predict bioavailability and are generally classified into two categories corresponding to two defined concepts of bioavailability: chemical activity and accessibility.[35] Chemical activity describes the potential for partitioning uptake into organisms and is closely related to the concentrations of the freely dissolved chemicals in the environmental media. It is similar to bioavailability termed by Semple *et al.*,[36] which they defined as the fraction of a chemical readily available for uptake by an organism. Instead, accessibility is an operational parameter representing the mass quantity of contaminants and it corresponds to bioaccessibility as defined by Semple *et al.*[36] as the fraction of a chemical potentially available to an organism.

According to the two concepts of bioavailability, a suite of chemical techniques have been developed to predict the bioavailability of organic contaminants. Many reviews have summarized the current chemical techniques for estimating the bioavailability of organics and suggest that these techniques can adequately estimate chemical body residues in biota and their toxic effects.[26,27,29,32,37] Chemical activity is

a mechanistically defined concept based on equilibrium partitioning theory.[35] Various passive-sampling techniques have been applied to measure the chemical activity of nonpolar organics, *e.g.*, semi-permeable membrane devices,[38] solid phase microextraction,[39] polyoxymethylene,[40] polyethylene devices,[41] and polymethyl methacrylate.[42] Meanwhile, passive samplers have also been designed for concentrating polar organic contaminants in water, such as the polar organic chemical integrative sampler (POCIS),[43] Chemcatcher,[44] and organic-diffusive gradients in a thin film aquatic passive sampler (*o*-DGT).[45] The operationally defined accessibility of organic contaminants is commonly estimated using non-exhaustive bioaccessibility-directed extractions, including mild solvent extraction,[46] cyclodextrin extraction,[47] supercritical fluid extraction,[48] and sorbent-assisted desorption (*e.g.*, Tenax and XAD extraction).[49,50] Though the two bioavailability-based extraction techniques (partitioning-based passive sampling and bioaccessibility-directed extraction) measure different components of the matrix, their estimates have both been shown as good indicators of body residues of organic contaminants in biota.[27,29,35] Compared with mimicking the bioaccumulation of chemicals in organisms, the use of bioavailability-based extraction techniques in predicting adverse effects is less investigated.[51,52] The limited studies have implied that incorporating bioavailability-based dose metrics into toxicity evaluations may significantly improve the accuracy of ecological risk assessment.[4,28–30,53]

Incorporating bioavailability into EDA analysis

Bioavailability influences the apparent toxicity of a chemical tremendously, thus it is imperative to consider bioavailability in the cause diagnosis of environmental mixtures.[54,55] Nevertheless, conventional EDA seldom takes bioavailability into consideration when using exhaustive extraction, which accordingly biases the toxicity composition of individual compounds in the mixture.[56] However, an ignorance of bioavailability may lead to an overestimation of the toxicity of some hydrophobic contaminants that have high toxicity but poor bioavailability. Consequently, the toxicity contribution from more bioavailable polar contaminants in the same mixture may be masked and overlooked, resulting in a failure of EDA applications.[6,15,54] To obtain more realistic exposure scenarios, bioavailability is recommended to be integrated into EDA procedures. Such an incorporation of bioavailability would aid EDA to reduce bias in assessing the toxicity of contaminants with limited exposure potential and would subsequently increase the accuracy in cause diagnosis.

The most straightforward way to consider the bioavailability issue is to perform EDA using biological samples, yet this type of EDA practice is understudied nowadays due to some conceptual and technical challenges.[16,17,54,55] In the meantime, increasing numbers of studies have tried to develop EDA approaches for abiotic samples in combination with previously established bioavailability-based techniques. As shown in Fig. 1,

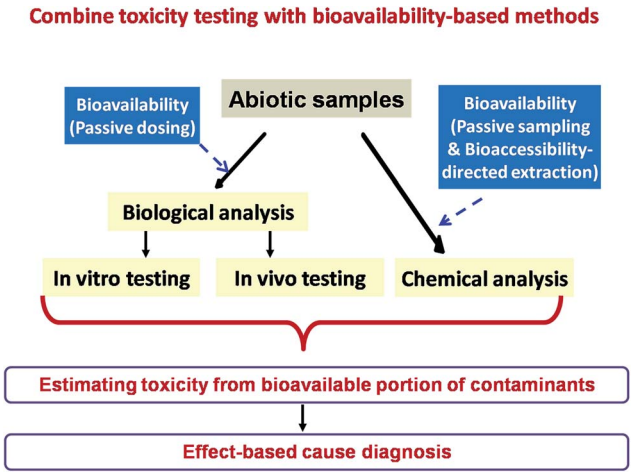

Fig. 1 A scheme to incorporate bioavailability-based extraction and dosing methods in effect-directed analysis.

