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A prospective whole-mixture approach to assess risk of the food and chemical exposome

T. Tralau[1]✉, M. Oelgeschläger[1]✉, J. Kugler[1]✉, D. Bloch[1], A. Braeuning[1], T. Burgdorf[1], P. Marx-Stoelting[1], V. Ritz[1], S. Schmeisser[1], A. Trubiroha[1], S. Zellmer[1], A. Luch[1], G. Schönfelder[1,2], R. Solecki[1] and A. Hensel[1]

Many widely used chemicals result in ubiquitous human exposure from multiple sources, including diet. Legislation mainly deals with the toxicological evaluation of single substances owing to a methodological and conceptual lack of alternatives, and does so within defined silos subject to over 40 distinct regulations in the EU alone. Furthermore, much of the research and many of the initiatives concerned with the assessment and evaluation of chemical mixtures and their potential effects on human health rely on retrospective analysis. Here we propose an approach for the prospective identification, assessment and regulation of mixtures relevant to human health. We address two distinct aspects of toxicology—which chemicals actually do occur together, and how potential mixture-related health hazards can be predicted—with an adapted concept of the exposome and large-scale hazard screens. The proactive use of the likelihood of co-exposure, together with the new approach of methods-based testing, may be a timely and feasible way of identifying those substances and mixtures where hazards may have been overlooked and regulatory action is needed. Ideally, we would generate co-exposure patterns for specific consumer groups, depending on lifestyle and dietary habits, to assess the specific risk of identified mixtures.

Over 350,000 chemicals and mixtures of chemicals are registered for production and use worldwide[1] that during their life cycle can be released into the environment, enter the food chain or otherwise contribute to human exposure. Food, in particular, has been shown to be a dominant source of exposure[2]. The potential adverse effects from continuous and chronic exposure to mixtures of multiple chemicals, particularly from food, medicine and cleaning products, are of concern[3]. Legislation has been implemented to protect consumers and workers from unintended exposure to potentially dangerous chemicals—in Europe, the combination of the European Commission (EC)'s legislation on registration, evaluation, authorization and restriction of chemicals (REACH, European Parliament and Council Regulation (EC) number 1907/2006) with plant protection products (European Parliament and Council Regulation (EC) number 1107/2009) and biocides (European Parliament and Council Regulation (EC) number 528/2012) provides a comprehensive regulatory framework. Real-life exposure comprises the simultaneous or consecutive (co)-exposure to chemical substances originating from various sources, including pesticides, pharmaceuticals, personal care products and, most importantly, diet[4,5]. This includes foodstuffs as well as environmental and food process contaminants, residues of pesticides, veterinary medicines and chemicals from food contact materials, food additives and others[6].

Existing legislation provides extensive information on the respective substances within their remit. However, it generally addresses the effects of single substances only, and this inherently carries the risk of missing potential mixture-induced effects. Even without considering adverse effects from long-term exposure, potential mixture effects have been associated with genotoxicity and developmental and reproductive toxicity, as well as cancer, hepatotoxicity or neurotoxicity[7–13]. Consequently, there is political demand for the introduction of combined exposure in risk assessment. The EC, for example, enforced a claim by legal action in 2012[14] for the need to assess mixtures and has consequently prioritized the issue of mixture assessment within its Green Deal[15].

Combinatorial effects of chemical substances in humans have been an issue for a long time. Multiple drugs taken at the same time, for example, can lead to beneficial or adverse interactions[16,17], occurring at the pharmacodynamic or pharmacokinetic level[18,19]. With environmental chemicals, unlike in pharmacology, owing to the different sources of exposure and lifestyle habits, including diet, many more molecules can interact at the same time throughout an entire lifetime. In addition, with environmental chemicals we are dealing with much lower dosages and potentially longer windows of exposure than in the case of drug therapy.

In the light of numerous published examples of cumulative toxicity, including cumulative hepatotoxicity for combinations of pesticides[20,21], the question is therefore no longer whether combinatorial effects exist, but whether current regulatory measures are sufficiently protective, and how to address this if they are not (for a rough estimate, please refer to Supplementary Fig. 1). With regard to public health protection, it is this latter issue that is crucial.

