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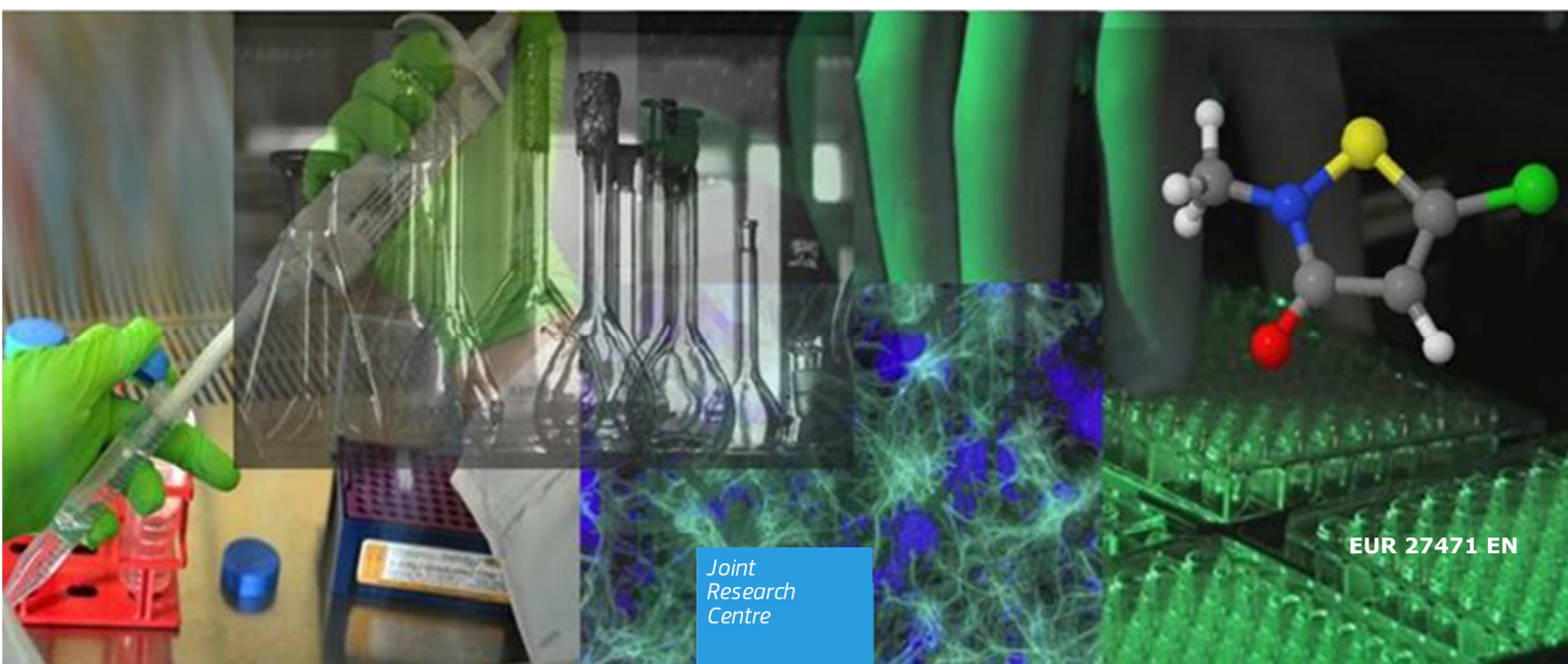
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Scientific methodologies for the assessment of combined effects of chemicals – a survey and literature review

Use of novel and alternative methods in the assessment of effects from combined exposure to multiple chemicals

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Table of contents

Abstract

Exposure of humans and wildlife to chemicals via food, consumer products, the environment etc. can imply exposure to an infinite number of different combinations of chemicals in mixtures. It is practically impossible to test all these possible mixtures experimentally and it is therefore needed to find smart strategies to assess the potential hazards using new tools that rely less on *in vivo* testing and incorporate instead alternative experimental and computational tools. In this report the current state of the art for the application of these alternative tools for assessing the hazard of chemical mixtures is briefly reviewed. The focus is hereby on the adverse outcome pathway (AOP) concept, *in vitro* methods, omics techniques, *in silico* approaches such as quantitative structure activity relationships (QSARs) and read-across, toxicokinetic and dynamic energy budget (DEB) modelling, and on integrated approaches to testing and assessment (IATA).

