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Review

Effect-directed analysis and beyond: how to find causal environmental toxicants

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Abstract

Humans and wildlife are exposed to complex environmental mixtures. Identifying causal toxic pollutants in environmental samples remains challenging because of the high complexity of sample mixtures and the unknown nature of the potential toxicants. In the field of environmental chemistry and toxicology, this pursuit of causal toxicants leads us to the method of effect-directed analysis (EDA), an integrated method comprised of three iterative modules: (1) bioassays to guide component prioritization; (2) fractionation to reduce the mixture complexity; and (3) chemical analysis to identify the toxicants. In this commentary review, we try to provide a concise guideline for EDA beginners by summarizing good practices from successful EDA studies, categorized by sample-toxicity pair selection, efficient separation, and chemical analysis. We also discussed the practical challenges faced with current EDA practices. Based on these above, we try to provide suggestions and perspectives for future EDA studies. Specifically, we discussed the potential of applying EDA on human biological examples to identify the environmental causes of human diseases. We proposed future collaboration between environmental chemists and toxicologists, environmental health scientists, epidemiologists, physicians, and social scientists.

Keywords: untargeted analysis; exposome; environmental chemistry; pollutants; toxicology; EDA

Introduction

One of the ultimate goals of environmental chemistry is to find the causal toxicants responsible for human and ecological toxicities. Since Percivall Pott's study in 1775 linking chimney sweeps' carcinoma and exposure to polycyclic aromatic hydrocarbons (PAHs),[1] scientists and physicians began to link environmental exposures to human diseases. Environmental exposures are known to play vital roles in the development of diseases (like cancers and amyotrophic lateral sclerosis),[2,3] but most environmental contributors remain unknown because of the complexity of human exposure and the long pre-symptomatic intervals. The search for environmental causes of diseases has become more convincing when the model of gene–environment interaction (Disease = Gene × Environment or $D = G \times E$) is widely accepted.[4,5] More recently, the "exposome" has become the new focus of environmental exposure studies,[6,7] as it is defined as the measure of all the exposures of an individual in a lifetime and how those exposures relate to health. The search for "functional exposome," the totality of the biologically active exposures relevant to disease development,[8] resonates with the investigation of causal toxicants. Aside from human adverse health effects, biologists and ecologists have been asking similar questions about causal toxicants as wildlife (animals, plants, and prokaryotes) may suffer from unexplained environmental toxicity at both sublethal and lethal doses.[9-11] Since wildlife are more often directly exposed to the environmental sources and fates of contaminants than human beings, corresponding studies have focused on causal toxicants in environmental samples such as water, sediment, soil, and air particulates. In this field of environmental chemistry and toxicology, the pursuit of causal toxicants leads us to the method of effect-directed analysis (EDA).

As early as the 1980s,[12,13] EDA-type of studies were introduced with the term "bioassay-directed chemical analysis," there are a few synonyms (EDA, bioassay-directed fractionation, and bioassay-directed identification), which vary slightly but share the same goal: identifying the toxicity drivers in complex environmental samples via fractionation. Different from the conventional paradigm of "toxicity from measured chemicals," EDA methods acknowledge that environmental toxicities were rarely explained by the well-known target compounds, and focus on prioritizing toxicity drivers even if they are unknown. Toxicity identification evaluation (TIE) and EDA aim to identify these toxicants using different bioassay approaches. While EDA typically uses *in vitro* bioassays and focuses on sophisticated fractionation, TIE stresses bioavailability and *in vivo* toxicities. Except for whole organism endpoints, TIE also has a broader range of application that is not limited to organic contaminants. Phase I of TIE is toxicity characterization, which uses simple sample manipulation steps such as pH adjustment, aeration, and chelation to explore the properties of the toxicants. The two approaches complement each other, and a hybrid method combining Phase I of TIE and EDA can be

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comprehensive and powerful. Further comparison of TIE and EDA has been discussed by Burgess *et al.*[14]

