21st Century Approaches for Evaluating Exposures, Biological Activity, and Risks of Complex Substances: Report of an ICCA-LRI Workshop Co-organised with the European Commission's Joint Research Centre

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This report presents the proceedings from the International Council of Chemical Associations Long-Range Research Initiative Workshop "21st Century Approaches for Evaluating Exposures, Biological Activity, and Risks of Complex Substances"; co-organised with the European Commission, Joint Research Centre. The inherent properties of complex substances present technical and scientific challenges when evaluating their bioactivity, exposures and risks, especially when applying new approach methodologies (NAMs) and innovative exposure modelling tools for regulatory purposes. The workshop presentations and discussions exemplified how such challenges can be addressed. Opportunities were identified to meet prevailing knowledge gaps. To enhance the applicability of NAMs for the assessment of complex substances (and combined exposures to multiple chemicals), defined and constant exposure levels should be ensured during in vitro testing and quantitative in vitro and in vivo extrapolations enabled. Also, NAMs that allow evaluating potential interactions of chemicals in mixtures should be the focus of further research. Concerted action is necessary to integrate NAMs into tiered risk assessment frameworks for complex substances and mixtures. Implementing such a framework will necessitate a shift in the risk assessment practice by emphasising exposure-driven assessments, identifying mechanisms of effects, and focusing on collecting data that are most relevant for risk assessment and management.

Key words: Multi-constituent substances; UVCBs (substances of unknown or variable compositions, complex reaction products and biological substances); new approach methodologies (NAMs); mode-of-action (MoA); adverse outcome pathway (AOP); integrated approach to testing and assessment (IATA); exposure assessment; combined exposure

1. Introduction

Complex substances include multi-constituent substances (MCSs) and *substances of unknown or variable compositions, complex reaction products and biological substances* (UVCBs) (ECHA, 2012a, b; USEPA, 2015; see Glossary Section 10 for definitions of key terms). While MCSs are well-defined substances that possess more than one main constituent, UVCBs are comprised of a complex mixture of individual components that could not practically be formed by deliberate physical mixing. UVCBs may range in complexity from predictable alkyl chain variations (e.g. surfactants) to less predictable multi-constituent biologicals (e.g. fragrance oils), and the number of their components can range from only few to many thousand. Due to their complexity, UVCBs cannot be represented by unique structures or molecular formulas, but are described by a broader indication of the nature of their components. Each of the individual components of MCSs and UVCBs may possess different physico-chemical and fate properties (Clark et al., 2013).

Approximately 10% and 20% of the substances registered under *Regulation (EC) 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals* (REACH; EP and Council, 2006) have been declared as MCSs and UVCBs, respectively (ECHA, 2017a). Similarly, approximately 25% of the substances listed on the *United States Environmental Protection Agency (USEPA) Toxic Substances Control Act Inventory* are flagged as UVCBs (USEPA, 2015, 2019).

The inherent properties of UVCBs and MCSs present technical and scientific challenges when evaluating their biological activity, exposures and risks (Clark et al., 2013). These challenges are especially pronounced when striving to apply 21st

century approaches, or new approach methodologies (NAMs), for their hazard and risk assessment (Kienzler et al., 2016). Similar challenges are also encountered during the risk assessment of combined exposure to different substances.

NAMs include e.g. *in vitro* and *in chemico* methods, *in silico* tools and 'omics technologies (National Research Council, 2007). The development of NAMs for evaluating the biological activity of chemicals is progressing rapidly. Good progress is also being made in the development of new exposure assessment tools. Many NAMs and innovative exposure assessment tools are now sufficiently far advanced to be applied for the risk assessment of chemicals and chemical products for priority setting, regulatory decision-making and product stewardship (Berggren et al., 2015, 2017). Nevertheless, the specific challenges related to UVCBs and MCSs still impair the use of NAMs for the exposure, hazard and risk assessment of complex substances.

Against this background, the International Council of Chemical Associations (ICCA) Long-Range Research Initiative (LRI) convened an international workshop *21st Century Approaches for Evaluating Exposures, Biological Activity, and Risks of Complex Substances* that took place on 19 and 20 June 2019 in Stresa, Italy. This workshop, that was co-organised with the European Commission, Joint Research Centre (JRC), brought together approx. 50 experts from academia, government, authorities, industry, and non-governmental organisations from Canada, Europe, Japan and the USA. This report presents the proceedings of the workshop.

Kathy Plotzke (*Dow, USA*) opened the workshop, also on behalf of **Elke Anklam** (*European Commission, JRC, Belgium and Italy*). It was the aim of the workshop to bring together relevant stakeholders to enhance dialogue on the development and use of NAMs for the risk assessment and risk management of complex substances. The workshop addressed all aspects of risk assessment, i.e. hazard identification, hazard characterisation (dose-response evaluation), exposure assessment, and risk characterisation. Specific topics included case examples to identify challenges in using NAMs to support regulatory and product stewardship decisions related to complex substances, consideration of uncertainty during risk evaluation, specific approaches to improve the risk assessment of complex substances, and advancements made under the LRI programmes. Further, the hazard and risk assessment of combined exposure to substances was addressed. The workshop presentations and facilitated panel discussions aimed at enhancing the application of NAMs for regulatory and product stewardship evaluations of complex substances, also by fostering future collaborations and promoting a common understanding of the opportunities and challenges of additional research needs (see https://sites.google.com/site/iccalriworkshop2019/workshop-materials for workshop programme and background material).

2. Session I: Setting the stage: New technologies - perspectives from users and agencies

2.1. The vision for the workshop: What is the potential of these approaches?

Elisabet Berggren (*European Commission, JRC, Italy; workshop co-chair*) denominated prevailing challenges that still impair the use of NAMs to evaluate complex (or mono-constituent) substances. Challenges include the translation of long-term external exposure to internal doses at target organs and how to translate those to actual *in vitro* exposures; the prediction of toxicokinetics especially with regard to metabolites; and how to identify the most relevant modes-of-action (MoAs) affecting human health. However, increasingly knowledge on MoAs, and their key events, structured in adverse outcome pathways (AOPs) assists in an improved mechanistic understanding of human disease compared to traditional animal studies (Kienzler et al., 2016). Such knowledge is being used to develop integrated approaches for testing and assessment (IATAs; OECD, 2016a, b). Further, more sophisticated approaches are being developed to perform quantitative *in vitro* to *in vivo* extrapolations (QIVIVE) enable the prediction of thresholds for effects in humans (McNally et al., 2018).

Within the public-private partnership SEURAT-1 (*Safety Evaluation Ultimately Replacing Animal Testing*; Gocht et al., 2015), an *ab initio* work flow for exposure-driven testing and assessment was developed. This *ab initio* work flow does not rely on any new animal testing. Hypotheses are formulated based on existing data, *in silico* modelling and biokinetic considerations and are followed up by targeted non-animal testing (Berggren et al., 2017). Generally, the *ab initio* work flow can also be applied for the assessment of complex substances. A major challenge when

assessing complex substances is the determination of the relevant chemical space, i.e. all possible (relevant) molecules and multi-dimensional conceptual spaces representing the structural diversity of these molecules (Awale et al., 2017). During substance grouping and read-across, as an important means to avoid animal testing (ECHA, 2017b), the chemical space should be enhanced with descriptors of biological similarity (Berggren et al., 2015). In addition, following the *ab initio* workflow, targeted testing applying non-animal methods can be directly applied to complex substances when read-across or bridging arguments not are convincing.

Dr. Berggren asked how much we actually know about the protection levels applied today as a consequence of chemicals legislation and whether these could be reached more efficiently and also with a broader coverage. She concluded her presentation with the following recommendations for a more efficient and more complete future assessment of chemicals and mixtures:

- Improve use of the knowledge about chemical space
- Develop 'greenlight testing' for chemicals and mixtures of little concern (i.e. an abbreviated testing strategy to confirm absence of bioactivity and, hence, negligible risk potential)
- Ban undesired substances (instead of testing them)
- Implement exposure-driven testing and assessment for (complex) substances and mixtures
- Enhance legislative coherence across regulated sectors
- Prioritise substance evaluation based on real concerns

2.2. Combining complexities of novel methods and co-exposures: How to distil for use in risk management in industry and agencies

Heli M. Hollnagel (*Dow, Switzerland; workshop co-chair*) identified four tasks to be addressed to advance the use of NAMs for the hazard and risk assessment of complex substances.

Task 1: Think of combined exposure as the normal. The assessment of complex substances is highly relevant for environmental and human health protection. By comparison, single substance exposure is not even given in an experimental setting since also mono-constituent substances are generally administered as mixtures containing one predominant substance (and further components such as vehicles, impurities, etc.) and always result in combined exposure of the organism to feed, air, medium and the test material. The scientific limitations of the 'single substance assessment model' in predicting real-world exposure scenarios have to be communicated to the public, to regulators and within the scientific community.

Task 2: Identify necessary advancements to enable full exploitation of NAMs. By yielding more data more economically and in shorter-time frames, NAMs can help overcome many technical and scientific limitations of traditional hazard and risk assessment approaches. Such limitations include high animal use and time expenditure for properly designed studies to assess combined exposures, shortcomings in addressing the spatial and temporal variance of real-world exposures including component interactions, and the need to determine individual dose-responses for each toxicological endpoint. To facilitate the full exploitation of NAMs, agreement has to be sought between regulators and regulated on the ultimate aim of risk assessment and when exposure to a (simple or complex) substance can be considered safe.

Task 3: Consider uncertainties – and conservatisms alike. Traditional hazard and risk assessment approaches also have inherent uncertainties in predicting environmental and human health effects. Such uncertainties have to be considered when comparing the outcomes of NAMs to those obtained using traditional methods. The provisions for the acceptance of NAMs should not be more restrictive than those for traditional methods, but should aim at making available 'fit-for-purpose' methodologies and tools. While traditional *in vivo* studies are based on a pre-integrated view of the live animal, NAMs generally only consider one or few details of the complex processes occurring in an organism. QIVIVE and quantitative AOPs can fill the gap between the two by integrating information, and their use should be enhanced to promote the applicability and acceptance of NAMs.