Furthermore, an expert survey was performed to collect up to date information and experience on the current use of different approaches for assessing human and environmental health risks from exposure to chemical mixtures, with a view to informing the development of a consistent assessment approach. An online survey was performed among experts in the field of combined exposure assessment in the period of January to March 2014, addressing both, human health and environmental risk assessment. Fifty-eight experts from 21 countries, different stakeholder groups and sectors of legislation participated in the survey. The main sectors where most experience is already gained in assessing mixtures are in the area of plant protection products and chemicals under REACH. These were also rated highest regarding the priority for performing mixture assessments. Experts have experience with the whole mixture as well as the component-based approaches applying them to both, intentional and unintentional mixtures. Mostly concentration addition (CA) based methods are used for predicting mixture effects. Regarding the use of novel and alternative tools in the risk assessment of mixtures, expert opinions are split between those applying them (often more in a research context) and those that generally think these tools are valuable but their use is currently limited because of lack of guidance, lack of data, or lack of expertise. A general need for clear guidance for combined exposure assessments was identified.

Overall, a high potential in applying novel tools and scientific methodologies for the assessment of chemical mixtures can be identified. They allow deriving meaningful information on individual mixture components or whole mixtures, enabling a better understanding of the underlying mechanisms of mixture effects. Their main strengths lie in their integrated use and smart combination to put different aspects regarding the hazard from combined exposure to multiple chemicals into context. In order to benefit from these tools in the hazard assessment of mixtures, more guidance on their use is needed to facilitate a more widespread application.

1. Introduction

Humans and the environment are continuously exposed to a multitude of substances via different routes of exposure. Some of these chemical mixtures are intentional and thus have a known composition, e.g. personal care products, food additives and pesticide formulations. However, in many cases, mixtures are unintentional and (largely) of unknown composition, e.g. the combination of substances in surface water, drinking water and air.

While many pieces of EU legislation are in place to protect humans and the environment against adverse effects of chemicals including mixtures, in many cases it remains unclear how to consider the combined exposure to chemical mixtures in the risk assessment. Current regulatory requirements do not generally address the exposure to a single substance by multiple pathways and routes of exposure, following its possible different uses (i.e. the so-called "aggregate exposure"). Exposure to multiple components from one or different exposure pathways (i.e. combined exposure) might also pose a health problem even if the individual components are present at levels below their respective NOAELs (No Observed Adverse Effects Levels), as these levels are derived from single compound testing. However the different existing legislations do often not take into account such risks or do not provide clear guidance on how to perform risk assessment for aggregate and combined exposures. A detailed review of regulatory requirements and related guidance can be found in Kienzler et al. (2014).

In order to reflect the actual exposure scenarios, there is a need to develop a consistent, cross-sectorial approach to deal with the combined exposure to multiple chemicals. In order to develop such a harmonised approach it is important to consider the current scientific state of the art in this area. The objective of this report is to give an overview of the current practices, tools and scientific developments in assessing risks from combined exposure to chemical mixtures. For this purpose, the report provides some general background information (Section 2), a brief overview of new scientific tools in relevant areas of (eco)toxicology based on current literature (Section 3) and presents the results of a recent survey among experts in the field of mixture risk assessment (Section 4).

2. Introduction to main concepts and terminology in the assessment of mixtures / effects from combined exposure

The hazard of chemical mixtures can be assessed as a whole (whole-mixture approach), or based on the individual components of the mixture (components-based approach). Whole mixture effects can be assessed by testing the mixture itself, but can also be based on data generated with a mixture of similar composition (i.e. close in composition regarding components and proportions). If adverse effects are found in relevant toxicity studies, a quantitative assessment can be carried out directly from these data. This approach allows consideration of any unidentified materials in the mixtures and any interactions among mixture components, but it does not identify the chemicals responsible for the mixture effects or interactions, and does not provide any information on the toxicity of individual mixture components. Moreover, this approach is restricted to mixtures that do not significantly change in their composition, and is therefore not recommended as a general approach (SCHER, SCCS, SCENIHR, 2012).

