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Pioneering an effect-based early warning system for hazardous chemicals in the environment

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ABSTRACT

Existing regulatory frameworks often prove inadequate in identifying contaminants of emerging concern (CECs) and determining their impacts on biological systems at an early stage. The establishment of Early Warning Systems (EWSs) for CECs is becoming increasingly relevant for policy-making, aiming to proactively detect chemical hazards and implement effective mitigation measures. Effect-based methodologies, including bioassays and effect-directed analysis (EDA), offer valuable input to EWSs with a view to pinpointing the relevant toxicity drivers and prioritizing the associated risks. This review evaluates the analytical techniques currently available to assess biological effects, and provides a structured plan for their systematic integration into an EWS for hazardous chemicals in the environment. Key scientific advancements in effect-based approaches and EDA are discussed, underscoring their potential for early detection and management of chemical hazards. Additionally, critical challenges such as data integration and regulatory alignment are addressed, emphasizing the need for continuous improvement of the EWS and the incorporation of analytical advancements to safeguard environmental and public health from emerging chemical threats.

1. Introduction

The global chemical industry is large and growing. In 2020, an estimated 29,000 unique chemicals were registered for commercial use in the EU alone, with global chemical production projected to double between 2017 and 2030 [1,2]. Researchers assert that this extensive production of chemicals exceeds the planetary safe operating space and surpasses society's capacity to conduct appropriate safety assessments [3]. Additionally, many of these chemicals are explicitly engineered to target biological processes, finding applications in biocides, and pharmaceuticals. Notably, 62 % of the EU's total chemical consumption, equivalent to 345 million tonnes, was classified as posing a health hazard [1].

The production, use, and disposal of chemicals are managed by

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national and international bodies to protect the environment and human health. The Stockholm Convention on Persistent Organic Pollutants under the United Nations Environmental Programme entered into force in 2004 [4] and regulates chemicals that are persistent, bio-accumulative, toxic, and subject to long-range transport. In Europe, the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) is the legislative framework for chemicals management that entered into force in 2007 and also includes chemicals in imported goods. However, while chemical restrictions are an important tool for risk management, they are typically reactive in nature. Although the REACH program intends to prevent the widespread use of problematic chemicals, ongoing examples of contamination and exposure cases indicate oversights or inefficiency, such as the global pollution issue of per-and polyfluoroalkyl substances (PFAS) [5] or the ongoing exposure to regulated phthalates [6]. Additionally, restrictions often fail to address all potential risks associated with chemical mixtures [7]. Finally, the restriction of chemicals may be followed by their replacement with potentially regrettable alternatives, which may introduce similar hazards or poorly understood risks [8].

Consequently, there is a need for detecting potential environmental and health problems associated with chemical production and use at an early stage. Most of the research-based initiatives focus on identifying contaminants of emerging concern (CECs), using *in-silico* and/or experimental screening methods [9,10]. Recent advancements in analytical chemistry have allowed for more precise, selective, and sensitive quantifications of chemical contamination, however, information on the toxicity of chemical mixtures and their bioavailability is often lacking during chemical screening [11]. Complementarily to the approaches focusing on chemical identities, effect-based methods (EBMs) can help to identify undesired effects of a chemical or mixture, i.e. assess the toxicity of bioactive compounds, aiming to determine the biological impact of a given sample through the utilisation of bioassays [12]. Based on observed effects, high-resolution non-target screening (NTS) chemical analyses can make attempts at identifying the cause of the effect in effect-directed analyses (EDAs) [13].

In Europe, the EU Water Framework Directive has implemented a *Watch-List* approach that supports the monitoring and assessment of emerging substances in the aquatic environment. Under the Common Implementation Strategy of the Directive, an activity on EBMs, including bioassays and biomarkers, has been carried out [12,14]. Recently, EBMs for the detection of estrogenicity have been proposed to be included in the new European Commission proposal directive as well as a new definition of Environmental Quality Standards based on effect-based trigger values (EBTs) [15,16]. A focus on early detection and action is also evident in the EU's one substance – one assessment strategy [17, 18].

As part of a comprehensive chemical risk management, the incorporation of Early Warning Systems (EWSs) emerges as a proactive solution. While EWSs are still at the stage of conceptual and technical development, existing methods for the early detection of potential environmental problems will be central elements of an EWS. In this review, we discuss the use of EBMs and their systematic integration into an EWS for hazardous chemicals. Specifically, this review i) addresses key scientific advancements in effect-based approaches in environmental monitoring, including important components such as sample preparation and EDA ii) lays the groundwork for EBMs for the early warning of hazardous chemicals and iii) discusses future perspectives for the application of an effect-based EWS. Emphasis is placed on environmental matrices, particularly aqueous ones, due to their common role as pollution vectors.

2. Purpose and concept of an early warning system (EWS)

The overall purpose of an EWS is the early detection of a potentially problematic chemical, raising an alert that can be followed up with minimum delay by more specific studies and/or regulatory actions. While EWSs have been widely applied for the detection of natural hazards, their application in chemical hazard and risk assessment is less common. The goal of a chemicals-based EWS is to introduce capabilities for promptly identifying emerging chemical threats, ideally in any environmental or human media without significant delays.

For that purpose, an EWS will likely encompass a range of assessment tools and methods designed to promptly detect emerging chemical threats (see Table 1). Its primary objective is to recognize harmful chemical substances and situations with the potential to cause adverse effects on human health or the environment at the earliest possible stage. An EWS should be designed to communicate the information to relevant authorities, ensuring that appropriate action can be taken as fast as possible [20]. In the EU, several activities exist on a national level aiming to detect hazardous chemicals, such as the expert-based SamTox in Sweden; however, the need for a common EWS at EU-level has recently been recognised and discussions on its scope are currently ongoing [21,22].

EWSs should be able to analyse and process signals from the field as inputs, potentially in real-time, such as instances or clusters of health effects that are linked to chemical exposure and/or contamination. Given that new hazards may be infrequent or emerge after prolonged latency periods [23], a close monitoring strategy in space and time will be needed for signal detection. Furthermore, confirmation of a certain detected signal reduces the risks of false positives and might indicate a more serious issue spread over space and/or time.

Fig. 1 illustrates a potential framework of an EWS for chemicals and delineates its vital functions, based on discussions at EU-level [24]. An EWS begins with representative and correct sampling and sample preparation steps, as further discussed in Section 3, followed by the detection of signals, which could be the occurrence of a chemical, or an adverse effect. While literature data is one source of signals, with the strength of quality assurance through a peer-review process, the publication of scientific data is arguably a protracted process. Therefore, real-time monitoring and screening programs are important sources for an EWS. Following signal acquisition, these signals are assessed for their originality, verifying that they meet the purpose of being an early warning. As a strengthening measure of the signal assessment, the third stage of the proposed EWS gathers information related to the signal's risks, potentially followed by an assessment and prioritisation of these risks. As an output of the process, the signals are eventually communicated to the final user enabling mitigation measures. A feedback loop ensures that the EWS stays updated with the newly identified contaminants along with the associated actions.

