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Review

Integrating Bioassays and Non-Target Screening: A Review of Effect-Directed Analysis for Unknown Toxicants

Yujun Tong [1,2], Huizhen Li [1], Fei Cheng [1,3], Jingru Zhang [2] and Jing You [1,*]

[1] Guangdong Key Laboratory of Environmental Pollution and Health, College of Environment and Climate, Jinan University, Guangzhou 510443, China
[2] Laboratory of New Pollutants Risk Assessment and Control, Guangdong Provincial Academy of Environmental Science, Guangzhou 510045, China
[3] State Key Laboratory of Advanced Environmental Technology, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
* Correspondence: youjing@jnu.edu.cn

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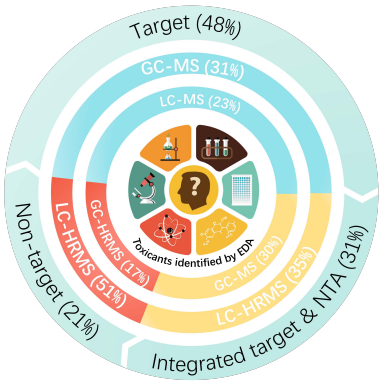
Highlights

- EDA is increasingly combined with NTA for identifying unknown toxicants
- HRMS-based analysis enhances the reliability of compound identification
- Standardized EDA-NTA databases facilitate cross-study comparison and data integration
- Data-driven effect attribution will enhance future mixture risk assessment

Abstract: The widespread use of chemicals continues to pose significant risks to ecosystems and human health. While toxicological mechanisms of many known pollutants remain incompletely understood, the emergence of numerous unknown or poorly characterized contaminants intensifies the urgent need for robust identification strategies. Effect-directed analysis (EDA) has shown effective in detecting bioactive compounds in complex environmental matrices, yet traditional EDA approaches mainly relying on target analyses are inherently limited in uncovering new or unexpected toxicants. Integrating emerging non-target analysis (NTA) techniques with EDA offers a transformative approach, enabling comprehensive profiling of unknown compounds and improving accuracy and efficiency of environmental risk assessment. Despite this potential, the lack of standardized workflows has constrained the widespread application of NTA in EDA. The present review summarized recent developments in integrating NTA with EDA, covering key aspects from sample collection and preparation to fractionation, instrumental analysis, and toxicity confirmation, with a focus on chemical analysis for various matrices. We discuss key methodological challenges such as matrix effects and confidence levels in structural elucidation. By synthesizing current knowledge and identifying future directions, this work aims to provide actionable guidance for the identification of new or less-concerned toxicants in mixtures, ultimately advancing environmental monitoring and public health protection with integrative EDA and NTA approaches.



1. Introduction

Rapid population growth and accelerated industrialization have led to complex chemical mixtures in the environment, posing a significant challenge for identifying principal toxicants responsible for ecological effects. Conventional chemical-based pollution control strategies are effective in monitoring priority pollutants, yet possibly fail to capture primary contributors to the toxicity of complex mixtures due to the ignorance of unknown toxicants. In contrast, bioassays directly measure the overall toxic effects of environmental samples on test organisms, but they alone provide limited information on the identities of the causative contaminants.

Effect-directed analysis (EDA) integrates bioassays with chemical analysis to iteratively simplify complex mixtures and eventually identify the most relevant toxicants, providing an effect-based way to prioritize pollutants in mixtures. Stepwise strategies such as toxicity identification evaluation (TIE) are capable of classifying toxicants and identifying inorganic toxicants, including ammonia, cyanide, and selected metals, but they show significant limitations when faced with complex organic mixtures. Therefore, EDA has been used in combination of TIE for chemical mixtures in which organic contaminants are regarded as the dominant toxicants [1]. The EDA employs repeated chromatographic fractionation coupled with toxicity testing, enabling effective simplification of mixtures and target identification of biologically active components.

Since its introduction in the 1980s, EDA has been widely applied for the identification of organic toxicants in diverse matrices [2]. However, with the increasing emergence of new pollutants and their transformation products [3], conventional target analysis shows insufficient for identification of organic toxicants with unknown identities. Advances in high-resolution mass spectrometry (HRMS) have enabled non-target analysis (NTA) to emerge as a powerful tool for discovering numerous environmental contaminants which are previously unrecognized. While NTA is advantageous in revealing unknown compounds, on the other side it increases the challenges of prioritizing contaminant of toxicological concerns for environmental management due to the tremendously enlarged chemical space. Ecotoxicology effects can help focusing the goals of NTA. Thus, integrating NTA with EDA is expected for the identification of toxicologically relevant unknown organic pollutants, providing a scientific basis for their source tracking and risk management. Despite the potential of integrative EDA and NTA methods, the shift toward NTA-enhanced EDA necessitates a more rigorous focus on data fidelity. However, variabilities in research objectives, sample types, and analytical platforms have hindered an establishment of standardized workflows and restricted their broader implementation.

To facilitate method standardization and promote the application of the integrative EDA and NTA methods, the present review provides a specialized perspective on the transition to NTA-enhanced strategies through (1) comprehensively reviewing the available methods for identifying key toxicants using EDA; (2) summarizing recent advances and limitations of NTA application with EDA; and (3) proposing a workflow for conducting NTA-based EDA in environmental samples, with goals of informing future standardization and broader adoption of these approaches.

2. Literature Search and Summarization

Although concepts resembling EDA were proposed as early as the 1980s, the systematic development and widespread application of EDA began in 2003. At that time, inspired by the strategies in pharmaceutical screening, an integrated "fractionation–bioassay–identification" approach was introduced into environmental science to identify biologically active compounds in complex mixtures [2]. Since then, EDA has gradually expanded beyond common environmental applications to different research fields including toxicology, public and occupational health, pharmacology, and forensic science, serving as a critical bridge between chemical occurrences and biological effects in mixture samples.

To ensure a rigorous and transparent review, we conducted a comprehensive literature search following the PRISMA guidelines (Figure 1). Given this development timeframe of EDA techniques, the bibliographic data were retrieved from the Web of Science (WoS) Core Collection via Advanced Search using the following search string: [(Topic = ("effect-directed analysis" OR "effect driven analysis" OR "bioassay-directed analysis" OR "effect-oriented analysis")) AND (Document Type = ("ARTICLE" OR "REVIEW")) AND (Language = ("ENGLISH")) AND (Publication Date = (2003.01.01–2025.10.31))]. In addition, a supplementary manual search was performed in Google Scholar. Only peer-reviewed journal articles and reviews written in English were included to ensure the quality and relevance of the collected literature. The inclusion criteria were strictly defined to encompass studies that (i) utilized an integrated fractionation-bioassay-identification framework, (ii) focused on environmental or biological matrices and (iii) provided primary data or significant methodological advancements in chemical identification. In contrast, studies were excluded if they lacked any bioassay-driven components or focused solely on target monitoring without a discovery-based fractionation step.

Through this systematic screening, a total of 476 publications, including 83 methodology-, 264 research-, and 129 review-articles, were acquired (Tables S1–S8 in the Supplementary Materials I). A temporal analysis showed that only 82 EDA-related publications appeared

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between 2003 and 2012, while the remaining 394 papers have been published since 2013. The distribution of research-based publications is summarized in Figure 2. There is a notable increase in field research-oriented studies in the past decade and this sharp rise of EDA applications for real-world samples demonstrated the transition of EDA from conceptual exploration in the 2000s to methodological maturity since the 2010s (Figure 2a). Within these EDA research, studies focusing on non-target identification greatly increased in recent years, from 10 until 2012 to 137 publications in the subsequent 12 years, highlighting the growing integration of EDA with NTA techniques.

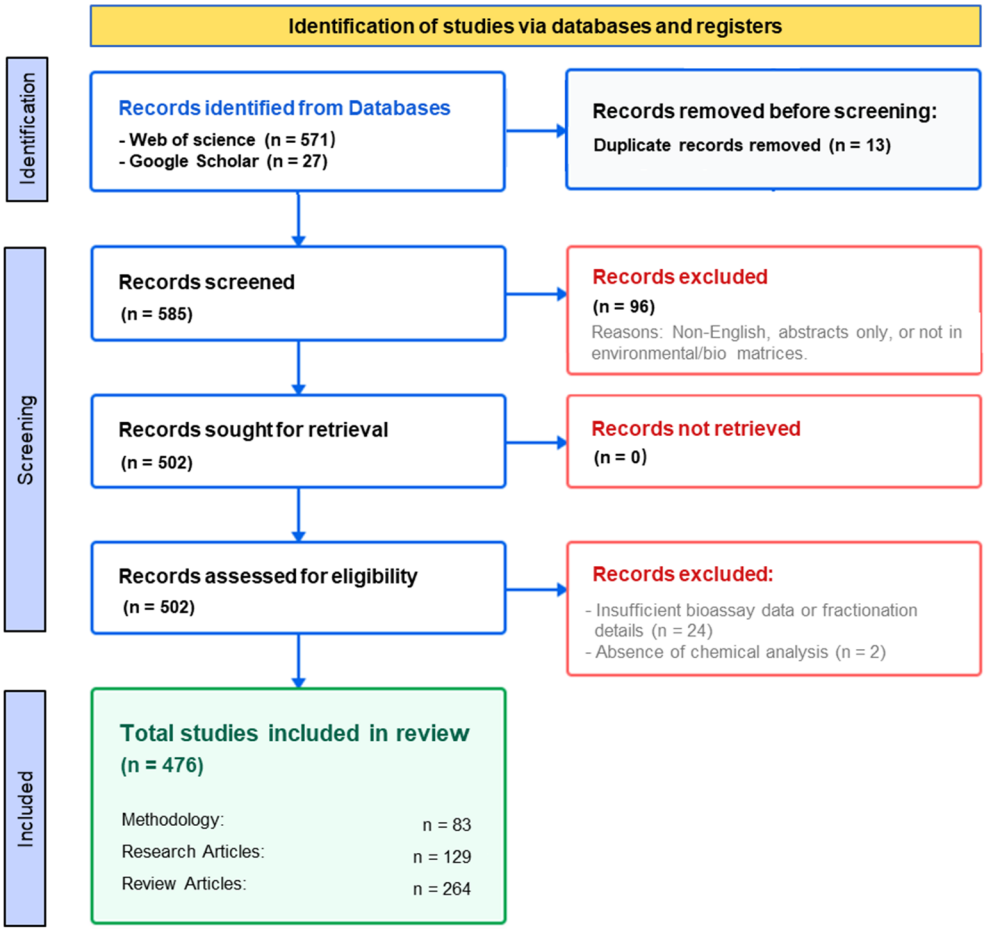
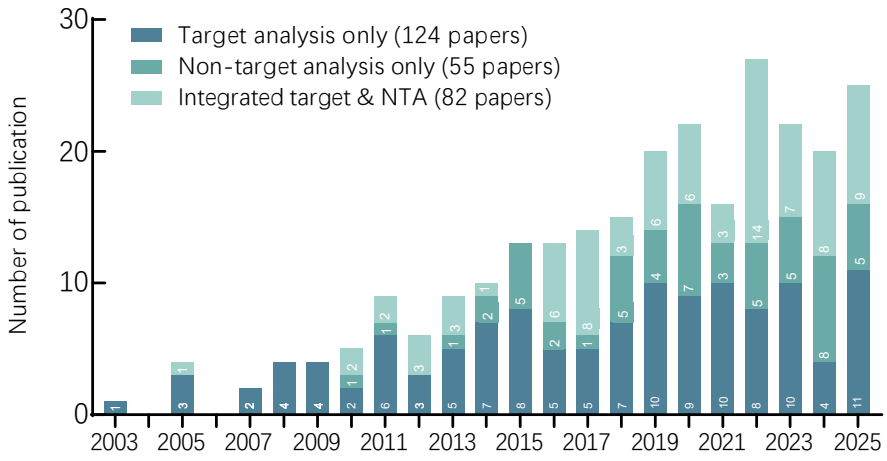
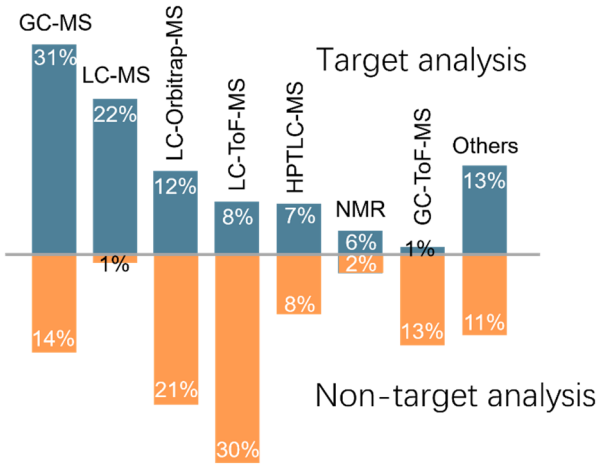


Figure 1. PRISMA flowchart of systematic literature search and selection processes for effect-directed analysis (EDA) studies (2003.01.01–2025.10.31).