Another approach, which is generally used when the components of the mixture are known, is to mathematically predict the combined action of the components. The choice of the mathematical approach to use depends mainly on considerations whether the mixture components act by the same mode of action (MoA) or whether they are acting independently (Groten et al., 2001). Its optimal use is therefore dependent on the knowledge of the composition of the mixture and the corresponding MoA of the individual components, or on the information regarding their association with groups of chemicals demonstrating similar or identical MoA (assessment groups). Such information may be based on chemical structures and structure-activity relationships (either qualitative or quantitative), molecular modelling, structural alerts or on toxicological responses or effects (SCHER, SCCS, SCENIHR, 2012).

Three basic types of action for combination of chemicals are usually addressed by: (i) dose or *concentration addition* (CA), applied to substances with a similar MoA; (ii) *independent action* (IA) or response addition, applied to substances with a dissimilar MoA; and (iii) interactions between substances in the mixture. The term interaction includes all forms of joint action that deviate from the above additivity concepts. Hence, the combined effect of two or more substances is either greater (synergistic, potentiating, supra-additive) or less (antagonistic, inhibitive, sub-additive, infra-additive) than that predicted on the basis of dose addition or response addition. Both, CA and IA, are based on the assumption that substances do not influence each other's toxicity by interacting at the biological target site, and they have been suggested as default approaches in regulatory risk assessment of chemical mixtures (SCHER, SCCS, SCENIHR, 2012), although chemical mixtures are rarely composed of either only similarly or of only dissimilarly acting substance. SCHER, SCCS, SCENIHR (2012) recommend applying CA if no MoA information is available, as it is regarded as more protective.

Another way of addressing risks form combined exposure is to apply an additional mixture assessment factor (MAF), which could be easily implemented in single substance RA. Detailed information on the ongoing discussion can be found in Backhaus (2015). A generally applicable MAF is hard to find due to the huge variety in mixture scenarios and the need to be protective but not overly conservative. Therefore, Backhaus (2015) investigated further possibilities to develop a protective MAF concept based on addressing the most important uncertainties that are supposed to be covered by a suitable MAF. These uncertainties are incomplete knowledge of the mixture composition (compounds and concentrations), incomplete knowledge on hazard of mixture components, possible synergistic interactions, and uncertainties related to the sole use of CA. An exercise was performed to consider four different exposure scenarios with mixtures of 15-42 components. It was observed that single substance RA and risk management and mitigation significantly lowered the risk of the mixture, however was

insufficient to ensure protection against mixture effects. The Maximum Cumulative Ratio (Price and Han, 2011) resulted as an adequate approximation for a MAF, which ranged from 2 to 17 in the investigated examples, highlighting the need for considering specific exposure scenarios. A scheme is suggested to determine the value of MAF depending on the information available on the mixture (e.g. known number of components of the mixture, information on individual risk quotients of the components, information on interactions). Applying a MAF in the RA of single substances is however difficult since appropriate risk management and risk mitigation measures might need to be developed for scenarios in which many actors contribute to an overall risk with chemical emissions that individually have a risk quotient below 1. The conclusion of the report is that the risk quotient of an individual chemical should not only be viewed as a measure of risk in itself but also as a measure of the contribution of the compound to the overall risk and a combined exposure scenarios, overcoming the concept that chemicals with a risk quotient below 1 are automatically safe even in complex exposure scenarios.

For further information on the underlying concepts please refer to e.g. Kortenkamp et al. (2009), SCHER, SCCS, SCENIHR (2012) or Kienzler et al. (2014). Here, recent literature regarding the approaches for mixtures of similarly and dissimilarly acting compounds are further addressed, as well as regarding the considerations of interactions in the risk assessment of chemical mixtures.