Task 4: Enhance the willingness to accept NAMs. The opportunities in advancing the traditional risk assessment paradigm towards exposure-driven assessments using NAMs, and the implications of failing to do so, need to be communicated both to the public and to experts in academia, governments, authorities, and the private sector. Reservations need to be identified and addressed e.g. by training for new skills and by implementing necessary adaptations of processes and tools.

An important issue to be addressed in pursuing all of these tasks is that the establishment of dose-response relationships remains challenging for complex substances. Many complex substances do not exhibit linear dose-response relationships, predominantly because the biochemical MoAs largely involve non-linear binding and reaction kinetics, and in some cases also because synergistic or antagonistic interactions between different components occur (see Section 4.1; presentation Dr. Benfenati).

When characterising the potential hazards of combined substance exposures, it has to be determined how minor components of a given mixture shall be evaluated. The whole mixture approach (OECD, 2018) aims at reflecting actual effects at given component ratios and at tested concentrations. Uncertainties related to dose-response extrapolations and the sensitivity of test systems can impair the predictivity of test results. Whole mixture approaches are especially useful if the exact composition of the mixture is not known. By comparison, component-based assessments aim at considering all components of the mixture, rendering the assessment of mixtures with unknown (often) minor components difficult, as they may not have been identified or quantified. The outcomes of component-based assessments can be biased by decisions on how to deal with analytes below the detection limit, and their predictivity is often impaired by erroneous assumptions about the underlying dose-response relationships. Most likely, the whole mixture and component-based approaches yield different results when applied for the same combined exposure, and the implications of such divergence have to be addressed.

Importantly, combined substance exposure should be tested at appropriate dose levels. A consensus paper of the US *Society of Toxicology* and the *Society of Environmental Toxicology and Chemistry* (Teuschler et al., 2002) recommended including doses at or below the no-observed effect levels for individual mixture components. With respect to component-based assessments, *"the mixture components that are tested and their relative proportions in the mixture also should reflect those seen in environmental samples. In addition, the impact of the unidentified materials in the mixtures should be considered"* (Teuschler et al., 2002).

Taken together, facilitating the regulatory use of NAMs requires a realignment of the risk assessment paradigm to move away from 'tick-box testing' (Combes and Balls, 2005) and traditional toxicity testing methods. Testing needs should be based on exposure and risk assessment needs; NAMs should be evaluated by their fitness-for-purpose, and the existing methods expanded and enhanced, as necessary. Dr. Hollnagel emphasised that the solutions to most topical policy areas (e.g. circular economy, sustainability, food security, climate protection, novel diseases) include chemicals in one way or the other. Therefore, it is advisable that policy makers consider risk-benefit assessments for industrial chemicals just as these have been implemented for pharmaceutical and other substances.

2.3. Guidance on harmonised methods for human and animal health, and ecological risk assessment of combined exposure to multiple chemicals: An EFSA perspective

Jean-Lou Dorne (*European Food Safety Authority (EFSA), Italy*) introduced the EFSA Scientific Committee (SC) *Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals* (EFSA SC, 2019). This Guidance presents a harmonised framework for risk assessment of combined exposure to multiple chemicals with tiered approaches for both component-based and whole mixture assessments (see Section 2.2; presentation Dr. Hollnagel).

Problem formulation takes on a particular importance in the context of combined exposure to multiple chemicals because the demarcation of the problem is generally more complex than for single substances (Solomon et al., 2016). A dialogue between (eco)toxicologists and exposure assessors is recommended to set up an analysis plan.

Exposure assessment is undertaken in a tiered approach that is primarily based on occurrence data while also considering exposure estimates and consumption data. Often, the occurrence and consumption tiers do not match. In these cases, values may need to be attributed from one tier to another along with other pooling techniques. Hazard assessment can be performed by a component-based approach or by whole mixture approach.

During risk characterisation, the ratio of combined exposure to the quantitative metric for combined toxicity is generated for the given species, population, community, etc. If this ratio indicates that there is no safety concern, the assessment can be concluded. If the ratio indicates a safety concern, additional data may be needed to refine the risk

characterisation or to implement specific risk management measures. Uncertainties are identified in each stage of the framework and an overall uncertainty analysis is integrated in the risk characterisation.

The EFSA SC (2019) Guidance includes three case studies showing how the harmonised framework for risk assessment of combined exposure to multiple chemicals can be applied, i.e. (1) human health risk assessment of combined exposure to hepatotoxic contaminants in food; (2) animal health risk assessment of botanical mixtures in an essential oil used as a feed additive for fattening in chicken; and (3) quantifying interactions for hazard characterisation in worker honey bees.

During hazard assessment, the collection of available data is facilitated by EFSA's chemical hazards database OpenFoodTox (https://www.efsa.europa.eu/en/microstrategy/openfoodtox) that encompasses 10,000 toxicological endpoint studies and 12,000 risk assessment summaries from previously published EFSA scientific opinions, statements and conclusions (Dorne et al., 2017). Further, application of next-generation physiologically-based kinetic modelling provides toxicokinetic information to support grouping and read-across (Paini et al., 2019).

In conclusion, publicly available exposure and hazard data and open-access models are pivotal to support food and feed safety assessments. Research needs relate to the development of specific models to assess risks in farm animals; the refinement of QIVIVE approaches; and the development of *in vitro* approaches to assess inter-individual and population-dependent enzyme isoform variabilities.

3. Session II: Screening, biological profiling, and more complex risk evaluations

3.1. Development of a novel approach for analysing the biological actions of chemicals based on the deep phenotyping method

Tadahaya Mizuno (*Tokyo University, Japan*) presented a novel profile data analysis method, the *Orthogonal Linear Separation Analysis* (OLSA) method, that was developed by a simple modification of factor analysis with principle component analysis. As compared to the traditional, hypothesis-based approach to risk assessment, profile data analysis is a non-targeted approach that is driven by the availability of large amounts of 'omics data. 'Omics technologies allow converting biological response information of a specimen into numeric information that can be analysed with statistical analysis tools and compared to information available in public databases (ontologies). Thereby, the recorded 'omics profile is matched with known patterns of pathophysiological alterations. 'Omics technologies are being used to investigate mechanisms of carcinogenicity and to identify candidate drugs (Kosaka et al., 2013). For many pharmaceutical candidates, the overall effect is composed of a spectrum of more basic effects. For a comprehensive understanding, the overall composite 'omics response has to be decomposed to identify the underlying basic effects. OLSA has been designed to assist in this decomposition of effects (Mizuno et al., 2019). OLSA does not only target gene expression profiles, but response profiles, i.e. effect levels relative to the controls, and it allows detecting slight differences in the effects caused by different chemicals. OLSA allows weighting the basic components of the overall effect and inferring the transcription factors driving specific basic effects (Mizuno et al., 2019).

The applicability of OLSA has been demonstrated using transcriptomics data of cultured MCF7 cells treated with 318 compounds, where OLSA contracted 11,911 genes to 118 basic effects (factors) in a Connectivity Map (https://www.broadinstitute.org/connectivity-map-cmap). Ontological evaluation of the main genes constituting the factors detected significant enrichment of the ontology in 65 of 118 factors. In further analysis of the Connectivity Map, one factor discriminated two Hsp90 inhibitors, whereas traditional clustering analysis did not. Further, the suggested mechanism of cardiotoxicity of topoisomerase inhibitors was correctly predicted. Five novel compounds were predicted to induce autophagy, and further analyses confirmed that four of these compounds actually induced autophagy (Mizuno et al., 2019).

A model case addressing the decomposition of multiple effects of a mixture of two chemicals showed that OLSA allowed determining the effects of each chemical without any data for the individual chemicals. Use of OLSA facilitates an understanding of the MoAs leading to a substance-induced effect. When decomposing effects, OLSA may contribute to detecting latent toxicity (e.g. latent oestrogen-receptor stress inducibility). OLSA can be used to evaluate all types of 'omics data, and the application of OLSA does not require specific hardware. However, large amounts of data

are required to achieve satisfactory results. Otherwise, the OLSA response is split into small response spaces thereby diminishing the chances for detecting unexpected responses.

Furthermore, OLSA uses linear (orthogonal) effect analysis, such that the basic effects are assumed to act independently, whereas it is currently not possible to identify such dependencies as antagonism or synergism. However, many factors are being detected by linear analysis, and their biological annotation is underway; thereafter, non-linear analyses will become meaningful.

3.2. Fate-directed toxicity testing and risk assessment of UVCBs (UVCB-FATETOX)

Philipp Mayer (*Technical University of Denmark, Denmark*) presented the ongoing Cefic LRI project ECO42 *Fate-Directed Toxicity Testing and Risk Assessment of UVCBs* (UVCB-FATETOX; http://cefic-lri.org/projects/eco42-fate-directed-toxicity-testing-and-risk-assessment-of-uvcbs/). The work packages of UVCB-FATETOX focus on combining state-of-the-art dosing methods and analytical techniques with UVCB fate testing; fate-directed ecotoxicity and bioaccumulation testing; and the development of fate-directed hazard and risk assessment approaches for persistent UVCBs.

A major challenge in assessing UVCBs is to achieve defined and maintain constant exposures. For example, during *in vitro* testing the effective exposure concentration is often much lower than the nominal (total applied) concentration due to medium binding, and the exposure may decrease over time due to sorptive and evaporative losses (Birch et al., 2019). Medium binding and loss processes are highly constituent-specific. Therefore, they do not only alter the mixture exposure levels but also the composition of the mixture. Generally, medium binding and sorptive losses increase with increasing octanol-water partition coefficients, whereas evaporative losses are correlated with air-water partition coefficients and with temperature (37° C versus 20° C) (Birch et al., 2019).