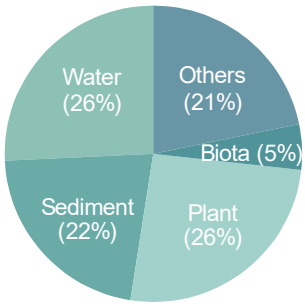


(a) Publication trends

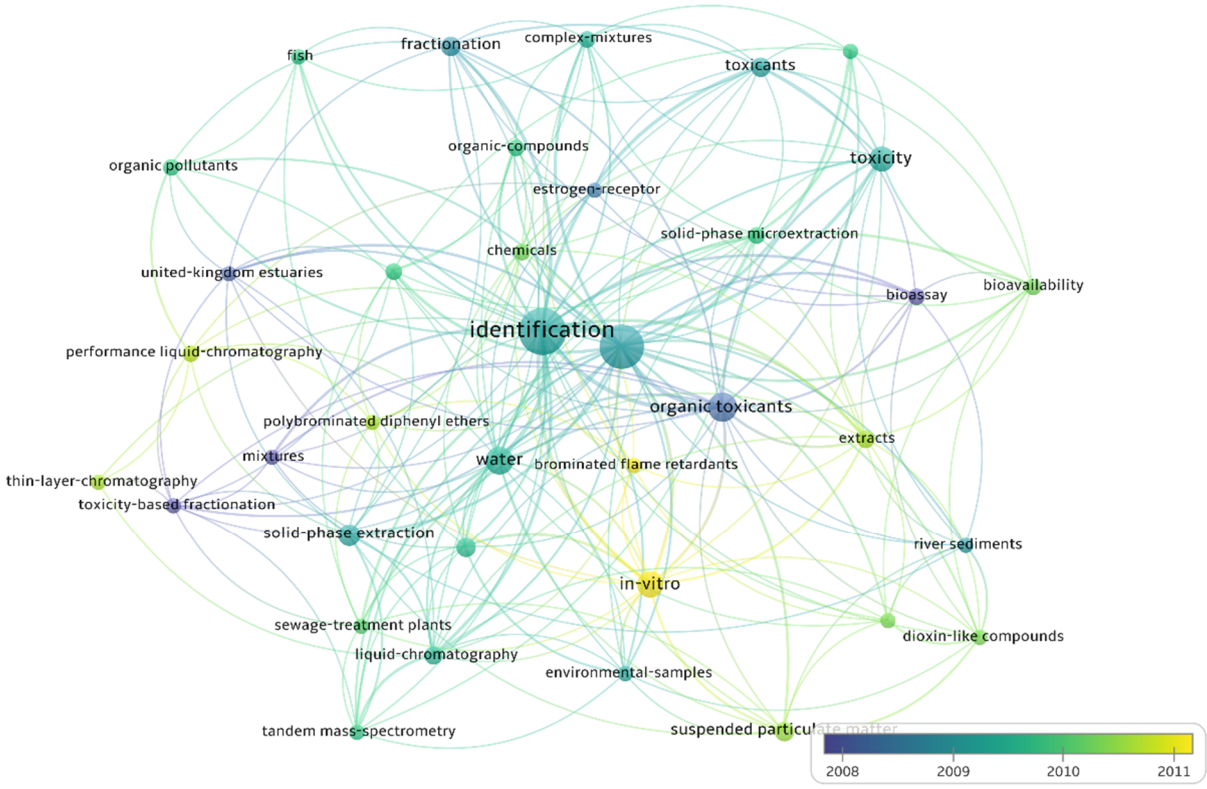
Figure 2. *Cont.*



(**b**) Instrument trends

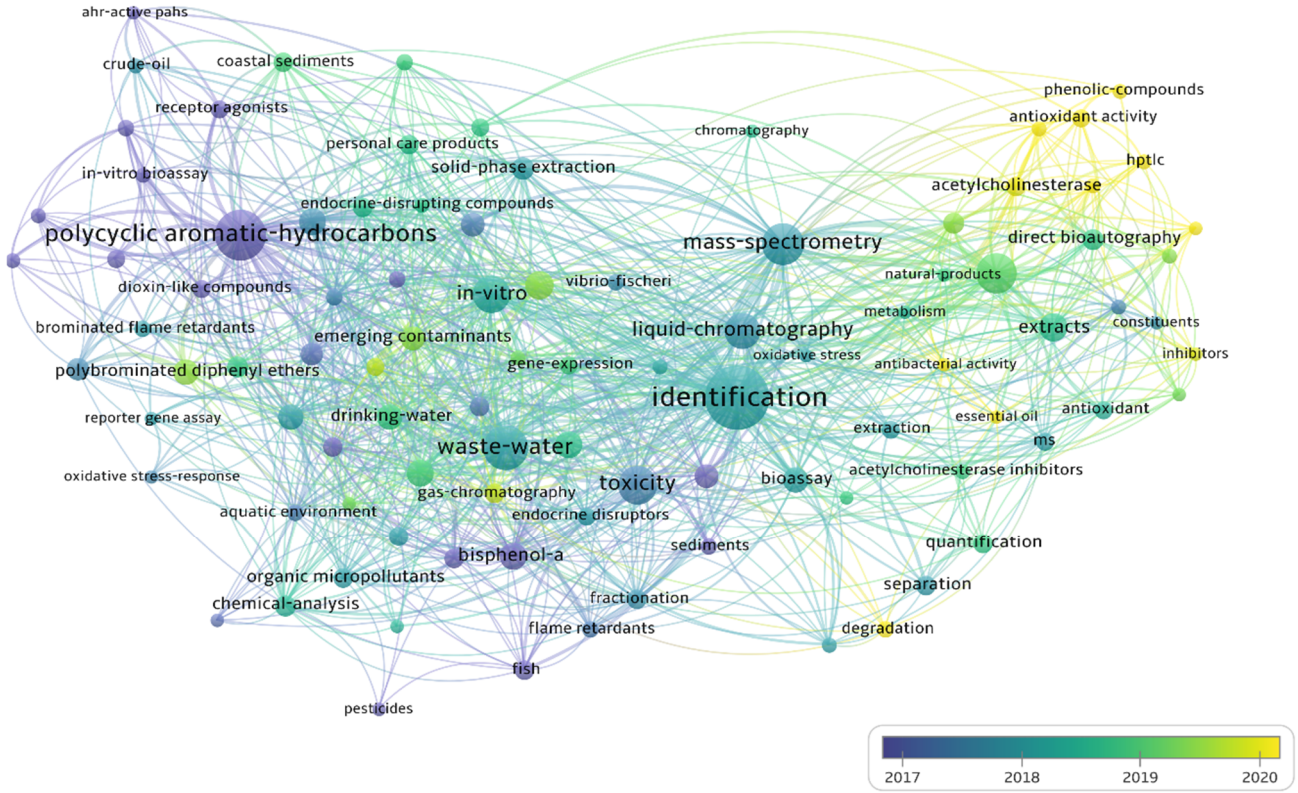


(**c**) Sample type



(**d**) Keywords (2003–2012)

Figure 2. *Cont.*



(**e**) Keywords (2013–present)

Figure 2. Summary of research articles in effect-directed analysis (EDA) published from 2003 to 2025. (**a**) Number of EDA publications using target and non-target analyses annually, (**b**) various analytical instruments applied in EDA publications, (**c**) various environmental matrices studied for EDA samples, and (**d**,**e**) research hotspots in EDA publication over the last two decades (2003–2012 and 2013–present, respectively).

Rapid advancements in analytical instrumentation have been pivotal for screening and identifying unknown pollutants within EDA frameworks (Tables S5–S7). Gas and liquid chromatography coupled with mass spectrometry (GC-MS, LC-MS) remain the most commonly used chemical identification methods, and recently high-resolution MS (HRMS) techniques, including time-of-flight (ToF), orbitrap, and Fourier-transform ion cyclotron resonance (FT-ICR)-based HRMS, have markedly enhanced instrumental capability to identify unknown compounds [4]. Among them, LC-HRMS is particularly advantageous in NTA efforts due to its broad applicability and high chemical coverage (Figure 2b). In addition, other spectroscopic and structural elucidation tools such as high-performance thin-layer chromatography-ultraviolet detection (HPTLC-UV), nuclear magnetic resonance (NMR), as well as GC coupled with various detectors (e.g., electron capture detector (ECD), flame ionization detector (FID), atomic emission spectrometer detector (AED), and Fourier transform infrared spectroscopy (FTIR)) have also been applied for chemical identification in some studies.

The types of samples were also expanded considerably in recent years (Tables S3–S4). While early studies primarily focused on sediments and wastewater, recent EDA research covered diverse environmental and consumer-product matrices, such as commercial disinfectants, indoor dust, atmospheric particulate matters, epoxy resin coatings, landfill leachates, food-contact materials, plastic toys, coal and petroleum coke, microplastics, cigarette leachates, and fermented plant products [5–21]. The applications of EDA for biological samples have grown rapidly as well. Among 261 EDA research studies, approximately 31% focused on biological matrices, with plant extracts accounting for 26%, reflecting the influence of pharmacological techniques on EDA applications (Figure 2b).

Co-occurrence analysis of chemical identification keywords revealed clear evolutionary trends in EDA research over years (Figure 2d,e). Between 2003 and 2012, studies primarily focused on methodological exploration and feasibility evaluation, with regional research hotspots mainly concentrated in the Europe. At that time, technological and instrumental limitations constrained the EDA scopes of bioassay endpoints and identifiable compounds. In contrast, in the past decade we have witnessed substantial advances in instrumental analysis and multi-omics technologies. These breakthroughs enabled EDA to be utilized for diverse sample types and bioassay endpoints, along with a dramatical increase of chemical space. The keywords have expanded from traditional

environmental matrices to biological and consumer-product samples closely linked to human activities, and from single organism-level apical endpoints to molecular pathways and omics-level bioassays. The advance of emerging sample preparation techniques and high-throughput analytical platforms has facilitated the identification of a large number of new pollutants and even previously unknown bioactive compounds (e.g., *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine quinone (6PPDQ) [22], highlighting the value of EDA as a frontier methodology for environmental and health risk assessments. As shown in Table S1, over 80 review articles have been published concerning EDA methods and applications, such as the bioavailability issues in EDA [23] and the integration of EDA and TIE techniques [1]. Therefore, we do not cover the whole EDA workflow in the present study, but specially focus on the chemical analysis methods used in EDA, including instrumental analysis and sample preparation.

3. Workflow and Key Considerations in NTA-EDA Integration

While used in different matrices, the application of NTA in EDA remains at an early stage, necessitating the establishment of a systematic workflow along with the identification of key challenges. This workflow can be divided into four phases (Figure 3). Phase 1 involves sample preparation, including organic solvent extraction followed by solvent exchanging to biocompatible media for initial toxicity screening. Samples exhibiting biological activity advance to Phase 2, where fractionation reduces matrix complexity and enriches bioactive components, with the fractions re-screened for toxicity to identify active constituents. Phase 3 employs advanced analytical instrumentation to profile compounds within bioactive fractions. Finally, Phase 4 entails toxicity confirmation using commercial or synthetic standards, encompassing mode of action (MoA) verification, analytical confirmation, effect confirmation, and risk assessment, thereby quantifying the contribution of individual pollutants to observed biological effects. Implementing NTA-EDA requires a realistic assessment of its considerable operational demands, which stem from high capital costs, prolonged timelines, and a reliance on multidisciplinary expertise. Financially, the burden is substantial, as a single HRMS platform often requires a starting investment exceeding $500,000, with further recurring costs of specialized bioassay kits and high-purity chemical standards. On the computational side, raw data from individual samples can reach several gigabytes, and the terabyte-scale datasets generated in large-scale monitoring necessitate high-performance computing clusters and advanced bioinformatics pipelines for processing. Consequently, the time commitment is also extensive. A full study, from initial sampling to final validation of a new toxicant, typically spans 6 to 18 months. Moreover, successful execution of this workflow depends heavily on the integration of expertise across analytical chemistry, ecotoxicology, and data science, a requirement that currently restricts the use of NTA-EDA largely to leading research institutions.