bioavailability can be dealt with throughout the EDA process. Bioavailability-based extraction techniques (partitioning-based passive sampling and bioaccessibility-directed extraction) have been used for collecting contaminant mixtures from environmental samples before fractionation and toxicity testing, while bioavailability-based dosing techniques have been used as a replacement for traditional solvent-dosing methods for conducting toxicity testing of fractionalized extracts. As a result, more ecologically relevant EDA methods that consider the bioavailability of the contaminants have been established.[6,15,54,55] In the following sections, the current status and challenges for integrating bioavailability into EDA practices are reviewed for biological and abiotic samples sequentially.

The EDA of biological samples

While rarely performed, the EDA of biological samples already directly take bioavailability into consideration by using biological samples, such as tissue homogenates of organisms and body fluids (*e.g.*, blood plasma, bile, and urine). As listed in Table 1, a variety of tissue and body fluid samples have been used in EDA. Similar to a general EDA procedure, a whole-process EDA of biological samples consists of sample extraction, cycled extract fractionation and bioassays, chemical analysis, and toxicant identification and confirmation. Simon *et al.*[17] reviewed the applications and recent innovations in the EDA of biological samples and claimed that the use of biological samples is advantageous due to the direct consideration of bioavailability by covering the complete exposure routes of contaminants from the environment to the biota.

The difficulty in preparing extracts for EDA analysis is one of the main aspects hindering the application of biological samples.[17,54,55] In general, extracting biological samples poses challenges related to the small sample size and high matrix interference, *e.g.*, lipids and natural hormones. Therefore, developing cleanup methods to effectively remove the lipids and hormones from biological extracts without sacrificing

Table 1 Examples of some effect-directed analysis studies of biological samples

	Sample	Bioassay endpoint	Biological analysis	Chemical analysis	Candidate toxicants	Ref.
Mammal	Plasmas of polar bears	Thyroid hormone (TH)-like activity	125I-T4-TTR binding assay	HR-GC-EI-MS	4-Hydroxyheptachlorostyrene	92
	Blood plasma of polar bear cubs	TH-like activity	125I-T4-TTR binding assay	LC-ESI-TOF-MS	4-Nonylphenol; monohydroxylated octachlorinated biphenyls (OH-PCBs): 4′-OH-CB201 and 4,4′-OH-CB202	93
	Blubber of baikal seal, liver of common cormorant, raccoon dog, finless porpoise	Androgen receptor (AR) antagonistic activity; dioxin-like and AR antagonistic activities;	AR-CALUX; DR-CALUX	GC-EI-MS	1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p*,*p*′-DDE); PCDD/Fs and coplanar-PCBs	94
	PDMS-passive sampling in the blubber of dugong	Aryl hydrocarbon receptor (AhR); Nrf2-mediated oxidative stress response; p53-mediated DNA damage response; NF-κB-mediated response to inflammation	CAFLUX; AREc-32; p53-bla; NF-κB-bla	HRGC-HRMS		61
Turtle	PDMS-passive sampling in the blood of green turtles	AhR-mediated activity; Nrf2-mediated oxidative stress response	CAFLUX assay; AREc32 assay	GC-ECD; HRGC-HRMS	PCDD/Fs, PCBs, PBDEs and OCPs	60
Fish	Hepatic tissue extracts of white sucker	AhR-mediated activity; estrogenic/androgenic activity	EROD activity measurement; ligand responsive estrogen and androgen receptor-binding bioassay, sex steroid-protein binding assay	RP-HPLC fractionation	PCDD/Fs accounted for MFO activity of liver contaminants of log K_{ow} > 5 but multiple nondioxin AhR ligands of log K_{ow} 2 to 5 also caused significant induction. Compounds of log K_{ow} 2 to 5 in livers of exposed fish exhibited significant competition for the AR, ER, and SSBP	95 and 96
	Bile from male bream	Estrogenic activity	ER-CALUX	RP-HPLC fractionation; GC-MS analysis	Natural hormones 17β-estradiol, estrone, and estriol accounted for the majority of estrogenic activity. In addition, ethynylestradiol, triclosan, chloroxylenol, and chlorophene were detected	97
	Bile/gonads of rainbow trout and roach exposed to wastewater treatment effluents	Estrogenic activity	Yeast estrogen receptor transcription (YES) bioassay	RP-HPLC fractionation; GC-MS analysis	Natural hormones (17β-estradiol [E2], estrone [E1], estriol [E3]) and synthetic hormones (ethynylestradiol [EE2]) estrogen hormones; nonylphenol (NP), shortchain NP polyethoxylates, 17 β-dihydroequilenin (DHQ)	98
	Bile of rainbow trout exposed to wastewater treatment effluents	Antiandrogenic activity	YAS bioassay	RP-HPLC fractionation; GC-MS	Di(chloromethyl)-anthracene, dichlorophene	99
	Bile of rainbow trout exposed to wastewater treatment effluents	Antiandrogenic activity	Yeast (anti-YAS) and mammalian-based (AR-CALUX) androgen receptor transcription screens	RP-HPLC fractionation; GC-iontrap MS	Chlorophene, triclosan, chloroxylenol, dichlorophene, resin acid isomers, 2-naphthol, oxybenzone, 4-nonylphenol, bisphenol-A, 1-hydroxypyrene, oxybenzone, 2,2′-dihydroxybiphenyl, 9,10-di(chloromethyl) anthracene	100
	Flounders homogenate	Genotoxicity	AMES fluctuation assay	GC-EI-MS	4-Nonylphenol; musks: cestolide/phantolide, musk xylene	63