Status quo in assessment of chemical mixtures

Risk assessment of combined exposures comprises the risks arising from human exposure to multiple substances from single or multiple sources, via single or multiple routes. Conceptually, there are two basic approaches: the whole-mixture approach and the component-based approach.

[1]German Federal Institute for Risk Assessment (BfR), Berlin, Germany. [2]Charité–Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, HumboldtUniversität zu Berlin and Berlin Institute of Health, Berlin, Germany. ✉e-mail: Tewes.Tralau@bfr.bund.de; Michael.Oelgeschlaeger@bfr.bund.de; Josephine.Kugler@bfr.bund.de

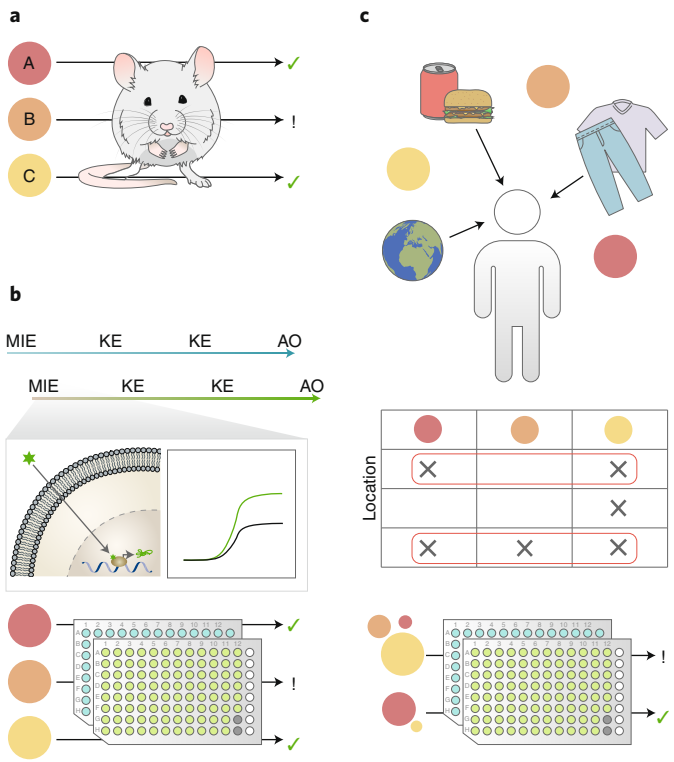

Fig. 1 | Risk assessment approaches. a, In the classical approach, risk assessment relies on testing of substances (red, orange and yellow circles) in animal tests, from which it is possible to conclude whether a risk has been identified (exclamation mark) or not (green tick), but not the mode of action of the substance. **b**, New assessment methods (NAMs) are based on adverse outcome pathways (AOPs) for which molecular initiation events (MIE), key events (KE) and adverse outcomes (AO) are defined. These methods rely on in vitro assays, which can often be used in a high-throughput fashion and convey information about the mode of action. Especially for MIE and KE, these in vitro assays often rely on reporter cell lines, in which an easily detectable reporter gene is expressed upon substance stimulus. **c**, To analyse possible mixtures of substances, our proposed approach of experimental regulatory toxicology combines parts of the exposome concept with the NAMs. From usage patterns, product data and surveys and analytics (top), it would be possible to create a correlation matrix in which likely co-exposures can be estimated (middle – indicated by red boxes for mixtures in two different locations). These possible mixtures could then be tested in established NAMs (bottom).

Whole-mixture approach. The whole-mixture approach tests the final mixture as experienced during exposure, and so information on the toxicity of the individual components or the specific nature of possible interactions cannot be derived. Moreover, whole-mixture approaches assess constant mixtures that do not notably vary in their composition. Given the diversity and variation of mixtures that contribute to human exposure and the small number that can be tested, the applicability of whole-mixture approaches has so far been technically limited[22]. Not only would comprehensive in vivo testing of respective cocktails require almost infinite numbers of experimental animals, but even for single-substance testing, in vivo reproducibility for complex endpoints lies within a range of 10% to 57% (refs. [23,24]). For mixture effects with varying effect superimpositions and effect sizes, error propagation and statistical multiple testing fallacy raise the bar even higher. Also, given such reliability of in vivo experiments, questions are raised about the necessity and extent of animal experimentation and euthanization.