The integration of EDA with NTA occurs throughout all the phases, but several methodological considerations are critical. Non-discriminatory and non-destructive pretreatment is essential to minimize analyte loss. Environmental matrices often require significant enrichment to achieve effective detection of trace pollutants, yet excessive sample loading can introduce matrix effects, leading to false positives in both bioassays and instrumental analysis. Thus, appropriate sample volumes are determined empirically, balancing detection sensitivity, matrix interference, and downstream assay requirements. While target screening of compounds with known MoA or well-defined toxic endpoints can enhance the efficiency of toxicant identification, the limited interpretability of single apical bioassay endpoint requires a shift toward comprehensive multi-endpoint MoA pathway evaluation. In this context, the evolution of bioassay technology has been vital in expanding the scope of NTA-EDA. High-throughput *in vitro* screening now allows for rapid examination of various responses relating to specific molecular initiating events, such as receptor-mediated endocrine disruption, acting as a “biological filter” to narrow down candidate lists for NTA. Furthermore, the integration of omics approaches, such as toxicogenomics, provides molecular fingerprints that help verify whether the chemicals identified by HRMS are indeed responsible for the observed biological pathways. Novel endpoints, including behavioral changes in zebrafish or high-content imaging, further capture sublethal effects that guide NTA toward identifying chronic exposure, low-dose pollutants. Additionally, environmental parameters, such as pH, ionic strength, and the presence of metals or inorganic salts, can influence extraction efficiency, compound stability, and bioassay responses, and therefore should be carefully controlled and documented.

Fractionation strategies directly impact NTA outcomes. In sediment samples, normal-phase liquid chromatography (NPLC) effectively recovers and separates non-polar compounds while reducing matrix complexity; reversed-phase liquid chromatography (RPLC) can subsequently refine polar compound separation [24–26]. Conversely, water samples often benefit from an RPLC-first approach followed by NPLC to achieve comprehensive coverage. Selection of fractionation methods must consider sample type, bioassay sensitivity, and physicochemical properties of target analytes (e.g., molecular mass, log K_{ow}).

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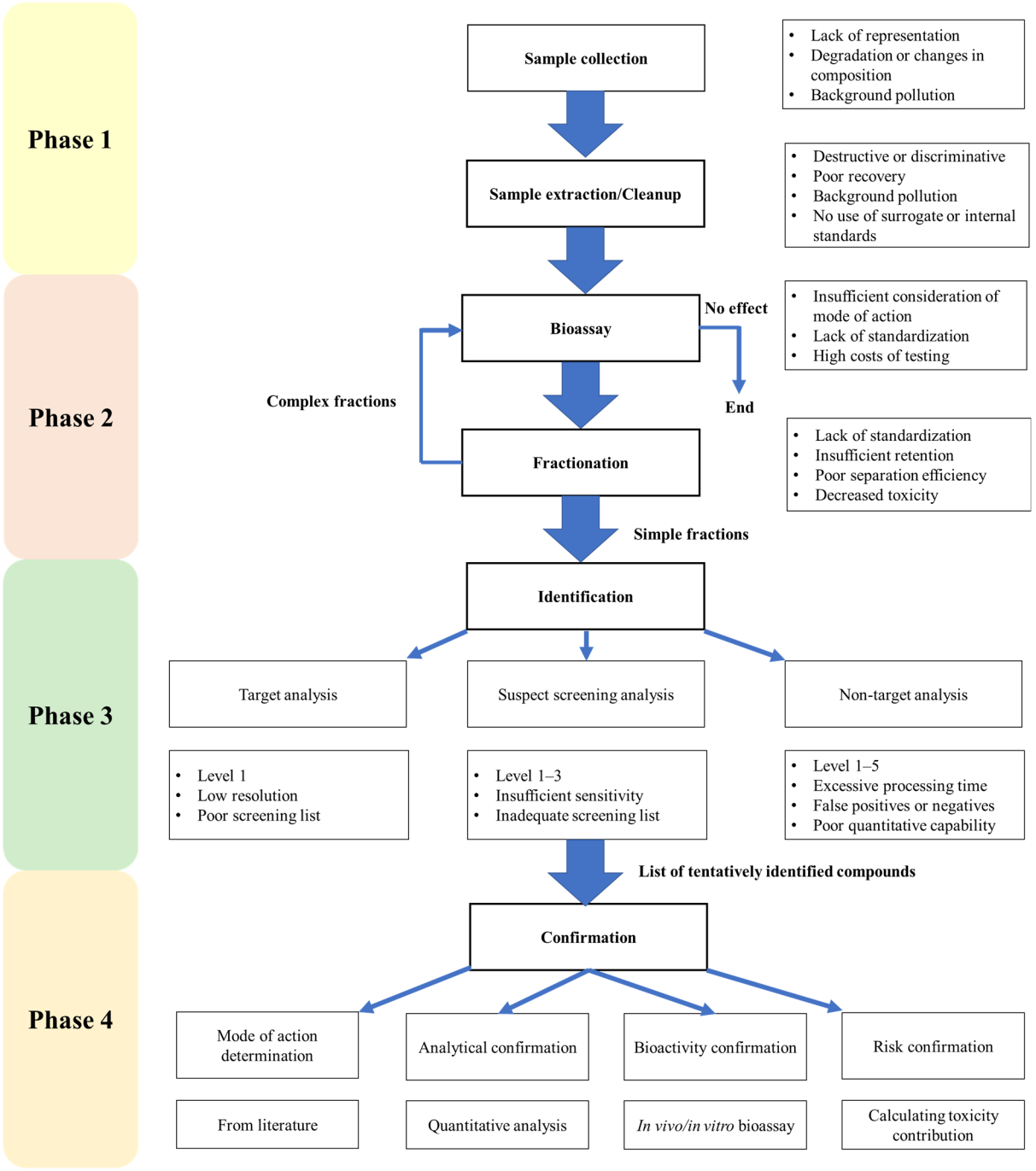

Figure 3. A generic analytical workflow integrating nontarget analysis (NTA) and effect-directed analysis (EDA). Possible reasons for the inherently incomplete analytical procedures are also listed.

Identification of bioactive substances is the most critical step in NTA-EDA integration. Unknown bioactive substances are screened via target, suspect, and non-target approaches against various spectral databases. However, practical implementation often faces multiple challenges Specifically, the reproducibility and comparability of EDA results across different laboratories remain challenging. Studies have shown that variations in fractionation schemes (e.g., using different HPLC column chemistries or mobile phase gradients) can lead to the inconsistent partitioning of toxicants into different fractions, complicating the tracking of biological activity. Furthermore, the selection of bioassay endpoints (such as specific reporter gene assays vs. broad apical toxicity tests) often results in different sets of identified "drivers of toxicity" for the same sample. Even when using identical HRMS raw data, the use of different data processing software and algorithms may lead to significantly different candidate lists [27]. To deal with these issues, future development of NTA in EDA should focus on (1) advanced algorithms and deconvolution software to enhance data processing efficiency; (2) standardized chromatographic, mass spectrometric, and elution conditions coupled with rigorous quality

assurance/quality control (QA/QC) protocols; (3) improved interoperability between software platforms, instrument vendors, and open-source spectral databases to facilitate resource sharing; (4) effect-driven identification strategies to prioritize biologically relevant compounds.

The practical integration of NTA-EDA is best illustrated by its successful deployment throughout the entire analytical workflows, from sampling to toxicant confirmation. In studies of urban runoff toxicity, researchers progressed from site-specific water collection to identify 6PPDQ. By combining multi-stage EDA with custom chemical synthesis, they addressed the challenge of "unknown unknowns" and showed that the synthetic compound exhibited the lethality matching that of the original samples [22]. Similarly, in surface water monitoring, the extraction of bulk samples coupled with parallel fractionation methods helped overcome the matrix-induced signal suppression. This approach enabled the isolation of antiandrogenic 1,3-diphenylguanidine and benzotriazoles, whose identities were confirmed by matching the fractionation patterns and retention times of authentic standards with the environmental isolates [28]. Finally, in aquatic sediment assessments, multi-step HPLC fractionation was utilized to separate polar bioactive constituents from complex sediment extracts, therefore resolving interference from hydrophobic mixtures. Finally, through dose-response comparisons and relative potency (REP) analysis, oxygenated polycyclic aromatic compounds (PACs) were successful identified as major aryl hydrocarbon receptor (AhR) agonists [29]. Collectively, these cases show that procedural bottlenecks, regardless of relating to sample complexity, signal interference, or compound identification, can be systematically overcome through tailored pretreatment, advanced deconvolution, and independent validation. Collectively, NTA represents a crucial tool for identifying novel toxicants and provides an essential reference for understanding their contribution to complex toxicity profiles.

4. Instrumental Identifications of Toxicants in EDA

When using EDA to identify key toxicants, the primary challenges are the detection of toxicants and the structural elucidation of compounds in the bioactive fractions which are still a mixture. Depending on analytical objectives and technique availability, the identification of bioactive substances in the toxic fractions is usually achieved through three strategies, including target analysis, suspect analysis, or NTA.

4.1. Target Analysis

In EDA, target analysis involves the qualification and quantitation of environmental pollutants using a target list of chemicals with reference standards. Thanks to toxicological research, modes of toxicological action of commercialized chemicals are relatively well understood. Therefore, based on chosen endpoints, researchers can select certain pollutants as the target analytes according to prior knowledge and monitoring results. Target analysis often provides higher analytical sensitivity and accuracy, allowing for the quantification of pollutants at relatively low exposure concentrations. This approach can be implemented using various instruments. For example, GC-MS, which is suited for analyzing volatile organic compounds, have been a major detection technique in the early stage of EDA. Pollutants form molecular fragments in electron ionization (EI) mode, which are matched with specific mass spectrometry databases through spectral library searches to deduce similar compound information and infer compound structure. Meanwhile, LC-MS is suitable for analyzing non-volatile polar compounds and can complement the limitations of GC-MS. Concentration data obtained from reference standards allow for the calculation of the contribution percentage of target substances in the mixture effects. Cha et al. [30] quantified AhR agonists in the extracts of blubber, liver, and muscle of dolphin and whale from the Korean coastal waters using GC-MS and LC-MS/MS, and found that polar AhR agonists significantly contributed to the total AhR-mediated potency. However, the target analysis strategy based on a priori knowledge can only identify a limited number of pollutants of interest, potentially limiting the interpretation of overall bioactivity. Dusza et al. [31] conducted a target analysis of 13 of endocrine disrupting compounds (EDCs) in human amniotic fluid and found that they contributed little to the observed endocrine-disrupting activity, highlighting the need for the identification of unknown bioactive compounds.

4.2. Suspect Analysis

Suspect screening in EDA involves screening potential compounds using prior knowledge in the absence of reference standards. To achieve the screening, lists of compounds of interest are first established based on precise mass measurements and structural information, and candidate compounds in samples can then be identified by combining these lists with database matches [32,33]. Suspect screening in EDA often targets certain classes of compounds that may induce similar modes of action or potential metabolites of known parent compounds. Compared to target screening, an expanded list for suspect screening is more efficient and cost-effective, on the other hand, suspect screening provides more accurate structural elucidation information than non-target screening. Therefore, suspect screening has gradually increasingly used in EDA for chemical identification. Lopez-Herguedas et al. [34] screened possible toxicants in hospital wastewater using GC-MS and LC-Orbitrap-MS coupling with the NIST17 MS library (267,376 compounds) and the NORMAN database

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(40,059 compounds), respectively, and 94 suspect substances were identified and the toxicity contributions of 25 compounds were further confirmed using authentic standards. In another EDA study, Cheng et al. [35] conducted suspect screening using GC-MS with a Compound Composer database containing 942 organic compounds and found cypermethrin and musks as key neurotoxicants in urban sediments in South China. A suspect list with 228 compounds was established for liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QToF-MS) screening contaminants from chemical industrial areas and six suspect compounds were identified with toxic potency [36].

Overall, the ability of suspect analysis to identify substances depends on the size of the constructed database. For suspect analysis, library matching based solely on precise mass measurements is often insufficient for accurate identification, instead, further evaluation of its mass spectrum, mode of action (MoA), concentration, and risk contribution is generally required to confirm a candidate as a toxicant. While suspect screening is efficient to identify known pollutants and their degradation products based on a predetermined chemical list, it is less effective for screening unknown pollutants, a task that requires the stronger screening capabilities of non-target analysis.

4.3. Non-Target Analysis

While suspect screening relies on predefined compound lists and is guided by prior assumptions regarding specific chemical classes, true NTA is fundamentally data-driven. The NTA does not pre-define any targets, instead, it processes all detected features within a sample, utilizing molecular formula assignment, in silico fragmentation simulation, and spectral deconvolution to elucidate structures of entirely unknown pollutants [37,38]. It should be noted that many EDA studies adopted a hybrid approach, transitioning from data-driven discovery to suspect-list refinement for structural annotation.