Table 1 (*Contd.*)

	Sample	Bioassay endpoint	Biological analysis	Chemical analysis	Candidate toxicants	Ref.
Invertebrate	Blue mussels	AhR-mediated activity; estrogenic activity	DR-CALUX; YES bioassay	NP-HPLC fractionation; GC-EI-MS and GC-NCI-MS	Halogenated aromatics (PCBs, PCDDs, PCDFs) and nonhalogenated aromatics; estrogenic response < limit of detection	101
	Homogenates of lugworms and crabs	Thyroid hormone-like activity	125I-T4-TTR binding assay	LC-ESI-ToF-MS	4-Nonylphenol; perfluoroalkyl substances (PFASs): 7*H*-perfluoroheptanoic acid [7*H*-PFHpA], perfluorobutane sulfonate [PFBS], perfluorooctyl phosphonic acid [PFPA]; musks: cestolide/phantolide, galaxolide/tonalide/traseolide, musk ambrette	63
	Marine mussels	Feeding rate	Measurement of added food removal (standard procedure)	NP-HPLC fractionation; GC-MS	Unresolved complex mixture of aromatic hydrocarbons	64

potential toxicants is critical to expanding their applications in EDA. An accelerated membrane-assisted cleanup (AMAC) method has been proposed for cleaning lipid-rich biological samples prior to chemical and/or biological analyses. Streck *et al.*[57] showed that this AMAC method is effective in removing >90% of lipid-like matrix components and could satisfactorily recover most compounds (>70%). Simon *et al.*[58] used a combination of dialysis, gel permeation chromatography (GPC), and normal phase-high performance liquid chromatography (NP-HPLC) to eliminate the co-extracted lipids and hormones from fish tissues. This method enabled the use of *in vitro* bioassays in EDA to unravel the anthropogenic toxicants for the noted endocrine-disrupting (ED) effects of thyroid hormone (TH)-like and (anti)androgenic activities. In addition, a cleanup method coupling solid phase extraction (SPE) with liquid–liquid extraction (LLE) was developed and used in the EDA of blood plasma with the *in vitro* TH disrupting effect as the endpoint. With the help of the validated SPE-LLE method, an EDA of polar bear plasma samples found that the OH-PCBs in plasma explained 60–85% of the detected TTR-binding potency.[59] Passive samplers were innovatively introduced for extracting biological samples for EDA and this method could significantly reduce the interfering matrix components. Jin *et al.*[60–62] employed polydimethylsiloxane (PDMS) polymer as a partitioning-based passive sampler to collect neutral organic chemicals accumulated in the blood of green turtles and the blubber of marine mammal dugong. The results showed that most of the dioxin-like activity (>70%) in bioassays could be explained by EDA-deducted toxicants, but the cause of the oxidative stress response is not clear yet.