Strategies to achieve defined, constant exposures include the avoidance or minimisation of losses by non-depletion testing using gas-tight glass vials instead of open wells or flasks (Mayer et al., 2000); modelling or measurement of the effective exposure; and, importantly, controlling the effective exposure via passive dosing. Passive dosing can be achieved by loading a bio-compatible silicone with the test material (donor) and adding these to the vial or well-plate for controlling freely dissolved concentrations during the test (Smith et al., 2010a, b). While passive dosing was initially used for testing individual chemicals, it has more recently been extended to control the level and composition of increasingly complex mixtures (Schmidt et al., 2013; Birch et al., 2018) and UVCB mixtures (Bera et al., 2018; Hammershøj et al., 2019a). Further, a simple and versatile headspace passive dosing method has been developed that allows assessing hydrophobic and volatile chemicals in aquatic toxicity tests at and below their saturation level (Trac et al., 2019).

As shown for hydrocarbon species of certified reference material diesel, differences in water solubility (aliphatic versus aromatic substances) drive the hydrocarbon patterns in water (determined via two-dimensional gas chromatography (GCxGC)). Passive dosing yielded hydrocarbon patterns resembling the water-accommodated fraction, whereas spiking of the original concentration at regular intervals amplified the more hydrophobic constituents.

The relevance of biodegradation assays can be increased by conducting them at low concentrations that better reflect real-world exposure scenarios (Birch et al., 2018). Recently, Hammershøj et al. (2019b) used passive dosing to vary initial test substance concentration and mixture composition to assess biodegradation kinetics (based on the ratio between biotic and abiotic test systems). When tested at low concentrations, biodegradation kinetics were found to correlate with test substance concentration but not with the number of mixture constituents. The simultaneous testing of several chemicals at low concentrations accelerated the generation of biodegradation kinetics data, while improving the environmental relevance of the findings as compared to assessments conducted with single chemicals at much higher concentrations (Birch et al, 2018; Hammershøj et al., 2019b).

Preliminary findings from an ongoing study addressing two hydrophobic UVCBs (diesel oil and lavender oil) show that passive dosing also allows generating initial UVCB concentrations and that biodegradation kinetics can be followed with GC – mass spectrometry (MS). For one of the UVCBs, reduced biodegradation kinetics were observed at the highest concentration, which coincided with metabolic inhibition. These findings confirm the importance of testing at low concentrations.

With respect to *in vivo* bioaccumulation testing, a key challenge is that hydrophobic UVCBs cannot be

readily assessed using standard flow-through test methods. However, the most recent revision of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 305 (Bioaccumulation in fish; OECD (2012)) states that bioconcentration factors (BCFs) can be derived from a depuration rate constant measured after dietary exposure and an estimated uptake rate constant. For a set of well-studied reference chemicals, the derived BCFs, determined in a benchmarked single-feeding-dose depuration experiment, agreed very well with published literature values. Internal benchmarking of the BCF test with benchmark chemicals with well-characterised BCFs allows correcting for growth dilution and reduces inter-individual variability due to, e.g., variability in the delivered dose of chemicals due to different feeding behaviour (Chen et al., 2018). This experiment also demonstrated that the approach facilitates the measurement of the bioaccumulation potential of multiple constituents in a mixture in one single *in vivo* experiment. In further experiments, constituents of pine oil and cedarwood oil were determined using single-dose dietary exposure to rainbow trout and internal benchmarking.

Dr. Mayer explained how concentrations of the components of a mixture in the donor relate to those in water during passive doing. Passive dosing is based on phase partitioning from a dominant donor into water and has similarities to sediment-water partitioning. Each mixture constituent partitions according to its own partition coefficient. Therefore, the mixture composition in water is not the same as in the donor. The more polar mixture components will be amplified in water, whereas the more hydrophobic components will have the lowest concentrations. However, if equilibrium is achieved in an organism, the mixture composition in lipid tissues will be similar to that in the passive dosing donor.

3.3. Bioactivity-exposure profiles of fruit and vegetable extracts: 21st century approaches for evaluating biological activity, exposures and risks of complex substances

Melvin E. Andersen (ScitoVation, USA) presented the findings from a study comparing the biological activity of extracts from thirty organically grown fruits and vegetables with effect profiles recorded for chemicals included in the USEPA ToxCast program (Wetmore et al., 2019). Prior to testing, all extracts were screened for the presence of agrochemicals and heavy metals (and biological toxins, if relevant). *In vitro* testing of the extracts was performed using the BioMAP® assay platform that includes a panel of eight different human primary cells primed with endogenous pathway activators to identify phenotypic perturbations related to proliferation, inflammation, immunomodulation, and tissue remodelling (yielding a total of 87 BioMAP readouts). The BioMAP reference profile database and predictive informatics tools were used to compare the bioactivity profiles of the extracts with those of the ToxCast chemicals (Wetmore et al., 2019).

Overall, the extracts caused many more BioMAP responses than did individual ToxCast chemicals. Generally, extracts showed varied, frequently composite dose-responses in a concentration- and cell type-dependent manner, indicating that the overall effects were most likely caused by superimposed effects of different compounds present within the extracts. In evaluating dose-response on a molar-basis, the relative extract concentrations causing responses were 10- to 50-fold less potent than were the agrochemicals. Similarly, when testing was constrained to the maximum testing concentrations used with ToxCast chemicals, produce extracts did not show activity in as many assay endpoints. Using intake-adjusted measures of dose, however, the bioactivity potential was much higher for produce extracts than for agrochemicals, consistent with the comparatively small amounts of agrochemical residues present on conventionally grown produce. Some extracts showed significant similarity to specific ToxCast chemicals, e.g. apple extracts showed a similar bioactivity profile to tin (II) chloride, broccoli extracts a similar profile to mitomycin C and mancozeb, and garlic a similar profile to the anthelminthic oxibendazole (Wetmore et al., 2019). The BioMAP responses seen by Wetmore et al. (2019) were consistent with those from another ToxCast assay (Romanov et al., 2005).

Notably, most *in vitro* assays, such as those from the BioMAP panel, do not take metabolism into account. Preparations enabling a certain level of metabolism (e.g. adding metabolically competent cells or microsomes in the testing conditions) or using 'omics to infer activation of metabolically relevant pathways could be added to the assays. However, the value of such adaptations has to be balanced against the challenge that the study design becomes complex and technically demanding. The current BioMAP assay platform is well-characterised, and any alterations will necessitate re-assessing its applicability.

In conclusion, consensus should be sought within the scientific community, regulators and regulated industries on how bioactivity profiles relate to adversity considering that simple perturbations do not necessarily reflect adversity (National Research Council, 2007). Just as recommended for individual compounds, risk context-dependent testing of mixtures using NAMs needs to proceed across different levels. In Level 1, purely computational screening could prioritise possible effects and likely exposures of the mixture for further assessments. In Level 2, high-throughput *in vitro* screening could derive human equivalent, intake-adjusted doses and interrogate possible MoAs. In Level 3, fit-for-purpose *in vitro* mechanism-designed assays would be used to verify and extend information on MoAs and permit estimating intake-adjusted human equivalent doses for major components of the mixture. Lastly, in Level 4, more complex assays would move forward when there were specific concerns about high exposures or about specific toxicity concerns (Andersen et al., 2019).

3.4. Session II Panel Discussion: Screening, biological profiling, and more complex risk evaluations

Facilitators: **Océane Albert** (*Cefic, Belgium*); **Kunifumi Inawaka** (*Japan Chemical Industry Association, Japan*); panellists: **T. Mizuno**, **P. Mayer**, **M.E. Andersen**

Toxicity profiling, using e.g. the OLSA method or the BioMAP panel, are promising tools for the screening of complex substances. Are the results also relevant for regulatory hazard and risk assessment? Which level of confidence is needed to use NAMs in a given regulatory context? For example, while NAMs allow detecting biological activity, how does this relate to adversity in humans or wild-life populations? How should negative screening results be taken forward during risk assessment? – The answers to these questions depend on the particular test material. For many mixtures and MCSs (but generally not for UVCBs), the individual components, as well as their toxicity profiles, are already known. In such cases, the positive or negative results from NAM screening, along with targeted assessments of MoAs for the mixture and major components, should be sufficient for hazard and risk assessment, especially if the findings are consistent with the available evidence on the individual components.

However, if the components of a complex substance or mixture and/or their toxicity profiles are not well-known, screening approaches should primarily be used to prioritise further testing. Benchmarking against reference materials and reference mixtures will serve to increase confidence in NAMs regardless of whether they are used for decision-making or prioritisation. It was proposed to establish a stepwise prioritisation framework for regulators.

Standardisation of NAMs is pivotal to increase the relevance of results and facilitate their applicability. For example, it may be challenging to adequately incubate abiotic and *in vitro* test systems with the complex substances and mixtures. Mandatory steps to improve exposure data in general and confirm *in vitro* levels of exposure to the test materials should be integrated in the study protocols.

Case studies are recommended to show how NAMs can be used for different risk assessment scenarios and to address prevailing knowledge gaps and uncertainties related to the testing of complex substances. Case studies might also be useful to increase confidence in specific results of the NAMs, such as baseline toxicity (minimum toxicity) in aquatic toxicity (see also Escher and Hermens (2002); ECETOC (2013)). If screening tools do not reveal any effects up to the level of baseline toxicity, there should be little or no concern.

Innovative risk assessment approaches should address ecological and human exposure assessment before hazard assessment. If a substance is not released into the environment and/or humans are not exposed externally or internally, the corresponding testing for ecotoxicity, local or systemic toxicity is unlikely to yield results that will be useful for risk assessment.

Workshop participants recommended that all stakeholders should engage in discussions on how exposure-driven priority-based risk assessment approaches could be implemented in a regulatory setting. In this regard, some workshop participants further advised that the notion of 'concern' should be defined. Concern was not seen as a biological concept, but as a 'mental model' so that different stakeholders would have different levels of concern with regard to the use of chemical substances.