Component-based approach. The component-based approach is more common, but requires greater information on substance identity, concentration and toxicity, including the assumed or established mode of action (MoA). For chemicals with similar MoAs (action on the same target), dose addition in combination with toxicity factors or assessment groups is used[5,25,26] Dissimilar MoAs (action on different targets contributing to the same endpoint) are addressed as independent action or response and effect addition[5]. Response addition refers to the sum of probabilistic risks, whereas effect addition refers to the sum of biological responses. Therefore, response addition is based on any measurable response, even if it is not adverse. In contrast, effect addition focuses on adversity, hence omitting any findings below the no observed adverse effect level (NOAEL). Yet even for legislation or substances for which there are extensive data available, data gaps—as well as unclear human relevance of the existing data—remain problematic[27].

One of the best-known examples of a successful component-based approach is the assessment of phthalate toxicity, which was a serious food safety issue[28]. As an assessment group, a majority of phthalates share the same MoA. This makes the group accessible to the use of potency factors along with dose additivity[29]. However, there are two aspects to consider. First, mixtures consisting of substances with a shared MoA and/or a common adverse outcome tend to be an exception. For most other scenarios, independent MoAs are more likely. Therefore, the toxicological assessment of more complex mixtures often relies on effect additivity, supported by various approaches for risk or hazard prioritization[30]. Second, considering the combination of substances in dose ranges near their NOAEL or below can lead to an underestimation of adversity, since applying dose additivity, for undetectable effects ($0+0=0$) is not applicable[31,32].

However, it should be noted that the minimum detectable effect range of in vivo studies lies between 10% and 30% (ref. [33]). Thus, undetectable effects in classical single-substance studies may well become apparent under conditions of combined exposure, owing to increased systemic stress, increased susceptibility, decreased resilience, interacting toxicokinetics or modulated toxicity responses[34]. Metals, in particular, have been reported to show increased effects when applied as mixtures, with indications of (synergistic) low-dose metal injuries[35]. Likewise, plant protection products are complex mixtures of up to dozens of substances that can exhibit increased effect levels when compared with the original active substances[36].

Therefore, the premise that the compounds do not interact with each other, either on a physico-chemical or physiological level, or with regard to their toxicokinetics and toxicodynamics, is a simplification that keeps assessments feasible. Yet mixture effects may be additive, less than additive (antagonistic) or over-additive (synergistic). Indeed, a recent systematic review of the effects of pesticide mixtures found dose additivity and synergistic interactions[37]. Such observations typically result from the combination of toxicological effects on unrelated pathways contributing to the same endpoint, thereby resulting in a strong increase in potency, be it in xenobiotic mixtures or their well-tested natural counterparts—predatory venoms[38].

In the light of the plethora of potential targets, the generally asserted low frequency of synergism and the claim that it rarely exceeds a potency of four- to tenfold are debatable[22,39]. In fact, a systematic review of 194 binary pesticides and 136 antifouling mixtures reported occurrences of synergism in 7% and 26% of cases, respectively[39]. Although one would expect this rate to rise with more active substances, the impact of synergism on overall toxicity decreases with more chemicals, because for each *n*-fold synergism, it only takes *n* more substances of similar strength to arrive at the same toxicity. Moreover, known synergism often results from interference in key biochemical pathways of xenobiotic metabolism, with the respective drivers thus accessible to systematic screening[39].