Despite many studies referencing or applying EDA, this approach is still considered challenging and rarely streamlined into routine environmental monitoring programs (outside EU, at least). The practical difficulties of EDA are (1) the complexity of environmental samples and chemical interactions, (2) the low concentrations of toxicants in the samples, and (3) the unknown nature of the causal toxicants. In the early days, EDA was questioned for its effectiveness and efficiency,[15] but the later developments and applications showed its feasibility and potential as an essential paradigm for environmental chemistry. Especially since the 2010s, EDA has been "revived" based on the development of non-targeted analysis (NTA) supported by high-resolution mass spectrometry (HRMS),[16] data analyses adapted from metabolomics and proteomics, and high sensitivity/high-throughput *in vitro* bioassays.[17] While successful EDA studies have been conducted by various research groups in different continents, the most pioneering and productive groups are from Europe. The top contributors of EDA have been listed based on a bibliometric analysis (Figure 1a and b). It can be seen from this analysis that environmental journals are not the only sources of EDA studies, as the field of natural products analysis and isolation also uses the same methodology.

Aim of this critical review

Previous sources[19-22] have covered the many aspects of EDA in recent years. Especially, the 2016 review from Brack *et al.* contains a comprehensive and well-organized technical summary of EDA methods. Therefore, in this review, we are not trying to repeat the existing effort of all those great works. Instead, we hope to provide a commentary review based on what has been known about EDA, discuss more recent advances, and provide an outlook on how to connect EDA with human exposure/exposome. We hope to make this review concise and easy to read, for a broader audience including but not limiting to, environmental chemists, toxicologists, epidemiologists, physicians, and social scientists.

Good practices from successful EDA cases

EDA is an integrated method designed to prioritize and identify the causative toxicants in environmental mixtures, which is comprised of three iterative modules: (1) *in vitro* bioassays to guide component prioritization; (2) fractionation to reduce the mixture complexity; and (3) chemical analysis to identify the toxicants (Figure 2). Before recommending "good practices" from EDA studies, the first question is how to define "successful" EDA cases. The 2003 review by Brack summarized that successful EDA studies should: (a) identify and confirm toxic pollutants responsible for the observed toxicities; (b) use properly selected toxicological endpoints, either *in vitro* bioassays or *in vivo* organisms; and (c) be applied to "pollution hot spots" but not for the general screening purpose.[19] Over the years, the criteria (a) and (b) remain valid, while the criterion (c) is open to debate as the technologies have greatly advanced (eg, HRMS and high-throughput *in vitro* bioassays) and could reduce the time and cost of EDA. If the high-throughput bioassays can be applied to regular monitoring or screening, then EDA could be feasible when toxicities are observed. In the following discussion of successful EDA studies, we will examine the good research questions/toxicological endpoints and the key factors for the successful identification of toxicants. Studies since 2016 will be given more consideration, as they reflect more recent technologies and trends, and were not included in earlier reviews.[20]

Identification of suitable samples and biological endpoints

First, it is crucial to locate suitable subjects for EDA studies. Because EDA studies could be challenging and time-consuming, we hope to identify toxicants of relevance and importance. Practically, EDA complete the "missing link" model of sample → chemical → toxicity (Figure 3). By identifying the causal toxicants (chemicals), EDA builds the link between a sample and its measured toxicity. When the sample-toxicity pairs are repetitively observed, but target compounds cannot explain the correlation, EDA comes into play. Good EDA subjects originated from real-world phenomena and observations, such as the coho mortality in US Pacific Northwest.[23] Since this sample-toxicity pair was observed and concluded during long-term ecological monitoring, collaborations between environmental chemists and biologists/ecologists are necessary for solving these questions.

Ideally, the samples of interest for EDA should be *abundant and stable*. Since the nature of the toxic chemicals is unknown, it usually takes more experiments to tease out their physicochemical properties and behaviors than targeted analysis. Phase I of TIE provides an excellent framework for such pre-screening trials, including pH.[14] Furthermore, explorations are necessary to optimize the separation mechanisms and detailed parameters in fractionation, resulting in larger amounts of samples. Scientists need to collect or generate stable and reproducible samples to keep the results from bioassay and chemical analysis consistent. While varying concentrations could help with later toxicant confirmation,[24] they could cause difficulty for reproducible fractionation and identification. When a treatment or a fractionation step removes the toxicity, it could also provide clues on the property of the toxicant or simplify the fractionation.[25]