EBMs hold significant promise for both signal detection and strengthening, by identifying the combined effects of all known and unknown chemicals in a sample, going beyond the limitations of chemical analysis alone [25]. Recent advancements in EBMs have focused on the use of bioanalytical tools that assess the response of biological entities, either *in vivo* using whole organisms, or *in vitro* with cellular bioassays, targeting ecologically relevant endpoints in well-plate formats. EDA takes the capabilities of bioanalytical tools one step further, by combining bioassays with fractionation and chemical analysis, allowing for the detection of a wide range of potentially toxic compounds in a non-discriminative manner [26]. As of today, EBMs and EDA have mainly found applications in research, with limited adoption by the water sector and regulatory bodies. As summarized in Table 2, EBMs and/or EDA have been applied to water, soil and sediment, with a few publications discussing the integration of EBMs in contaminant water monitoring. Integrating such techniques into monitoring strategies, particularly for water quality, offers information for use in an effective EWS (Table 2). So far, contaminant-related EWSs have mainly been described for specific cases or pollutants such as heavy metals or endocrine disrupting compounds whereas state-of-the-art analytical techniques such as EBMs, EDA and NTS will enable a more comprehensive approach to early warnings of potential new pollutants.

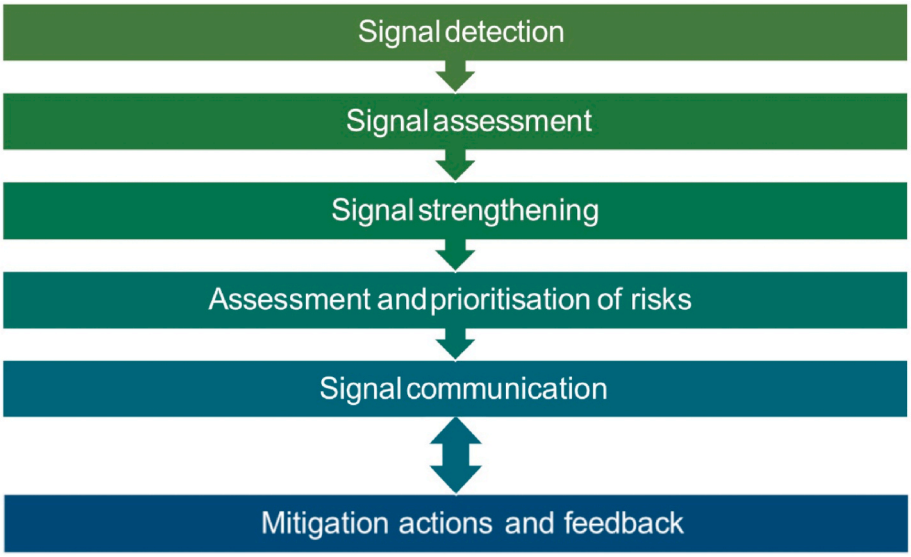

Fig. 1. Key stages of the proposed early warning system (EWS) for the mitigation of hazardous chemicals.

Table 1
Key terms used in this review.

Term	Common abbreviation	Definition
Adverse outcome pathway	AOP	A sequence of events linking perturbation at the molecular level to an adverse outcome at an individual or population level
Contaminants of emerging concern	CEC	Chemicals that are potentially hazardous but typically not yet restricted or regulated
Early warning system	EWS	An integrated system of tools for identification of a problematic chemical, that enables timely action to reduce associated risks
Effect-based methods	EBMs	High-throughput *in vitro* bioassays (primarily mammalian cell models) and well plate-based *in vivo* assays (small organisms) for effective environmental quality assessment [19]
Effect-based trigger value	EBT	Thresholds of chemical concentrations, above which adverse responses are expected in organisms
Effect-directed analysis	EDA	A systematic technique to identify the causative agents behind biological responses by fractionating complex chemical mixtures
Mode of action	MoA	Biological responses that a chemical can induce when interacting with living organisms
Non-target screening	NTS	Analytical technique used to detect and identify a wide range of chemical compounds in a sample using high-resolution mass spectrometry [9]. Suspect screening is a subset of NTS that links molecular structures with chemical databases to identify possible matches. In this review, NTS encompasses both suspect and non-target screening
Relative potency value	REP	A measure of the potency of a substance to produce a biological response compared to a reference sample

Table 2
Examples of effect-based approaches and EWS-related initiatives for various environmental matrices.

Matrix	Effect-based approaches	Iniatives related to early warning systems (EWSs)
Surface water	A European-wide demonstration program was carried out for effect-based monitoring of micropollutants in surface waters [27] US Great Lake monitoring [28] US surface water screening [29] A recent review report on EDA in surface waters [30]	EWS for hydrological hazards in European surface waters [31] Harmful algae hazardous effects monitoring [32] EWS for estrogenic effects in surface water [15]
Drinking water	EDA has been utilised to investigate tap water along the Yangtze River [33]	Application of EWS for heavy metals [34] EWS applied for heavy metal pollution accident in drinking water source [35]
Soil	Bioassays conducted for soils affected by mining [36]	Early warning technologies for crop pest monitoring [37] EWS for cadmium in rice production [38]
Sediment	Seasonal assessment of EDA in riverbank samples [39] Aryl hydrocarbon receptor (AhR) agonists in sediments [40] Application of EDA to determine estrogens in electronic waste area [41]	EWS for trace metals in karst aquifers [42] Biological EWS based on bivalve monitoring [43]

3. Sample acquisition and preparation for an effect-based EWS

A robust EWS for hazardous chemicals starts with the accurate and reliable detection of relevant signals (Fig. 1), which strongly depends on representative samples that have not been compromised during the sampling and preparation steps, for example through contamination or compound losses. The sampling acquisition and preparation steps are recognised as one of the limiting factors for the detection of low contaminant levels [44]. The importance of these steps is particularly emphasized in EBMs, where chemical enrichment becomes crucial, aiming to increase concentrations to detectable levels while minimising potential interference from the matrices under consideration [45]. In drinking water samples, an enrichment up to 100 times might be needed for detectable effects [46,47]. Another important point is to ensure sample representativeness and integrity. While this is important for any sampling procedure, unspecific responses in the context of an effect-based EWS might increase challenges to identify and exclude sampling artefacts. Quality assurance/control measures are essential to integrate into sampling strategies, including field blanks (in addition to

laboratory blanks) that reflect the sampling procedure.