To perform high-throughput toxicity testing with small sample masses, *in vitro* bioassays are regarded as compatible methods to evaluate the adverse effects of biological extracts and the fractions. As shown in Table 1, the ED effects are the most concerned toxicity endpoints in the EDA of biological samples, including TH-like, estrogenic, (anti)androgenic, and aryl hydrocarbon receptor (AhR)-mediated activities. In addition, genotoxicity has also been used as an endpoint for *in vitro* bioassays in EDA.[63] Comparatively, *in vivo* bioassays are hardly used in the EDA of biological samples due to the small sample size. Donkin *et al.*[64] applied the feeding rate of green mussels *Mytilus edulis* as the EDA toxicity endpoint, and found that the unresolved complex mixture of aromatic hydrocarbons impaired the feeding behavior of the mussels. Advances in nontarget analysis research along with ever-improving mass spectrum techniques have greatly benefited toxicant identification and confirmation in the EDA of biological samples, making it a promising approach for diagnosing the toxicants in biota with unknown identity. However, the conceptual drawbacks in the EDA of biological samples along with the technical challenges mentioned above have hampered its development.[55] First, the body residues of contaminants in biota are governed by an *in vivo* toxicokinetic process, but this is species-dependent, which prevents the findings being extrapolated from one species to others, making the assessment less generalized. Second, a great number of organic contaminants are metabolizable and only metabolites are accumulated in

organisms. The toxicity of the metabolites may be totally different from the parent compounds. A better understanding of the distribution pattern and biotransformation pathway of suspect toxicants is critical for toxicity elucidation. These conceptual drawbacks restrict the EDA of biological samples to be merely used for specific questions instead of being generally applicable, although it takes advantage of being able to easily incorporate bioavailability into EDA practices.

The EDA of abiotic samples

The gradual maturing of bioavailability-based techniques (bioaccessibility-directed extraction and partitioning-based passive sampling and passive dosing) in recent years has provided effective tools to incorporate bioavailability into the EDA of abiotic samples.[6,56] To achieve this, the challenge of enriching enough extracts for cycled fractionation and bioassays needs to be overcome. Most passive-sampling techniques use small-scale extraction methods, *e.g.*, solid phase microextraction,[27,29] so the amount of extracts is too limited to conduct fractionation and bioassays in EDA, although it is satisfactory for chemical analysis.

In vitro toxicity testing has been extensively used in EDA practice due to its high-throughput, highly specific endpoints, and low sample amount requirement. However, *in vitro* bioassays are not environmentally relevant and are difficult to be extrapolated to the adverse outcomes at the organism and population levels. Moreover, *in vitro* bioassays cannot account for the *in vivo* toxicokinetic process of test contaminants, *i.e.*, uptake, distribution, biotransformation, and elimination. Therefore, it is preferable to use more environmentally relevant *in vivo* bioassays in cause diagnosis.[65] To facilitate the use of *in vivo* bioassays in EDA practices, it is necessary to set up a miniaturized testing system. Moreover, developing bioavailability-based techniques to gain a relatively large amount of samples in the extraction step serves as another solution. So far, the methods to incorporate bioavailability into the EDA of abiotic samples can be separated into three types, namely bioaccessibility-directed extraction, and partitioning-based passive sampling and passive dosing (Table 2).