The bibliometric analysis (Figure 1c) also showed that many EDA studies focused on pollution hotspots such as wastewater effluents or contaminated sediments. These samples are usually stable and abundant because they are the sources and sinks of various environmental pollutants. Besides the pollution hotspots, some environmental samples with direct/long-term exposures to humans need special attention. For instance, studies have shown that unregulated and unmonitored disinfection by-products (DBPs) in drinking water played major roles in measured toxicities,[26,27] but there are still no exemplary EDA studies on this despite the call for future work.[28]

For many EDA studies, the biological endpoints were not "chosen," but pre-determined by the research question, such as unexplained environmental toxicities or exposure-related diseases. Both *in vitro* and *in vivo* bioassays were adopted as the toxicological endpoints for EDA studies. *In vivo* assays, such as zebrafish and sea urchin embryo tests, may have better environmental relevance as the effects on whole organisms simulate those in real-world exposures.[29,30] Also, *in vivo* assays can be used to measure toxicities even when toxicological mechanisms are not clear.[30,31] Downsides of *in vivo* bioassays include higher cost, lower throughput, and more variables, and it's unethical to test human toxicity *in vivo*. In contrast, cell line-based *in vitro* bioassays are good alternatives for EDA. Their high sensitivity, specificity, and high-throughput potential make them suitable for EDA studies with many fractions and subfractions. Reporter gene assays for receptor binding (estrogen receptor [ER], androgen receptor [AR], transthyretin binding receptor, and peroxisome

Figure 1. Bibliometric analysis on EDA. The analysis used R package Bibliometrix.[18] Searched key words: "EDA" OR "bioassay-directed fractionation" OR "bioassay-directed identification" OR "bioassay-directed chemical analysis." Searched time span: 1980–2022. Total number of documents: 680. (**A)** summary of "most relevant authors". (**B)** summary of "most relevant sources". (**C)** thematic map network by keywords.

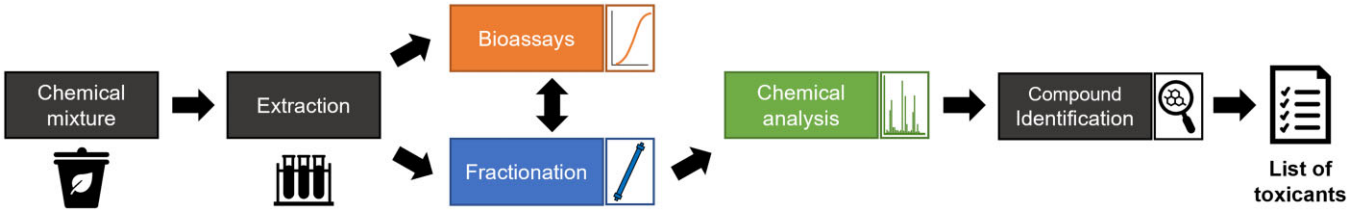

Figure 2. Scheme of EDA on environmental samples.

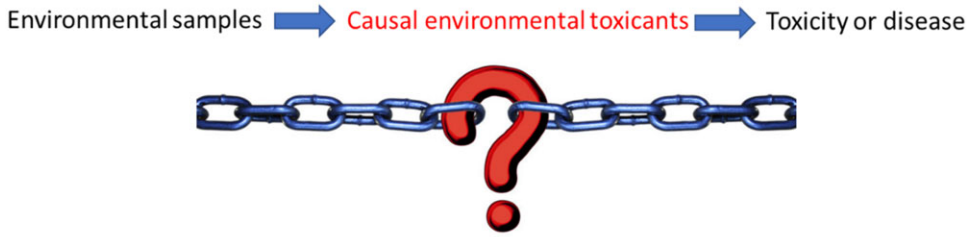

Figure 3. Conceptual "missing link" model that could be solved by EDA.

proliferator-activated nuclear receptors [PPAR]) have been successfully applied to EDA studies.[32-35]

Toxicological endpoints could affect the results of EDA, as EDA-traced *specific, strong, and reproducible toxicities* are more likely to succeed. More specific toxicities with known adverse outcome pathways (AOPs), such as ER or AR antagonistic effects, will be triggered by more particular compounds, resulting in less active fractions for identification. Less specific toxicities such as cytotoxicity, mutagenicity, and genotoxicity could be more difficult to identify as they could originate from many compounds, and the toxicity chromatogram (bioassay results in the order of fractions) would have many peaks. It is common and sometimes good to have multiple fractions showing biological effects, but too many active fractions (eg, >50% fractions) will result in too many unknowns to identify. A couple of rules of thumb could help decide if a fraction is still too complex to start identification. On the number of features, it is optimal to have active fractions containing <10 features (after blank subtraction and statistical analysis), which are manageable for manual examination. On the number of fractions, at least 10 fractions for high-performance liquid chromatography (HPLC) and three fractions for open column chromatography would be good starting points.