3.1. Sampling techniques

With regard to water samples, sampling methods can be categorised broadly into grab (discrete) and passive sampling. Grab sampling techniques are common for water samples, but might encounter limitations in sample representativeness due to fluctuations in water concentrations [48]. Furthermore, large volumes might be needed to concentrate toxic chemicals to effect-relevant levels. Passive sampling techniques can overcome some of these limitations due to their capacity to concentrate large volumes over extended periods, resulting in lower detection limits [49]. These devices consist of a sorptive medium that is deployed in the monitored matrix (e.g., water, sediment), allowing the analytes of interest to diffuse into the sorbent. Analyte concentrations are subsequently calculated on a time-weighted average basis. Thus, passive sampling methods are generally not designed to capture sudden peaks in contaminant levels, which might be a disadvantage for their use for early warnings. However, individual samples will have to be collected with a high temporal resolution to ensure that contamination peaks are captured, which is a significant consideration in the context of EWSs. Grab samples can also be planned after events that could trigger contaminant peaks, such as precipitation-induced contamination or chemical spills.

In their integration over time, passive samplers first take up chemicals in a linear way, then entering a curvilinear phase and eventually reaching equilibrium between sampler and sampling medium [50]. These uptake phases are sampler- and compound-specific and depend on environmental conditions, such as temperature. Thus, the calibration of a passive sampler is not straightforward and requires equilibrium partitioning coefficients and/or uptake rates [51]. While no absolute concentrations are necessary for subsequent use of extracts in EBMs, it should still be noted that a passive sampler does not collect a substance beyond its equilibrium with the surrounding environment.

As detailed by Booij et al. [52], passive sampling allows measurements of the concentrations of freely dissolved compounds, whereas grab sampling yields total concentrations (if particles are included) or total dissolved concentrations (freely dissolved and colloidally bound). The freely dissolved part of a chemical is directly linked to bioavailability, i.e., the uptake by and reaction with biological systems, as tested in EBMs. Establishing a link between the bioavailable fraction of a chemical and its effect in a bioassay is a strong advantage of passive sampling over other sampling strategies. Based on these principles, several studies have connected passive sampling with effect-based monitoring [53–55].

Sorptive capacities of passive samplers vary according to the physical-chemical properties of the compounds, resulting in different materials used for e.g., hydrophobic and hydrophilic compounds as well as molecules with specific physical-chemical properties such as PFAS [51,56,57]. For the use in EBMs for EWSs, it is important to note that a passive sampler phase typically only covers a certain part of the chemical domain, and several passive sampler materials should be used in combination. Furthermore, extracting sorbed compounds from a passive sampler may lead to the co-extraction of sorbent material, which has been shown for silicone extractions [58] Potentially toxic matrix components need to be removed to avoid effects in the bioassay. Material blanks are essential, to be able to distinguish a positive signal in the sample from an effect potentially caused by the passive sampling material and blank contamination.

For solid samples such as soil and sediment, composite samples improve spatial or temporal coverage of an area without increasing sample number [59], but face the same issue of integrating over potentially varying concentrations. For outdoor and indoor air sampling, both active and passive air sampling methods are common [60]. Active air samplers can provide separate gas- and particle-phase samples from a clearly defined sampling volume, whereas passive air samplers generate time-integrated data. While they have advantages due to their simplicity and low cost, they face the same limitations as described above. In particular, the frequently used polyurethane foam might contribute a background to the extract that could interfere with EBMs [61]. However, air samples collected with polyurethane passive samplers have been successfully used in toxicity testing [62], although not with a specific early warning purpose. Overall, a combination of methods and materials may be necessary for accurate contaminant signal detection without overlooking short-term changes. In cases where diurnal variations must be monitored, composite samples may also be necessary.

3.2. Sample preparation

Following sampling, the subsequent steps typically involve stable sample transport and storage, homogenisation of the samples, extraction of the compounds, clean-up, and concentration [63]. The preparation of samples intended for bioassay analysis comes with certain particularities different from those for chemical analysis. Addition of surrogate standards should be avoided as they can cause false positives in bioassays. To avoid degradation of contaminants of interest, sample preservation might be necessary, which can be achieved by storage at low temperatures (<-20°C), or pH adjustment to 3 or lower during sample storage.

The appropriate method for sample preparation primarily depends on the type of matrix, the properties of the compound (e.g., polarity, volatility, solubility) and the expected concentration levels [59,64]. Common methods for preparation of aqueous samples include solid phase extraction (SPE), which utilises a sorbent phase to selectively retain contaminants, and liquid-liquid extraction, which partitions contaminants using two immiscible liquids. There is a pressing need for the development and optimisation of automated SPE devices designed for the extraction of large-volume water samples [59,65], which is especially relevant in the context of early warning. Recent advancements allow for the extraction of large volume water samples on-site, utilizing mobile SPE stations [66], which can minimise the issues associated with sample transport [67–69]. Other common techniques involve solid phase microextraction (SPME), which uses a fibre coated with sorbent films, and thin-film SPME, utilised for organic compounds with low volatility [65].

There is a trade-off between the concentration of samples and matrix interference. Such issues can be addressed by applying thorough clean-up steps aiming to eliminate interfering substances [70]. However, it is important to recognize that the clean-up process itself will change the composition of the samples, with risks of false negatives. Every step of sample pre-treatment also increases the risks of contamination and thus false positives should be monitored closely through procedural blanks [71].

Method validation for an effect-based EWS should encompass analytical and toxicity recovery [45], as outlined in Fig. 2, and thus address the risk of false negatives. During this process, it is advisable to initially identify a set of candidate analytes as positive controls. These should not only represent various compound groups pertinent to the matrix but also exhibit known activity in one or more of the selected bioassays. The solution should be spiked to the matrix or reference material before extraction, followed by the extraction and clean-up chosen for this effect-based approach. Ideally, the compounds of this solution should be fully recovered in terms of their concentration (target analysis), presence (non-target screening) and toxicity (bioassay testing) (Fig. 2). If fractionation is intended, the sum of the fractions should match the initially added compounds, amounts and toxicity, respectively. The procedure should ensure that biological effects are not caused by the solvent, i.e., negative controls need to be included as well. The fractionation and chemical analyses relate to EDA that will be further addressed in Section 5.