Bioaccessibility-directed extraction

Bioaccessibility is the fraction of chemical potentially available to an organism. Although this operational concept is not fully clear in theory, the bioaccessible fraction (rapid desorption fraction) estimated by various bioaccessibility-directed extraction methods has been shown as a promising measure for the bioaccumulation potential and noted toxicity of contaminants to the exposed organisms.[28,66–68] Compared with partitioning-based passive sampling (*e.g.*, solid phase microextraction), which normally exposes the sampling materials to abiotic samples for a long period to achieve equilibrium, bioaccessibility-directed extraction (*e.g.*, Tenax extraction) takes the advantage of merely requiring a single time point treatment (usually <48 h), making it more applicable for less persistent contaminants.[27] More important, bioaccessibility-directed extraction makes it easier to obtain a relatively large amount of extracts compared to partitioning-based extraction, which is a premise to incorporate bioavailability into EDA. Accordingly, the first study to integrate bioavailability into sediment EDA used a bioaccessibility-directed extraction technique, *i.e.*, large volume Tenax extraction.[69,70] In order to gain enough sediment extracts for fractionation and bioassays, the regular 24 h single time point Tenax extraction method was upscaled by a factor of 125.[69] Inhibiting the growth of benthic algae *Scenedesmus vacuolatus* was selected as the toxicity endpoint. The established large volume Tenax extraction method was used in the EDA procedures for three sediment samples taken from the Elbe River Basin and the results were compared with those obtained using exhaustive accelerated solvent extraction (ASE). The patterns of toxicity composition in fractions of the extracts were different in the bioaccessibility-directed and exhaustive extraction methods, while exhaustive ASE overestimated the toxicity contribution from the poorly bioavailable compounds.[70] Recently, Hong *et al.*[71] compared the EDA results for coastal sediment extracts by Tenax and Soxhlet extractions using a battery of *in vitro* H4IIE-luc bioassays. The authors found that the major AhR-active PAHs were C4-phenanthrene and C1- and C3-chrysene. The levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent-PAHs in the sediment extracts by Tenax extraction (60%) better explained the bioassay-derived TCDD-EQ concentrations than those by Soxhlet extraction (31%), showing the superiority of using bioaccessibility-directed extraction in EDA analysis.

Overall, the incorporation of bioaccessibility-directed extraction into EDA could effectively improve key toxicant prioritization for more accurately estimating the exposure of likely toxicants in complex mixtures. Many bioaccessibility-directed extraction methods have been developed to date and are suitable for sediment, soil, and dust samples. The selection of the method should ensure that the desorption of contaminants from the environmental medium is the rate-limiting step and determines the bioaccessible fractions of the contaminants, such that the extraction procedure does not significantly affect the collected mixture.[55] In addition, to guarantee the applicability of the method, strict quality control procedures should be included to avoid positive errors caused by impurities in the extraction materials during biological and chemical analyses.

Partitioning-based passive sampling

The chemical activity determines the fraction of a chemical readily available for uptake by an organism and is represented by the freely dissolved chemical concentrations. Compared with bioaccessibility-directed extraction, partitioning-based passive sampling is theoretically more favorable since it directly mimics the partitioning process of chemicals from an environmental medium to organisms. In reality, however, partitioning-based passive sampling is less applied in EDA due to the limitations related to the small sample masses and long equilibrium time.

Passive-sampling techniques have been extensively used to assess the risk of contaminated sediments.[26,31,32] Li *et al.*[72]

Table 2 The methods to incorporate bioavailability into the effect-directed analysis of abiotic samples

	Sample matrix	Extraction method	Dosing method	Biological analysis	Chemical fractionation and analysis	Ref.
Bioaccessibility-directed extraction	Spiked sediment	Large volume Tenax extraction	Solvent dosing with dimethyl sulfoxide (DMSO)	Inhibition of cellular reproduction of green algae *Scenedesmus vacuolatus*; immobilization of brine shrimp *Artemia salina*	GC-MS	69
	Field sediment	Large volume Tenax extraction	Solvent dosing with DMSO	Growth inhibition of the green alga *S. vacuolatus*	NP-HPLC (cyanopropyl (CN), nitrophenyl (NO) & porous graphitized carbon (PGC) columns) fractionation; GC-MS screening	70
	Coastal sediments contaminated by spilled crude oil	Tenax extraction	Solvent dosing	H4IIE-luc *in vitro* bioassay (cytotoxicity; aryl hydrocarbon receptor (AhR)-mediated potency)	GC-MS analysis	71
Passive sampling	Water	Polar organic compound integrative sampler (POCIS) & semi-permeable membrane device (SPMD)	Solvent dosing	Estrogenic, (anti-) androgenic, pregnane X receptor-like (PXR) and dioxin-like activities	Combination of NP-SPE and RP-HPLC fractionation	76
	River water near a pharmaceutical factory	POCIS with HLB sorbent	Solvent dosing	Steroid-like activities, including glucocorticoid, antimineralocorticoid, progestogenic, PXR-like and estrogenic activities	RP-HPLC fractionation; HPLC-MS-MS analysis	74
	River water	POCIS-Apharm, POCIS-Bpesticide, low-density polyethylene membranes (LDPE) and silicone strips	Solvent dosing with ethanol	Androgen receptor antagonist screen (YAS)	RP-HPLC fractionation; GC-MS and HPLC-QTOFMS analysis	77
	Coastal waters	POCIS with sepra *ZT* sorbent and silicone rubber	Solvent dosing after evaporating off the solvents of hexane/acetone	Pulse amplitude modulation (PAM) fluorometry assay with marine microalgae *Dunaliella tertiolecta* and the endpoint is photosystem H efficiency (phi PSII) in marine microalgae	RP-UPLC microfractionation technique using 96-well plates; HPLC-TOF-MS analysis	78
	Water	Blue rayon (BR) as a passive sampler that selectively adsorbs polyaromatic compounds	Solvent dosing with DMSO	Ames fluctuation assay with strains TA98, YG1024 and YG1041 with and without S9 activation	Multiple-step HPLC fractionation with MCX, C18 & phenyl-hexyl columns; HPLC-APCI-MS-MS analysis	80
	Water	LDPE	Solvent dosing with DMSO	Zerbrafish embryo tests with multiple endpoints (mortality, edema and notochord distortion)	GPC and cyano-propyl column NP-HPLC fractionation; GC/MS/MS screening	75

