REVIEW

Tracking complex mixtures of chemicals in our changing environment

Beate I. Escher[1,2]*, Heather M. Stapleton[3], Emma L. Schymanski[4]

Chemicals have improved our quality of life, but the resulting environmental pollution has the potential to cause detrimental effects on humans and the environment. People and biota are chronically exposed to thousands of chemicals from various environmental sources through multiple pathways. Environmental chemists and toxicologists have moved beyond detecting and quantifying single chemicals to characterizing complex mixtures of chemicals in indoor and outdoor environments and biological matrices. We highlight analytical and bioanalytical approaches to isolating, characterizing, and tracking groups of chemicals of concern in complex matrices. Techniques that combine chemical analysis and bioassays have the potential to facilitate the identification of mixtures of chemicals that pose a combined risk.

Chemicals are the basis of life, but some anthropogenic organic chemicals pose inherent dangers. Pesticides, industrial chemicals, pharmaceuticals, and other synthetic chemicals can enter the environment and the food chain, causing unwanted effects and disease. Medical research indicates that as much as two-thirds of chronic human disease risk cannot be explained by genetics alone and may result from the environment or gene-environment interactions (*1*). Furthermore, the *Lancet* Commission on Pollution and Health has estimated that 16% of global premature deaths are linked to pollution (*2*). These statistics highlight the need for research to elucidate the complex links among exposure to chemicals, environmental quality, and health.

Concentrations of many legacy chemicals are decreasing after national and international actions led to near-global phase-out of these chemicals (*3*). However, the number of new chemicals is rising, with the Chemical Abstract Service Registry growing from 20 million to 156 million chemicals between 2002 and 2019. Regulation of problematic chemicals can take decades; once enacted, such rules can lead to chemical substitutions that are less well characterized. There have been several cases in which the replacement chemical had properties, including toxicity, similar to those of the chemical it was intended to replace. Notable examples include plasticizers, flame retardants, chlorinated paraffins, and polyfluoroalkyl substances.

More recent industrial and agricultural chemicals, pharmaceuticals, and personal care products are not generally persistent, but they are ubiquitous as a result of their continuous use and global sources. When degraded, the resulting transformation products may be more persistent and may occur at higher concentrations than their parent compounds (*4*). Generally, degradation leads to transformation products that are more water-soluble and less toxic; however, some transformation products are more toxic than their parent (*5*).

The mixture challenge

Historically, chemical pollution was often attributed to a defined group of industrial chemicals. Today, awareness is increasing that we are exposed to a true cocktail of chemicals, only a fraction of which have been identified. There is no equity in the global distribution of these pollutants; developing countries may be at highest risk, given that large-scale production is moving to these countries and adding to their challenges in developing chemical regulations and infrastructure such as wastewater treatment (*2*). Awareness of the need to deal with complex chemical mixtures has increased with the introduction of the exposome concept, which integrates all human exposure from chemical and nonchemical stressors in relation to adverse health effects in humans (*6*) and can be expanded to any biota.

Chemicals can contribute to toxicity in a complex mixture even if they are present below their own effect threshold and/or analytical detection limit (*7*). Chemicals with the same modes of toxic action tend to follow the mixture concept of "concentration addition," whereas those with different modes of action act according to "independent action" (*8*). At low effect levels and low, environmentally realistic concentrations, concentration-effect curves are linear and the two models converge. Synergy is generally limited to mixtures with a small number of components at high concentrations (*9*) but becomes less relevant for low doses (*10*). Because synergy rarely leads to more than a factor of 10 increase in effect for any synergistic combination and only a few components will interact, it is of lower priority for environmental mixtures. It is safe to assume that most environmental mixtures with tens of thousands of chemicals of diverse modes of action at low concentrations will act according to the simple additive model, but the big unknown is the contribution of unidentified chemicals to the effect of environmental mixtures. Therefore, we discuss bioanalytical tools as a quantitative measure of mixture effects in monitoring studies.

Although this review focuses on anthropogenic organic chemicals, the relevance of mixtures is exacerbated by metals, inorganics, and particles (e.g., plastics, nanomaterials). Moreover, chemical mixtures can act jointly with multiple stressors caused by external factors such as oxygen levels, increasing temperature, and ocean acidification.