4. Session III: Improved methods for using read-across approaches on new data streams

4.1. Strategies to apply in silico models to evaluate mixtures

Emilio Benfenati (*Istituto Mario Negri, Italy*) explained that *in silico* models should address the

challenges related to the hazard and risk assessment of mixtures (e.g. changes in composition over time or in different environments, interaction of mixture components). Importantly, different substances within the mixture can exhibit different behaviours, and it has to be decided how substances and/or behaviours can be clustered. During supervised clustering, the software refers to families and substances within the families as pre-defined by an expert. Unsupervised clustering refers to the application of programmes which automatically split substances into clusters, using structural features.

Generally, *in silico* models for the evaluation of mixtures can predict information on individual compounds (see below Examples 1-3) or enable a direct or stepwise prediction of combined effects and/or compound interactions (Example 4).

4.1.1. Example 1: Botanical extracts: Multiple properties; need for fast, automated processing

A broad spectrum of botanical extracts is available that exhibit very diverse compositions and a large variety of individual substances. Botanical extracts are widely used in cosmetics. In the EU, animal testing is prohibited under the Cosmetic Products Regulation (EP and Council, 2009). Therefore, *in silico* modelling (Fitzpatrick et al., 2018; Toropov et al., 2019; Vian et al., 2019) combined with predictions of thresholds for toxicological concern (Yang et al., 2017) is especially suitable for the evaluation of botanical extracts used in cosmetics. Multiple *in silico* tools should be applied in parallel to increase confidence in the prediction of a specific type of effect.

In silico modelling of botanical extracts allows identifying families of extracts by supervised clustering and characterising their common properties as well as uncommon properties of individual family members. Such clustering does not only rely on structural similarity, but also considers e.g. the presence of specific structural alerts. This increases confidence in the predictions e.g. when used for grouping and read-across. For example, MetaPrint2D-React predicts xenobiotic metabolism through data-mining and statistical analysis of known metabolic transformation (https://omictools.com/metaprint2d-tool). *In silico* models have been applied to predict the properties of approximately 20,000 individual components of botanical extracts (Raitano et al., 2019).

4.1.2. Example 2: Food and feed additives, botanicals: Need for fast, automated processing

In silico models are also available that enable unsupervised clustering of families of mixtures and those family members for which experimental data are available, e.g. istChemFeat (https://chm.kode-solutions.net/products_istchemfeat.php) and the KNIME analytics platform (www.knime.com). The models compare the biological activity of family members and strive to identify the mechanistic reasons for divergences in biological activity.

4.1.3. Example 3: Surfactants: Mixtures with undefined composition: Information as range and key features

Surfactants are mixtures whose components have different numbers of carbons with different levels of saturation. Since many surfactants are used in consumer products (e.g. shampoos) for which down-the-drain release is relevant, they need to be assessed for human health and ecological effects. *In silico* models are available to predict such effects. They address the unknown composition of the mixtures by generating and calculating all possible compositions. Further, *in silico* models must allow considering that one component of the surfactant can influence the properties of another. Frequently, such interactions occur via toxicokinetic processes, for example when one component modifies the skin permeability or metabolism of another component. *In silico* models should also allow simulating how the composition of a mixture changes quantitatively and qualitatively when it is taken up into an organism (external versus internal exposure) or reaches different environmental compartments.

4.1.4. Example 4: The role of MoAs in mixture assessment: Compound interactions

Different *in silico* models have been developed for predicting MoAs related to fish acute toxicity (Toropov et al., 2017), endocrine disruption (Manganelli et al., 2019), genotoxicity (Vian et al., 2019), and skin sensitisation (Toropov et al., 2019). When substances have the same MoA, differences in their biological activity reflect their relative potency. The overall toxicity of a mixture in a given tissue corresponds to the sum of the single toxic units (defined as response per concentration). Three scenarios of this concentration addition model (Loewe and Muischnek, 1926) are possible:

- Additivity: Toxicants are concentration additive;

- Antagony: Toxicants are less than concentration additive;
- Synergy: Toxicants are more than concentration additive.

The 'null model' assumes additivity, i.e. no interaction. Synergisms and antagonisms are defined in relation to it, as upwards or downwards deviations from the null model predictions, respectively.

Recent advancements in the development of *in silico* models of relevance for mixture assessment include machine learning and advanced statistical models (Toropov et al., 2018). Such models allow introducing external knowledge (e.g. on MoAs, metabolism, etc.) to inform the system. If sufficient data (e.g. on toxic units and MoAs) are available in the system, modelling can be undertaken in an unsupervised manner.

Dr. Benfenati also addressed several challenges encountered in developing and applying *in silico* tools:

- Selection of representative chemicals: Since in silico models are generally used for risk assessment, the representative chemicals should encompass those compounds that drive toxicity.
- Prediction of metabolites by in silico modelling: While this is indeed challenging, tools are available some of which are quite sophisticated. Preferably, tools should be used that are freely available and have been integrated into a common platform e.g. VEGA (https://www.vegahub.eu).
- The number of different in silico tools needed to obtain a relevant result: This depends on the purpose of the evaluation and how in silico modelling is embedded in a tiered testing strategy. Importantly, the number of necessary tools depends on the consistency of the outcomes of the modelling. If conflicting data are obtained, further modelling may be needed.
- Challenges in updating *in silico* models as new relevant data become available: Tools are being developed that allow integrating new data 'seamlessly' to prevent the need to re-write the model when new data are integrated. In this regard, software inconsistencies may make it difficult to exchange data between different models.

In conclusion, while exposure to mixtures is the 'normal' real-world scenario, mixture assessment is a complex task requiring complex in silico tools. Challenges with respect to their development relate to data availability, the identification of deviations from concentration addition, and the availability of information on e.g. species differences of effects.

4.2. Navigating towards data-driven read-across approaches: Generalized Read-Across (GenRA), a workflow module within the EPA CompTox Chemicals Dashboard

Grace Patlewicz (*National Center for Computational Toxicology, USEPA, USA*) presented *Generalized Read-Across* (GenRA), a data-driven, harmonised read-across workflow that has recently been made publicly available within the *EPA CompTox Chemicals Dashboard* (Helman et al., 2019; https://comptox.epa.gov/) that covers 875,000 chemicals including property data, integrated biological data for thousands of chemicals, and further relevant information (Williams et al., 2017).

For data-poor substances included in CompTox, GenRA assists researchers in identifying source analogues, or nearest neighbours, and in predicting their toxicity (target chemicals). Predictions are expressed as the similarity-weighted activity of nearest neighbours based on chemistry and bioactivity descriptors. Results are presented as binary outcomes reflecting the presence or absence of toxicity together with quantitative measures of uncertainty. GenRA allows users to identify analogues in different ways, to quickly assess the availability of *in vivo* data for the analogues and to visualise these data in a matrix in order to determine their consistency and concordance before making a GenRA prediction (Helman et al., 2019).

GenRA builds on previously published frameworks for developing category or analogue approaches and for the assessment of read-across (Wu et al., 2010; Blackburn and Stuard, 2014; OECD, 2014; Patlewicz et al., 2015, 2017, 2019). The evaluation of existing frameworks also served to establish how uncertainties of the read-across predictions should be characterised and how GenRA can be linked to other NAMs within IATAs (Webster et al., 2019).

Ongoing research to enhance GenRA includes the development of user-friendly tools to summarise and aggregate the toxicity effect predictions and the inclusion of further information to define and refine the analogue selection (e.g. physico-chemical similarity, metabolic similarity, reactivity similarity). Ongoing research work also aims at introducing dose-response information into GenRA to refine the scope of predictions beyond

binary outcomes, transitioning from qualitative to quantitative predictions of effect levels or points of departure.

4.3. Using NAMs to underpin category and read-across approaches for complex substances under regulatory programmes

Hans Ketelslegers (*Concawe, Belgium*) explained that petroleum substances are UCVBs that can include thousands to millions of molecules. A total of 185 different CAS numbers have been assigned to petroleum substances, that, however, mainly reflect differences in production processes and not necessarily in chemistries.

An abundance of historical data is available on the mammalian toxicity of petroleum substances. Prior to REACH (EP and Council, 2006), these data have been collated e.g. by the *Petroleum High Production Volume Testing Group* in response to the USEPA's High Production Volume Challenge Program (Gray et al., 2013; McKee et al., 2013; Murray et al., 2013a; see also https://petroleumhpv.org) or under the previous EU Dangerous Substances Directive (Council, 1967). The available data show that specific physical properties (boiling point) and chemical structures (specific types of hydrocarbons) can determine the toxicity potential of petroleum substances (Murray et al., 2013b; Roth et al., 2013). Some lighter petroleum substances have the potential to elicit respiratory irritation when inhaled at high concentrations, dermal irritation upon repeated exposure, acute effects on the central nervous system, and chemical pneumonitis if the liquid substances enter the lung (Gray et al., 2013). By contrast, depending on the presence and weight concentration of 3-7 ring polycyclic aromatic hydrocarbons (PAH), some unrefined heavier petroleum substances can elicit dermal carcinogenicity, repeated-dose effects (mainly liver and thymus weight changes), and developmental and reproductive toxicity (Gray et al., 2013).

Concawe has applied these historical scientific insights to build read-across hypotheses and used all historical data to fulfil requirements for relevant endpoints directly and to extrapolate for completion of data gaps via read-across. The historical data are currently being challenged by the European Chemicals Agency (ECHA) that questions the appropriateness of selected exposure routes on account of hazard-based considerations; the quality of non-standard test data; compositional descriptions of the UVCBs; and/or the above-mentioned 3-7 ring PAH hypothesis. Concawe is prepared to defend the historical data while acknowledging that data gaps remain and need to be addressed to comply with the REACH Regulation.

Generally, animal data continue to be the default to fulfil systemic toxicity information requirements. Notwithstanding, the REACH Regulation also prescribes that animal testing may only be undertaken as a last resort. Further, the 3Rs principle to replace, reduce and refine animal testing is implemented in *Directive 2010/63/EU on the protection of animals used for scientific purposes* (EP and Council, 2010). Concawe considers NAMs appropriate tools to minimise animal testing. However, the available NAMs are not always applicable to (petroleum) UVCBs. For example, the *ECHA Read-Across Assessment Framework considerations on MCSs and UVCBs* (ECHA, 2017c) require that "*grouping of substances on the basis of structural similarity must take account of all constituents, and the predictions within proposed groups must likewise consider the impact of all constituents*" (ECHA, 2017c). In practice, this premise is not feasible for UVCBs where, generally, the spectrum of constituents is not fully known (as the name UVCB implies).