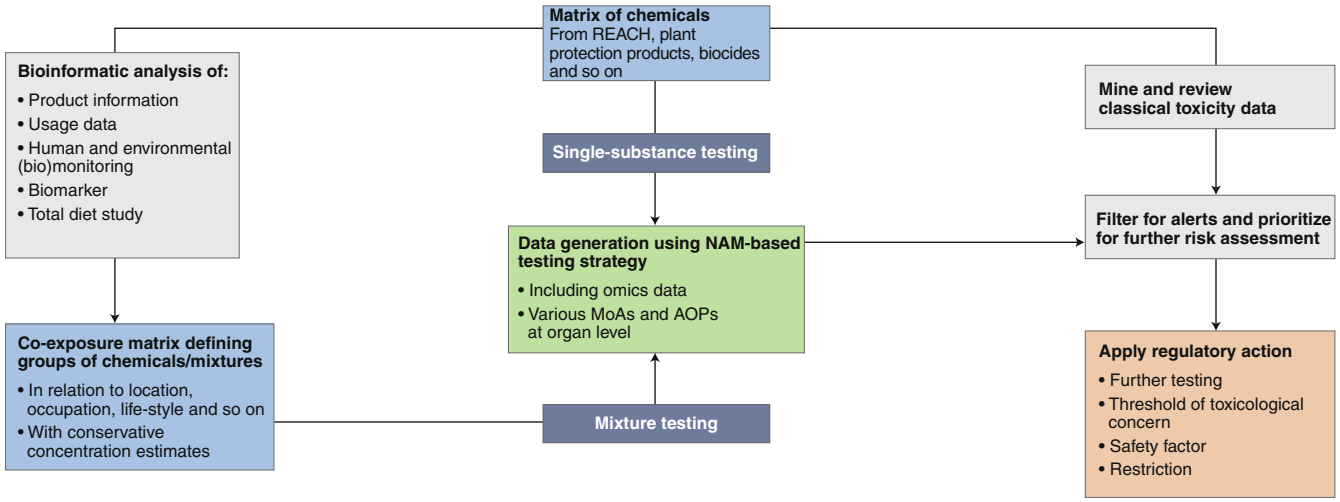

Fig. 2 | Experimental regulatory toxicology. The starting point is a matrix of chemicals (top blue box) populated with substances registered under REACH, plant protection products and biocides. From various resources, information about possible co-exposure will be gathered (left grey box) to define groups of chemicals that occur together (left blue box). Established mixtures and their respective single substances will be tested using NAM-based testing strategy (dark blue boxes either for single-substance or mixture testing). Data generated by this approach (green box) will be complemented with classical toxicity data (right grey boxes) such that further regulatory actions can be applied (orange box). The colours used indicate either matrices of chemicals (blue), bioinformatic tools and processes (grey), NAM-based testing (dark blue), data on substances generated by NAM-based testing (green) or regulatory action (orange).

Regulatory concepts of chemical mixture assessments

A number of documents describe the methodology, terminology and relevant EU legislative requirements that address aspects of mixture toxicity. There are also related guidance documents and international frameworks, such as an overarching framework from the Organisation for Economic Co-operation and Development and the European Food Safety Authority (EFSA)[5,25]. Although extensive and thorough, the strategies outlined therein remain an extension of single-substance assessments, are not harmonized and are restricted to single frameworks. This also holds true for less-data-driven, 'quick fix' concepts such as adding an additional mixture assessment factor[40]. Besides being purely hazard-driven, such a factor would, again, be sector-specific and of limited cross-legislation compatibility. Thus, while the aforementioned approaches might work for set commercial mixtures, they fail to serve the wider remit of protection from effects originating from exposures across legislative silos.

Although several EU-founded projects deal with mixture risk assessment, they are missing an overall framework for the translation of results into regulatory action[41]. Finally, and most importantly, none of these projects can solve the problem of relying on retrospective assessments, which are a practical compromise, but remain a second choice scientifically.