2.1. Similar vs dissimilar mode of action (MoA) and grouping of chemicals

As mentioned above, usually mixtures of components with similar MoA are addressed based on the concept of concentration addition (CA) and compounds with different MoA are addressed based on the concept of independent action (IA). For deciding on the right concept, the distinction of (dis)similar MoA and the related grouping of chemicals in a mixture are crucial. Typically two main approaches are used for deciding if mixture components act in a similar or dissimilar way and to perform related groupings of mixture components: (1) investigating whether components follow a common MoA, or (2) whether they elicit common phenomenological effects or affect the same target organs.

EFSA's PPR Panel (2014) developed a methodology for forming cumulative assessment groups (CAGs) for pesticides in the context of setting maximum residue levels for pesticides in food. The proposed methodology follows a phenomenological approach based on organ or system toxicity. Thus all pesticides causing a specific effect are included in one CAG even if the underlying MoA is unknown. Due to the low exposure levels of residues, interactions are not expected to occur and the PPR Panel based the approach on concentration addition. EFSA's PPR Panel (2013) further discussed the assessment of pesticides with dissimilar MoA, however restricting its considerations to substances with dissimilar MoA but common adverse effects on the same organ or system. The PPR Panel concluded from the reviewed literature that no case showed more conservative predictions of combined toxicity using IA based approaches where at the same time the predictions were also more accurate than based on CA. The use of IA for predicting combination effects requires demonstration that modes of action of individual substances in a mixture are strictly independent, a condition that can rarely be met in practice. In addition, CA can be applied with existing data and has less data requirements than IA (Kortenkamp et al., 2009). The PPR Panel therefore recommended using cumulative risk assessment methods derived from CA also for the assessment of mixtures of pesticides with dissimilar modes of action, provided they produce a common adverse outcome.

Junghans et al. (2006) investigated the suitability of the two concepts of CA and IA to predict the combined effects of realistic environmental mixtures. The exercise was performed on a realistic exposure scenario for agricultural field run-off water considering

25 pesticides. Effects on the reproduction of the freshwater algae *Scenedesmus vacuolatus* were well predicted by CA, in accordance with the finding that toxicity was dominated by a group of similarly acting photosystem II inhibitors, even if the mixture included also pesticides with diverse and partly unknown MoA. Predictions based on IA slightly underestimated mixture toxicity, however, the difference in predictions based on CA and IA was rather small (factor of 1.3). The authors concluded that CA provides a precautionary but not overprotective approach for combined effect predictions of pesticide mixtures under realistic exposure scenarios, irrespective of the similarity or dissimilarity of their mechanisms of action.

Apart from the combined effects of pesticides, the combination effect of endocrine disruptors is relatively well researched. Kortenkamp (2007) reviewed literature on combination effects of endocrine disruptors (EDs). Examples in the literature clearly demonstrate that combinations of EDs with similar effects are able to act together at doses that when used alone do not lead to observable effects. The experimental evidence is in line with the assumption of dose addition. For EDs it seems best to follow a phenomenological approach to produce workable grouping criteria. There are arguments against using a toxicity equivalency factor (TEF) approach or other CA based approaches for EDs since it would ignore potential interactions, however, there is overwhelming evidence showing that groups of estrogenic, anti-androgenic, and thyroid-disrupting chemicals act together in an additive way. For the time being it is proposed to group EDs according to their ability to provoke similar effects rather than according to similar mechanism of action. Given that the TEF concept unrealistically assumes parallel dose-response curves it should not be used. Dose addition should be preferred for calculating quantitative additivity expectations. Further research might trigger adaptations to such a temporary approach. Comparatively little is known about combined effects of EDs belonging to different classes, how these might interact and produce combined effects. Also combinations with chemicals which are not producing the same effects under analysis but that can modulate the effects of other chemicals should be investigated further since such effects cannot be predicted by CA. It should be explored whether the direction of modulation could be anticipated qualitatively e.g. by analysing interaction at the level of metabolism and transport. Further research should particularly focus on combinations of EDs that belong to different categories.