To address the challenges in the regulatory assessment of petroleum substances, Concawe has conducted the Cat-App project *New Technologies to Underpin Category Approaches and Read-across in Regulatory Programmes* (www.concawe.eu/cat-app). This project aims at determining the biological activity and toxicogenomic profiles of petroleum substances (tested as extracts) in human cancer and pluripotent stem cell lines. These biological responses will then be correlated to analytical data to find trends and similarities supporting chemical-biological grouping and read-across assessments. The biological fingerprint for each extract is statistically established and visualised (Marvel et al., 2018) striving to group extracts with similar profiles and to rank them by bioactivity profiles as well as in the chemical-biological space. First results indicate that, at a high level, the biological profiles are similar within categories of petroleum substances and distinct between them. However, at a more detailed level, significant variation is also observed within a category and overlap between categories which are 'close' from a petroleum refinery perspective. This is expected, as petroleum substances form a continuum based on their physico-chemical profiles. The findings are further confirmed when the biological profiles are observed in context of the chemical analytical profiles: Across petroleum substance categories, the concentration of 3-7 ring

PAH in substances showed a strong correlation with bioactivity. Additionally, all of the highest expressed genes are involved in PAH metabolism-related pathways. Together, these findings further support the historical toxicological database on petroleum substances and the related PAH hypothesis (Concawe, 2018).

The Cat-App data shall assist in the grouping of petroleum substances. This will help to build intelligent testing strategies in which higher-tier animal testing shall only be performed on representative substances from each group selecting those that are expected to be most responsive in the respective animal test (worst-case). Based thereupon, the data gaps for the other substances of a group shall be filled by read-across, and results will be applied in a conservative approach (worst case classification and labelling following the 'bridging principle' (EP and Council, 2008)).

ECHA and the Member State competent authorities have challenged the Cat-App grouping approach expressing the opinion that it lacks a predictive aspect. However, in line with the 3Rs principle and the state-of-the-art *in vitro* testing, the Cat-App project was not intended to generate data for toxicological predictions (replacement of animal testing), but only to underpin grouping and read-across (leading to reduction). To address the authorities' concerns, the hypothesis that the weight concentration of the 3-7 ring PAH correlates with specific endpoint toxicity (here: developmental toxicity) has been further investigated in a battery of *in vitro* mouse embryonic stem cell assays combined with toxicogenomics profiling (Kamelia et al., 2017). The findings from this study strongly confirmed the hypothesis that developmental toxicity of petroleum substances is related to the concentration and types of PAH present in them, thereby increasing confidence in its scientific relevance and adding a predictive aspect in the selection of a worst-case substance for *in vivo* testing and subsequent read-across. The outcome of the Cat-App project and further NAM efforts of Concawe are being used to draw up a tiered testing strategy for human health risk assessment of petroleum substances which aims at reducing animal testing needs under REACH.

Dr. Ketelslegers concluded his presentation by expressing the hope that the REACH process will be used as an opportunity to make significant progress with the application of NAMs in a regulatory context. Traditional 'tick-box testing' (Combes and Balls, 2005) is applied under REACH on a substance-by-substance basis to assess intrinsic hazard potential. However, this approach disregards exposure potential under real-life conditions. One straightforward way to avoid unnecessary animal testing, as required by the REACH Regulation and Directive 2010/63/EU, is to include real-life exposure data at an early stage in the process and advance the regulatory assessment to a holistic, result-driven approach. Additionally, NAMs can help to further reduce and eventually replace animal testing. To progress this, the acceptance of NAMs for regulatory purposes can be achieved in a step-wise process, starting with their inclusion in informed tiered testing strategies. However, the full exploitation of NAMs will only be possible if academia, industry and regulators join forces to find consensus on an acceptable (and practical) way forward to meet this goal.

4.4. Session III Panel Discussion: Improved methods for using read-across approaches on new data streams

Facilitators: **Robert A. Barter** (*ExxonMobil Biomedical Sciences, Inc., USA*); **Bruno Hubesch** (*Cefic, Belgium*); panellists: **E. Benfenati**, **G. Patlewicz**, **H. Ketelslegers**, **Reinhard Kreiling** (*Clariant Produkte (Deutschland) GmbH, Germany*)

How should structural similarity, specific physico-chemical and fate properties and the results from cell-based assays be weighted to justify grouping and read-across? – Basing the grouping of substances on chemical similarity alone is too restrictive. Further physico-chemical properties should be included as relevant for the scope of the risk assessment. Depending on the exposure scenario, relevant further parameters for grouping can be water solubility, persistence in environmental compartments, etc. Adding biological information from cell-based assays will help to overcome the difficulties in proving similarity between substances where chemical data alone fall short (as is the case for UVCBs). This should be integrated in a chemical-biological approach to grouping and read across.

Which components of a mixture should be considered relevant for grouping and/or be included in extracts (of e.g. petroleum substances) when assessing biological activity in NAMs? – It should be ensured that those components with the highest risk potential are considered during grouping and when assessing extracts, which can be identified using screening assays.

Which obstacles stand in the way to the full exploitation of NAMs? – For every NAM, its relevance, reliability and applicability domain need to be known so that these tools, embedded in integrated testing strategies, serve to minimise animal testing (Henry, 2003). The standards applied for the regulatory acceptance of NAMs, and their data, should not be higher than those applied for the acceptance of the traditional animal tests, and their data. Since integrated testing strategies include both NAMs and the traditional methods, their relevance and reliability also need to be determined.

The regulatory acceptance of NAMs (for the assessment of both complex and mono-constituent substances) does not only require scientific efforts, but also proper communication to disseminate their functionality and added value to all those responsible. The risk assessment of both complex and mono-constituent substances should be advanced from a hazard identification-based approach towards exposure-driven assessments, applied in a holistic way. All workshop participants agreed that international meetings, such as the present ICCA-LRI workshop, provide valuable opportunities to identify and address both scientific knowledge gaps and 'gaps in acceptance'.

5. Session IV: Exposure methods to evaluate fate, transport, and dosimetry

5.1. Mixture Touch: A web platform for assessments of complex mixtures using comprehensive two-dimensional gas chromatography (GCxGC) coupled with mass spectrometry

Yasuyuki Zushi (*National Institute of Advanced Industrial Science and Technology, Japan*) explained that while the legislative concept of risk assessment and management and the development of instrumental analysis tools have evolved in parallel, the application of instrumental analysis tools for regulatory risk assessment is lagging behind. Since the 1950s, risk-based regulations have been implemented in all major jurisdictions (McClellan, 1999). By comparison, MS was invented in 1912, GC-MS for the analysis of single organic substances appeared in the 1960s, and it has been used in environmental studies since the 1970s (Touchstone, 1993). In the 1990s, GCxGC and high-resolution time-of-flight MS (HRTOFMS) were developed and have enabled non-targeted analyses since the 2000s (Zushi et al., 2015, 2016). Currently, efforts are required to introduce non-targeted analysis tools into the regulatory risk assessment of mixtures.

While target analysis uses a selected ion monitoring mode (so that prior information on the target compounds is necessary), non-target analysis allows assessing all ions present in a sample (by using a scan mode). Therefore, non-targeted analysis is especially suitable for the risk assessment of complex substances since it only requires limited information on their components (Krauss et al. 2010; Diaz et al., 2012; Zushi et al., 2014).

An example for a powerful non-targeted analysis tool is GCxGC. GCxGC consists of a linear combination of GC thereby enhancing separation and detection performance when analysing semi-volatile compounds. The outcome of the analysis is provided as two-dimensional map, which is advantageous for visual analysis. GCxGC has been coupled with HRTOFMS to achieve an even more exhaustive characterisation of the test items. Due to the abundance of data obtained with GCxGC-HRTOFMS, specific data processing methods are required to extract relevant data. Data processing methods include target screening tools, such as the T-SEN (*Defined Domain Two-dimensional Peak Sentinel Program*) that allows identifying and visualising target peaks in chromatograms based on a personal database that includes different relevant spectra (Zushi et al., 2013). Using T-SEN, that can also be applied for sequential automatic quantification, target compounds are identified very accurately, even if mixtures are very complex, e.g. sediments without clean-up (Zushi et al., 2014).

A further data processing tool that is applicable for GCxGC-HRTOFMS is non-negative matrix factorisation (NMF) deconvolution (Zushi et al., 2015, 2018) that allows deconvoluting the mass spectrum of each peak into individual spectra, as relevant. The NMF deconvolution tool has been applied for the evaluation of GCxGC-HRTOFMS data from river water samples from the basin of Tokyo Bay (Zushi et al., 2016). The NMF-based deconvolution yielded almost three-fold more peaks than when the National Institute of Standards and Technology library (https://chemdata.nist.gov) was used to identify peaks. Hence, NMF deconvolution enhanced the detection performance of non-target analysis (Zushi et al., 2016). More recently, Linear Free Energy Relationship has been applied to predict the chemical properties of substances evaluated using GCxGC (Nabi et al., 2014; Zushi et al., 2019).

While GCxGC-HRTOFMS and the advanced data processing tools are generally suitable for evaluating mixtures, they are cutting-edge technologies and their application requires a high level of expertise. The web platform Mixture Touch (http://www.mixture-platform.net/Mixture_Touch_open/) has been developed to facilitate the use of advanced data processing tools for the assessment of complex mixtures to allow GCxGC-HTROFMS data to be analysed and interpreted by non-expert users. Exemplarily, Mixture Touch has been applied to evaluate short-chain chlorinated paraffins. The separation patterns presented by Mixture Touch were similar with those from previous GCxGC assessments. Mixture Touch also provided information on e.g. the long-range transport potential and terrestrial bioaccumulation potential of the short-chained chlorinated paraffins (Fenner et al., 2005; Korytár et al., 2005). Recently, the environmental fugacity model (Mackay et al., 1996) has been integrated into Mixture Touch to provide information on the 'escaping tendency' of a chemical in a mixture in the environment.