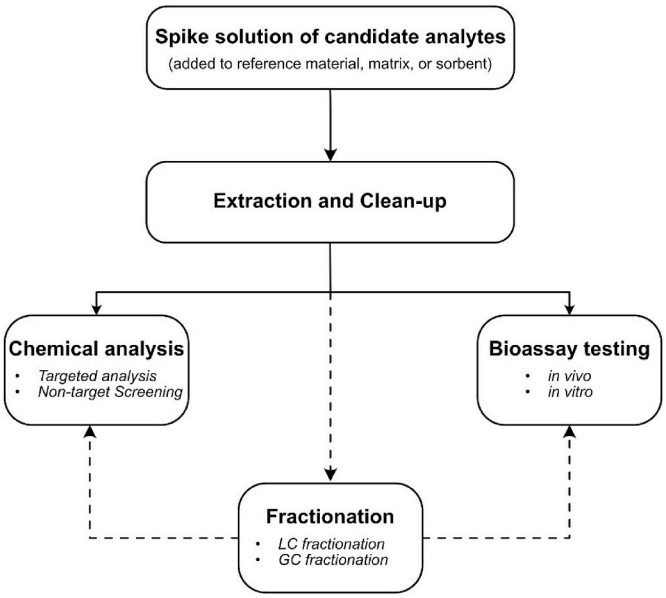

Fig. 2. Proposed elements of a method validation procedure for sample preparation in the context of effect-based methods (EBMs) and effect-directed analysis (EDA).

4. Effect-based monitoring for EWSs

Bioassays are designed to measure the potency or biological activity of a substance or a mixture of substances by evaluating its effects on an organism, tissue, or cells, thus facilitating toxicity profiling. Quantal bioassays assess binary responses, such as cell death or survival, while graded bioassays measure a spectrum of responses, such as growth inhibition. Moreover, bioassays differ in their mode of action (MoA), including enzyme, receptor and cytotoxicity assays. While a large variety of bioassays exist for various MoAs, some examples that can be relevant in the context of an EWS are presented in Section 4.1 and Table 3. Concepts developed for toxicity identification evaluation in the USA are often directed at whole organism endpoints in the toxicity tests [72].

Bioassays can also vary based on the level of biological organisation, including *in vitro* bioassays, which are conducted in controlled settings using isolated biological components like cells and enzymes, and *in vivo* bioassays, which involve whole living organs or organisms [73]. While *in vivo* bioassays were historically viewed as more biologically relevant, there has been a paradigm shift towards *in vitro* testing in recent years, due to stricter regulations, as well as recent technological advancements bolstering their reliability [12]. Additionally, methods are being developed to extrapolate *in vivo* ecotoxicological data through *in vitro* testing [74]. One such method is the use of adverse outcome pathways, which provide a framework for understanding the linkage between initial molecular events and adverse outcomes on ecosystems [75–77]. Lastly, *in vitro* assays can exhibit higher throughput, rapid response times, and cost-effectiveness [78].

A key technique in EBMs is the reporter gene assay, where genetically modified cells express a detectable reporter protein (e.g., luciferase) regulated by a specific biological process [79]. For instance, if a reporter gene assay targets androgen receptor activity, a responsive DNA sequence is introduced upstream of the gene for a signalling protein. This DNA, typically in plasmid form, is added to cultured cells exposed to environmental samples or compounds of interest. If the sample contains compounds activating the receptor, a complex forms, binding to the DNA sequence and inducing signalling protein expression. Measurement of the signalling protein production, using luminescence or fluorescence, provides a proportional quantitative indication of the overall activity toward the analysed endpoint, reflecting the impact of all compounds present.

In the context of EWSs, (ultra)high-throughput screening of bioassays is crucial, because timely detection is required and due to the necessity to scrutinize large numbers of samples and complex chemical mixtures. High throughput has traditionally been achieved through optical detection techniques, while more recent efforts have focused on automation [80]. There can be a trade-off between throughput and biological relevance of the bioassays, however, recent advancements have helped overcome this.

Various metrics for use in EWSs and prioritisation can be derived from EBMs, such as the effect concentration, which represents the concentration necessary to induce a specific biological effect. From effect concentrations, bioanalytical equivalent concentrations (BEQs) can be derived and compared with concentrations of identified compounds [81]. Bioassays are also crucial for the determination of EBTs, which can help identify acceptable bioassay responses, enabling their use in regulation.

4.1. Bioassays based on MoAs

4.1.1. Endocrine disruption

Endocrine disrupting chemicals include substances that are capable of interfering with hormonal systems, leading to various adverse health effects, such as alterations in sperm quality/quantity, immune system function, growth, cardiovascular problems, increased cancer risk, and more [82]. Endocrine disruptors can induce adverse effects at very low concentrations; therefore, chemical analysis often fails to account for potential risks when concentration levels are below chemical detection limits. However, many effect-based methods for endocrine disruptive effects have much higher sensitivity and can thereby quantify e.g. estrogenic effects at environmentally relevant concentrations, far below the chemical detection limit. While our understanding of the specific mechanisms disrupting these biochemical processes is limited, it is recognised that endocrine disrupting chemicals can operate through various MoAs and interact with different receptors [83]. These include stimulation or inhibition of:

i) Endogenous hormone biosynthesis or degradation
ii) Endogenous receptor expression or degradation
iii) Hormone-receptor binding
iv) Hormone-signalling pathways
v) Binding to circulating hormone-binding proteins

There is a large variety of assays that can be used to monitor endocrine disruption and capture the complexity of their MoAs. Table 3 provides an overview of key biological targets for bioassay testing, focusing specifically on endocrine disruptors within an EWS. A non-exhaustive list of chemicals that can reportedly interact with these receptors is presented. The table shows that a wide variety of chemicals can be detected with these bioassays, including bisphenol A and bisphenol derivatives, POPs, personal care products, pesticides, polycyclic aromatic hydrocarbons (PAHs) etc. Some assays are rather specific, e.g. PCBs binding to the constitutive androstane receptor (CAR), while others respond to multiple chemicals. Bioassay testing has traditionally focused on estrogens, androgens, and thyroid agonists and antagonists, however, in recent years, peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptors have gained increasing attention, because of their crucial roles in regulating metabolism and homeostasis.

4.1.2. Mutagenicity/Genotoxicity

Chemicals exhibiting mutagenic and/or genotoxic MoAs are often deemed more severe than any other potential adverse effects, due to the potential risk to human health. When evaluating the capacity of chemical substances to interact with genetic material, it is crucial to establish the various outcomes this contact can induce. Exposure to substances

Table 3
Key biological targets for bioassay testing of endocrine disruptors in an early warning system (EWS).