In many EDA studies, the target compounds usually offer limited explanations for the observed adverse effects, with most of the toxic contributions arising from unknown substances. Therefore, NTA, as a powerful means of identifying unknown pollutants, has been gradually gaining prominence in EDA. Appropriate analytical tools (such as GC×GC, FTIR, UPLC) should be chosen according to research goals and the nature of the compounds (non-polar or polar) and matrices, and sometimes a combined use of multiple analytical methods is required [39].

The rapid development of NTA in recent years, facilitated by HRMS providing precise mass measurements, has made the analysis of more unknown substances possible. The HRMS narrows down the range of possible molecular formulas, though different mass analyzers which have varying mass errors. In general, Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has the smallest error, followed by Orbitrap-MS, while ToF-MS exhibits relatively higher error [40]. A primary bottleneck in EDA is that current NTA remains largely non-quantitative, which complicates the transition from chemical identification to assessing compounds' actual contribution to mixture risk. This limitations arises from the scarcity of reference standards and the high variability of instrumental response factors, which can span several orders of magnitude depending on molecular structure, making peak area-based risk estimates highly uncertain [41]. Quantifying the "toxic units" in a complex mixture requires precise concentration data, yet the lack of authentic standards often prevents traditional mass balance analysis. To address this, semi-quantitative NTA strategies now utilize machine learning and quantitative structure-activity relationship (QSAR) modeling to predict response factors based on physicochemical properties. These computational frameworks allow for the estimation of exposure concentrations and corresponding risk contributions even for unknown pollutants, significantly improving the risk assessment of environmental mixtures [42]. Cheng et al. [42] developed a multimodal learning-based semiquantitative method and incorporated it to the Event-Driven Taxonomy (EDT)-Screening strategy, which increased the explained risk contribution from 7.1% to 82%, mainly from semiquantitative nontarget compounds. Consequently, the selection of NTA instrumentation should be optimized to align with these diverse physicochemical requirements and quantitative modeling requirements.

4.3.1. NTA Based on GC-MS

The GC-MS, which is particularly suitable for the analysis of volatile and semi-volatile organic compounds, was a major technique in the early days of EDA development. In electron impact ionization (EI) mode, the fragment ions of analytes are matched with the NIST mass spectrometry database to infer compound structures. While selective ion mode is usually used for quantitative analysis with authentic standards, gas chromatograph-EI-MS (GC-EI-MS) in scan mode is appropriate for NTA. For instance, Schulze et al. [43] adopted a hybrid approach, using GC-MS in full scan mode to detect non-target features followed by suspect matching against the NIST05 database to identify a photo-transformation product of a pharmaceutical. In specific scenarios, atmospheric pressure laser ionization sources (APLI), which exhibit exceptional selectivity and sensitivity towards aromatic non- or low-polar compounds (e.g., polycyclic aromatic hydrocarbons, PAHs), have been applied in the screening of environmental samples for non-target bioactive compounds, such as dioxin-

like compounds in sediment [44]. Considering the properties of compounds, GC-MS is suitable for analyzing thermally stable, non-polar, or weakly polar compounds, thereby limiting its scope. For example, Grote et al. [45] found that it was unable to successfully identify toxic fractions in sediment extracts using GC-MS, possibly due to the toxicants being thermally unstable or polar. Furthermore, GC-MS screening is heavily relied on databases, which makes it challenging to identify compounds outside the database.

GC-HRMS technology has been successfully applied in the identification of non-target toxicants in EDA (Table S9 in the Supplementary Materials II). Due to a higher analytical efficiency of ToF-MS in full scan mode and its relatively lower cost compared to other HRMS, it is more widely used. Dahl et al. [46] used GC-ToF-MS to identify genotoxic or estrogenic petroleum hydrocarbons in sediments influenced by the wood industry. Froment et al. [47] screened for acetylcholinesterase inhibitors in petrogenic produced water components. Lee et al. [48] used GC-ToF-MS to analyze unmonitored AhR-active compounds in marine sediments. Alternatively, Orbitrap-MS enables multi-stage mass spectrometry analysis of compounds, offering significant advantages for the analysis of trace substances and emerging pollutants in samples. Dusza et al. [49] used GC-Orbitrap-MS to reveal non-polar endocrine-disrupting compounds with dioxin-like, (anti-)androgenic, and (anti-)estrogenic activity in human amniotic fluid. Ma et al. [50] employed GC-Orbitrap-MS to identify aryl hydrocarbon receptor (AhR) agonists in sediment samples from an electronic waste recycling site in China. With the increasing use of GC-HRMS in NTA, the development of HRMS library becomes important and the transfer of low-resolution NIST MS database to HRMS database through deep learning seems a viable way [51].

Although fractionation in EDA has already simplified the composition of samples, traditional one-dimensional GC may still struggle to adequately separate overly complex samples, which has led to the emergence of comprehensive two-dimensional GC. The GC×GC has gained increasing recognition for NTA in EDA due to its powerful separation capability and peak capacity, which better meet the analytical demands of complex samples compared to one-dimensional GC. Moreover, GC×GC provides the high resolution, which can be further enhanced through chemometric methods if required. Xu et al. [6] combined EDA with GC×GC/ToF-MS-based NTA to successfully identify 11 compounds eliciting AhR response in smoked cigarette leachate. Radovic et al. [52] used GC×GC/ToF-MS to identify AhR agonists and androgen receptor (AR) antagonists in crude and refined oils, including some complex compounds not detected by other traditional methods. Muusse et al. [53] integrated GC-ToF-MS and GC×GC-ToF-MS to identify AhR agonists in roadside snow samples, although some AhR agonists remained unidentified. While useful, data from two-dimensional GC×GC-HRMS is not only voluminous but also highly complex in terms of structural information, requiring the use of efficient data processing algorithms and software for interpreting two-dimensional chromatograph to obtain the necessary chromatographic and mass spectrometric information.

4.3.2. NTA Based on LC-MS

While GC-MS based screening is suitable for moderately polar and non-polar substances, LC-MS compensates for the limitations in analyzing polar compounds and non-volatile compounds. The assembled electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) sources enable the LC-MS to analyze compounds with a wide range of polarity. In addition, compared to well-developed GC-EI-MS, LC-MS spectra generally have fewer fragment ions, poorer reproducibility, and fewer standardized spectrum database. Consequently, in LC-MS, unknown compound analysis often involves full unknown identification and relies less on database match.

In recent years, reports of NTA based on liquid chromatography in EDA have primarily used LC-QToF-MS and LC-Orbitrap for the screening of non-target compounds in various environmental matrices, followed by quantitative analysis of these compounds using LC-MS (Table S10 in Supplementary Materials II). Zhou et al. [9] discovered glucocorticoid receptor antagonists with high bioavailability in indoor dust using LC-QToF-MS-based NTA. Cha et al. [54] used LC-QToF-MS to identify novel polar AhR agonists accumulating in the liver of Korean black-tailed gulls. Stütz et al. [55] used HPTLC to separate samples and then identified genotoxic transformation products of metformin through LC-QToF-MS after hypochlorite treatment. Jonker et al. [56] identified that bisphenol A analogs were the main contributors to estrogenic activity in the plastic components of electronic devices utilizing LC-QToF-MS. Compared to ToF-MS, Orbitrap-MS offers higher resolution and mass accuracy, extending a broader scanning range [57]. Schreiner et al. [5] utilized LC-Orbitrap-MS to identify acetylcholinesterase inhibitors in meal replacement powder. Barrett et al. [58] used LC-Orbitrap-MS to recognize antibacterial compounds in wastewater treatment plant sludge, discovering that triclosan was the primary inhibitor affecting the growth of *E.coli*. Hashmi et al. [59] investigated progestogenic and glucocorticoid activities in river water and successfully identified the major drivers mediated by progesterone receptor (PR) and glucocorticoid receptor (GR) using LC-Orbitrap-MS. These studies highlighted the great potential of LC-HRMS for screening non-target contaminants in complex matrices. Notably, comprehensive two-dimensional LC has recently been applied in EDA, enabling high-throughput screening of

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acetylcholinesterase inhibitors via LC×LC fractionation coupled with parallel ToF-MS identification [60].

4.3.3. NTA Based on Other Tools

During non-targeted full-scan analysis using GC-MS and LC-MS, false positive identifications of compound structures may occur when the characteristic information of the MS ion fragments is insufficient. Additionally, for a more comprehensive analysis of the elemental composition, molecular formula, and structural elucidation of unknown substances, auxiliary techniques such as NMR and FT-ICR-MS are sometimes important.

The NMR is a non-destructive analytical method that can provide qualitative, semi-quantitative, and quantitative characterize chemical structures and has been recently utilized in the analysis of complex environmental samples. Móricz et al. [61] utilized NMR to identify unknown antibacterial and antioxidant compounds as well as acetylcholinesterase (AChE) inhibitors in *Solidago virgaurea* root extracts. However, due to the inability to isolate compounds individually and relatively low sensitivity of NMR, improved techniques have emerged. For instance, Azadniya et al. [62] employed preparative layer chromatography-NMR to analyze non-target compounds in *Salvia miltiorrhiza* and uncovered some unidentified AChE inhibitors.

The FT-ICR-MS, with a ultra-high resolution (450,000~650,000 @ m/z = 500), can separate mass spectral peaks with differences of less than 3 mDa, allowing for precise inference of potential chemical formulas, which is significant in EDA identification [63]. Dong et al. [64] used FT-ICR-MS to elucidate the molecular weight components causing toxicity in chloramine and chlorinated drinking water, effectively revealing unknown high molecular weight disinfected by-products responsible for the toxicity. Bataineh et al. [65] employed FT-ICR-MS as a non-target identification tool for polar mutagenic PAHs in sediment, confirming the identities of suspect compounds with the PubChem database. With the exact mass weights and molecular structures estimated by FT-ICR-MS, aromatic amines were identified as important drivers of genotoxicity in $PM_{2.5}$ dissolved organic matters [66]. Additionally, researchers developed classifier models to predict retention behaviors of candidate compounds in non-target analysis of environmental samples, aiming to narrow down the list of potential candidates in EDA toxicity confirmation [67,68].

The integration of HRMS into EDA requires a strategic trade-off between analytical precision and practical throughput. While QToF platforms offer superior scan speeds and favorable cost-efficiency for high-throughput suspect screening, their moderate resolving power often limits its ability to distinguish isobaric interferents in complex environmental matrices. Orbitrap technology occupies a critical middle ground, delivering high mass accuracy and resolution that balances identification confidence with manageable data complexity. In contrast, FT-ICR-MS provides unmatchable ultra-high resolution which is essential for deciphering molecular formulas in extremely complex mixtures such as dissolved organic matter, yet its high cost and the vast volume of data generated pose significant barriers to routine adoption in EDA workflows. Consequently, selecting an HRMS platform involves more than optimizing sensitivity, instead, it demands aligning the instrument's resolving power with the chemical complexity of the sample matrix and the available computational capacity for data deconvolution.

Different analytical methods have their own advantages and limitations in addressing various sample matrices and target compounds. The combined use of multiple techniques holds promise for maximizing the detection of non-target active substances. Ma et al. [50] simultaneously utilized GC- and LC-Orbitrap-MS to identify AhR agonists in electronic waste-related sediment. Tian et al. [22] employed a combination of GC-QToF-MS, LC-QToF-MS/MS, LC-Orbitrap-MS, and NMR and identified the key toxicant 6PPDQ in the tire tread wear particle leachates causing acute mortality of coho salmon. Lee et al. [69] utilized GC- and LC-QToF-MS to identify non-target AhR agonists with different polarities in sediment. Similarly, Bengtström et al. [7] identified AhR agonists in recycled pizza boxes using both GC- and LC-QToF-MS. These examples demonstrated the advances of NTA techniques and their use in EDA, creating novel opportunities that may ultimately lead to the identification of previously unknown contaminants with adverse potency to ecosystem and human health.

As noted above, different researchers used varied chromatography and mass spectrometry conditions and data processing methods and parameter changes may significantly alter analytical results. As a result, reproducibility and comparability of NTA results in the literature are challenging, calling for standardizing NTA methods. Successful screening relies heavily on effective strategies, such as software for feature extraction from HRMS data. Advancements in online databases, instrument performance, and computer technology will drive progress in this field.