Apart from most of the recommendations and current practices of focusing on combined effects on chemicals with similar MoA, recently also the relevance of combinations of dissimilarly acting compounds was highlighted. Based on the relatively well studied adverse effects of mixtures of pharmaceuticals, Hadrup (2014) suggested that chemicals with dissimilar mechanisms of action could be of bigger concern in the toxicological risk assessment of chemical mixtures than chemicals with a similar mechanism of action. Examples such as e.g. in cancer and HIV treatment, show that pharmacological combination therapy targeting different mechanisms of action is more effective than a strategy where only one mechanism is targeted. Another argument is that also in many diseases several organ systems concomitantly contribute to the pathophysiology, implying that a grouping based on common target organs may be inadequate. In further considerations, it should be however considered that in pharmacology usually higher doses are applied, whereas at lower concentrations some specific effects might not occur.

Goodson et al. (2015) reviewed actions on key pathways and mechanisms related to carcinogenesis for 85 chemicals. The analysis suggested that the cumulative effects of individual (non-carcinogenic) chemicals acting on different pathways and a variety of related systems, organs, tissues and cells, could in combination produce carcinogenic synergies. Additional basic research on carcinogenesis and low-dose effects of chemical mixtures is needed. However, the concept of assessing combined effects strictly based on grouping chemicals according to their MoA/AOP, might need to be revisited in order not to underestimate cancer-related risks. Risk assessment for combined exposure

should consider synergies of chemicals acting via dissimilar processes, acting on different targets and tissues, and consider synergies between certain pathways.

Overall, evidence in the literature supports the application of concentration addition as a first, protective approach. It is therefore also the default approach to start from in several international recommendations and frameworks, independent of components' similar or dissimilar mode of action. However, once a detailed risk assessment for a mixture is performed, chemical grouping should be considered and based on common target organs and/or common mode of action (MoA). The choice of the approach depends strongly on the context of the risk assessment as well as on the information on which to base the grouping of components. Irrespective of the starting point for grouping, it is recommended to use all available information on the mixture and its components: physico-chemical properties, structural alerts, (Q)SAR and read-across information, evidence from omics, *in vitro* (high throughput screening or other) or *in vivo* experimental data, depending on availability.

2.2. Interactions

Toxicological interactions modulate toxicokinetic and/or toxicodynamic mechanisms of individual chemicals. Toxicokinetic interactions could be e.g. induction of metabolising enzymes, alterations in uptake mechanisms, all processes linked to influence of individual chemicals on ADME of others. Toxicodynamic interactions can be based on e.g. modulation of homeostasis or repair mechanisms.

Boobis et al. (2011) performed a literature review, identifying 90 studies demonstrating synergisms in mammalian test systems performed at low doses (i.e. close to the point of departure, POD) for individual chemicals. Only in 6 of the 90 studies useful quantitative information on the magnitude of synergy was reported. In those six studies the difference between observed synergisms and predictions by CA did not deviate by more than a factor of 4.

Cedergreen (2014) performed a systematic literature review for binary mixtures within three groups of environmentally relevant chemicals (pesticides, metals, antifouling agents). Synergy was defined as a minimum two-fold deviation from CA predictions. Synergy was found in 7%, 3% and 26 % of the pesticide, metal and antifoulant mixtures, respectively. The extent of synergy was rarely more than factor of 10. Based on some more in depth analysis Cedergreen concluded that true synergistic interactions between chemicals are rare and often occur at high concentrations. Using standard models as CA is regarded as the most important step in the RA of chemical mixtures.

Kamo and Yokomizo (2015) performed a modelling exercise addressing three theoretical scenarios of non-interacting chemicals, directly and indirectly interacting chemicals. The results showed that combined effects obey CA only when the MoA of the components of the mixture are exactly the same. However, nonlinear effects vanished when the chemical concentrations were low, suggesting that the current management procedure of assuming CA is rarely inappropriate because environmental concentrations of chemicals are generally low.