The strength and reproducibility of measured toxicity are also crucial for EDA. In different batches of bioassay testing, the same or similar samples should show reproducible responses. If larger variations were observed, reference compounds and positive controls could help normalize the effects.[36] Stronger toxicities are also preferred in EDA studies, because the treatment and fractionation could cause toxicant loss, and stronger toxicities are more likely to survive the lower recovery and show up in finer fractions. The bottom line is to see good toxicity "signal-to-noise ratios," so that we know we are chasing a real correlation between samples and toxicities. In summary, with abundant samples and reproducible sample-toxicity pairs, we are on the right track of successful EDA.

Another critical aspect of EDA is sampling and sample preparation, which could determine the environmental relevance and the technology roadmap of later steps. Environmental samples like sediment, soil, and water must be extracted and concentrated for chemical analysis and bioassays. While aggressive extractions enable detection of more compounds from the samples, bioaccessibility-based extractions can better reflect the observed toxicity,[37-39] and the sampling volume/concentration ratios are usually determined by the sensitivity of the bioassays and the strength of the toxicities. These considerations on sampling and sample preparation of EDA have been recently thoroughly reviewed by Huang *et al.*[40]

Efficient separation by multi-column or high-throughput EDA

The basic idea of EDA is to reduce the complexity of mixtures by removing non-toxic components to enable the identification of remaining toxicants.[19] Therefore, fractionation is a critical EDA step that connects chemical analysis and bioassays. In fact, insufficient fractionation could be one of the major reasons for failed EDA identification efforts. Well-designed fractionation procedures not only separate the toxicants from the rest of the samples, but also inform the properties of the toxicants (eg, polarity, functional groups, and molecular mass range). A challenge for "*ad hoc*" EDA studies without prior knowledge is the design of the fractionation method, as the properties of toxicants in the mixture are usually unknown. The TIE Phase I steps[14] or a generic HPLC fractionation method[41,42] would be a good first pass. Fractionation strategies should be developed based on the properties of sample extracts. For instance, soil and sediment need gel permeation chromatography to remove natural organics with larger molecular masses,[43] and the relatively non-polar analytes fit well with initial normal phase (NP) separation.[42,44] For water samples with relatively polar chemicals, reverse phase (RP) enrichment and separation are default choices. For very complex samples, the most effective fractionation strategies are (1) multi-level/multi-column fractionation[23,35,45] and (2) high-throughput fractionation with finer micro-fractionation.[31,46]

Although additive effects are common in the concentration ranges of trace organic contaminants, synergistic and antagonistic effects have been observed in EDA studies.[36,47] Weiss *et al.*[36] showed the masking effects of anti-androgenic compounds on the overall androgenic potential measured by AR CALUX assay. Muz *et al.* found synergistic effects from different mutagens in surface water. When good separation methods are applied, and rigorous controls are present, these effects could be elucidated by EDA.

Like the philosophy of using GC×GC MS or adding ion mobility spectrometry, multiple column fractionation adds extra separation dimensions and sometimes even achieves orthogonal separations, multiplying the peak volumes in chromatography.[42,48] With the most common RP chromatographic separations, various stationary phases such as cyanopropyl (CN), phenyl, pentafluorophenyl (PFP), etc. can be selected to diversify the separation mechanisms. Furthermore, combining NP and RP could expand the chemical spaces of separation, but attention needs to be paid to solvent exchange/evaporation because sample loss and immiscibility could occur in these steps. Except for the commonly used RP and NP setting, hydrophilic interaction liquid chromatography (HILIC), mix-mode chromatography, and size exclusion chromatography (SEC) could also be adopted to the multi-column methods for special needs. Especially, some more polar compounds (persistent and mobile organic contaminants) could have been ignored[49] and they might explain some toxicity losses in previous EDA studies. GC-based fractionation was also developed for the EDA of non-polar samples.[50] Since multi-column methods could go up to three to four rounds of separations, it adds more steps and extends the whole procedure of fractionation. Therefore, full-method blanks and a visual check of the chromatograms would be necessary to ensure separation reproducibility and efficacy. Proper automation (eg, HPLC sequences) would be helpful as these processes could be time-consuming.