5. Sample Preparation in NTA-EDA

Sample preparation marks the beginning of the EDA workflow. While specific pollutants may be of interest (e.g., highly toxic or emerging contaminants), non-discriminatory sample preparation is essential to avoid loss of active substances in the integrative EDA-NTA. By reviewing current research related EDA incorporating NTA, we summarized some guiding principles for preparing samples from various matrices (Table S11 in Supplementary Materials II).

5.1. Water

Water samples are the most common non-biological samples studied in EDA and the commonly used water sample collection and preparation methods are listed in Table S11. Grab sampling has been widely applied due to its efficiency and convenience but it only reflects the instantaneous pollution characteristics of the sampled water. Large volume solid-phase extraction (LVSPE) involves in-situ enrichment of large volume water samples using equipment deployed in the field, such as Schulze et al. [70] developing a LVSPE device capable of enriching up to 50 L of water. This method provides sufficient analytes for subsequent biological testing, and thus it may eliminate the need for subsequent enrichment steps. However, its long sampling duration and high cost limited its widespread application. Instead, passive sampling techniques, which can reflect the long-term pollution characteristics of water bodies, are increasingly being utilized. Passive sampling is based on the theory of equilibrium partitioning and can reflect the bioavailability of pollutants in the environmental samples while obtaining the time-weighted concentration of pollutants. Gardia-Parège et al. [71] employed polar organic chemical integrative samplers (POCIS) to collect environmental mixtures downstream of a landfill. Using multiple passive samplers in combination enables the simultaneous collection of both polar and non-polar toxicants. Zwart et al. [72] deployed adsorption-based speedisk (SD) and partitioning-based silicone rubber (SR) passive samplers in wastewater to simultaneously obtain polar and non-polar toxicants. Booij et al. [73] deployed POCIS and SR passive samplers in coastal waters. Additionally, researchers developed blue rayon passive samplers, which exhibit extremely high selectivity for aromatic compounds, to identify non-target toxicants in surface water [74–76]. However, most passive sampling techniques can only acquire limited sample volumes, which may be insufficient to meet the analytical requirements of EDA after fractionation.

After sampling, as pollutants in water samples are typically present at trace levels, an enrichment step is required for subsequent bioassay and chemical analysis. solid phase extraction (SPE), the most widely used method for enrichment and removal of matrix interferences in water samples, achieving compound enrichment through pretreatment with sorbents, sample loading, and elution. In all 36 studies targeting toxicity identification in water samples, over 80% of the studies used SPE for sample enrichment, among which 52% of these studies opted for hydrophilic-lipophilic balance (HLB) as the sorbent due to its favorable enrichment efficiency for both polar and non-polar compounds. Besides HLB, other commercial sorbents are also commonly used for water sample enrichment to obtain the interested components. Non-polar polymeric sorbents typically possess characteristics such as hydrophobic and chemical-inertness, making them particularly suitable for adsorbing hydrophobic compounds and studies requiring high sample loading capacities, such as non-polar divinylbenzene-based neutral polymeric sorbents. Stütz et al. [77] utilized bond elut plexa (BEP) cartridges containing non-polar sorbents for the enrichment of drinking water, surface water, and treated wastewater, identifying potential AChE inhibitors. Oberleitner et al. [78] also employed BEP cartridges for the enrichment of surface water, comprehensively evaluating the multi-toxic effects of unknown organic micropollutants. Meinert et al. [79] used poly(styrene-divinylbenzene) for SPE of organic compounds in groundwater and identified various genotoxicants. Other sorbents suitable for hydrophobic compounds are also considered in different scenarios. Zwart et al. [80] used bridged ethylene hybrid (BEH) for the extraction of organic contaminants from surface water and wastewater and Froment et al. [47] employed C18 for petrogenic produced water. Furthermore, SPE methods utilizing mixed sorbents can effectively capture organic compounds with various properties ranging from non-polar to polar, neutral to charged [81]. The filter cartridges used in LVSPE containing top-to-bottom non-polar sorbents (HR-X), weak anion exchanger (HR-WAX), and weak cation exchanger (HR-WCX), which have been widely applied in the identification of non-target toxicants in water samples [28,59,60,82–85]. Mijangos et al. [86] utilized SPE cartridges containing top-to-bottom Bond Elut Plexa and weak anion exchanger Strata X-AW for wastewater enrichment. These works indicated that the choice of SPE sorbents should be considered based on application scenarios, the polarity of target components, adsorption capacity, among other aspects. In a study revealing disinfection by-products (DBPs) with cytotoxicity and genotoxicity in drinking water, XAD resin was used for the extraction of a large volume of water followed by the identification of unknown compounds [64]. Liao et al. [87] evaluated the recovery of DBPs extracted using XAD resin and found extremely low recovery for certain chemical classes. Recent studies have also reported that compared to XAD resin, SPE with HLB exhibit higher recovery for DBPs [88], indicating limitations of the XAD resin for some contaminants and more absorbents should be considered in SPE method for water samples.

For some water samples with highly complex matrices, additional pretreatment may be necessary before SPE. Pinzón-Espinosa et al. [89] initially performed liquid-liquid extraction (LLE) on refinery wastewater under different pH conditions, followed by sequential SPE using HLB and WAX absorbents. Fang et al. [90] pretreated sediment porewater by low-speed centrifugation, flocculation, and solvent extraction, and reported this procedure to be the most effective method

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for obtaining representative extracts for EDA. For specific purposes, such as obtaining components containing different polarities, sequential liquid-liquid extraction (LLE) can be conducted for polarity-based separation, followed by SPE extraction of individual components [91]. Additionally, it can be observed that to minimize substance loss during extraction steps, none of these studies utilized additional purification steps (Table S11).

5.2. Sediment and Soil

For sediment and soil samples, which can effectively reflect the spatial and temporal scales of pollution levels, the sampling and extraction procedures for both target and non-target analyses are similar, but it is important to avoid actively adding chemicals or background contaminants. As shown in Table S11, among the 31 sediment/soil-related studies that simultaneously combined EDA with NTA, 87% utilized Soxhlet extraction or accelerated solvent extraction (ASE) for organic compound extraction, while the rest employed liquid shaking, ultrasonic, or microwave extraction [38,92–97]. Additionally, the use of continuous extraction or parallel multiple extraction methods is beneficial for comprehensively obtaining various pollutants [92,98–100].

To eliminate interference from matrix components on the subsequent bioassay or instrumental analysis, further purification is often required for sediment/soil extracts. For example, copper is commonly used to eliminate sulfur and can be directly added to the extraction process. However, in these studies, only 39% of researchers performed additional purification on samples. Dialysis-based passive diffusion methods have also been used for the purification of sediment extracts, where the extracted material is transferred to a semi-permeable membrane for dialysis. Bataineh et al. [65] combined polyethylene-foil bags with accelerated Solvent Extraction (ASE) to simultaneously extract and purify sediment samples, demonstrating comparable retention for polycyclic aromatic compounds. However, the application scenarios for such analysis targeting specific categories of substances are limited. For instance, Xiao et al. [44] specifically considered the dioxin-like effects of sediment extracts and used acidic silica gel to remove acid-labile compounds (e.g., PAHs), according to the U.S. EPA Method 8290. In contrast, NTA aims to cover more compounds, so the extraction and purification processes should be non-discriminatory. Higley et al. [101] utilized an accelerated membrane-assisted clean-up (AMAC) technique to purify sediment samples and Li et al. [93] used gel permeation chromatography (GPC) with Bio-Beads SX-3 to remove high molecular weight humic acids and sulfur from sediment extracts, only collecting clean components containing the small molecular compounds. Qu et al. [102] employed activated silica gel for the purification of sediment extracts, obtaining non-discriminatory components. Combining these methods can preserve compounds to a large extent while simplifying sample compositions. However, within complex mixtures, the loses of unknown or unconcerned chemicals are inevitable. Thus, refraining from sample purification, where feasible for analytical purposes, provides the most comprehensive representation of the environmental chemical composition.

5.3. Biota

Biota samples typically contain complex matrices such as proteins, lipids, and endogenous metabolites, greatly interfering with the analysis of xenobiotics. To date, 9 studies combining NTA and EDA for animal samples have been published, and different sample preparation methods were used. Overall, the choice of sample preparation strategies depends on the type and content of the interferences, the polarity of analytes, the specificity of toxicity endpoints, and instrumental analysis techniques.

Liquid animal samples typically have low lipid content, and thus relatively simple extraction and purification methods are sufficed. Loewenthal et al. [38] employed agitation, vortex, precipitation, and filtration to remove proteins from whole blood. Jonkers et al. [103] utilized protein denaturation followed by mixed cation exchange (MCX) absorbent-based SPE as purification for fetal calf serum. Dusza et al. [49] used an HLB SPE cartridges for enriching contaminants in human amniotic fluid, followed by a purification with dispersive LLE and precipitation separation. Simon et al. [104] employed organic solvents to denature proteins in polar bear plasma, followed by SPE using WCX absorbent and LLE. Nielen et al. [105,106] reported that two rounds of SPE with C18 and NH_2-absorbent, sequentially, were efficient for enrichment and purification of urine samples. If polar compounds are of particular interest, the supernatants can be directly analyzed on LC-MS, yet for GC-based screening, exchanging to nonpolar solvent is required.

Solid animal samples often contain high levels of endogenous lipids and hormones, which may interfere the measurement of biological activity as well as chemical identification, thus sample purification is generally required. Concentrated sulfuric acid was used for sample treatment, offering excellent purification efficiency but with significant destructiveness, thus it is only suitable for some acid-stable compounds (Wong et al. [107]). Instead, non-destructive lipid removal techniques such as dialysis, GPC, and adsorption chromatography show superiority in extracting more compounds while minimizing biological matrix interference [108]. A combination of these methods can further reduce complexity. Cha et al. [54] conducted Soxhlet extraction on the homogenized, dehydrated, and filtered liver of black-tailed gulls, combined with HPLC with Phenogel 100A column to

remove lipid. Alvarez-Muñoz et al. [98] used ultrasonic probe assisted solvent extraction to treat clam samples, followed by freezing to remove lipid precipitates from supernatants. Klöppel et al. [109] used methanol for sponge extraction without further purification. The difficulty in establishing standardized sample preparation methods for animal samples necessitates the development of in-house approaches, and thus strict QA/QC procedures are necessary to ensure data quality.

5.4. Plant

Unlike environmental samples, plant samples used in EDA often focus on specific bioactive substances. Due to their typical origin from plant tissues, parts, or whole plants, complex secondary metabolites, proteins, and carbohydrates require multiple extraction, purification, and separation steps, with ultrasonic extraction and volume immersion being the most common extraction methods [61,110]. Among the 68 studies combining EDA and NTA for plant samples, 91% of the studies chose HPTLC as the fractionation method after simple purification of plant extract, e.g., filtration and centrifugation. Integrating NTA into EDA holds promise for discovering emerging bioactive substances with potential medicinal values in plant samples.

5.5. Air and Dust

Atmospheric samples are collected using various methods like commercial filters, adsorbents, or custom-designed samplers. Extracts from unpurified air samples can be directly used for EDA analysis. However, when sampling with materials like polyurethane foam that exhibit strong matrix effects, it is recommended to minimize interference through purification. Currently, there are no integrative EDA-NTA studies specifically for atmospheric samples. Regarding dust samples, only two relevant studies have used solvent combinations to effectively extract both non-polar and polar compounds. To prevent pollutant loss, both unpurified and limited purification methods like SPE fractionation are viable. For instance, Zhou et al. [9] utilized ASE and Tenax to extract all or bioaccessible pollutants from indoor dust without additional purification. Conversely, Jonkers et al. [103] employed Envicarb SPE cartridges to purify dust extracts for a comprehensive screening of emerging bioactive chemicals. Excessive matrix effects may lead to higher instrument detection limits or obscured toxic effects, necessitating a balanced approach to purification to mitigate matrix interference during analysis.

5.6. Other Matrices

The integrative EDA-NTA methods have also been used for other samples which were of contact with humans. The pretreatment methods for these samples are similar to those for other types of samples mentioned above. For liquid samples, LLE and SPE remain the most commonly used extraction techniques. For instance, Krstić et al. [111] used LLE to extract bioactive substances for assessing the antioxidant, antibacterial, and enzyme inhibition activities of apple and grape juices in the German market. In addition, SPE with HLB absorbents has been used to enrich bioactive compounds from various samples such as smoked cigarette leachate, photo-transformation products of diclofenac, herbal mixtures and sport supplements, and road snow samples [6,43,53,112,113]. However, for screening all components with different physicochemical properties in the samples, the single SPE absorbent is limited. Wang et al. [17] conducted toxicant identification on aged microplastics and their filtrates using SPE and concentrated components with different polarities through the combined use of multiple adsorbents. For semi-solid and solid samples, common extraction methods including ultrasonic extraction and ASE, were used for samples such as lignite [15], paper and board food-contact materials [7], plastic baby teethers [8], and feed [105]. The application potential of integrative EDA and NTA provides new insights for addressing diverse environmental samples and emerging chemicals that have not yet been regulated.