Receptor	Function	Endogenous ligands	Target genes and functions	Chemicals that may induce a positive response	Detection method(s)	References
Estrogen receptor (ER)	Female sexual development	Estradiol, estriol, and estretrol	Oxytocin, Vitellogenin	Bisphenols (e.g. bisphenol A (BPA), tetrachlorobisphenol A (TCBPA), tetrabromobisphenol A (TBBPA)), parabens, phthalates, benzophenone derivative, dioxins, polyaromatic hydrocarbons (PAHs), per- and polyfluoroalkyl substances (PFAS)	ER assays, lyticase yeast estrogenic screen, ERα-CALUX, MELN cells, planar yeast estrogenic screen, VM7Luc4E2	[84–88]
Progesterone receptor (PR)	Female sexual development	Progesterone	Maintaining the menstrual cycle and uterus lining during pregnancy, bone formation as well as being a neurosteroid in the brain	Musk compounds, BPA, herbicides, insecticides, fungicides	Autobioluminescent yeast bioassays (e.g., BLYrPRS)	[89]
Androgen receptor (AR)	Male sexual development, anabolic steroid	Testosterone, dihydrotestosterone (DHT)	Anabolic metabolism, cell proliferation, cell migration, production of seminal fluid	Dioxins, polychlorinated biphenyls (PCBs), phthalates, BPA	Yeast androgen bioassays, cell proliferation assays, receptor binding, reporter gene	[90–92]
Aryl hydrocarbon receptor (AhR)	Xenobiotic metabolism, cellular differentiation, stem cell maintenance, immune responses, neurogenesis, circadian rhythm	5α-THB and 5β-THB	Cytochrome P450s (CYPs)	PCBs, dioxins, BPA, PAHs, pesticides	DR-CALUX, H4IIE-luc cells	[93–97]
Thyroid hormone (TR)	Metabolism, heart rate	Thyroid hormones (THs)	CYPs as well as various transcription factors and regulatory proteins	PCBs, BPA, dioxins, furans, polybrominated diphenyl ethers (PBDEs) and other flame retardants, phthalates pesticides, perchlorates, phytoestrogens, PFAS	TTR-TRβ, CALUX	[98]
Glucocorticoid receptor (GR)	Metabolism, water and electrolyte balance, immune response, normal bone development, maintenance of the cardiovascular system, stress response, reproduction	Cortisol	Anti-inflammatory, metabolic, stress response, developmental (e.g. Klf9, Zbtb16)	Bisphenols, organotins, metabolites of certain organochlorine pesticides, phthalates	Reporter gene, receptor binding, flow cytometry, imaging assays	[99,100]
Peroxisome proliferator-activated receptors (PPARs) with Retinoid X receptors (RXRs)	Lipid, fatty acid and cholesterol metabolism	Lipids (PPARs) Retinoic acid (RXR)	Cytochrome P450s (CYPs), glutathione S-transferase (GST)	BPA, organotins, phthalates	DPI-ELISA (DNA–Protein-Interaction Enzyme-Linked Immunosorbent Assay), Luciferase reporter gene, receptor binding	[101,102, 102]
Pregnane X receptor (PXR)	Xenobiotic and pharmaceutical metabolism	5 β-cholestane-3α, 7α, 12α-triol steroids	Cytochrome P450s (CYPs)	Pharmaceuticals (e.g. antibiotics, sedatives, antineoplastics), BPA, PCBs	Reporter gene (e.g., luciferase, β-galactosidase, receptor binding	[103]
Constitutive androstane receptor (CAR)	Xenobiotic detoxification	Androstane and derivatives	CYP2B, CYP3A, CYP2Cs, OATP2, MRP2, UGT1A1	PCBs	Reporter gene, receptor binding, transactivation assays	[104,105]

that damage DNA can lead to genetic instabilities and/or changes in gene expression patterns, which in turn can result in various disorders, such as cancer [106]. Mutagenicity refers to persistent and heritable alterations in the quantity and composition of genetic material. Genotoxicity can encompass mutagenicity; however, not all genotoxic substances are mutagenic, as exposure to them may not induce changes in the genetic material.

The assessment of genotoxicity is a key component of the evaluation of surface water quality. Numerous EBMs permit the evaluation of genotoxicity. Several priority substances of the EU Water Framework Directive have mutagenicity or genotoxicity properties (e.g. PAHs, benzene). Annex VIII of the directive identifies compounds "that possess carcinogenic or mutagenic properties" as among the main pollutants. In vitro EBMs for the detection of mutagenic and clastogenic potentials are used under REACH (Council Regulation (EC) No 440/2008).

Several EBMs can be used to assess genotoxicity in the presence or absence of an external metabolic activation, e.g. by the use of S9-mix in the Ames or micronucleus tests, Comet assay, P53 assay, SOS-umu test, SOS-chromo test and others. No single test can detect all genotoxic mechanisms. Therefore, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use recommends using a combination of mutagenicity tests, known as a battery of tests. Examples of tests that can be used for mutagenicity and genotoxicity tests in the context of EWSs, are presented in the following. The selection of tests can vary depending on the aim of the study or monitoring programme. The typical format of a standard test battery involves:

i. A bacterial reverse gene mutation test (e.g., Ames test), to identify potential genetic changes and detect genotoxic substances
ii. Assessment of genotoxicity in mammalian cells *in vivo/in vitro*

The Ames test, also known as the "reversion assay," is a common method for assessing chemical compounds' mutagenic potential using specific bacteria strains. In the early 1970s, this simple and fast bioassay used Salmonella typhimurium/E. coli strains with histidine operon mutations [107,108]. These strains, termed auxotropic mutants, lack the ability to synthesize histidine and thus cannot grow on histidine-deficient agar; however, exposure to mutagenic compounds or spontaneous mutations can restore histidine synthesis and colony formation. For the Ames tests several strains can be used to detect different types of mutations. The Ames test and the Ames Fluctuation Test are standardised according to International Standardisation Organisation (ISO) (ISO 16240:2005, ISO 11350:2012)

The Comet assay is a sensitive and economical technique for checking DNA strand fractures in eukaryotic cells. The process entails embedding cells within agarose gel and subsequently lysing them with detergents and salt. The comet tail, an elongated representation of the cell, is generated when fragmented DNA migrates more rapidly through an agarose matrix under the influence of an electrical current [109]. This technique is employed in human monitoring studies and ecotoxicological studies in various sentinel organisms [110–114].

Chromosome aberration tests can be used to identify potential risks associated with mutagenic xenobiotics [115]. These involve mammalian cells (e.g., human peripheral blood lymphocyte cultures) exposed to suspected genotoxic compounds in the presence or absence of S9 mix. Exposure to mutagenic xenobiotics can cause DNA damage, including double-strand breaks and structural chromosome aberrations.