Table 2 (*Contd.*)

	Sample matrix	Extraction method	Dosing method	Biological analysis	Chemical fractionation and analysis	Ref.
Passive dosing	Sediment	Exhaustive accelerated solvent extraction (ASE)	Passive dosing with silicone rods *vs.* solvent dosing with DMSO	Growth inhibition test with the green algae *S. vacuolatus*	Multistep NP-HPLC fractionation (NO, CN and PGC columns); GC-MS analysis	82
	Sediment	Exhaustive ASE	Passive dosing with silicone rods *vs.* solvent dosing with DMSO	Growth inhibition test with the green algae *S. vacuolatus*	Multistep NP-HPLC (NO, CN and PGC columns) and RP-HPLC (C18) fractionation; GC-MS analysis	81
	Sediment	Exhaustive microwave-assisted sonication extraction	Passive dosing with PDMS film	Behavior toxicity and enzymatic activity of *Chironomus dilutus*	Two-step RP-HPLC fractionation; GC-MS screening	89
Bioaccessibility-directed extraction & passive dosing	Sediment	Large volume Tenax extraction *vs.* ASE	Passive dosing with silicone rods *vs.* solvent dosing with DMSO	Growth inhibition test with green algae *S. vacuolatus*	Multistep NP-HPLC fractionation (NO, CN and PGC columns); GC-MS analysis	90

demonstrated that passive sampling was better suited with *in vitro* bioassays than exhaustive extraction in sediment toxicity evaluation. A recent study by Vethaak *et al.*[73] showed that *in vitro* bioassay testing with passive sampler extracts was a promising tool to assess sediment toxicity together with a consideration of bioavailability. While they are rarely used in sediment EDA nowadays, the successful applications of passive sampler extracts in toxicity testing provides the possibility to apply this technique in sediment EDA, but further work is needed before it can be practically applied.

Alternatively, passive samplers, *e.g.*, POCIS, have been used to collect organic contaminants from water samples for EDA. Creusot *et al.*[74] placed POCIS in the upstream and downstream of a pharmaceutical factory. The extracts of POCIS were evaluated for steroid-like activities using *in vitro* bioassays and showed high glucocorticoid, antimineralocorticoid, progestogenic, and pregnane X receptor (PXR)-like activities and weak estrogenic activity. Further EDA tests revealed that dexamethasone, spironolactone, and 6-alpha-methylprednisolone were the main contributors to the corticosteroid activity, while levonorgestrel was responsible for the progestogenic activity. To evaluate the contribution from less polar compounds, other passive samplers have also been tried. Bergmann *et al.*[75] deployed low-density polyethylene (LDPE) at the Portland Harbor superfund megasite, Oregon, the U.S., where PAHs have been traditionally considered as the reason for fish embryo toxicity. The EDA of LDPE extracts showed that fatty acids and dithiocarbamates, which were not previously monitored, were the main contributors to the zebrafish embryo toxicity. The use of passive sampling (LDPE) can help to diagnose the toxicants in the bioavailable fraction instead of the total extract, thus improving the accuracy of toxicant identification.