Remaining uncertainties in the application of GCxGC-HRTOFMS and the advanced data processing tools for mixture assessment include variabilities in the estimations, e.g. statistical errors caused by the modelling and instrumental error. These uncertainties are accessible by error propagation. Further, it is currently not possible to address the dynamics of mixture effects in the environment.

5.2. Non-targeted analysis research at the USEPA and related topics

Annette Guiseppi-Elie (*USEPA, Office of Research and Development (ORD), USA*) presented current research work undertaken at the USEPA ORD. The ORD serves to provide a scientific foundation for the USEPA's mission to protect human health and the environment. Further, the ORD makes available scientific evidence to support federal and regional work related to e.g. chemical safety; sustainable and healthy communities; and safe and sustainable water resources. UVCBs are considered in a number of these activities.

The 2016 update of the Toxic Substances Control Act (US Government, 2016) requires a risk-based evaluation of existing and new chemicals. In this regard, the National Academy of Sciences, Engineering and Medicine (2017) envisions the use of computational exposure data to translate high-throughput data into risk-based chemical priority-setting. The ORD contributes to meeting these goals by developing high-throughput screening tools for predicting exposure and hazard potential. Further, the ORD research related to the exposome provides valuable data to support the risk-based evaluation of chemicals.

At the ORD, non-targeted analysis (with a focus on MS) is used for a broad spectrum of purposes including exposure surveillance to identify chemicals present in water, products, dust, body fluids, etc. (Phillips et al., 2018); the prioritisation of substances and mixtures (Rager et al., 2016); exposure forensics to identify chemical signatures of exposure sources; and the discovery of relevant biomarkers in model organisms (Catron et al., 2019).

In 2015, the USEPA initiated the *Non-Targeted Analysis Collaborative Trial* (ENTACT) to evaluate the ability of non-targeted analysis tools to consistently and correctly identify unknown chemicals in samples of e.g. water and soil (Ulrich, 2019). The project aims at establishing the chemical space of the different tools as well as their sensitivity, accuracy and inter-laboratory variability. Further, it shall be determined how sample complexity affects performance.

As a part of ENTACT, 1269 unique ToxCast substances were combined to produce 10 synthetic mixtures containing 95-365 substances each (Sobus et al., 2019). Blinded non-targeted analysis was performed on each mixture using liquid chromatography coupled with high-resolution MS, followed by an unblinded evaluation to identify limitations of the applied tools. Overall, at least 60% of spiked substances could be observed using selected methods. An overall reproducibility rate of 75% was recorded for substances spiked into more than one mixture. However, there was considerable discordance in substance identification when comparing a subset of the results derived from two separate reversed-phase chromatography methods. Sobus et al. concluded that single non-targeted analysis methods, even when optimised, can likely characterise only a subset of ToxCast substances and, hence, also of 'contaminants of emerging concern' (Sobus et al., 2019). Further experiments with standard reference method dust are ongoing.

In conclusion, 21st century exposure science requires NAMs, including higher-throughput monitoring techniques. Experiments using non-targeted analysis tools for top-down exposomics (based upon biomonitoring) or bottom-up exposomics (based upon environmental sampling (Rappaport, 2011)) are shedding light on novel

chemicals that might be relevant for exposure assessments. Thereby, they present a viable 'first-pass' monitoring option. However, tools and instruments must be selected and implemented with care, and the results should be interpreted as provisional exposure data. Further efforts are needed to establish reference methods. Overall, the successful implementation of non-targeted analysis tools for exposure assessment requires a collaborative team approach.

5.3. Ecological risk assessment of UVCBs under the Canadian Chemicals Management Plan

Marc Fernandez (*Environment and Climate Change Canada (ECCC), Canada*) shared experiences in regulating UVCBs within the Chemicals Management Plan implemented under the Canadian Environmental Protection Act (Government of Canada, 1999).

UVCBs are considered single substances under Canadian Environmental Protection Act. Challenges in the ecological risk assessment of UVCBs include fit-for-purpose substance identification and characterisation to describe the identity and proportion of individual components. Enhanced knowledge of UVCB composition is derived from in-house data collection, voluntary surveys within industry, and enumeration and modelling approaches e.g. as described by Kutsarova et al. (2019).

It is especially challenging that the presence of specific components of a UVCB in a given environmental compartment can be affected by their physico-chemical properties (e.g. water solubility or vapour pressure) which may be affected by the co-presence of components. This should be considered during exposure characterisation as the original composition of a UVCB can change when it is released into waste streams. Since monitoring involves discrete substances, it is complex and potentially ambiguous to link monitoring data to a released UVCB. It can be difficult to match component concentrations in the environment to ecological toxicity data that are generally obtained for the entire UVCB. Often, UVCB exposure and fate can only be modelled, using both available data and assumptions on the specific composition of the UVCB.

Due to such complexities of UVCB characterisation, exposure and hazard assessment, complex risk assessment results need to be effectively communicated to stakeholders for comment and to implement adequate risk management measures when and where required. Information or evidence may be available for both the UVCB as a whole and (some of) its components. It may be practicable to present the outcome of the risk assessment in the form of a weight-of-evidence framework that helps combine these different types of evidence in a logical and transparent manner.

Dr. Fernandez referred to the hitherto unpublished conclusions from a European Centre for Ecotoxicology and Toxicology of Chemicals / Research Institute for Fragrance Materials workshop *Developing a Strategy to Improve the Environmental Risk Assessment of Difficult to Test Multi-Component Substances* (http://www.ecetoc.org/event/developing-strategy-improve-hazard-risk-assessment-difficult-test-multi-component-substances/). The participants from that workshop concluded that a tiered or multi-pronged approach for the ecological risk assessment of UVCBs is needed. While there have been few modifications to standard test guidelines to make them suitable for reliable testing of UVCBs, all testing should ensure that the concentrations of the key or main UVCB components represented in the test matrix are identified and controlled. For non-petroleum organic UVCBs, an approach based on representative chemicals, consistent with the hydrocarbon block method (Comber et al., 2014), might be suitable. Representative chemicals should be chosen as single (lead) chemicals or several chemicals that conservatively represent a subclass (e.g. single MoA) or fraction of the UVCB.

The representative chemical approach provides one or more chemical structures for modelling and/or empirical data collection to complement whole substance information in a weight-of-evidence approach. The ECCC has developed a representative chemical selection tool to assist in the conservative selection of representative chemicals for each subclass within a UVCB. The tool ranks intra-class bioavailability, persistence and ecotoxicity of the UVCB constituents and can be integrated with hazard and exposure predictions (Fernandez et al., 2017a, b). MoAs are identified using a weight-of-evidence approach, usually by using a combination of MoA modelling predictions along with any relevant and reliable sub-lethal toxicity data.

Dr. Fernandez presented preliminary findings from a UVCB mapping exercise conducted by the ECCC. The mapping exercise aimed at identifying major chemical classes of organic UVCBs in order to focus further work on the most relevant UVCB

classes within the tripartite *Health and Environmental Sciences Institute Sub-committee on Ecological Risk Assessment for Multi-component Substances / UVCBs* (https://hesiglobal.org/committees/uvcb/). The mapping exercise included approx. 400 organic UVCBs that were a subset of approx. 4,000 existing substances meeting the criteria for further regulatory action under the Chemicals Management Plan. UVCBs were mapped by chemical structure and/or functional class. The UVCB groups (i.e. categories) were charted based on total number of UVCBs in each group and total import / manufacture volumes (kg/year) of the UVCBs included in each group (Table 1).

Table 1: UVCB mapping exercise: Substance number and volume cross-walk[1]

Ranking	Total Grouping Volume Imported/Manufactured per Year	Number of Substances per Grouping
1	Sulfite liquors and cooking liquors	Terpenes and terpenoids
2	Linear and branched alkylbenzene sulfonic acids and derivatives	Quaternary ammonium compounds
3	Individual (not possible to group)	Resins and rosins
4	Alkanes, alkenes, and alkynes	Fatty acids and salts
5	Fatty acids and salts	Aliphatic amines
6	Fatty amides	Linear and branched alkylbenzene sulfonic acids and derivatives
7	Arenes	Individual (not possible to group)
8	Terpenes and terpenoids	Pigments and dyes
9	Resins and rosins	Naphthenic acids and their salts
10	Quaternary ammonium compounds	Fatty amides

The 'top ten' groups from the mapping exercise showed a range of different groups in terms of chemistries, functionality, and sectors involved. Each of these groups may have different challenges associated with risk assessment (e.g. level of complexity, variation and uncertainty in composition, presence of poorly soluble, ionogenic or surface-active components, single or multiple MoAs). Understanding the range of complexity, uncertainty and variation in the components and other challenges of the main UVCB groups will help tailor future testing thereby improving the hazard and risk assessment in a realistic and relevant direction.

In summary, the first step of the mapping exercise has served to identify examples of the types of UVCB groups (by volume and number of substances) that may be identified as priorities by regulators and their associated challenges in performing hazard and risk assessment. Based thereupon, the next step of the mapping exercise will be to focus efforts on devising the best approach to risk assessment for typical UVCBs of concern in general. The key data streams and parameters, that are especially important for the weight-of-evidence risk assessment framework are fate, persistence, bioaccumulation, exposure, toxicity and risk quotients of representative components of the UVCB and any applicable whole substances data. Dr. Fernandez also noted that the human health risk assessment approach is outside ECCC's mandate, and that fate and exposure are addressed differently in ecological versus human health risk assessment. Therefore, the weight-of-evidence risk assessment framework as described in this presentation only applies to ecological risk assessment.