6. Toxicity Confirmation in NTA-EDA

The reliability of NTA-integrated EDA critically depends on the accurate identification of candidate toxicants. Following initial screening and prioritization, the identification of “known unknowns” is often achievable, yet complex environmental mixtures frequently contain co-eluting or low-abundance compounds that are only partially resolved by instrumentation. Consequently, the confidence in structural elucidation varied considerably across studies, with researchers typically being guided by a five-level confidence assignment framework [114]. Nevertheless, a significant challenge arises when candidate toxicants identified in this manner are not commercially available, preventing conventional confirmatory analysis.

One primary approach involves the use of standard spiking and custom synthesis to verify whether candidate compounds replicate the toxic potency of original fractions. For instance, the identification of 6PPD-quinone was confirmed by synthesizing the compound in high-purity and demonstrating that its dose-response curve matched the acute lethality of urban runoff samples [22]. Meanwhile, effect reconstitution represents another strategy, in which a synthetic mixture of all identified candidates is tested to calculate the “explained toxicity”. A case study on anti-androgenic contaminants in surface water successfully reconstructed the observed toxicological effects by combining identified benzotriazoles and substituted phenols, which accounted for the majority of the total bioactivity [28]. Finally, MoA validation using

mixture toxicity models, such as concentration addition (CA) model, can provide a mechanistic confirmation [115]. This approach was effectively applied in aquatic sediment assessments, where the predicted joint toxicity of oxygenated PACs was compared against the whole-mixture response, confirming them as the dominant AhR agonists [29]. Through integrating these quantitative confirmation strategies, EDA can rigorously link chemical identity to biological effects even in the absence of commercial chemical standards.

Nevertheless, structural confirmation alone does not capture all bioactive hazards. Counterions, adjuvants, dispersants, and other mixture components may evade detection yet contribute to toxicity. Therefore, toxicity confirmation in EDA should integrate quantitative and qualitative data. Candidate identification via NTA must be coupled with bioassay validation to establish causality between the detected compounds and the observed effects. This integrated approach not only improves the prioritization of risk-driving substances but also addresses limitations inherent in single-method strategies, enhancing the discovery of new bioactive chemicals in complex environmental matrices.

7. Perspectives

The major bottleneck in linking chemical identity to biological effect lies in the limited coverage and completeness of spectral databases. Many emerging contaminants lack reference standards and are absent from the existing libraries, creating a blind spot in environmental hazard assessment. To overcome this, there is an urgent need for comprehensive, high-quality mass spectral libraries that encompass both parent compounds and transformation products across diverse environmental matrices. Advances in computational approaches and data science will play an increasingly important role. Development of advanced algorithms for automated deconvolution, machine learning-based pattern recognition, and predictive toxicity modeling can substantially accelerate the interpretation of large HRMS datasets. Coupled with standardized instrumental methods and QA/QC protocols, these strategies can improve reproducibility and comparability of analytical results among laboratories.

From a methodological standpoint, future research should focus on: (1) High-throughput, effect-guided NTA workflows, enabling real-time prioritization of bioactive compounds; (2) Integration of mixture toxicity assessment, accounting for adjuvants, salts, metals, and pH-dependent interactions that modulate bioavailability and biological effects; (3) Bridging chemical and biological data, through mechanistic modeling, in silico toxicity prediction, and combined MoA and risk assessment frameworks; (4) Scalable and cost-effective analytical platforms, including miniaturized sample preparation, automated fractionation, and rapid HRMS acquisition.

Ultimately, the pathway to regulatory adoption depends on translating analytical successes into environmental policy. A prominent example is the identification of 6PPDQ, where NTA-EDA findings prompted immediate monitoring initiatives and new tire-additive regulations in North America. Similarly, the European Water Framework Directive has begun in including effect-based methods to complement traditional chemical monitoring. As these methods become more standardized and spectral databases expand [42], NTA-EDA is expected to provide a reliable framework for environmental monitoring and risk assessment globally.

Supplementary Materials

Additional data and information can be downloaded at: https://media.sciltp.com/articles/others/260210150239631 7/GES-25120047-SI.zip. Table S1. Overview of all published effect-directed analysis (EDA) studies from 2003 to 2025. Table S2. Yearly statistics of effect-directed analysis (EDA)-related research. Table S3. Classification of sample matrices in effect-directed analysis (EDA) studies. Table S4. Proportion of sample matrices investigated in effect-directed analysis (EDA) studies. Table S5. Inventory of analytical instruments for chemical identification in effect-directed analysis (EDA) studies. Table S6. Application of targeted screening instruments in effect-directed analysis (EDA) studies. Table S7. Application of non-targeted screening instruments in effect-directed analysis (EDA) studies. Table S8. Annual statistical overview of targeted and non-targeted screening in effect-directed analysis (EDA) studies. Table S9. Nontarget gas chromatography high-resolution mass spectrometry methods applied in effect-directed analysis (EDA). Table S10. Non-target liquid chromatography high-resolution mass spectrometry (LC–HRMS) methods applied in effect-directed analysis (EDA). Table S11. Summary of sample preparation and fractionation workflows used in effect-directed analysis (EDA) integrated with non-target analysis (NTA).

Author Contributions

Y.T.: Conceptualization, Investigation, Writing—original draft preparation; H.L.: Funding acquisition, Methodology, Supervision; F.C.: Data curation; J.Z.: Funding acquisition, Supervision; J.Y.: Conceptualization, Funding acquisition, Methodology, Supervision, Writing—Reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Use of AI and AI-Assisted Technologies

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References

1. Li, H.; Zhang, J.; You, J. Diagnosis of Complex Mixture Toxicity in Sediments: Application of Toxicity Identification Evaluation (TIE) and Effect-Directed Analysis (EDA). *Environ. Pollut.* **2018**, *237*, 944–954.
2. Brack, W. Effect-Directed Analysis: A Promising Tool for the Identification of Organic Toxicants in Complex Mixtures? *Anal. Bioanal. Chem.* **2003**, *377*, 397–407.
3. Guo, J.; Deng, D.; Qiu, J.; et al. Biodirected Identification of Untargeted Toxicants in Industrial Wastewater Guides the Upgrading of Water Treatments. *Environ. Sci. Technol. Lett.* **2021**, *8*, 474–481.
4. Gwak, J.; Lee, J.; Cha, J.; et al. Molecular Characterization of Estrogen Receptor Agonists During Sewage Treatment Processes Using Effect-Directed Analysis Combined with High-Resolution Full-Scan Screening. *Environ. Sci. Technol.* **2022**, *56*, 13085–13095.
5. Schreiner, T.; Eggerstorfer, N.M.; Morlock, G.E. Ten-Dimensional Hyphenation Including Simulated Static Gastro-Intestinal Digestion on the Adsorbent Surface, Planar Assays, and Bioactivity Evaluation for Meal Replacement Products. *Food Funct.* **2023**, *14*, 344–353.
6. Xu, E.G.; Richardot, W.H.; Li, S.; et al. Assessing Toxicity and *In Vitro* Bioactivity of Smoked Cigarette Leachate Using Cell-Based Assays and Chemical Analysis. *Chem. Res. Toxicol.* **2019**, *32*, 1670–1679.
7. Bengtström, L.; Rosenmai, A.K.; Trier, X.; et al. Non-Targeted Screening for Contaminants in Paper and Board Food-Contact Materials Using Effect-Directed Analysis and Accurate Mass Spectrometry. *Food Addit. Contam. Part A* **2016**, *33*, 1080–1093.
8. Berger, E.; Potouridis, T.; Haeger, A.; et al. Effect-Directed Identification of Endocrine Disruptors in Plastic Baby Teethers. *J. Appl. Toxicol.* **2015**, *35*, 1254–1261.
9. Zhou, Q.; Shen, Y.; Chou, L.; et al. Identification of Glucocorticoid Receptor Antagonistic Activities and Responsible Compounds in House Dust: Bioaccessibility Should Not Be Ignored. *Environ. Sci. Technol.* **2022**, *56*, 16768–16779.
10. Qi, H.; Zhao, B.; Li, L.; et al. Effect-Directed Analysis of Toxic Organics in $PM_{2.5}$ Exposure to the Cellular Bioassays *In Vitro*: Application in Shanxi of China. *Ecotoxicol. Environ. Saf.* **2022**, *237*, 113501.
11. Łata, E.; Fulczyk, A.; Ott, P.G.; et al. Thin-Layer Chromatographic Quantification of Magnolol and Honokiol in Dietary Supplements and Selected Biological Properties of These Preparations. *J. Chromatogr. A* **2020**, *1625*, 461230.
12. Riegraf, C.; Reifferscheid, G.; Moscovici, L.; et al. Coupling High-Performance Thin-Layer Chromatography with a Battery of Cell-Based Assays Reveals Bioactive Components in Wastewater and Landfill Leachates. *Ecotoxicol. Environ. Saf.* **2021**, *214*, 112092.
13. Stiefel, C.; Lindemann, B.; Morlock, G.E. Non-Target Bioactive Compound Profiles of Coffee Roasts and Preparations. *Food Chem.* **2022**, *391*, 133263.
14. Ristivojević, P.; Morlock, G.E. Effect-Directed Classification of Biological, Biochemical and Chemical Profiles of 50 German Beers. *Food Chem.* **2018**, *260*, 344–353.
15. Meyer, W.; Seiler, T.-B.; Christ, A.; et al. Mutagenicity, Dioxin-Like Activity and Bioaccumulation of Alkylated Picene and Chrysene Derivatives in a German Lignite. *Sci. Total Environ.* **2014**, *497*, 634–641.
16. Bell, A.M.; Keltsch, N.; Schweyen, P.; et al. UV Aged Epoxy Coatings-Ecotoxicological Effects and Released Compounds. *Water Res. X* **2021**, *12*, 100105.
17. Wang, X.; Zhang, Y.; Zhao, Y.; et al. Inhibition of Aged Microplastics and Leachates on Methane Production from Anaerobic Digestion of Sludge and Identification of Key Components. *J. Hazard. Mater.* **2023**, *446*, 130717.
18. Ahmad, R.; Cho, E.; Rakhmat, S.; et al. Characterization of Structure Isomers of Ethylbenzalkyl Dimethyl Ammonium Chlorides and Quantification in Commercial Household Disinfectant Products. *Environ. Technol. Innov.* **2023**, *29*, 102979.
19. Rosenmai, A.K.; Bengtström, L.; Taxvig, C.; et al. An Effect-Directed Strategy for Characterizing Emerging Chemicals in Food Contact Materials Made from Paper and Board. *Food Chem. Toxicol.* **2017**, *106*, 250–259.
20. Schönlau, C.; Larsson, M.; Dubocq, F.; et al. Effect-Directed Analysis of Ah Receptor-Mediated Potencies in Microplastics Deployed in a Remote Tropical Marine Environment. *Front. Environ. Sci.* **2019**, *7*, 120.
21. Krüger, S.; Urmann, O.; Morlock, G.E. Development of a Planar Chromatographic Method for Quantitation of Anthocyanes in Pomace, Feed, Juice and Wine. *J. Chromatogr. A* **2013**, *1289*, 105–118.
22. Tian, Z.; Zhao, H.; Peter, K.T.; et al. A Ubiquitous Tire Rubber–Derived Chemical Induces Acute Mortality in Coho Salmon. *Science* **2021**, *371*, 185–189.
23. You, J.; Li, H. Improving the Accuracy of Effect-Directed Analysis: The Role of Bioavailability. *Environ. Sci. Process. Impacts* **2017**, *19*, 1484–1498.