Maintaining the composition of all the contaminants in the original samples is preferable during transferring the contaminants in the environment to the exposure medium for bioassays. Since there is no passive sampler suitable for all compounds with various properties, using a combination of passive samplers designed for extracting polar and nonpolar organics (*e.g.*, POCIS and LDPE) is favorable. Creusot *et al.*[76] simultaneously deployed POCIS and a semi-permeable membrane device (SPMD) in river water, where the sediment was contaminated by endocrine-disrupting chemicals (EDCs). Different distribution patterns of ER, (anti-)androgenic receptor, PXR-like and dioxin-like activities were detected in extracts of the sediment with POCIS and SPMD, indicating the presence of varying active chemicals in different compartments. Similarly, Liscio *et al.*[77] used a combination of four passive samplers (POCIS-Apharm, POCIS-Bpesticide, LDPE, and silicone strips) to obtain a holistic profile of EDCs in surface water. The extracts of individual passive samplers were diagnosed by EDA combining HPLC fractionation with an *in vitro* bioassay of the androgen receptor antagonist screen (YAS). Anti-androgenic contaminants were identified as the main toxicants in the extracts of POCIS-Apharm and silicone samplers, but the contaminants had different polarity. A combination of POCIS and silicone rubber sheets was also been in an EDA test with impaired photosystem H efficiency in marine microalgae as the

toxicity endpoint.[78] In this study, a novel microfractionation technique using 96-well plates was implemented for the extracts of passive samplers. Chemical and biological analyses were conducted on the same plates, and the results showed that several herbicides were responsible for the noted effect on the microalgae. Altogether, these studies showed the need to use multiple passive samplers simultaneously to achieve a holistic exposure assessment and to consider the toxicity contributions of various contaminants. This calls for the development of novel sorption materials for passive samplers to enable them to be able to extract a broad range of chemicals at the same time.[79]

A high-selectivity sampler could help to address some specific questions and improve the accuracy in cause diagnosis by reducing the complexity of the extracts. In order to find out the polyaromatic mutagens in surface water, blue rayon was used as a passive sampler, which selectively adsorbs polyaromatic compounds.[80] The Ames fluctuation assay was performed using strains TA98, YG1024, and YG1041 with and without S9 activation. The use of S9 activation in bioassays took the metabolism-induced change of mutagenicity into consideration. Analytical screening of the mutagenic fractions unraveled amino and nitro-compounds as the potential mutagens.[80]

Partitioning-based passive dosing

In EDA, bioavailability is not only dealt with in the extraction step, but also in the dosing step for the fractionalized extracts for toxicity testing. Organic solvents, such as dimethylsulfoxide and methanol, are generally used as carriers to dose the extracts into the bioassay medium in conventional EDA. However, solvent dosing, which transfers the extracts into the bioassay medium, may change the composition of contaminants in the original samples. Recently, partitioning-based passive dosing has been employed in EDA to take bioavailability into consideration.[6,54–56,81,82]

Passive-dosing techniques are based on chemical partitioning among phases and have been proposed to maintain constant concentrations of hydrophobic organic compounds in water.[83] Compared with solvent dosing, passive dosing is advantageous in compensating for chemical losses caused by test chamber absorption, volatilization, degradation, and organismal uptake. Thus it provides more accurate measures of the solubility, partitioning coefficients, and adverse effects of hydrophobic organic compounds in water.[84,85] Due to its high biocompatibility, high permeability, and relative small mass transfer coefficients, PDMS (silicone) is the most commonly used polymer material in passive dosing.[86]

A passive-dosing procedure consists of loading and releasing steps. Two methods have been used to load the fractionalized sediment extracts onto PDMS polymers. One is the preloading method, in which the extracts are added into the PDMS precursor before curing the PDMS polymer,[87] while the other involves postloading the extracts, which can be achieved by soaking PDMS in a methanol/water solution containing the extracts and then loading the chemicals onto PDMS through equilibrium partitioning[88] or directly loading all the solution of the fractionalized extracts onto the PDMS polymer.[89] Regardless of the loading methods, the extracts are required to be transferred to the dosing polymer without preferential losses. Then, PDMS polymers loaded with the extracts serve as a source to release chemical mixtures into the testing medium through partitioning.[84] In addition to maintaining constant concentrations of contaminants in the test medium, passive dosing imitates the partitioning of chemical mixtures in the sediment–water–organism system, which assists in re-establishing the composition of chemical mixtures in the bioassay medium the same as in the original samples.[6,56] Therefore, passive dosing is superior to traditional solvent dosing, which transfers all the contaminants into the bioassay medium without considering their bioavailability.