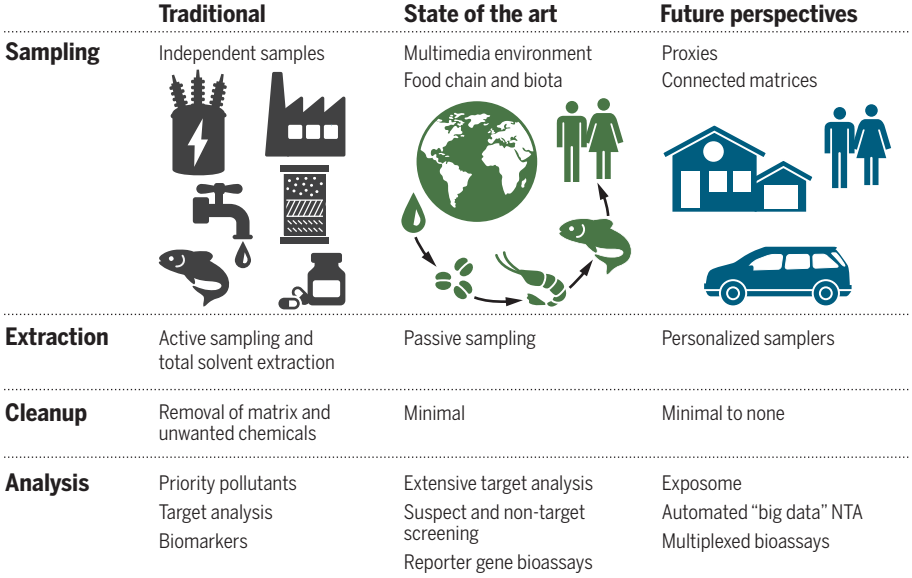

Fig. 1. Past, present, and future sampling and (bio)analysis strategies for complex environmental mixtures in the environment and in humans. NTA, non-target analysis.

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[1]Department of Cell Toxicology, Helmholtz Centre for Environmental Research–UFZ, DE-04318 Leipzig, Germany. [2]Environmental Toxicology, Center for Applied Geoscience, Eberhard Karls University Tübingen, DE-72074 Tübingen, Germany. [3]Nicholas School of the Environment, Duke University, Durham, NC 27708, USA. [4]Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, 4367 Belvaux, Luxembourg.
*Corresponding author. Email: beate.escher@ufz.de