The micronucleus assay is a method designed to investigate genotoxicity by detecting micronuclei in the cytoplasm of cells during the development phase and it is useful for the evaluation of chromosome structure. It has been included in the Organisation for Economic Co-operation and Development (OECD) guidelines for chemical testing [116]. The micronucleus assay is standardised according to ISO (ISO 21427–1:2006, parts 1 and 2).

In general, genotoxicity tests can be used to assess the combined action of potentially hazardous compounds present in surface waters in complex mixtures highlighting synergistic, additive and antagonist effects at sub-lethal concentrations [107]. EBTs could be unnecessary because the qualitative detection of mutagenicity in a waterbody is a signal (Yes/No) of status. Therefore, the establishment of a threshold is of limited practical utility after definition of a suitable enrichment procedure [14].

Overall, it is recommended to employ a test battery comprising the Ames test, Comet assay, and micronucleous assay, to comprehensively assess genotoxicity in EWSs for emerging and traditional chemicals, including mixture effects.

4.1.3. Neurotoxicity

Neurotoxicity refers to the ability of chemical, biological, or physical agents, to induce adverse functional or structural alterations in the nervous system. Developmental neurotoxicity is particularly concerned with the effects of toxicants on the developing nervous system of organisms. The developing brain and nervous system is more sensitive to toxic effects than the mature brain and nervous system. The number of neuroactive compounds released into the ecosystems has increased over the past few years, and there is, therefore, a growing interest in assessing the related potential risks for environmental and human health [117]. A literature study comparing 30 different MoAs estimated that neuroactive compounds formed the largest category (13 %) [118]. Moreover, the neuroactive compounds in ecosystems can be present at low levels, and can also form synergistic mixtures with unknown effects.

The selection of *in vitro* assays for neurotoxicity could be guided by adverse outcome pathways relevant for eco-neurotoxicity [14]. The assessment of (developmental) neurotoxicity is possible on non-mammalian cells since the mechanisms underlying the development and function of the nervous system are well conserved across the phylogenic tree. Many biochemical processes are identical in mammals and in non-mammalian species. For example, the zebrafish *(Danio rerio)* represents a suitable model for chemical testing, owing to its small size, embryonic transparency, and rapid development. Integrating data (*in vitro* and *in vivo*) from diverse sources, can provide a comprehensive understanding of neurotoxic effects of chemical pollutants, both in terms of ecological and human health [119]. Behavioural analyses, such as the study of locomotion, have been proposed as critical in detecting potential neuroactive effects [120]. Furthermore, the spontaneous tail coiling in zebrafish embryos might represent another important endpoint in neurotoxicity assessment [121]. This method allows a very quick screening since it can be performed within 24 h.

The main neurotoxicity endpoints for zebrafish that can be used in EWSs are:

i. Locomotor activity. Monitoring swimming behaviour in zebrafish is key for neurotoxicity detection, with locomotion responses comparable to mammals [122]. Selderslaghs et al. [123] elaborated a new method for locomotor analysis, with an assessment at 96, 120, 144, 168 and 192 h post fertilisation using a camera with behavioural tracking software.
ii. Acetylcholinesterase. Screening zebrafish for neurotoxicity induced by chemicals on the cholinergic system is common, and is linked with several cognitive functions and processes [124].
iii. Lateral tail movements. These are the first spontaneous behaviours observed in zebrafish embryos. The tail coiling test consists of the evaluation of spontaneous tail coiling frequency in zebrafish embryos, as a possible indicator of neurotoxic compounds [125].

4.2. Quality control of bioassays

Quality assurance and control considerations are crucial to reduce the risk of false positives or negatives in EBMs and thus ensure the accuracy and reliability of the results (Fig. 2). Bioassays can exhibit variability between tests, which can be mitigated by incorporating a biological reference material or a chemical standard with known activity. This can help with calibration of bioassays and improve their comparability. Evaluation of accuracy, precision, sensitivity, and robustness is essential during method validation, along with assessing potential matrix effects and technical considerations [126]. The overall quality can be assessed by a z-factor developed for bioassays, based on high and low level reference materials [127]. The OECD and the ISO provide test guidelines for commonly used bioassays (e.g., estrogenic effects, androgenic effects, genotoxicity, acute toxicity in fish cells) to ensure method consistency and reliability.

5. Effect-directed analysis (EDA)

5.1. Principle of EDA

Introduced in the early 1980s, EDA aims to identify drivers of toxicity in environmental samples, employing a fractionation strategy [70,128]. Utilizing NTS approaches, EDA is a powerful tool for characterising known toxicants and identifying unknown chemicals in complex environmental mixtures [129]. While EBMs contribute to an EWS through toxicity alerts, EDA takes this signal a step further by attempting to identify the specific toxicant, which can be essential for risk assessors and regulators. Recent progress in high-resolution fractionation methods coupled with NTS has significantly enhanced the efficiency and effectiveness of EDA, reducing the time required for fractionation to a matter of seconds [130]. This development positions EDA comparably to EBMs in terms of time requirements, as both methodologies utilize

similar *in vitro* assays.

In the EWS context, EDA plays a crucial role in identifying toxic CECs through effect-based experiments, fractionation, and analytical chemistry tools, particularly high-resolution mass spectrometry [128]. In a typical EDA workflow, environmental extracts undergo testing for toxicological effects using one bioassay or a series of bioassays. The extract displaying effects is then fractionated based on physicochemical characteristics such as polarity, vapour pressure, and molecular size. Each fraction is individually tested for its ability to reproduce the observed effect using the same bioassays. The active fractions are selected for further analysis to characterize the responsible toxic compound(s) in NTS approaches. Finally, the presence of these chemicals is validated by comparing with analytical standards. Similar to EDA approaches, toxicity identification evaluation systems have been applied, mainly outside of Europe. The concept is essentially the same, but toxicity tests include several phases and endpoints have mainly been the survival, growth, and reproduction of whole organisms [72].

Over the last forty years, a variety of EDA methods have been developed and applied to identify the key contributors to toxicity across a range of environmental media, including sediments, soils, sludge, air particulate matter, effluents, surface water, and groundwater [45,59]. However, the major challenge in applying and implementing EDA lies in analysing biota samples, with limitations including selection of bioassays and toxicity tests, sample preparation and clean-up steps, selecting representative organs containing toxicants, identifying unknown chemicals, confirming identified chemicals, and the limitations as a routine monitoring tool [131].

5.2. Fractionation methods

Fractionating a mixture of chemicals is a key component in EDA, to reduce the complexity of the samples and assess chemical mixture effects [13,45]. Fractionation should avoid any losses of relevant compounds (causing false negatives) as well as contamination (causing false positives), while achieving a stepwise separation based on physicochemical properties of the response-inducing compounds.