24. An, S.-A.; Hong, S.; Lee, J.; et al. Identification of Potential Toxicants in Sediments from an Industrialized Area in Pohang, South Korea: Application of a Cell Viability Assay of Microalgae Using Flow Cytometry. *J. Hazard. Mater.* **2021**, *405*, 124230.
25. Weiss, J.M.; Simon, E.; Stroomberg, G.J.; et al. Identification Strategy for Unknown Pollutants Using High-Resolution Mass Spectrometry: Androgen-Disrupting Compounds Identified through Effect-Directed Analysis. *Anal. Bioanal. Chem.* **2011**, *400*, 3141–3149.
26. Creusot, N.; Budzinski, H.; Balaguer, P.; et al. Effect-Directed Analysis of Endocrine-Disrupting Compounds in Multi-Contaminated Sediment: Identification of Novel Ligands of Estrogen and Pregnane X Receptors. *Anal. Bioanal. Chem.* **2013**, *405*, 2553–2566.
27. Portolés, T.; Pitarch, E.; López, F.J.; et al. Use of Soft and Hard Ionization Techniques for Elucidation of Unknown Compounds by Gas Chromatography/Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1589–1599.
28. Muschket, M.; Di Paolo, C.; Tindall, A.J.; et al. Identification of Unknown Antiandrogenic Compounds in Surface Waters by Effect-Directed Analysis (EDA) Using a Parallel Fractionation Approach. *Environ. Sci. Technol.* **2018**, *52*, 288–297.
29. Lübcke-von Varel, U.; Machala, M.; Ciganek, M.; et al. Polar Compounds Dominate *In Vitro* Effects of Sediment Extracts. *Environ. Sci. Technol.* **2011**, *45*, 2384–2390.
30. Cha, J.; Hong, S.; Lee, J.; et al. Identification of Mid-Polar and Polar Ahr Agonists in Cetaceans from Korean Coastal Waters: Application of Effect-Directed Analysis with Full-Scan Screening. *Environ. Sci. Technol.* **2023**, *57*, 15644–15655.
31. Dusza, H.M.; Janssen, E.; Kanda, R.; et al. Method Development for Effect-Directed Analysis of Endocrine Disrupting Compounds in Human Amniotic Fluid. *Environ. Sci. Technol.* **2019**, *53*, 14649–14659.
32. Huang, J.; Cheng, F.; He, L.; et al. Effect Driven Prioritization of Contaminants in Wastewater Treatment Plants across China: A Data Mining-Based Toxicity Screening Approach. *Water Res.* **2024**, *264*, 122223.
33. Houtman, C.J.; Brewster, K.; Ten Broek, R.; et al. Characterisation of (Anti-)progestogenic and (Anti-)androgenic Activities in Surface and Wastewater Using High Resolution Effectdirected Analysis. *Environ. Int.* **2021**, *153*, 106536.
34. Lopez-Herguedas, N.; Mijangos, L.; Alvarez-Mora, I.; et al. Suspect Screening of Chemicals in Hospital Wastewaters Ssing Effect-Directed Analysis Approach as Prioritization Strategy. *Molecules* **2023**, *28*, 1212.
35. Cheng, F.; Li, H.; Ma, H.; et al. Identifying Bioaccessible Suspect Toxicants in Sediment Using Adverse Outcome Pathway Directed Analysis. *J. Hazard. Mater.* **2020**, *389*, 121853.
36. Guo, J.; Deng, D.; Wang, Y.; et al. Extended Suspect Screening Strategy to Identify Characteristic Toxicants in the Discharge of a Chemical Industrial Park Based on Toxicity to *Daphnia Magna*. *Sci. Total Environ.* **2019**, *650*, 10–17.
37. An, S.-A.; Lee, J.; Cha, J.; et al. Characterization of Microalgal Toxicants in the Sediments from an Industrial Area: Application of Advanced Effect-Directed Analysis with Multiple Endpoint Bioassays. *Environ. Int.* **2023**, *173*, 107833.
38. Loewenthal, D.; Dagan, S.; Drug, E. Integrating Effect-Directed Analysis and Chemically Indicative Mass Spectral Fragmentation to Screen for Toxic Organophosphorus Compounds. *Anal. Chem.* **2023**, *95*, 2623–2627.
39. Marić, P.; Ahel, M.; Senta, I.; et al. Effect-Directed Analysis Reveals Inhibition of Zebrafish Uptake Transporter Oatp1d1 by Caulerpenyne, a Major Secondary Metabolite from the Invasive Marine Alga *Caulerpa Taxifolia*. *Chemosphere* **2017**, *174*, 643–654.
40. Milman, B.L. General Principles of Identification by Mass Spectrometry. *TrAC Trend. Anal. Chem.* **2015**, *69*, 24–33.
41. He, L.; Cheng, F.; Wu, F.; et al. Identifying and Prioritizing Organic Toxicants in Treated Flowback and Produced Water from Shale Gas Exploitation Sites Using an Integrative Effect-Directed Analysis and Nontarget Screening Method. *Water Res.* **2025**, *277*, 123311.
42. Cheng, F.; Li, H.; Lou, X.; et al. Event-Driven Taxonomy (EDT) Screening: Leveraging Effect-Based Spectral Libraries to Accelerate Semiquantitative Nontarget Analysis of Ahr Agonists in Sediment in the Era of Big Data. *Environ. Sci. Technol.* **2025**, *59*, 14359–14371.
43. Schulze, T.; Weiss, S.; Schymanski, E.; et al. Identification of a Phytotoxic Photo-Transformation Product of Diclofenac Using Effect-Directed Analysis. *Environ. Pollut.* **2010**, *158*, 1461–1466.
44. Xiao, H.; Brinkmann, M.; Thalmann, B.; et al. Toward Streamlined Identification of Dioxin-Like Compounds in Environmental Samples through Integration of Suspension Bioassay. *Environ. Sci. Technol.* **2017**, *51*, 3382–3390.
45. Grote, M.; Altenburger, R.; Brack, W.; et al. Ecotoxicological Profiling of Transect River Elbe Sediments. *Acta Hydroch. Hydrobiol.* **2005**, *33*, 555–569.
46. Dahl, M.; Survo, S.; Välitalo, P.; et al. Identification of Toxicants from a Highly C10–C40-Contaminated Sediment Influenced by the Wood Industry: Petroleum Hydrocarbons or Biogenic Organic Compounds? *Environ. Toxicol. Chem.* **2019**, *38*, 936–946.
47. Froment, J.; Langford, K.; Tollefsen, K.E.; et al. Identification of Petrogenic Produced Water Components as Acetylcholine Esterase Inhibitors. *Environ. Pollut.* **2016**, *215*, 18–26.
48. Lee, J.; Hong, S.; Kim, T.; et al. Identification of Ahr Agonists in Sediments of the Bohai and Yellow Seas Using Advanced Effect-Directed Analysis and in Silico Prediction. *J. Hazard. Mater.* **2022**, *435*, 128908.
49. Dusza, H.M.; Manz, K.E.; Pennell, K.D.; et al. Identification of Known and Novel Nonpolar Endocrine Disruptors in Human Amniotic Fluid. *Environ. Int.* **2022**, *158*, 106904.
50. Ma, Q.; Liu, Y.; Yang, X.; et al. Effect-Directed Analysis for Revealing Aryl Hydrocarbon Receptor Agonists in Sediment Samples from an Electronic Waste Recycling Town in China. *Environ. Pollut.* **2022**, *308*, 119659.

51. Cheng, F.; Escher, B.I.; Li, H.; et al. Deep Learning Bridged Bioactivity, Structure, and GC-Hrms-Readable Evidence to Decipher Nontarget Toxicants in Sediments. *Environ. Sci. Technol.* **2024**, *58*, 15415–15427.
52. Radovic, J.R.; Thomas, K.V.; Parastar, H.; et al. Chemometrics-Assisted Effect-Directed Analysis of Crude and Refined Oil Using Comprehensive Two-Dimensional Gas Chromatography–Time-of-Flight Mass Spectrometry. *Environ. Sci. Technol.* **2014**, *48*, 3074–3083.
53. Muusse, M.; Langford, K.; Tollefsen, K.E.; et al. Characterization of Ahr Agonist Compounds in Roadside Snow. *Anal. Bioanal. Chem.* **2012**, *403*, 2047–2056.
54. Cha, J.; Hong, S.; Gwak, J.; et al. Identification of Novel Polar Aryl Hydrocarbon Receptor Agonists Accumulated in Liver of Black-Tailed Gulls in Korea Using Advanced Effect-Directed Analysis. *J. Hazard. Mater.* **2022**, *429*, 128305.
55. Stütz, L.; Leitner, P.; Schulz, W.; et al. Identification of Genotoxic Transformation Products by Effect-Directed Analysis with High-Performance Thin-Layer Chromatography and Non-Target Screening. *JPC J. Planar Chromatogr. Mod. TLC* **2019**, *32*, 173–182.
56. Jonker, W.; Ballesteros-Gómez, A.; Hamers, T.; et al. Highly Selective Screening of Estrogenic Compounds in Consumer-Electronics Plastics by Liquid Chromatography in Parallel Combined with Nanofractionation-Bioactivity Detection and Mass Spectrometry. *Environ. Sci. Technol.* **2016**, *50*, 12385–12393.
57. Krauss, M.; Singer, H.; Hollender, J. LC–High Resolution MS in Environmental Analysis: From Target Screening to the Identification of Unknowns. *Anal. Bioanal. Chem.* **2010**, *397*, 943–951.
58. Barrett, H.; Sun, J.; Gong, Y.; et al. Triclosan Is the Predominant Antibacterial Compound in Ontario Sewage Sludge. *Environ. Sci. Technol.* **2022**, *56*, 14923–14936.
59. Hashmi, M.A.K.; Krauss, M.; Escher, B.I.; et al. Effect-Directed Analysis of Progestogens and Glucocorticoids at Trace Concentrations in River Water. *Environ. Toxicol. Chem.* **2020**, *39*, 189–199.
60. Ouyang, X.; Leonards, P.E.; Tousova, Z.; et al. Rapid Screening of Acetylcholinesterase Inhibitors by Effect-Directed Analysis Using LC × LC Fractionation, a High Throughput *In Vitro* Assay, and Parallel Identification by Time of Flight Mass Spectrometry. *Anal. Chem.* **2016**, *88*, 2353–2360.
61. Móricz, Á.M.; Ott, P.G.; Habe, T.T.; et al. Effect-Directed Discovery of Bioactive Compounds Followed by Highly Targeted Characterization, Isolation and Identification, Exemplarily Shown for *Solidago Virgaurea*. *Anal. Chem.* **2016**, *88*, 8202–8209.
62. Azadniya, E.; Morlock, G.E. Bioprofiling of *Salvia miltiorrhiza* via Planar Chromatography Linked to (Bio)assays, High Resolution Mass Spectrometry and Nuclear Magnetic Resonance Spectroscopy. *J. Chromatogr. A* **2018**, *1533*, 180–192.
63. Byer, J.D.; Siek, K.; Jobst, K. Distinguishing the C3 Vs SH_4 Mass Split by Comprehensive Two-Dimensional Gas Chromatography–High Resolution Time-of-Flight Mass Spectrometry. *Anal. Chem.* **2016**, *88*, 6101–6104.
64. Dong, H.; Cuthbertson, A.A.; Plewa, M.J.; et al. Unravelling High-Molecular-Weight Dbp Toxicity Drivers in Chlorinated and Chloraminated Drinking Water: Effect-Directed Analysis of Molecular Weight Fractions. *Environ. Sci. Technol.* **2023**, *57*, 18788–18800.
65. Bataineh, M.; Lübcke-von Varel, U.; Hayen, H.; et al. HPLC/APCI-FTICR-MS as a Tool for Identification of Partial Polar Mutagenic Compounds in Effect-Directed Analysis. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1016–1027.
66. Zhang, Q.; Ma, H.; Li, J.; et al. Unveiling Condensed Aromatic Amines as Noteworthy Genotoxic Components in $PM_{2.5}$ Dissolved Organic Matter. *Environ. Sci. Technol.* **2025**, *59*, 21015–21027.
67. Ulrich, N.; Mühlenberg, J.; Schüürmann, G.; et al. Linear Solvation Energy Relationships as Classifier in Non-Target Analysis—An Approach for Isocratic Liquid Chromatography. *J. Chromatogr. A* **2014**, *1324*, 96–103.
68. Ulrich, N.; Schüürmann, G.; Brack, W. Prediction of Gas Chromatographic Retention Indices as Classifier in Non-Target Analysis of Environmental Samples. *J. Chromatogr. A* **2013**, *1285*, 139–147.
69. Lee, J.; Hong, S.; Kim, T.; et al. Multiple Bioassays and Targeted and Nontargeted Analyses to Characterize Potential Toxicological Effects Associated with Sediments of Masan Bay: Focusing on Ahr-Mediated Potency. *Environ. Sci. Technol.* **2020**, *54*, 4443–4454.
70. Schulze, T.; Ahel, M.; Ahlheim, J.; et al. Assessment of a Novel Device for Onsite Integrative Large-Volume Solid Phase Extraction of Water Samples to Enable a Comprehensive Chemical and Effect-Based Analysis. *Sci. Total Environ.* **2017**, *581*, 350–358.
71. Gardia-Parège, C.; Kim Tiam, S.; Budzinski, H.; et al. Pesticide Toxicity Towards Microalgae Increases with Environmental Mixture Complexity. *Environ. Sci. Pollut. Res.* **2022**, *29*, 29368–29381.
72. Zwart, N.; Nio, S.L.; Houtman, C.J.; et al. High-Throughput Effect-Directed Analysis Using Downscaled *In Vitro* Reporter Gene Assays to Identify Endocrine Disruptors in Surface Water. *Environ. Sci. Technol.* **2018**, *52*, 4367–4377.
73. Booij, P.; Vethaak, A.D.; Leonards, P.E.; et al. Identification of Photosynthesis Inhibitors of Pelagic Marine Algae Using 96-Well Plate Microfractionation for Enhanced Throughput in Effect-Directed Analysis. *Environ. Sci. Technol.* **2014**, *48*, 8003–8011.
74. Gallampois, C.M.; Schymanski, E.L.; Krauss, M.; et al. Multicriteria Approach to Select Polyaromatic River Mutagen Candidates. *Environ. Sci. Technol.* **2015**, *49*, 2959–2968.
75. Gallampois, C.M.; Schymanski, E.L.; Bataineh, M.; et al. Integrated Biological–Chemical Approach for the Isolation and Selection of Polyaromatic Mutagens in Surface Waters. *Anal. Bioanal. Chem.* **2013**, *405*, 9101–9112.
76. Schymanski, E.L.; Gallampois, C.M.; Krauss, M.; et al. Consensus Structure Elucidation Combining GC/EI-MS,