Approaches to address interactions and unravel the mechanisms are shown e.g. in sections 3.6 and 3.7. In guidance by ECHA (2014) for biocidal products and EFSA's PPR Panel (2013) for pesticides, a deviation between CA predictions and measured mixture toxicities by a factor of 5 or more is regarded a synergistic/antagonistic interaction which has to be considered further. More generic approaches to address interaction in mixture hazard assessments look at the use of classical CA based methods and adding an additional safety factor to account for possible (unidentified) interaction effects. This might be an option for specific cases and compound classes as discussed e.g. in Backhaus et al. (2013), where an interaction factor of 2 for biocidal mixtures is proposed

based on the evidence that in the majority of cases synergistic effects will not lead to higher deviations from CA prediction.

Overall, evidence in the literature indicates that the interactions at lower concentration levels such as environmental concentrations are rare and if observed, leading to deviations from CA predictions that are relatively small. However, more knowledge could be gained from additional case studies covering different sectors to further underpin this.

3. New scientific tools for hazard assessment and how they could be used for assessing mixtures/effects from combined exposure

Exposure of humans and wildlife to chemicals via food, consumer products, the environment etc. can imply exposure to an infinite number of different combinations of chemicals in mixtures. It is practically impossible to test all these possible mixtures experimentally, especially *in vivo*. Therefore, smart strategies are needed to assess the potential hazards using new tools that rely less on *in vivo* testing and incorporate instead alternative experimental and computational tools. These tools are ideally simpler, faster, and more robust in providing the necessary toxicological information of defined and /or undefined mixtures.

In the following the applicability, benefits and limitations of the main current methods and concepts are discussed in the context of hazard assessment of mixtures based on recent literature.

3.1. Adverse Outcome Pathways (AOPs)

The Adverse Outcome Pathway (AOP) methodology is an approach which provides a framework to collect, organise and evaluate relevant information on chemical, biological and toxicological effects of chemicals. This approach supports the use of a mode (and/or mechanism) of action basis for understanding adverse effects of chemicals (OECD, 2013). The approach is based on the concept that toxicity results from the chemical first reaching and then interacting with an initial target or targets in the organism. Thus, an AOP is a sequence of events, starting from the molecular initiating event (MIE; at macromolecular level), via intermediate key events (KEs, at cellular or organ level) to the *in vivo* outcome of interest (adverse outcome, AO; at organism and population level).

As described in the sections below, the prediction and assessment of mixture effects often considers mechanistic information in order to determine whether mixture components follow a similar or dissimilar mode of toxic action and hence should be assessed together or not. This is most often used in order to group mixture components and to decide whether to apply CA or IA based approaches for mixture effect predictions (Borgert et al, 2004). Ankley et al. (2010) illustrated how effects caused by mixtures of chemicals that act via the same molecular initiating event or affect pathways that converge at common intermediate steps, can be aggregated for risk characterization. Thus AOPs provide a valuable framework for grouping mixture components based on the Mode of Action (MoA) into cumulative assessment groups (CAGs). Examples in the literature sometimes show a grouping of chemicals based on similar target organs, but often follow the AOP concept even if not presented as such. When chemicals are grouped based on their MoA, one needs to keep in mind that depending on the dose ranges, chemicals might produce different effects by different mechanisms (Borgert et al, 2004), thus following different AOPs. Thus the mechanistic considerations need to take due account of the relevant exposure concentrations.

In the Solutions project presented in Altenburger et al. (2015), water quality monitoring is performed by a combination of chemical and bioanalytical tools for targeted and non-targeted screening of components in environmental mixtures. The bioanalytical tools should capture MIEs and KEs in order to inform about the toxic pressure *in situ*. Effects at various biological levels should be assessed (molecular, cellular, organism and population level), which is in line with the AOP concept.

Using AOPs and toxicokinetic modelling, results from *in vitro* testing can be put into context and used to support mixture risk assessment with a reduced number of animal testing (section 3.2).