NAMs. US initiatives such as Tox21(ref. [42]; see also https://tox21.gov/) and ToxCast (ref. [43]; see also https://www.epa.gov/chemical-research/toxicity-forecasting) have demonstrated the practicality of testing thousands of substances in hundreds of high-throughput in vitro or in chemico assays—tested substances ranged from industrial chemicals, pesticides, food and feed additives and contaminants to medical products (see ref. [44]). From the tested chemicals, over 8,500 were directly related to food[45]. The most prominent example of NAM-based substance prioritization is the US Environmental Protection Agency endocrine disruptor screening programme (EDSP) for predictive model building, in which more than 10,000 unique chemicals were screened for potential endocrine-disrupting properties[46]. As a hazard prioritization tool, the programme (in combination with the AOP concept) has allowed strikingly accurate prediction models to be built[46]. The excellent performance of these models has prompted the US EPA to publish a Federal Register Notice on the intention to accept such models as an alternative to the current EDSP tier 1 oestrogen receptor binding, transactivation as well as uterotrophic screening[47], in which pesticides, industrial chemicals and environmental contaminants are tested. These assays may also be applied to monitor the biological activity of unknown mixtures in environmental samples from water[48] to biota and human specimens[49].

Biomonitoring the exposome. The ideal risk assessment would encompass the entirety of all exposures of an individual during their lifetime from conception onwards—the exposome[50,51]. Relevant routes comprise oral uptake, inhalation and dermal exposure to the corresponding compounds, both natural and man-made. An important part of the human exposome originates from dietary intake, a complex mixture of natural, intentionally added and unintentionally contaminating chemicals[51]. Although the exposome concept resulted in a paradigm shift from a single exposure–disease model to an agnostic analysis of environmental influences on human health, its complexity still poses major practical challenges regarding analytics and its correlation with human health[52,53].

Most studies on human exposure apply targeted analytical approaches, which only consider substances already identified as problematic. Only a few hundred chemicals are routinely assessable through targeted methods, with further limitations for short-lived compounds[54]. Total diet studies are promising as means of determining dietary exposure—generating data on food intake, nutrients, additives, process and environmental contaminants, mould toxins, pesticides, veterinary drugs and substances migrating from packaging into food. One prominent example in this context is the German MEAL study, which has been commissioned by the German Federal Institute for Risk Assessment[55]. Meanwhile, the European project HBM4EU aims to produce a comprehensive assessment of human exposure via biomonitoring. However, again these activities mainly focus on targeted analysis for known, pre-prioritized substances. Analytical chemistry-based exposome research is thus moving towards non-targeted analysis[56], but despite recent studies successfully assessing method performance and reproducibility for

10 synthetic mixtures with a total of more than 1,200 substances from the ToxCast library[57,58], the prediction and identification of metabolites in matrix-rich samples remain challenging[59]. Moreover, the concept critically relies on biomarkers (for biomarker repositories, see Supplementary Table 1) and human biomonitoring for substance prioritization and the identification of relevant mixtures.

A complementary means of passively determining cumulative exposure via the dermal and inhalation routes is silicone wristbands[60]. Absorbing organic chemicals in a partition-based fashion, such wristbands act as a personalized filter suitable for sampling individual environmental exposures.

A new proposal. Although understanding co-exposure patterns is unarguably an important step towards the investigation of the joint health effects of chemical mixtures, another major challenge for monitoring approaches lies in the assignment of biological meaning, as well as the reliance of monitoring approaches on biomarkers. Consequently, the selection of chemicals will inherently be retrospective, usually driven by technical feasibility as well as specific concerns[61]. Therefore, we propose an approach to proactively investigate the likelihood of exposure and its potential effects for possible mixtures and their single substances (Fig. 1).

Monitoring projects like HBM4EU, total diet studies, usage and sales data, and direct untargeted testing and analysis of environmental and human matrices have the potential to provide plenty of data about direct and indirect exposures. Fed into matrices and appropriate models, this could enable moderately conservative predictions of probable chemical combinations and concentration estimates to be made. On the basis of these data, groups of chemicals probably occurring together, and the single substances they comprise, can then be subjected to NAM-based screening bioassays. Data on MoA and possible synergistic effects will thereby be generated, facilitating hazard assessment. In such a way, we would be able to prioritize and filter critical mixtures and possible trigger substances for further regulatory action (Fig. 2).