A novel alternative or supplement to multi-step fractionation is high-throughput EDA (HT-EDA), which uses micro-fractionation and 96-well or 384-well plate-based bioassays to increase the resolution of separation.[31,46] Instead of collecting fractions corresponding to ∼1 min in HPLC, the micro-fractionation collects fractions corresponding to ∼10 s.[46,51] The fractionation can be performed in duplicates and used for both *in vitro* bioassays and HRMS analysis. The increased throughput and resolution improve EDA in several ways. First, the finer fractions further reduced the number of chemical features, making it easier to prioritize and identify toxicants. Second, less sample volume/mass is required for the micro-fractionation; therefore, it's especially suitable for biological samples with limited volumes. In a previous EDA study on polar bear cubs' plasma, sample volume of 1.7 mL has been successfully used to identify thyroid hormone disrupting compounds. With the cutting-edge nano-flow technologies,[52] it is possible to further reduce the sample volumes to tens of microliters of human plasma. Third, less solvent consumption and waste generation make it a "greener" method. To the best of our knowledge, HT-EDA is a cutting-edge method that is only developed and used at Vrije Universiteit Amsterdam,[31,46,51] and it has the potential to be adopted by more institutions and groups.

Targeted and untargeted chemical analysis

When bioactive fractions are found and confirmed, the next crucial step is the identification of causal compounds. Both targeted and non-targeted chemical analyses are important for EDA. Targeted methods are usually tested first to see if commonly known pollutants can explain observed toxicities.[34] If not, suspect screening and NTA with HRMS should be applied to the active fractions. Retention time and detection modes could inform the chemical properties of the features (eg, polarities and functional groups), therefore assisting the identification and exclusion. Compared with generic NTA studies, the unknown identification in EDA should be more "in-depth" because of the higher toxicological relevance and (ideally) fewer features. If MS^2 or MS^n fragmentation patterns are available, *in silico* fragmentation and spectra inspection should be applied to the unknowns.[53,54] Following the common practice of NTA, the structural identification of unknowns should be reported at different levels based on the available information, as suggested by Schymanski *et al.*[55] Within chromatography-mass spectrometric methods, multiple analytical techniques/polarities are recommended to cover a more comprehensive chemical space. For instance, Xiao *et al.*[56] used both GC-MS and LC-MS to detect AhR agonists in sediment samples. Eventually, for the "true unknowns" that could not be identified by mass spectrometry, other analytical methods such as UV–vis and NMR spectroscopy should be used to provide structural information, and isolation/purification would be need for pure and abundant compounds.

Practical challenges for EDA

Bioassay

One practical challenge on bioassays is the coverage of *in vitro* bioassays and corresponding AOPs. While *in vitro* assays are preferred biological endpoints because of their higher throughput and the potential to reduce/replace animal testing, not all the toxicities have corresponding bioassays. Cell line-based bioassays might not reflect toxicities at tissue/organ levels, and many diseases and observable adverse health effects cannot be assigned to a specific AOP. This is more likely a toxicological or medical question, but environmental chemists working on EDA are encouraged to consider it and consult toxicologists.

Another related question is the environmental relevance of bioassays, especially *in vitro* bioassays. Although EDA studies with *in vitro* assays have successfully identified new toxic contaminants, there are still uncertainties about how much the measured effects from *in vitro* and *in vivo* bioassays can be comparable/translatable.[57] For instance, bioaccessibility/bioavailability was not always considered when *in vitro* assays are used, resulting in biased toxicity estimation or compound prioritization.[58,59] Also, chronic effects or diseases such as cancers can hardly be surrogated by bioassays, as the measuring molecular initiating events (mutagenesis) may not represent the long term and complex development. One step further from *in vitro* bioassays, *in chemico* methods, which use key chemical reactions as replacements for animal bioassays, have been recently adopted to measure the toxicities of environmental samples.[60,61] As these methods are getting more popular, how to contextualize the *in vitro*/*in chemico* results in real-world environmental health studies will become an interesting question.