Column chromatography is the most prevalent fractionation technique, with liquid chromatography (LC) and gas chromatography (GC), targeting semipolar to polar and semipolar to nonpolar substances, respectively. Fractionation includes time-dependent fractions or signal-induced fractions using simultaneous UV-detection [13,132]. Recent advancements in fractionation allow for downscaling the process, improving possibilities of applying EDA in routine chemical screening [133]. Automated fractionation speeds up the process in EDA, combined with $\geq$96-well plates for replicable toxicity data [128]. The multi-well plate approach minimizes handling, solvent exchange, and evaporation steps, enhancing throughput and reducing risks of losses or contamination [134]. This method aligns with various *in vitro* and small-scale *in vivo* bioassays, including the zebrafish embryo toxicity assay.

Quality assurance measures to confirm that the fractionation process does not result in the loss of toxic components involve reconstituting fractions into a complete mixture; if there are no losses, the reconstituted sample should exhibit the same toxicity as the unfractionated one. However, it is worth noting during effect-based recovery evaluation that removing matrix or compounds may increase overall effects, as seen in mutagenicity and *in vitro* endocrine effects where the presence of antagonists may conceal an effect [135]. To enhance the accuracy of toxicity recovery evaluation, it is therefore advisable to complement it with analytical recovery assessment. This approach also helps identify compound groups and properties responsible for inadequate toxicity recovery.

5.3. Identification and confirmation of toxicants

Identifying toxicants in complex mixtures involves analytical techniques such as NTS. However, identifying compounds in a toxic fraction does not prove causation and confirmation steps are needed. These approaches include analytical confirmation, *in vitro* or *in vivo* effect confirmation, and hazard confirmation under realistic exposure conditions. The toxicity confirmation can follow a tiered approach; the first step focuses on confirming tentatively identified structures of toxicants by comparing mass spectra with standard compounds, and the second tier aims to confirm that the identified compounds are indeed causing the observed toxicity, for instance through correlation analysis, which investigates whether there is a consistent relationship between the concentration, or spiking tests, which involves spiking samples with additional amounts of the suspected toxicants and retesting [136].

A common challenge is the lack of analytical standards. Data sources provide varying levels of confirmation, from confirmed structures to tentative candidates with evidence for possible structures [45]. A set of suspect lists for high resolution mass spectrometry data evaluation and NTS guidance has been compiled under the umbrella of the NORMAN network [137,138]. In the context of EWSs, the need for signal validation needs to be balanced with the wish of an early alert that enables follow-up action.

5.4. Virtual EDA

Multivariate statistics, particularly partial least square analysis, can integrate chemical and toxicological data in EDA [139,140]. This method identifies co-varying chemical signals, acting as a "virtual fractionation" to pinpoint candidate chemicals explaining effects, even from unidentified sources. It enhances understanding beyond traditional methods and could be a valuable component in an EWS, but success may require many samples.

5.5. Iceberg modelling

Iceberg modelling describes comparisons between BEQs derived from bioassays (BEQ_{bio}) and chemical analysis (BEQ_{chem}) [141,142]. BEQ_{chem} is calculated from the measured concentration of a given compound in a sample and its relative effect potency [141], assuming additive effects of the chemicals sharing a common MoA. In this concept, BEQ_{bio} represents the total toxicity (the iceberg) whereas BEQ_{chem} only represents the visible part (the tip of the iceberg). For example, in a study on the influence of rain events on chemical pollution of rivers, 290 measured chemicals only explained 8 % of the total toxicity in the samples [143]. Thus, iceberg modelling provides a quantitative indication of how well toxicity-drivers can be identified. Samples with large differences between BEQ_{bio} and BEQ_{chem} (i.e. a small BEQ_{chem}/BEQ_{bio} ratio) could be prioritized for EDA and other follow-up action under an EWS.

6. Workflow of an effect-based EWS

A potential workflow of incorporating effect-based methods in an EWS for chemicals is presented in Fig. 3. After sampling the relevant matrices and conducting the appropriate sample preparation, the first step of the EWS is based on EBMs using bioassays, such as those detailed in Section 4. If the results are in compliance with EBTs or preliminary limit values associated to the specific bioassays, no further action is required. If the effect-based screening results exceed the EBTs or preliminary limit values, this can constitute an initial early warning signal, leading to further investigation for confirmation of the signal. This confirmation process will initially focus on strengthening the signal and will subsequently address the causality investigation, as also outlined in Fig. 1. Strengthening of the signal entails additional investigative actions, including existing data to assess the spatial and temporal occurrence as well as the intensity of the signal. It is important to assess whether this signal occurs at a local level or has broader implications, impacting the environment on a large scale. This investigation