3.2. *In vitro* methods

In vitro models usually consist of cell lines that are cultured in the laboratory under standard conditions. The main advantage of *in vitro* models is that they allow the study of biological responses under such controlled conditions, where *in vivo* models might be influenced by non-chemical stressors that can make the assessment of chemical induced effect(s) even more complex and challenging. In addition, most used cell lines can be cultured relatively easily, they can be used in a high throughput setting, which makes it possible to test for different effects and different combinations of compounds in parallel. As such, *in vitro* tools provide a pivotal role in toxicity pathway testing, as e.g. put forward by the NRC report on Toxicity Testing in the 21st Century: A Vision and a Strategy (NRC, 2007).

Most *in vitro* tools currently applied in risk assessment consist of cell lines that are designed to respond to specific effects, so called mechanistic assays. Generally, they respond to the activation of receptors and/or specific pathways, as a result of e.g. receptor activation or triggering of cellular repair mechanisms. As such, they can be used to elucidate the mechanism(s) of action of a compound or combination of compounds. By considering the effects in a broader context, e.g. as specific steps in an AOP, *in vitro* tests can provide important information on MoA/AOP, e.g. for subsequent grouping of chemicals or for prioritizing compounds for risk assessment (Caldwell et al, 2014).

The application of *in vitro* tools in the assessment of chemicals mixtures can be divided into two approaches: top-down and bottom up. In the top-down approach, *in vitro* assays are used to assess the overall amount of toxicity, effect, receptor activation etc. triggered by a complex mixture. This effect-based environmental monitoring is increasingly being applied to assess environmental mixtures, in part because of their ability to give an overall response to the mixture present, and in part because the compounds causing the effects were – and to a large extent still are – largely unknown (Tang et al., 2013, 2014). Because of this, *in vitro* tools are also widely used in identifying previously unknown or unconsidered effects, but are also used to identify the compound(s) responsible by combining sophisticated chemical analysis with *in vitro* measurements, in a process frequently called Effect Directed Analysis (EDA) (Burgess et al., 2013, Beyer et al., 2014).

While approaches like EDA start top-down, more bottom approaches are also utilized, in which many chemicals are screened for activity in a wide range of *in vitro* assays (Beyer et al. (2014). Several recent initiatives have been launched to profile the effects of a compound using a wide range of *in vitro* assays, like the ToxCast program from the US EPA, or the Tox21 by the National Institute of Health (NIH). These assays include assays focusing on specific pathways or effects, e.g. mitochondrial toxicity (Attene-Ramos et al., 2013a), cell viability and nuclear receptor assays including hormone receptors and metabolic pathways (Huang et al., 2011) and various other endpoints (Tice et al., 2013). While many of these initiatives initially focused on environmental chemicals, these approaches are promising for all chemical risk assessment of many compounds, including pharmaceutical, personal care products and food ingredients (Rovida et al., 2015). Regardless of the approach, linking the total mixture toxicity measured *in vitro* tools to compound concentrations is dependent on the mathematical model that is used to describe the overall predicted effects based on individual concentration. Similarly, *in vitro* tools can be used to assess the validity of specific models to predict mixture effects.

A major hurdle in the acceptance of results obtained by *in vitro* tools is the question how to translate *in vitro* findings to adverse *in vivo* effects (which is the actual protection goal). As *in vitro* studies generally cannot take into account some of the complexity of the whole organisms, including uptake, metabolism and feedback mechanisms, *in vitro*

to *in vivo* extrapolation (IVIVE) is currently an important research topic (see e.g. Adeleye et al., 2015). While *in vitro* assays can provide important information regarding the mode of action (the toxicodynamics), better predictions can be obtained by extrapolating the *in vitro* exposure conditions to the *in vivo* situation using toxicokinetic models (see section 3.6). Single *in vitro* assays cannot cover all the parameters necessary to make the translation to *in vivo*. Dedicated *in vitro* assays can help in identifying and quantifying the parameters needed to validate the uptake, metabolism and excretion models needed for the *in vitro* to *in vivo* translation.