Separation

As the core step of EDA, fewer challenges seem to present in separation. However, the impressions of EDA's heavy workload mostly come from the fractionation/separation. As discussed in the "good practice" section, multi-column and high-throughput fractionations are the most effective strategies for reducing sample complexities, but they are usually not standardized or automated. Details like retention reproducibility and solubility during

the solvent exchange are crucial for the later bioassays or chemical analysis, and these must be carefully and manually checked by experienced environmental chemists. Another inherent conflict of EDA fractionation was the chromatographic resolution versus the separation scale. To improve the separation resolution and minimize the feature numbers in the fractions, the sample injection volume and the column volume need to be small. However, larger injection volumes and column sizes are preferred by some bioassays, especially *in vivo* bioassays or *in vitro* assays that require many replicates. Higher flow/pressure or more repetition has to be used to solve this conflict, which usually means more time or resources.

Identification

Identifying unknown compounds is still a bottleneck for EDA and NTA studies. Unlike the more comprehensive NTA, successful EDA should result in fewer chemical features for identification. The EDA framework also provides more information on the unknowns, such as polarities, stabilities, and potential toxicity. Such information could help us further narrow down the scope of candidate compounds. Still, the unknown nature of causal toxicants, the vast chemical space with many synthetic chemicals, and the identification are the same, and specific challenges are discussed below.

The first challenge, shared by many NTA studies, is the compound coverage of detection methods. Since GC-MS and LC-MS have become the major analytical methods for EDA and NTA studies, mass spectrometry ionization modes largely affect the detected compositional space.[62,63] It is recommended to analyze abstracts and fractions with different polarities, ionization modes, and complementary methods, such as GC-EI and LC-ESI or LC-APPI and LC-ESI.[25] Even so, compounds not amenable to any of the mass spectrometry methods might still exist. The bottom line is that scientists working on EDA should know that all analytical techniques have their limitations, and there is no single method that could cover the vast space of synthetic chemicals.

The second challenge is the identification of the "true unknowns." Although there are usually fewer features in the fractions of EDA studies, "true unknowns" are more likely to present because obvious target chemicals and suspects might have been excluded, and what is leftover for identification could not be found in databases or publications. Alternative analytical methods (NMR, infrared), sample context, and expert knowledge could be helpful for the true unknowns.

There are EDA cases where the major causative toxicants could not be identified or most observed toxicity unexplained. Some of these "negative" results could come from fractionation or bioassays, such as toxicity loss or too many candidates from incomplete separation.[64,65] Some are caused by feature identification or structure elucidation, which means the toxicity was separated into one fraction, but causal chemical features could be specifically assigned.[66] Knowing these challenges and limitations will help the experimental design of EDA studies.

Suggestions and perspectives for future EDA

Recently developed chemistry and toxicology methods can be adopted into the existing framework to overcome the challenges and improve EDA further.

Based on the authors' expertise, we know many aspects on the analytical chemistry side could be developed. Alternative separation methods such as HILIC, supercritical fluid chromatography, and asymmetric field flow fractionation are rarely used in EDA, and they would provide additional dimensions for fractionation. Although not used for fractionation, ion mobility spectrometry could also offer additional property information for EDA studies.[67] When the separation resolution is satisfactory, ambient ionization mass spectrometry techniques such as direct analysis in real time and dielectric barrier discharge ionization can be applied to the toxic fractions for higher throughput.[68] Chemometric and computational MS methods should be integrated into the EDA workflow to assist the compound prioritization and identification. Specifically, methods like partial least-squares discriminant analysis and principal component analysis correlate the chemical features and toxicities quantitatively,[24,69-72] serving as a "virtual EDA" or "data-driven EDA" workflow that crosscheck the identifications. Cheminformatics and high-throughput chemical screening are also promising solution for expanding EDA to human exposome research.[73] Recent progress on clustering and classification of candidate chemicals can greatly help the identification of unknown toxicants.[74,75] These methods should be better recognized and applied more. The rapidly developing computational MS methods and more commonly used retention prediction methods would help structural elucidation and correlating the compounds with specific toxicities, and linking MS2 patterns with toxicities can greatly help EDA.[76,77] Not even a wild idea at this moment, machine learning methods could help compound prioritization and identification,[78] but it's important for environmental chemists to understand the basic statistical assumptions and limitations of those methods.