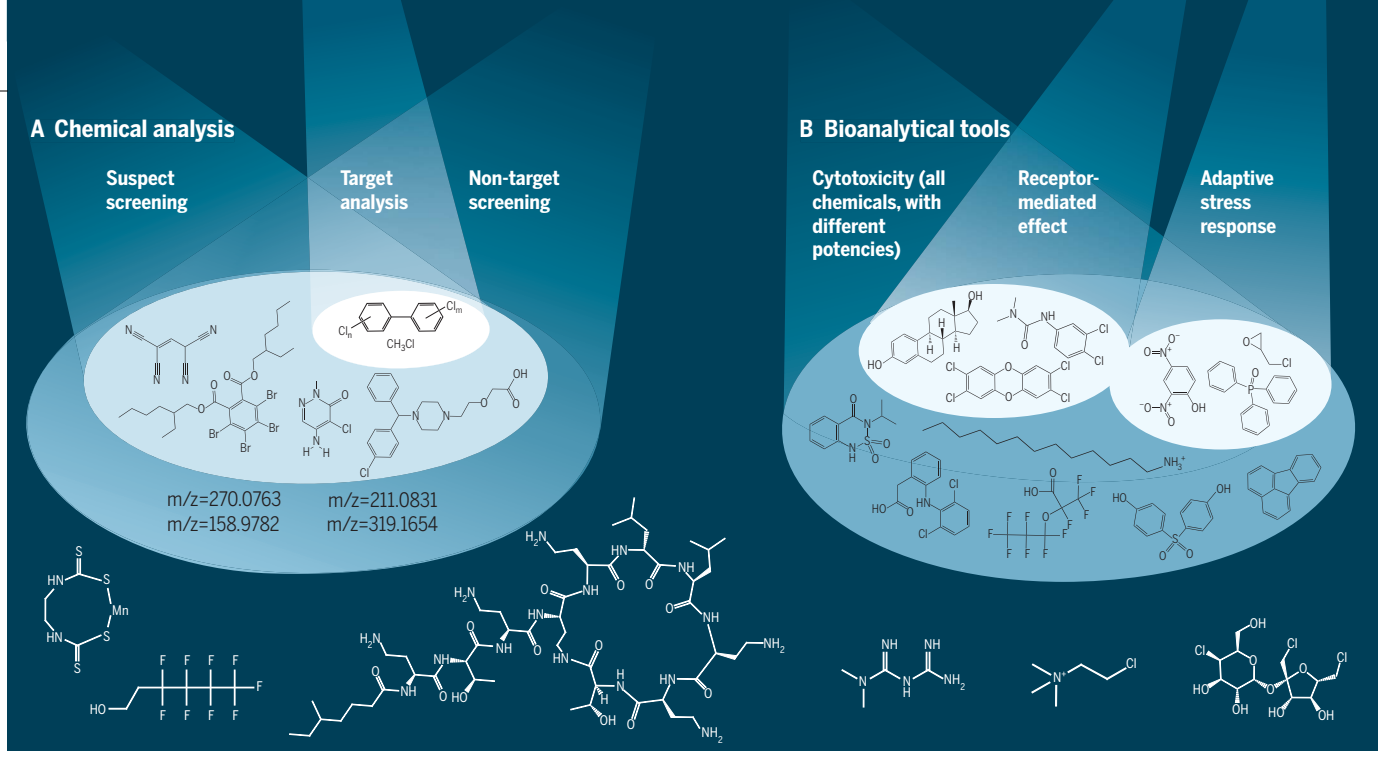

Fig. 2. Spotlight on the chemical universe. (**A**) Chemical analysis. (**B**) Bioanalytical tools. The overlap that exists between the chemicals "seen" by chemical analysis and bioanalysis is not depicted. The chemicals depicted are illustrative examples; selection is not comprehensive.

Monitoring chemicals

Early studies focused on hydrophobic persistent organic pollutants, which accumulate in soils, sediments, or lipid-rich tissues of organisms (Fig. 1A). Research has evolved to include a focus on polar contaminants as well, particularly in surface and ground waters used as drinking water sources (*11*).

Today, we recognize that there is a strong interconnectivity of chemicals in different environmental compartments (Fig. 1B). Some chemicals may preferentially accumulate in one environmental compartment over another and have different degradability in different compartments, but once emitted into the environment, they travel between all environmental compartments and along the food chain to humans. Simple exposure models and assessment of physicochemical properties of the chemicals of interest may make it easier to design sampling strategies that direct monitoring efforts toward relevant matrices.

Sampling and sample preparation

To identify chemical mixtures posing the greatest risk, sampling and monitoring plans must be developed to address variability in space, time, and composition, and to determine whether sampling should be continuous or discrete (*12*). Passive sampling techniques are alternative sampling methods applied to air, water, sediments, and biota in field or laboratory studies (*13*). Passive samplers are typically polymers (e.g., low-density polyethylene, polyurethane, or silicone) and offer time-integrative or equilibrium-based sampling.

Most environmental matrices and tissues cannot be directly analyzed because endogenous chemicals would disturb analysis. Pollutants are present in very low concentrations. Therefore, extraction is required to isolate and enrich pollutants, and cleanup steps are required to remove coextracted matrix (Fig. 1). Care must be taken not to alter the mixture composition during processing and to quantitatively track the enrichment. Recovery standards (e.g., isotope-labeled analogs) can be applied prior to chemical analysis, but they cannot be used for bioanalysis, for which independent recovery experiments must be conducted (*14*). Persistent organic pollutants can tolerate harsh chemical treatments to remove matrix components before analysis (Fig. 1A), but recent moves to focus on more labile contaminants require more directed sample preparation approaches. Because any cleanup is time-consuming and will lead to the loss of a fraction of chemicals, recent developments strive for minimal cleanup or none at all (Fig. 1C).