Structure Generation, and Calculated Properties. *Anal. Chem.* **2012**, *84*, 3287–3295.
77. Stütz, L.; Schulz, W.; Winzenbacher, R. Identification of Acetylcholinesterase Inhibitors in Water by Combining Two-Dimensional Thin-Layer Chromatography and High-Resolution Mass Spectrometry. *J. Chromatogr. A* **2020**, *1624*, 461239.
78. Oberleitner, D.; Stütz, L.; Schulz, W.; et al. Seasonal Performance Assessment of Four Riverbank Filtration Sites by Combined Non-Target and Effect-Directed Analysis. *Chemosphere* **2020**, *261*, 127706.
79. Meinert, C.; Schymanski, E.; Küster, E.; et al. Application of Preparative Capillary Gas Chromatography (pcGC), Automated Structure Generation and Mutagenicity Prediction to Improve Effect-Directed Analysis of Genotoxicants in a Contaminated Groundwater. *Environ. Sci. Pollut. Res.* **2010**, *17*, 885–897.
80. Zwart, N.; Jonker, W.; Broek, R.T.; et al. Identification of Mutagenic and Endocrine Disrupting Compounds in Surface Water and Wastewater Treatment Plant Effluents Using High-Resolution Effect-Directed Analysis. *Water Res.* **2020**, *168*, 115204.
81. Tan, B.; Xiong, J.; Li, H.; et al. Simultaneous Analysis of Current-Use Pesticides and Their Transformation Products in Water Using Mixture-Sorbent Solid Phase Extraction and High-Performance Liquid Chromatography–Tandem Mass Spectrometry. *J. Sep. Sci.* **2020**, *43*, 2409–2418.
82. Tousova, Z.; Froment, J.; Oswald, P.; et al. Identification of Algal Growth Inhibitors in Treated Waste Water Using Effect-Directed Analysis Based on Non-Target Screening Techniques. *J. Hazard. Mater.* **2018**, *358*, 494–502.
83. Tousova, Z.; Oswald, P.; Slobodnik, J.; et al. European Demonstration Program on the Effect-Based and Chemical Identification and Monitoring of Organic Pollutants in European Surface Waters. *Sci. Total Environ.* **2017**, *601*, 1849–1868.
84. Hug, C.; Krauss, M.; Nüsser, L.; et al. Metabolic Transformation as a Diagnostic Tool for the Selection of Candidate Promutagens in Effect-Directed Analysis. *Environ. Pollut.* **2015**, *196*, 114–124.
85. Tufi, S.; Wassenaar, P.N.; Osorio, V.; et al. Pesticide Mixture Toxicity in Surface Water Extracts in Snails (*Lymnaea Stagnalis*) by an *In Vitro* Acetylcholinesterase Inhibition Assay and Metabolomics. *Environ. Sci. Technol.* **2016**, *50*, 3937–3944.
86. Mijangos, L.; Krauss, M.; de Miguel, L.; et al. Application of the Sea Urchin Embryo Test in Toxicity Evaluation and Effect-Directed Analysis of Wastewater Treatment Plant Effluents. *Environ. Sci. Technol.* **2020**, *54*, 8890–8899.
87. Liao, X.; Allen, J.M.; Granger, C.O.; et al. How Well Does Xad Resin Extraction Recover Halogenated Disinfection Byproducts for Comprehensive Identification and Toxicity Testing? *J. Environ. Sci.* **2022**, *117*, 264–275.
88. Lau, S.S.; Forster, A.L.; Richardson, S.D.; et al. Disinfection Byproduct Recovery During Extraction and Concentration in Preparation for Chemical Analyses or Toxicity Assays. *Environ. Sci. Technol.* **2021**, *55*, 14136–14145.
89. Pinzón-Espinosa, A.; Kanda, R. Naphthenic Acids Are Key Contributors to Toxicity of Heavy Oil Refining Effluents. *Sci. Total Environ.* **2020**, *729*, 138119.
90. Fang, M.; Getzinger, G.J.; Cooper, E.M.; et al. Effect-Directed Analysis of Elizabeth River Porewater: Developmental Toxicity in Zebrafish (*Danio Rerio*). *Environ. Toxicol. Chem.* **2014**, *33*, 2767–2774.
91. Pochiraju, S.S.; Linden, K.; Gu, A.Z.; et al. Development of a Separation Framework for Effects-Based Targeted and Non-Targeted Toxicological Screening of Water and Wastewater. *Water Res.* **2020**, *170*, 115289.
92. Qi, H.; Li, H.; Wei, Y.; et al. Effect-Directed Analysis of Toxicants in Sediment with Combined Passive Dosing and *In Vivo* Toxicity Testing. *Environ. Sci. Technol.* **2017**, *51*, 6414–6421.
93. Li, H.; Yi, X.; Cheng, F.; et al. Identifying Organic Toxicants in Sediment Using Effect-Directed Analysis: A Combination of Bioaccessibility-Based Extraction and High-Throughput Midge Toxicity Testing. *Environ. Sci. Technol.* **2018**, *53*, 996–1003.
94. Tian, Z.; Gold, A.; Nakamura, J.; et al. Nontarget Analysis Reveals a Bacterial Metabolite of Pyrene Implicated in the Genotoxicity of Contaminated Soil after Bioremediation. *Environ. Sci. Technol.* **2017**, *51*, 7091–7100.
95. Feng, Q.; Yang, L.; Chen, J.; et al. Identification of the Estrogen-Active Compounds via Integrating Effect-Directed Analysis and Non-Target Screening in Soils of the Northeastern China. *Environ. Sci. Eur.* **2024**, *36*, 1–13.
96. Legler, J.; van Velzen, M.; Cenijn, P.H.; et al. Effect-Directed Analysis of Municipal Landfill Soil Reveals Novel Developmental Toxicants in the Zebrafish *Danio Rerio*. *Environ. Sci. Technol.* **2011**, *45*, 8552–8558.
97. Jondeau-Cabaton, A.; Soucasse, A.; Jamin, E.L.; et al. Characterization of Endocrine Disruptors from a Complex Matrix Using Estrogen Receptor Affinity Columns and High Performance Liquid Chromatography–High Resolution Mass Spectrometry. *Environ. Sci. Pollut. Res.* **2013**, *20*, 2705–2720.
98. Alvarez-Muñoz, D.; Indiveri, P.; Rostkowski, P.; et al. Widespread Contamination of Coastal Sediments in the Transmanche Channel with Anti-Androgenic Compounds. *Mar. Pollut. Bull.* **2015**, *95*, 590–597.
99. Guo, J.; Shi, W.; Chen, Q.; et al. Extended Virtual Screening Strategies to Link Antiandrogenic Activities and Detected Organic Contaminants in Soils. *Environ. Sci. Technol.* **2017**, *51*, 12528–12536.
100. Zaja, R.; Terzić, S.; Senta, I.; et al. Identification of P-Glycoprotein Inhibitors in Contaminated Freshwater Sediments. *Environ. Sci. Technol.* **2013**, *47*, 4813–4821.
101. Higley, E.; Grund, S.; Jones, P.D.; et al. Endocrine Disrupting, Mutagenic, and Teratogenic Effects of Upper Danube River Sediments Using Effect-Directed Analysis. *Environ. Toxicol. Chem.* **2012**, *31*, 1053–1062.

102. Qu, G.; Shi, J.; Li, Z.; et al. Detection of Tris-(2,3-Dibromopropyl) Isocyanurate as a Neuronal Toxicant in Environmental Samples Using Neuronal Toxicity-Directed Analysis. *Sci. China Chem.* **2011**, *54*, 1651–1658.
103. Jonkers, T.J.; Meijer, J.; Vlaanderen, J.J.; et al. High-Performance Data Processing Workflow Incorporating Effect-Directed Analysis for Feature Prioritization in Suspect and Nontarget Screening. *Environ. Sci. Technol.* **2022**, *56*, 1639–1651.
104. Simon, E.; van Velzen, M.; Brandsma, S.H.; et al. Effect-Directed Analysis to Explore the Polar Bear Exposome: Identification of Thyroid Hormone Disrupting Compounds in Plasma. *Environ. Sci. Technol.* **2013**, *47*, 8902–8912.
105. Nielen, M.W.; Elliott, C.T.; Boyd, S.A.; et al. Identification of an Unknown Beta-Agonist in Feed by Liquid Chromatography/Bioassay/Quadrupole Time-of-Flight Tandem Mass Spectrometry with Accurate Mass Measurement. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1633–1641.
106. Nielen, M.W.; Van Bennekom, E.O.; Heskamp, H.H.; et al. Bioassay-Directed Identification of Estrogen Residues in Urine by Liquid Chromatography Electrospray Quadrupole Time-of-Flight Mass Spectrometry. *Anal. Chem.* **2004**, *76*, 6600–6608.
107. Wong, H.; Giesy, J.; Siu, W.; et al. Estrogenic and Dioxin-Like Activities and Cytotoxicity of Sediments and Biota from Hong Kong Mudflats. *Arch. Environ. Contam. Toxicol.* **2005**, *48*, 575–586.
108. Simon, E.; Lamoree, M.H.; Hamers, T.; et al. Testing Endocrine Disruption in Biota Samples: A Method to Remove Interfering Lipids and Natural Hormones. *Environ. Sci. Technol.* **2010**, *44*, 8322–8329.
109. Klöppel, A.; Grasse, W.; Brümmer, F.; et al. Hptlc Coupled with Bioluminescence and Mass Spectrometry for Bioactivity-Based Analysis of Secondary Metabolites in Marine Sponges. *JPC J. Planar Chromatogr. Mod. TLC* **2008**, *21*, 431–436.
110. Krüzselyi, D. n.; Bakonyi, J.; Ott, P.G.; et al. Goldenrod Root Compounds Active against Crop Pathogenic Fungi. *J. Agric. Food Chem.* **2021**, *69*, 12686–12694.
111. Krstić, Đ.; Ristivojević, P.; Andrić, F.; et al. Quality Assessment of Apple and Grape Juices from Serbian and German Markets by Planar Chromatography—Chemometrics. *Molecules* **2022**, *27*, 3933.
112. Peters, R.J.; Rijk, J.C.; Bovee, T.F.; et al. Identification of Anabolic Steroids and Derivatives Using Bioassay-Guided Fractionation, UHPLC/TOFMS Analysis and Accurate Mass Database Searching. *Anal. Chim. Acta* **2010**, *664*, 77–88.
113. Houtman, C.J.; Ten Broek, R.; van Oorschot, Y.; et al. High Resolution Effect-Directed Analysis of Steroid Hormone (Ant)Agonists in Surface and Wastewater Quality Monitoring. *Environ. Toxicol. Pharmacol.* **2020**, *80*, 103460.
114. Schymanski, E.L.; Jeon, J.; Gulde, R.; et al. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci. Technol.* **2014**, *48*, 2097–2098.
115. Schmitt, S.; Reifferscheid, G.; Claus, E.; et al. Effect Directed Analysis and Mixture Effects of Estrogenic Compounds in a Sediment of the River Elbe. *Environ. Sci. Pollut. Res.* **2012**, *19*, 3350–3361.