On the toxicology/health side, with our limited expertise, we discuss more on new approaches and call for collaboration with the biology/toxicology community. First, we expect to see more EDA studies with endpoints from the perspective of system biology. The "omics" methods have revolutionized biomedical science and some are closely related to pollutant exposures. In fact, NTA and EDA studies have been adopting proteomics and transcriptomics as bioassays.[79-82] Metabolomic and transcriptomic analyses have helped prioritize the chemicals that contribute the most in mixtures.[82,83] Other new concepts and methods can provide biological endpoints for contaminant prioritization and identification. For instance, molecular gatekeepers are key metabolites that link exposure biomarkers with correlated clusters of endogenous metabolites.[84] *In silico* docking could be used to discover enzymatic targets of small molecule pollutants,[85] while protein affinity purification coupled with untargeted analysis could help identify unknown pollutants that bind to key receptors.[86] These new endpoints could have better health and environmental relevance, as they could "bypass" the AOPs in cell-based assays, therefore linking chemicals and diseases even when the etiologies are not clear. Second, closer collaboration with epidemiologists, physicians, and social scientists could help build the link between chemical exposure and human diseases. While there are many *in vitro* and *in vivo* bioassays, EDA studies only used a very limited subset of bioassays. Collaboration with broader range of toxicologists and physicians may help our field expand the biological endpoints, especially the ones closely related with human diseases. It is also true that some human diseases do not have clear etiology, which makes them less suitable for EDA. For these diseases, collaboration with epidemiologists and toxicologists could provide information/hypothesis for untargeted analysis on potential sources. In addition, social science and epidemiology studies also provide information on exposure scenarios and behaviors.

A new chapter of EDA: from environmental samples to human exposome

While most existing EDA studies focused on environmental samples (soil, sediment, water), it has the potential to be directly applied to biological samples such as serum and urine. Previous EDA works have been successful on polar bear plasma[34] and cow manure,[87] suggesting the potential feasibility of human biological samples. Applying EDA to the biotic compartment will cover the complete exposure pathway from contaminants desorption through bioavailability to internal concentrations. As discussed previously, such application of EDA could become a promising tool for linking human diseases to chemical pollution and exposures,[21] especially in the age of exposomes.[7]

However, the nature of human health studies added challenges to EDA work on humans exposome and specific environmental agents: first, humans have longer lifespan, so chronic diseases could be caused by repeated low dose exposures of environmental pollutants and thus difficult for EDA application; second, humans have more uncontrollable and complex exposure patterns, and it's difficult to conduct real controlled studies; third, biological samples like human serum and urine are usually limited in sample volume/mass. As highlighted by Vinggaard *et al.*,[21] "The potential of true EDA on human tissues remains to be explored." New methods and collaboration pathways are needed to address such challenges. Personal passive samplers like silicone wristbands may be tested in future EDA studies because their measured exposures have been shown to be related to internal doses.[88,89] HT-EDA can be perfect solutions for the limited sample volumes and complex matrices. To overcome the first challenge (exposure asynchrony and complex exposure pattern), as discussed earlier, environmental chemists need to develop new paradigms of collaboration with scientists from other fields. To overcome the second challenge (confounding), environmental scientists should adopt proven methods from epidemiology such as randomization, matching, and restrictions to improve the study designs and sample collections. Except for the well-known biological endpoints and related diseases (cancers, endocrine disruptions), we look forward to applying EDA to other diseases potentially caused by environmental exposures, including allergies and neurodegenerative diseases. The biological endpoints and AOPs would also need interdisciplinary collaborations. We look forward to such collaborations, and we hope the toxicologists, environmental health scientists, epidemiologists, physicians, and social scientists know about EDA and its great potential to diagnose environmental causes of human diseases.

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Zhenyu Tian (Conceptualization, Methodology, Visualization, Writing—original draft, Writing—review and editing), Madison H. McMinn (Conceptualization, Methodology, Visualization, Writing—review and editing), Mingliang Fang (Conceptualization, Methodology, Writing—original draft, Writing—review and editing)

Data availability

The data underlying this article are available in the article.

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Conflict of interest statement

None declared.

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