Water is an important source and sink of pollutants from wastewater treatment plants, urban runoff, and agricultural applications (*15*). If organic matter content is not too high, water can be directly injected into liquid chromatography instruments. This approach will likely grow in popularity as the sensitivity of analytical instruments increases. Solid-phase extraction is a versatile method for enrichment of chemicals from water samples that captures a large fraction of organic chemicals with generally good extraction efficacy in water, apart from limitations for small or ionized chemicals (*14*).

Chemicals in more complex matrices such as soil, sediment, plants, biota, and human tissue are often extracted with organic solvents. This approach may cause co-extraction of lipids and matrix components and requires tedious cleanups, including acid digests and gel permeation chromatography or silica gel columns, to remove the matrix and isolate the chemicals of interest (*16*). Certain chemicals (such as hormones, pyrethroid pesticides, and dioxins) pose a risk at very low concentrations, such that extensive cleanup and highly sensitive, dedicated target analyses are required to differentiate them from co-occurring chemicals present at higher concentrations. Thousands of chemicals can be detected in human blood, but xenobiotic chemicals are found at levels that are orders of magnitude lower than biological markers of human metabolism (*17*). Even with the current high dynamic range and high-resolution analytical methods, not all chemicals will be quantifiable because phenomena such as matrix suppression will interfere with analysis.

Because sampling of tissues from living organisms, particularly humans, can be logistically challenging and may pose ethical concerns, proxies are needed to evaluate exposure to mixtures (Fig. 1C). Rather than blood or tissue, noninvasive samples (e.g., hair, nails, urine, deciduous teeth) can be used as proxies for exposure, although knowledge of uptake rates and toxicokinetics is required (e.g., to relate urine concentrations to ambient exposure levels). Hand wipes were validated as a measure of personal exposure via correlations with biomarkers of exposure (*18*), but they only measure recent exposures and can be confounded by behavior such as hand-washing. Silicone wristbands have gained popularity as passive personal samplers (Fig. 1C) that can be worn for several days to weeks; these measure average integrated exposures by air

and possibly dermal and inadvertent dust ingestion pathways (*19*). Wristbands have been validated for several classes of semivolatile organic contaminants ubiquitous in indoor environments (*20*). House dust is often contaminated with a complex mixture of organic and inorganic contaminants that have been released from various building materials and consumer products into the home environment. Although increasing attention is given to the characterization of house dust using non-target analysis (NTA) to characterize indoor sources of human exposure, the use of wristbands may have the advantage of estimating exposure in multiple microenvironments in addition to the home. However, wristbands are limited to assessments of neutral organics and do not effectively characterize metal and dietary exposures.

Chemical analysis

The growth, evolution, and accessibility of high-resolution mass spectrometry (HR-MS) in environmental laboratories (*21*) has opened a Pandora's box of chemical complexity. HR-MS, often coupled with either gas or liquid chromatography, can detect tens of thousands of "features" (accurate molecular masses associated with unknown chemicals) in biological and environmental samples (*22*). Fragmentation information from tandem mass spectrometry (HR-MS/MS) is required to gain more information on structural features and assign more confident identifications (*23*). Methods for introducing analytes into a mass spectrometer strongly influence the range of chemical classes that can be detected in a sample; although different methods are complementary, they do not necessarily overlap. Although target analysis remains an essential component of chemical risk assessment, this approach illuminates only a narrow portion of chemical exposures and offers no information on unknown or previously unexpected contaminants that fall outside the targeted method (Fig. 2A). Suspect screening—using HR-MS data and lists of known chemicals of interest ("suspect lists") to identify contaminants without prior knowledge of their presence—widens the contaminant space and is currently a popular approach for providing semiquantitative analysis of complex mixtures. Even with the best NTA methods, some chemicals remain outside the spotlight, such as those that elute too early or late from the column, are poorly ionized by existing ionization methods, or are not yet interpreted correctly with current knowledge.

HR-MS/MS offers the possibility of performing routine target analysis, suspect screening, and discovery-based NTA in an all-in-one approach (Fig. 2A). With NTA, the limited scope of priority pollutants has been left behind, as even unknown masses can now be tracked in the environment (*21*), giving environmental chemists unprecedented power to detect new and emerging contamination and thereby covering a much larger chemical space (Fig. 2A). HR-MS/MS is now used for studies as diverse as daily monitoring of river water, tracing historical sediment contamination (*21*), characterizing indoor dust compositions (*24*), or performing retrospective screening of emerging contaminants (*25*).