5.4. Current EU activities on combined exposure and human biomonitoring

Stephanie K. Bopp (*European Commission, JRC, Italy*) presented current EU activities on combined exposure to chemicals and complex substances and related human biomonitoring initiatives. In 2015, the JRC conducted an expert survey inquiring current practices and experiences with assessing effects and risks from combined exposure. One of the questions showed that experts in the area clearly identified complex substances (e.g. UVCBs and

[1]Disclaimer: The mapping exercise was conducted for the purposes of discussion and method development. It combines data across the groups of UVCBs from the Domestic Substances List (DSL) nomination (1986) to 2011. It does not necessarily represent current priorities of ECCC or Health Canada, nor does it reflect an accurate estimate of the current production volume or use profiles for these UVCBs in Canada. Confidential business information has been masked or removed from the data as appropriate.

MCSs) under REACH as highest priority for risk assessment that needs to take mixture effects into account compared to other sectors (Bopp et al. 2015). JRC activities in the area of combined exposure to multiple chemicals build on past activities of the European Commission (Kortenkamp et al., 2009; SCHER, SCENIHR, SCCS, 2011). In particular, they support operational follow-up actions proposed in the Commission Communication *Combination Effects of Chemicals* (European Commission, 2012), i.e. to improve the understanding of how humans and the environment are exposed to mixtures and to examine opportunities for addressing knowledge gaps related to the MoAs of chemicals, the grouping of chemicals, the identification of those compounds that drive mixture toxicity, and the prediction of compound interactions (European Commission, 2012).

The Commission Communication also required promoting a more coherent approach to the generation, collection, storage and use of chemical monitoring data. In meeting this request, the *Information Platform for Chemical Monitoring* (IPCHEM; https://ipchem.jrc.ec.europa.eu/) has been established as reference access point for searching and accessing chemical occurrence data and to fill prevailing knowledge gaps on the environmental and human health implications of chemical exposure. IPCHEM includes modules covering human biomonitoring data; environmental monitoring data; food and feed monitoring data; and product and indoor air monitoring data. By providing a consistent data reporting format, IPCHEM also enhances data quality and comparability. IPCHEM has been designed to be interoperable with other information systems and web services to allow establishing a more comprehensive knowledge framework on chemical exposure.

To date, a systematic and integrated approach for the assessment of mixtures is unavailable (Kienzler et al., 2014; Evans et al., 2016; Bopp et al., 2019). Bopp et al. (2018) have summarised current EU research activities on combined exposure to multiple chemicals that aim at closing this gap:

EDC-MixRisk (https://edcmixrisk.ki.se) focused on integrating epidemiology and experimental biology to identify endocrine disruptor mixtures with adverse human health outcomes and the underlying mechanisms of effects. The findings from EDC-MixRisk shall be used to develop a framework for integrating epidemiological and experimental data to improve the risk assessment of mixtures.

EuroMix (https://www.euromixproject.eu) aimed at developing a tiered strategy (toolbox) for the risk assessment of chemical mixtures (including *in silico* and *in vitro* tools to assess toxicokinetics, hazard, exposure, and risk). The platform is available and several stakeholder groups have been trained and have tested it in case studies.

HBM4EU (https://www.hbm4eu.eu/) serves to coordinate and advance human biomonitoring in Europe, to generate evidence for causal links between (external and internal) exposure and adverse health outcomes, and to facilitate use of human biomonitoring data during chemical risk assessment. HBM4EU includes a specific work package on mixtures. Generally, human biomonitoring data are important for mixture assessments since they provide evidence for internal co-exposure integrated over time and for exposure sources, routes, and pathways (Evans et al., 2016). These data are useful for both aggregate and combined exposure assessments and support the identification of priority mixtures. Human biomonitoring data should be made available at the level of individuals (also from highly exposed and vulnerable sub-groups) with as little aggregation as possible to reflect real co-exposure. Additional contextual information on external exposure concentrations (e.g. in food or consumer products) can support the interpretation of internal concentrations and can also assist in the development and validation of physiologically-based kinetic models.

Solutions (https://www.solutions-project.eu) aimed at new and improved tools, models, and methods to support decisions in environmental and water policies. It developed holistic monitoring, modelling and assessment methods and tools for prioritising mixtures. To better address chemicals of emerging concern, it worked on the integrated use of effect-based tools, effect-directed analysis, non-target chemical screening, and models for spatially and temporally explicit exposure and risks.

EU-ToxRisk (http://www.eu-toxrisk.eu) is an integrated European flagship programme driving the development of animal-free, mechanism-based IATAs that include read-across, *in silico* and *in vitro* tools. The focus lies on assessments of repeated-dose toxicity in the kidney, liver, lung, and nervous system as well as developmental and reproductive toxicity. While EU-ToxRisk does not specifically address mixtures, the developed tools are expected to support their assessment.

In conclusion, prevailing difficulties and uncertainties in mixture assessment relate to

incomplete information on their composition; the determination of external versus internal concentrations; temporal aspects of exposure to mixtures; emerging chemicals (that can be addressed by non-targeted analysis and modelling), and, finally, non-detects (that can be addressed by worst / best case assumptions). Overall, improved data sharing, the complementary use of monitoring data and modelling, establishing links between external and internal exposure and human biomonitoring (just as monitoring in wildlife) will serve to enhance mixture assessments.

5.5. Modelling tools for predicting exposure, fate, and effects for complex petrochemical mixtures and UVCBs

Aaron Redman (*ExxonMobil Biomedical Sciences, Inc., Belgium*) presented challenges related to the exposure assessment and prediction of fate and effects of complex petrochemical mixtures and UVCBs, and suggestions for how these challenges could be met.

Challenges with respect to the exposure assessment of UVCBs relate to their variable composition and the circumstance that the physico-chemical properties of constituents vary with carbon number and chemical class. The distribution of UVCBs across environmental compartments varies depending upon the physico-chemical properties of the constituents. These challenges can be met by using multimedia modelling frameworks to predict the exposure to blocks (defined by small carbon number or boiling point interval and chemical class). Modelling frameworks include e.g. the spreadsheet tools PETRORISK (https://www.concawe.eu/reach/petrorisk) for environmental risk assessments for petroleum substances and PETROTOX (https://www.concawe.eu/reach/petrotox) to calculate the toxicity of petroleum products to aquatic organisms (Redman et al., 2014, 2017).

Predictions of environmental fate include predicting degradation in key environmental compartments (e.g. soil, air, water, sediment). However, in the actual environment, these compartments are not strictly separated, but interact, e.g. via clouds and wind. Predictions of fate can be improved by developing mechanistic biodegradation models using empirical data, provided that high-quality biodegradation data are available.

Challenges for hazard and risk assessment relate to the development of mechanistic QSARs to allow assessing multiple constituents with different physico-chemical properties (e.g. water solubility) and variable bioavailability (Cailleaud et al., 2019). The currently available modelling approaches assume a given MoA and use concentration addition for mixtures. During risk assessment, extrapolations are based on conservative predicted no-effect concentrations and, for comparison, to conservatively estimated emissions and exposures.

Specific challenges in using *in vitro* test systems for the environmental hazard assessment of UVCBs also relate to exposure, fate and toxicity. Exposure-related challenges include the need to determine the effective *in vitro* concentration, but also difficulties in ensuring stable *in vitro* exposure levels which, in turn, compromise QIVIVE. Fate-related challenges refer to the circumstance that chemicals continue to degrade both under real-world and experimental conditions. Further, macromolecules present in the test media can absorb test substances thereby reducing the proportion available to interact with the test system. Similarly, volatile substances are difficult to test *in vitro* (Fischer et al., 2018; Birch et al., 2019). Passive dosing is a means to address this challenge (see Section 3.2; presentation Dr. Mayer).

The interpretation of *in vitro* data to predict endpoint-specific toxicity of UVCBs is challenging also because most chemicals elicit baseline toxicity (unspecific cytotoxicity). Substance-specific effects evolving by a specific MoA need to be distinguished from such baseline toxicity (Klüver et al., 2016; Escher et al., 2019). Nevertheless, when testing conditions are well-defined and *in vitro* concentrations reflect *in vivo* concentrations, the *in vitro* data may be useful to predict *in vivo* effects. For example, the *in vitro* potencies of a spectrum of petroleum substances with different weight concentrations of 3-7 ring PAHs determined in the mouse embryonic stem cell test correlated well with available rat prenatal developmental toxicity data (Kamelia et al., 2017).

In summary, understanding the composition of complex substances, such as petroleum UVCBs, is pivotal for risk assessment. The selection of relevant constituents and fractions (such as the hydrocarbon block) of the UVCBs provide a sound basis for the evaluation. A consistent evaluation of effective exposures of *in vitro* systems is key to improving a broader utility of the *in vitro* data.

5.6. Session IV Panel discussion: Exposure methods to evaluate fate, transport, and dosimetry

Facilitators: **Athena M. Keene** (Afton Chemical, USA); R**obert Skoglund** (Covestro LLC, USA); panellists: **Y. Zushi, A. Guiseppi-Elie, S. Bopp, A. Redman**

Is the calculation of the Hazard Index (defined as the sum of each chemical component's Hazard Quotient, i.e. exposure/safe dose (OECD, 2018)) a useful tool for the evaluation of combined exposure to multiple chemicals? – It is a limitation of the Hazard Index approach that it does not account for interactions between different substances. Further, and as also stands true for most other tools, the validity of the predictions depends on the quality of the underlying data.

If non-targeted analysis tools are used for the assessment of mixtures in environmental samples, how are exogenous (synthetic) substances distinguished from endogenous (non-synthetic) substances? – This is indeed challenging also because the proportion of exogenous substances can be very small.