When passive dosing with silicone rods was used in EDA bioassay, the toxic fractions were distinct from those obtained with conventional solvent dosing for the same sediment extracts.[82] Accordingly, different key toxicants were identified by the two methods. While PAHs were regarded as the main reason for the toxicity to green algae when solvent dosing was applied, more polar triclosan was found responsible for the algal toxicity with the passive-dosing technique in a EDA study.[81] This is reasonable because the toxicity contribution of less bioavailable lipophilic compounds tend to be overemphasized when using solvent dosing, which transfers all the chemicals to the bioassay medium. On the contrary, passive dosing better represents the exposure of the mixtures in the original sediment samples and thus provides a more realistic diagnosis. Qi *et al.*[89] applied passive dosing to transfer the fractionalized extracts into water to conduct toxicity testing with the midges *Chironomus dilutus*. The lack of highly hydrophobic toxicants in this study calls for more efforts to validate the applicability of the current passive-dosing techniques in bioassays, especially in the progress of establishing environmentally realistic EDA methods to account for suspect toxicants with high hydrophobicity.

In addition to conducting toxicity testing with sediment extracts using exhaustive extraction, a combination of passive dosing with bioaccessibility-directed extraction[90] or passive-sampling techniques[91] is recommended for sediment EDA. Brack *et al.*[6] discussed the influence of dosing techniques for bioassays on the outcome of the EDA of sediment and water samples in detail through depicting the partition and dosing scenarios of chemical mixtures in the sediment, water, PDMS, bioassay medium, and biota. Bioavailability-based extraction has been suggested to be combined with passive dosing in EDA practice, which is, in theory, more environmentally realistic and reflects better the exposure of organisms in the environment. The selection of passive sampling and passive dosing techniques should be carefully designed for the EDA practices of different samples in order to avoid altering the chemical composition of the original mixtures during extraction and bioassays.

Conclusions and future perspectives

The role of the bioavailability of environmental contaminants in chemical-based risk assessment has long been noted. Extensive research in the past decades has provided a better

understanding of how bioavailability affects the toxicity of organic contaminants and how to incorporate bioavailability into effect-based cause diagnosis (*e.g.*, EDA), which is imperative in ecological risk assessment.

The use of biological samples in EDA is the most straightforward way to consider the bioavailability of environmental contaminants. While understudied, this method has been shown to be a promising way to identify previously unknown and/or unregulated but bioaccumulative toxicants, particularly the suspect EDCs in biota. This method, however, suffers from the restriction of the small sample amount and lack of evidence for the toxicants that have a tendency to be biotransformed. The development of effective extraction and cleanup methods for biological samples and a better understanding of the distribution pattern and biotransformation pathway of suspect toxicants are critical in generalizing the EDA of biological samples. For the EDA of abiotic samples, the integration of bioavailability/bioaccessibility-based extraction and passive dosing has been considered as a preferable strategy to transfer chemical mixtures from original environmental samples into the bioassay medium, providing a way to incorporate bioavailability into EDA tools for more accurately identifying the key toxicants.

Despite the studies presented in this review, there is still significant research potential in this area. To date, EDA methods with the consideration of bioavailability are too few and more studies on method development are critical to expand their practical uses. The applicability of bioavailability-based extraction and dosing techniques needs to be validated for their capability to maintain the composition of a contaminant mixture in raw samples and to mimic uptake by the organisms. Furthermore, a better understanding of the desorption and partitioning processes of various contaminants in environmental media and biota would reduce the bias in employing bioavailability-based extraction and dosing techniques, particularly for highly hydrophobic and ionic compounds. Lastly, validation of the proposed methods is critical to expand their applications for research and regulatory requirements. Future research is needed to standardize the procedures for incorporating bioavailability into EDA, which warrants achieving consistent and comparable results across laboratories.

Conflicts of interest

There are no conflicts of interest to declare.

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