Through the evolution of computational workflows based on exact mass and fragment matching, HR-MS/MS data can now be archived and used for plots showing the distribution of chemicals across time, space, or various matrices (Fig. 3) (*25*). The increasing popularity of NTA has seen a rapid increase of suspect lists to help find chemicals of interest, such as the CompTox Chemicals Dashboard (*26*) and the NORMAN Network Suspect List Exchange (www.norman-network.com/nds/SLE/). The CompTox Chemicals Dashboard, alongside PubChem (*27*), forms an important data source with its predicted physicochemical properties, connections to toxicity information, and data structures allowing access to metadata of mixtures in NTA via "MS-ready" data mappings (*28*). NTA, especially coupled with suspect screening of classes of compounds, can complement targeted analytical techniques but can only supply part of the picture of the chemicals causing toxicity in complex samples (Fig. 2).

Bioanalytical tools to capture mixture effects

Traditionally, whole-organism in vivo bioassays were used to evaluate the toxicity of wastewater effluent and sediment, but such bioassays suffered from limited sample throughput and an inability to distinguish the effects of pollutants from matrix components, salinity, or pH. This has changed with the advancement of cell-based in vitro bioassays, which are animal-protective and are amenable to high-throughput robotics (*29*). For example, the ToxCast/Tox21 program (*30*) seeks to develop predictive models to reduce or eliminate future in vivo testing. The program heralded a paradigm shift in toxicity testing, with in vitro methods now included in human health risk assessments to elucidate mechanisms of toxicity and to prioritize chemicals for further testing. The application of high-throughput in vitro assays toward ecological risk assessment and for monitoring and complex environmental samples is only emerging but has great potential to accelerate risk assessment and reduce animal testing (*31*).

In principle, sample extracts with little or no cleanup can be tested using in vitro bioassays, but care must be taken to avoid matrix effects. Samples that contain natural organic matter can suppress effects when cell-free receptors or enzyme systems are used, whereas co-extracted lipids and organic matter may lead to a decrease in sensitivity of cell-based bioassays because they act as an additional partitioning phase in the assay, decreasing the bioavailable fraction of more hydrophobic chemicals. A good understanding of the dosing process (*32*) and stringent quality control is vital when testing environmental samples in bioassays. Despite these practical limitations, bioassays are essential to capture the effects of all chemicals in mixtures. Every chemical will contribute to cytotoxicity, even if present below the instrumental detection limit or below the effect threshold of the single chemical (Fig. 2B).