How should mixtures be prioritised for risk assessment, e.g. based on exposure or hazard? – There is no consensus on how mixtures should be prioritised. Notwithstanding, the prioritisation depends upon the context of the risk assessment. A possible option would be to select those chemicals that a major proportion of the human population is exposed to and to evaluate e.g. the *in vitro* bioactivity of combinations of these chemicals. For example, the findings from the EDEN and ELFE cohort studies, that aimed at identifying the major substances that pregnant mothers are exposed to in France (Traoré et al., 2018), help prioritise combinations of substances (mixtures) for hazard assessment. Similarly, investigations on the exposome, especially when linked with the AOP concept (Escher et al., 2017), may assist in the prioritisation of mixtures. It is a limitation of some epidemiological studies that they provide occurrence data, but do not inform on the concentrations of the substances that the individuals were exposed to. In addition, exposure levels can vary considerably at different time points. Steady-state levels of bioaccumulative substances integrate temporal and spatial variation in environmental exposures. In contrast, for rapidly cleared substances, the same individuum or environmental medium may have to be monitored many times to inform on the temporal variation of exposure and the true average internal exposure or maximum peak exposures. Further, for many test methods, the sensitivity (detection limit) drives the investigations, whereas hazard assessment should be driven by the thresholds of toxicological concern (risks). Holistic, effect-based tools (e.g. to monitor the chemical status of water bodies), such as the ones proposed within the EU project Solutions (see Section 5.4; presentation Dr. Bopp) or the NORMAN Network on Emerging Pollutants (https://www.norman-network.net), can assist in the prioritisation of the risk assessment of substances in mixtures (Brack et al., 2018).

6. Session V: Facilitated discussion: Challenges and opportunities for implementing NAMs

Facilitator: **M. Andersen**; panellists: **A. Guiseppi-Elie**, **H. M. Hollnagel**, **P. Mayer**, **Erik Lebret** (*National Institute for Health and the Environment, and Utrecht University, Netherlands*)

NAMs can play a unique role in facilitating the exposure, hazard and risk assessment of complex substances and mixtures. However, prevailing scientific and conceptual challenges need to be solved to enable their full exploitation. These challenges should be addressed in view of the ultimate goal to ensure proper risk management for complex substances and mixtures.

Research work to enhance the applicability of NAMs should include the refinement and standardisation of approaches and procedures:

- To enable fit-for-purpose identification of complex substances and mixtures (and of representative components, if relevant);
- To ensure defined and constant exposure levels;
- To evaluate the potential interaction of compounds in mixtures with large numbers of constituents;
- To perform QIVIVE.

Concerted action is necessary to integrate NAMs into tiered risk assessment frameworks for complex substances and mixtures. Such frameworks should make use of all types of NAMs, i.e. *in silico* modelling, *in vitro* assays, 'omics, and grouping and read-across. NAMs should both be used to inform bioactivity potential and likely MoA of the test substance. The integration of NAMs into tiered risk assessment frameworks will allow minimising animal testing. *In vivo* studies will be restricted to the higher-tiers where only a limited number of substances will be evaluated (e.g. representative UVCBs from within a group or UVCBs identified as bioactive in the lower tiers), further focussing the types of *in vivo* studies on the likely MoAs.

The implementation of a tiered risk assessment framework for complex substances and mixtures does not only necessitate research work, but also a shift in the risk assessment practice. To provide an incentive for such a paradigm shift, the limitations of the traditional methods need to be communicated. Such limitations include high animal use and time expenditure for properly designed studies to assess combined exposures, shortcomings in addressing

the spatial and temporal variance of real-world exposures, and the need to determine individual dose-responses for each toxicological endpoint. Further, *in vivo* studies only enable targeted investigations, whereas non-targeted analysis, as enabled by different NAMs, is more appropriate for a comprehensive evaluation of complex substances whose spectrum of components may not be fully known.

The paradigm shift for risk assessment should place a focus on exposure-driven testing and assessment so that only such data are collected that are relevant for risk assessment and management. Such a paradigm shift has also been recommended following an international workshop convened among senior leaders from regulatory agencies including the USEPA, ECHA and Health Canada (Kavlock et al., 2018). Case studies were announced "*to explore new ways of describing hazard (i.e., pathway perturbations as a measure of adversity) and new ways of describing risk (i.e., using NAMs to identify protective levels without necessarily being predictive of a specific hazard)*" (Kavlock et al., 2018).

Similarly, case studies should be initiated to put the tiered risk assessment frameworks for complex substances and mixtures into practice. The selection of complex substances and mixtures for these case studies can be based upon e.g. presumption of environmental or human health effects or exposure potential (related to workplace exposure, consumer exposure; contaminated sites, etc.). Similarly, case studies can be selected by their relevance for topical issues (e.g. circular economy, sustainability of the water cycle). To begin with, evaluations should focus on combinations of representative substances, reflecting real-world exposures as far as technically possible, and ultimately aim at investigating the exposome.

7. Wrap-up of the workshop

The workshop co-chairs **E. Berggren** and **H. M. Hollnagel** closed the workshop thanking all participants for their valuable contributions. The discussions had identified the need for follow-up action to facilitate the use of NAMs for the risk assessment of complex substances (and of combined exposure to different substances) and opportunities to address prevailing knowledge gaps, e.g. by specific research action within LRI programmes. Further, the workshop had confirmed the unique value of the international multi-stakeholder setting to enhance the dissemination of relevant information, and the established networking would be carried forward in future events.

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10. Glossary

Analogue approach: During grouping (see definition), the analogue approach is employed between a few, very structurally similar substances for which it is not possible to establish a trend or a regular pattern (ECHA, 2017c).

Adverse Outcome Pathway (AOP): A logical sequence of key events triggered by chemical exposure and occurring at the molecular, cellular, organ, whole organism or population level (OECD, 2016b).

Category approach: During grouping (see definition), the category approach is employed between several substances that are grouped together based on defined structural similarity for one or more (toxicological or other) properties (ECHA, 2017c).

Combined exposure: Exposure from multiple sources, e.g. indoor air, emissions and waste, food, medicine, products of daily use (Bopp et al., 2015).

Exposome: All life-course environmental exposures (including lifestyle factors) from the prenatal period onwards (Wild, 2005; Rappaport, 2011).

Fraction (of a UVCB): An analytically separated fraction of its constituents, with similar polarity, functional group(s), molecular weight, etc. (ECCC, 2016).

Grouping (of chemicals): The general approach for considering more than one chemical at the same time (OECD, 2014).

Mode-of-action: The biologically plausible sequence of substance-specific key events, starting with exposure and proceeding through the interaction of the substance or its metabolites with a cell, through functional and anatomical changes leading to an observed effect supported by robust experimental observations and mechanistic data (Sonich-Mullin et al., 2001; Boobis et al., 2009; Fenner-Crisp and Dellarco, 2016).

Multi-constituent substance: A substance with more than one constituent, with each main constituent encompassing ≥ 10 weight%, but < 80 weight%; each main constituent is completely identified by IUPAC name, and typical minimum and maximum concentrations of each constituent are reported in the composition. The generic name format for a multi-constituent substance is "reaction mass of [main constituent 1] and [main constituent 2] and ..." (ECHA, 2012b).

New approach methodology (NAM): Any *in silico*, *in chemico* or *in vitro* technique that may provide data or information that could support regulatory decision making (ECHA, 2016).

Integrated Approaches to Testing and Assessment (IATA): Pragmatic, science-based approaches for chemical hazard or risk characterisation that rely on an integrated analysis of existing information in a weight of evidence assessment coupled with the generation of new information using testing strategies (OECD 2016b).

'**Omics**: The study of systemic genome responses to substances in cellular systems or whole organisms, including genomics, transcriptomics, proteomics, metabolomics, and epigenomics. 'Omics studies include data generation and storage, data processing and statistical analysis, and computational data interpretation using databases to match e.g. the recorded gene expression profile to specific pathophysiological patterns (Sauer et al., 2017).

Read-across: The approach to fill a data gap whereby a chemical with existing data values is used to make a prediction for a 'similar' chemical (Patlewicz et al., 2017).

Source analogue: A chemical that has been identified as an appropriate chemical for use in a read-across based on similarity to the target chemical and existence of relevant data.

Subclass (of a UVCB): A group of structurally similar components of the UVCB that ideally possess similar physico-chemical properties, bioavailability, persistence and ecotoxicity (ECCC, 2016).

Target chemical: A chemical which has a data gap that needs to be filled i.e. the subject of the read-across.

UVCB (substance of unknown or variable composition, complex reaction products or biological materials): A substance that cannot be sufficiently identified by its chemical composition, because (1) the number of constituents is relatively large and/or (2) the composition is, to a significant part, unknown and/or (3) the variability of composition is relatively large or poorly predictable (ECHA, 2012a). UVCBs include substances obtained from oil or oil-like sources; variations in carbon chain length, and enzymes (ECHA, undated).

Whole mixture approach: The whole mixture approach to combined exposure assessment considers a group of substances as if they were a single unit, with the assumption and limitation that the components and concentrations of the mixture do not vary significantly across individuals, over time, or between exposure routes, and that toxicity studies are conducted on the whole mixture (OECD (2018) citing National Academy of Science (2008); USEPA (2007); and ECETOC (2011)).

11. Abbreviations

AOP: Adverse outcome pathway; BCF: Bioconcentration factor; Cefic: European Chemical Industry Council; ECCC: Environment and Climate Change Canada; ECHA: European Chemicals Agency; EFSA: European Food Safety Authority; ENTACT: Non-Targeted Analysis Collaborative Trial; GCxGC: Two-dimensional gas chromatography; GenRA: Generalized Read-Across; HRTOFMS: High-resolution time-of-flight mass spectrometry; IATA: Integrated approach for testing and assessment; ICCA: International Council of Chemical Associations; IPCHEM: Information Platform for Chemical Monitoring; JRC: Joint Research Centre; LRI: Long-range Research Initiative; MCS: Multi-constituent substance; MoA: Mode-of-action; MS: Mass spectrometry; NAM: New approach methodology; NMF: Non-negative matrix factorisation; OECD: Organisation for Economic Co-operation and Development; OLSA: Orthogonal Linear Separation Analysis; ORD: Office of Research and Development; PAH: Polycyclic aromatic hydrocarbons; QIVIVE: Quantitative *in vitro* to *in vivo* extrapolation; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals; SC: Scientific Committee; SEURAT: Safety Evaluation Ultimately Replacing Animal Testing; USEPA: United States Environmental Protection Agency; UVCBs: Substances of unknown or variable compositions, complex reaction products and biological substances.

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Note: All websites were accessed in July 2019.

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