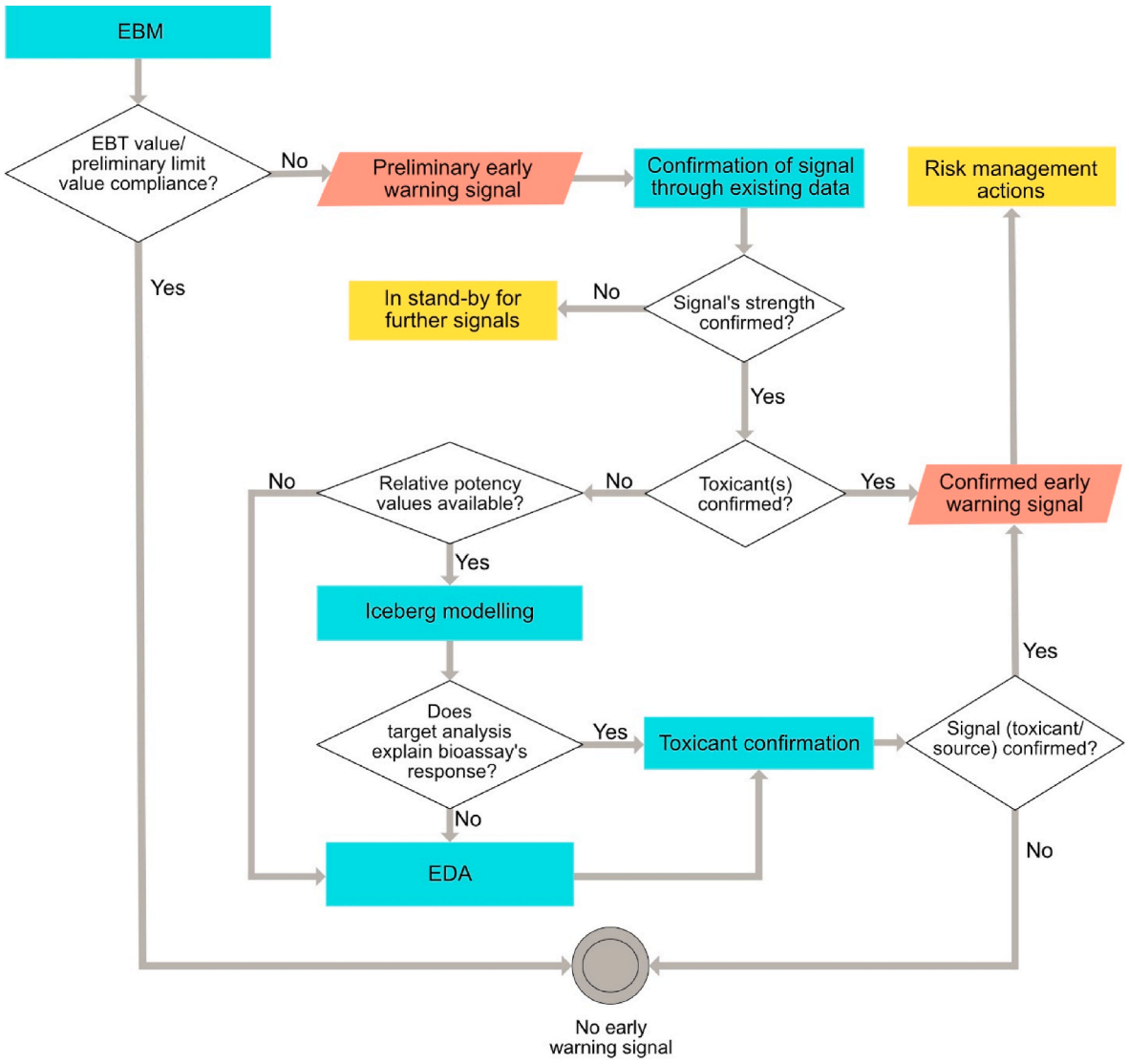

Fig. 3. The proposed workflow for an effect-based early warning system (EWS) for chemicals, after sampling and sample preparation. EBT: Effect-based trigger value. EDA: Effect-directed analysis.

necessitates retrieving as far as possible existing information concerning the pressures and activities within the area where the signal occurred. Such data can serve as additional evidence to reinforce the signal. Additionally, the generation of new monitoring data, while potentially time-consuming, may prove essential.

If the signal lacks sufficient support due to insufficient data or if the available data exhibit poor quality, the signal should be archived and placed on a waiting list until more robust evidence becomes available (Fig. 3). If the criteria for confirmation of the signal are met, the actions for toxicant discovery should take place to identify the contaminants responsible for the observed effects and the sources. Toxicant discovery can be conducted via iceberg modelling and EDA, as described in Section 5. If iceberg modelling cannot satisfactorily explain the observed effect, or if it is not possible to perform iceberg modelling, e.g. due to lack of relative potency values, EDA can be used to confirm the causes of the bioassay effects, through identifying the toxicant structures and verifying their correlation (Fig. 3).

In some cases, it is possible to anticipate the contaminants most likely linked to the activity detected by the bioassay. When such predictions are feasible, it becomes possible to initiate management actions without need for final proof of specific toxic driver(s) from the chemical analysis. It is important in EWSs to expedite the process and enable action despite time-consuming steps for chemical analysis and identification of compounds. Finally, in the case of a confirmed early warning signal through this process, the signal can be communicated to the relevant policy makers, enabling appropriate risk management actions (Fig. 3). These can involve regulatory measures, environmental remediation actions, enhanced monitoring efforts, and public health interventions, for example by conducting population exposure screenings.

7. Concluding remarks and future perspectives

The development of an EWS for hazardous chemicals in the environment represents a significant leap towards proactive environmental risk management. Although discussions regarding the design and implementation of such an EWS are still at an early stage, the vision of an effect-based EWS is increasingly attainable. This review has highlighted recent advancements in effect-based methods and EDA, facilitating their integration within an EWS framework.

The proposed workflow is an initial step toward an EWS for chemicals. Several limitations must be addressed to optimise the system's effectiveness. One primary challenge is the complexity of integrating biological and chemical data which can be labour-intensive and technically demanding. Innovative techniques such as NTS and EDA need

more harmonisation to ensure comparability and reproducibility. Implementing these techniques on a routine basis requires significant investment in technology, personnel, and training.

Timeliness is another concern, as delays in data collection, analysis, and response can undermine the primary goal of early detection. The optimisation of real-time technologies (e.g., auto-sampling or chemical sensors) and the enhancement of high-throughput EDA are expected to facilitate improvements in this direction. Ensuring the sensitivity and specificity of bioassays is crucial to avoid false positives or negatives. Multiplexed bioassays can hold significant promise in detecting multiple analytes in a single test. EBTs should also be carefully calibrated to avoid setting thresholds that are too high or too low.

Despite these challenges, the future of an effect-based EWS for hazardous chemicals holds considerable promise. Continuous advancements in bioanalytical tools and sampling techniques will likely enhance the system's capabilities. Future research is expected to improve data integration methods, enhancing the sensitivity and specificity of bioassays, and developing more robust methodologies. New approach methodologies (NAMs) are expected to expand the toolbox of EBMs, although their standardisation and validation should be improved.

The long-term sustainability of an EWS will depend on adapting to technological advancements while ensuring robustness. In EDA, ongoing research aims to enhance throughput through miniaturisation of bioassays and the application of multidimensional fractionation tools (GCxGC, LCxLC). Improved workflows for mass spectral data analysis are also expected to increase the rate of successful chemical identifications. Additionally, more comprehensive studies on the bioavailability and effects of chemical mixtures will provide deeper insights, leading to better-informed decision-making. While this review focuses on environmental samples, the potential for an effect-based EWS can be expanded to other matrices, such as products, food and human samples. However, more research is needed to validate EBMs and EDA in other matrices.

It is important to note that the success of an EWS depends on regulatory and policy alignment. Ensuring that the system is connected to existing policy frameworks and that regulatory bodies can respond promptly is essential. Constant technological investment accompanied with training and education is required, to ensure state-of-the-art technologies and improve the early detectability of problematic chemicals, for example through real-time monitoring and high-throughput analysis. Data integration should be implemented robustly, creating platforms that can handle the complex datasets generated by EBMs, NTS, and EDA. Developments in computational tools including machine learning will offer possibilities for further automation and efficiency in signal identification and the related processes (Fig. 1). Feedback from practical applications should be used in a systematic way to improve the EWS. Case studies testing the conceptual workflow of the effect-based EWSs in real-world applications will help evaluate the benefits and limitations, and apply modifications to the suggested workflow. These steps can form the basis for future guidelines for implementation of an effect-based EWS, aligned with the goal of proactive environmental and public health protection.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

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