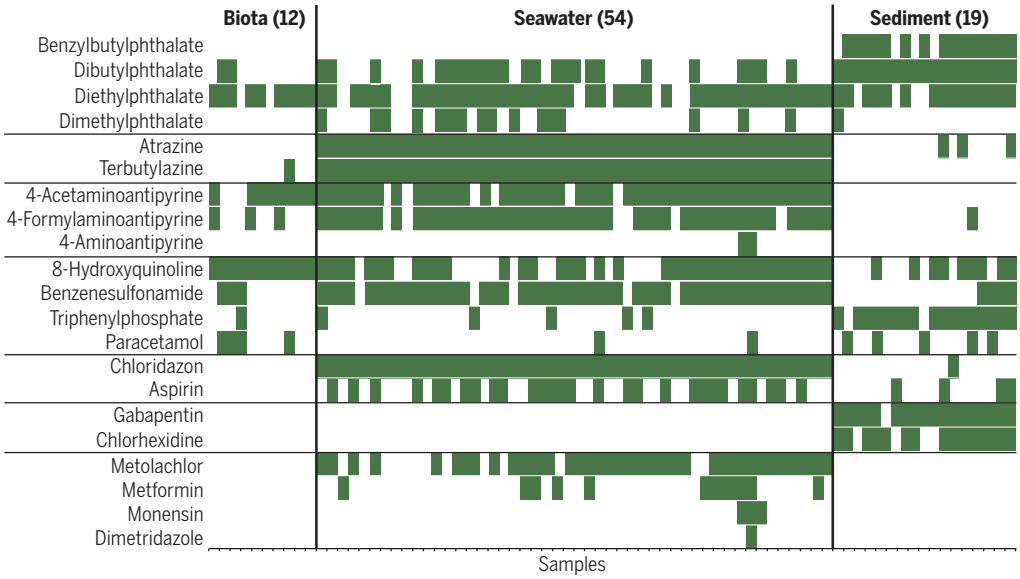

Fig. 3. Illustrative example of occurrence of chemicals in suspect screening of various matrices: biota (fish, mussels), seawater, and sediments. Green indicates presence with six or more matching HR-MS/MS fragments; white indicates absence or fewer than six HR-MS/MS fragments detected. [Created/modified from https://norman-data.eu/NORMAN-REACH/; details in (*25*)]

Reporter gene cell lines are popular in vitro assays and target one specific mode of action (MOA)—for example, binding to a hormone receptor, activation of metabolic enzymes through receptors such as the aryl-hydrocarbon receptor, or adaptive stress responses. This is accomplished by transient or stable transfection of a cell with a plasmid that contains multiple copies of the response element of the target receptor or transcription factor of interest, followed by multiple reporter genes. These encode reporter proteins that can be quantified easily, such as the enzyme luciferase. Cytotoxicity interference can mask specific effects, but this can be minimized by running a parallel cytotoxicity assay to avoid artifacts resulting from the so-called cytotoxicity burst (*33*). Concentration-effect curves are often linear at effect levels below 30%, which greatly simplifies mixture modeling and allows the calculation of bioanalytical equivalent concentrations (BEQ_{bio}) of complex mixtures in a sample (*34*). BEQ_{bio} relates the toxicity of a mixture to a well-characterized chemical for that MOA, and can be interpreted in terms of risk-scaled concentrations. These are easy to communicate to regulators and the public because they report mixture effects in easily understandable units—for example, "This water sample contains mixtures of chemicals that have the same effect as 6 ng of estradiol per liter of water," or "This fish has accumulated a chemical mixture that has the same effect as 3 pg of 2,3,7,8-tetrachlorodibenzodioxin per gram of fish." Effect-based trigger values based on acceptable BEQ_{bio} have been derived for diverse bioassays from drinking water guideline values and environmental quality standards and were proposed to be included in a future regulatory implementation of mixture effects in envrionmental monitoring (*35*).

Combining analytical and bioanalytical tools

Effect-directed analysis (EDA) or toxicity identification evaluation (TIE) can be used to identify risk drivers in complex mixtures and to separate bioactive chemicals that could otherwise be masked by matrix effects (*36*). A sample extract is separated by mass, hydrophobicity, or polarity by means of chromatography or physical separation into fractions, which are tested individually in bioassays. Each bioactive fraction is further fractionated until one or more bioactive subfractions are identified that contain chemicals that explain a majority of the observed effect(s). EDA has, for instance, helped to identify risk drivers in contaminated sites, industrial effluents, and sediments (*36*) or unknown chemicals that disturb thyroid function in blood of polar bears (*37*).

It is also possible to link measured concentrations and effects by modeling (*38*). The expected effects of quantified chemicals, expressed as bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}), can be predicted for all quantified and toxicologically characterized chemicals by multiplication of the detected concentration C_i with the relative effect potencies (REP_i) and adding the contributions from all chemicals in the mixture (Fig. 4). An equivalent concept is exposure-activity ratios of mixtures (EAR), where active concentrations are related to detected concentrations (*31*). These mass balance approaches apportion the effect of the known chemicals and identify chemicals that are mixture risk drivers among the known chemicals. For example, in Fig. 4A, the risk driver (light blue bar) would be chemical 2, because it has the highest relative effect potency (light gray bar) despite its low concentration (dark gray bar). If one compares the experimental BEQ_{bio} of the mixture with the BEQ_{chem} for matching bioassays, in many cases $BEQ_{bio} > BEQ_{chem}$, even if several hundreds of chemicals are quantified (*39*). The contribution of the unknown chemicals ($BEQ_{unknown}$) can be quantified using the simple effect balance equation $BEQ_{unknown} = BEQ_{bio} - BEQ_{chem}$. Certain receptor-mediated effects, such as activation of hormone receptors or inhibition of photosystem II, are triggered by a few chemicals that act in a highly specific manner. For these cases, it is often possible to close the mass balance and explain the entire biological effect of a sample (light green bar in Fig. 4B) by the predicted mixture effect of the known chemicals (dark blue bar). In contrast, for less specific effects, (nonspecific MOA in Fig. 4C), a large fraction of effect typically remains unidentified. For bioassays such as the fish embryo toxicity assay, activation of the oxidative stress response, or genotoxicity, the unknown effect may amount to as much as 90 to 99.99% of BEQ_{bio} (*39*). These mixture-modeling approaches historically suffered from a lack of toxicity data for individual chemicals, but with the large high-throughput screening databases becoming available and suspect screening lists expanding, more and more chemicals can be included in the BEQ_{chem} and EAR calculations and applied in monitoring studies (*40*).