Put into practice, the first step for such a systematic approach would be the establishment of a comprehensive chemical matrix populated with substances registered under REACH, plant protection products and biocide regulation. Then, information on co-exposure would be screened, including usage patterns, product data, surveys and analytics. Usage patterns may be obtained from consumption and usage databases such as total diet studies, national cohorts and biobanks, but also from surveys (for example, refs. [62,63]). Plant protection product tank mixtures and application patterns could be taken into account, as well as product data on the ingredients of marketed products. Available and newly generated surveys contribute to information, on food consumption and consumer behaviour, for example, as do sales data. Last, analytical data generated in total diet and human biomonitoring studies should be included. As a result, a matrix of substances will be generated, which are either marketed, applied or measured together. Next, this matrix would be integrated to group substances on the basis of common exposure patterns, while accounting for co-exposure probabilities. In addition to their systematic evaluation and interpretation by experts, multivariate statistical methods may be employed to elucidate relevant patterns. For example, co-exposure patterns for specific professions (such as farmers or painters) or specific groups of consumers (pregnant women or vegans, for example) may be derived.

The respective substances and their identified mixtures would then be tested using a NAM-based testing regime. The corresponding high-throughput bioassays should cover various relevant MoAs and AOPs at the organ level, ideally in test systems that allow physiologically based toxicokinetics (as contextually outlined in other papers) to be integrated sufficiently, and that consider compensatory mechanisms[64,44]. This regime should also include omics data, which may enable a more comprehensive analysis of MoAs or AOPs affected by the substances or their mixtures and thus help to facilitate a pathway-focused analysis of mixture effects[65]. Network approaches to identify potential interactions between substances have been used for quite some time in pharmacology and should similarly evolve in toxicology[66,67]. To help in the determination of substance risk assessment, a systematic review and evaluation of available toxicological information should also be conducted. Here, the REACH database should be mined for toxicological information as well as threshold values. Substance-specific information on MoA, AOPs and relevant physico-chemical properties should be collected. By integrating newly generated data with reviewed classical toxicological information, relevant mixtures could then be analysed with a specific focus on synergism, adverse effects and possible health hazards. Established tools of cumulative risk assessment would then assess mixtures flagged by this approach. Concomitantly, data from cohort studies such as the German National Cohort could be used as input for further plausibilization and refinement of assessments[68]. Where potential risks are identified, regulatory action should be taken. One option is the adaptation of specific safety factors or the driver-specific application of risk mitigation measures. Alternatively, management options such as the withdrawal of substances critical to the identified mixtures effect, or their restriction of use, may be considered.

Our proposal offers some distinctive benefits. Using the data generated, the somewhat patchy AOP network could be further refined, especially with respect to possible interactions between different pathways. The identification of chemical components as drivers of adverse effects and synergism within experimentally established hazardous cocktails may facilitate the assessment of possible mixtures effects in the future. Moreover, it would allow selective mitigation of such drivers by targeted regulatory action. This approach will be hazard-specific, as well as independent of retrospective analysis and legislative silos; it would proactively inform us of verifiable health threats through mixtures.

The proposed experimental regulatory toxicology approach exceeds the current practice of regulatory advisory boards for existing projects and, to some extent, challenges the regulatory status quo. It requires a translational transproject framework for cooperation with ensured data sharing. As an example, for food, this would include (but not be limited to) data from the WHO, FAO, the US Environmental Protection Agency, the EFSA, the European Chemicals Agency and the agricultural sector, as well as regional agencies and academic research. However, the effective evaluation of chemical mixture effects requires an integrated and systematic approach across different pieces of legislation[69,70]. On a final, more philosophical note—one question that emerges with this approach is: how much is 'enough' when it comes to data and translation to regulatory action? Indeed, we can never have 'enough' data from the regulatory perspective, but given the foreseeable difficulties arising from non-data-driven approaches, perhaps we should pragmatically settle for a solution that allows the worst culprits to be filtered out.

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Author contributions

J.K., M.O. and T.T. initially developed and drafted the concept. All authors contributed equally to the further conceptualization, writing and editing of the manuscript. All figures were generated by J.K.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence should be addressed to T.T., M.O. or J.K.

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