Because typical chromatograms from environmental samples contain tens of thousands of features, we cannot expect that the small subset of features that are identified and quantified contain all drivers of the mixture risk. Measured BEQ_{bio} values are real even if their causative agents cannot be fully explained.

Outlook

The number of chemicals identified in environmental samples using sophisticated instrumental analysis is steadily increasing, and we have developed better tools to investigate their combined effects and mechanisms of toxicity. However, research is drowning in disconnected details instead of capturing the bigger picture. It is still difficult to elucidate the drivers of chemical stress in the environment; the links among environment, wildlife, and people can only be made by applying common monitoring approaches.

Tracking chemicals and their transformation products in the environment and in our bodies at ever-lower concentrations is an immense (bio)analytical challenge: Sampling, extraction, chemical detection, and data analysis all need to be tuned to each other to obtain robust information. Complementing high-resolution mass spectrometry with bioanalytical tools, especially in vitro bioassays, can now yield information on effects related to all chemicals in a sample, equating to risk-scaled total concentrations. A smart combination of these tools has the potential to revolutionize environmental monitoring. Over time, the storage of NTA data coupled with results and metadata from bioassays will allow "big data" methods to take over and help tease out the relationship between signals

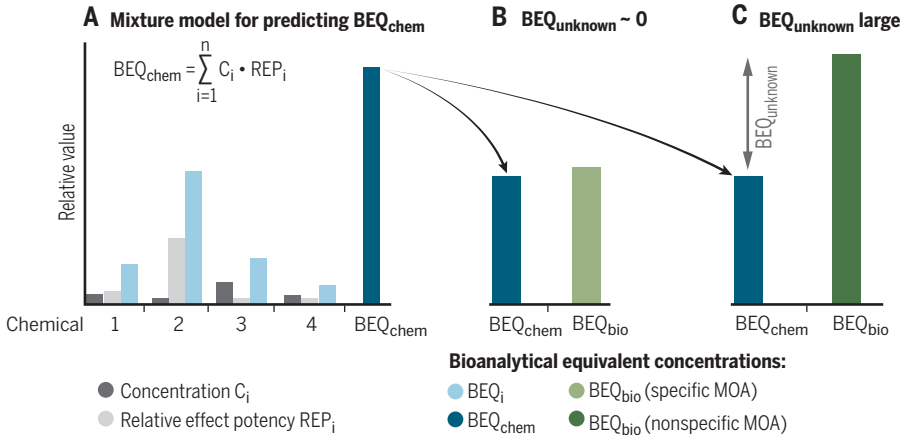

Fig. 4. Mixture toxicity models for linking detected concentrations with predicted and experimental mixture effects. (**A**) Example for calculation of BEQ_{chem} from concentrations C_i and relative effect potencies REP_i. (**B** and **C**) Comparison between the predicted mixture effects of the detected chemicals (BEQ_{chem}) and those directly measured in bioassays (BEQ_{bio}) allows derivation of the effect contribution of the unknown chemicals ($BEQ_{unknown}$). (B) For specific modes of action (MOA), BEQ_{chem} is often close to BEQ_{bio}. (C) For nonspecific effects, $BEQ_{unknown}$ is large.

and toxicity. Quantifying mixture effects is a way to capture all existing chemicals and their bioactive transformation products. Given the clear relevance of mixtures and the fact that thousands of chemicals are occurring in the environment and in our bodies, a shift in the existing regulatory paradigm toward mixture effects is urgently needed (*7*, *35*).

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