While it is difficult to predict the actual *in vivo* effects based solely on *in vitro* concentrations, *in vitro* tools might be used for regulatory screening purposes by relying on a threshold (both human and environmental) below which relevant effects are not expected to occur *in vivo*. Different approaches have been put forward, mainly focusing on endocrine pathways (Brand et al., 2013, Jarosova et al., 2014, Escher et al., 2015). Based on their ability to predict *in vivo* or chemical analysis results (Browne et al., 2015, Sonneveld et al., 2006), several *in vitro* tools are accepted by regulatory bodies for the assessment of single compounds and mixtures. E.g. the US EPA is currently considering the acceptance of ToxCast ER model data as alternative for the *in vivo* Uterotrophic Assay (Browne et al, 2015). Some regulation do already allow for the use of *in vitro* assay measurements for mixtures. For example, EU regulations 589/2014 and 709/2014 specifically allow the use of *in vitro* methods to give an indication of the total TEQ level in food and feed respectively, expressing the results as Bioanalytical Equivalents (BEQ), rather than the calculated TEQ based on individual congener concentrations analysed chemically.

3.3. Omics

Omics techniques allow a global analysis of gene transcripts (transcriptomics, also called gene expression profiling), of proteins (proteomics) and of small molecule metabolites (metabolomics) including their relative abundance (see e.g. Schirmer et al 2011; Villeneuve and Garcia-Reyero 2011). Omics are suitable to study effects at low doses which are more relevant for environmental mixture exposure due their high sensitivity. However, the effects observed at omics level need to be interpreted with care since the molecular responses do not necessarily lead to an adverse outcome at the physiological level (Borgert 2007; Beyer et al 2014). Furthermore, mechanistic information on the mode of toxic action and affected pathways can be derived, which makes the tools valuable in the context of mixture toxicity as well as single substance toxicity investigations.

Altenburger et al (2012) reviewed literature on the application of omics techniques in investigations of chemical mixtures. Among the 41 studies found (published 2002-2011), most were transcriptomics studies. Many studies investigate the mode of action of single substances and try to predict responses upon exposure to chemical mixtures. Omics techniques can help identifying toxicological mechanisms of individual compounds by a non-biased discovery driven approach (Beyer et al 2014). They can facilitate the identification of key molecular events and complex sequential events caused by stressors. They can support building a more complete overview of stress-response profiles (e.g. toxicity pathways), both for single stressors and mixtures; identify key MoAs; to mechanistically understand the potential for interactions between mixture components; the selection of robust biomarkers for mixture prediction models in ERA.

In the reviewed literature, Altenburger et al (2012) found no clear relationship between the exposure doses, the number of chemicals in a mixture and the number of related affected gene responses. In some cases responses of specific pathways upon exposure to individual compounds were replaced by more general stress response upon exposure to mixtures. However, by delivering more mechanistic information also on individual components, omics results can help in generating hypotheses on possible interactions

between mixture components (El-Masri, 2007). This can feed into the development of mechanistic models used to simulate results that can be tested by model-designed experiments. Dardenne et al. (2008a) used multi-endpoint bacterial gene profiling in combination with cluster and principal component analysis in order to explore to what extent compounds can be grouped according to their toxicological mechanism of action. Several clusters of MoA could be identified and results be improved by combining different clustering techniques. Projection of two environmental samples in the principal component analysis (PCA) plane allowed identifying the mixed mode of action of these samples, which can be useful for deriving first information on samples of unknown composition.

Several studies try to also quantitatively interpret the data, using the concepts of concentration addition and independent action as is usually done for apical endpoints. The major limitation for applying these concepts to omics studies is the usually limited number of tested concentrations (Altenburger et al., 2012). Another limitation that hampers the application of a classical toxic unit approach, is the difficulty of deriving effect concentrations (EC_x) since the maximum induction levels for different genes vary with different chemicals (depending e.g. on the cytotoxicity of a specific compound) (Dardenne et al., 2008b). Dardenne et al. (2008b) investigated the effects of individual substances and binary mixtures on 14 stress gene promoters. Mixture responses were fitted applying both, CA and IA models. In many cases both models were able to predict the mixture response from the individual compound responses. Differences between CA and IA predictions were rather small. Deviations from CA and IA occurred, sometimes with deviations being in opposite directions (i.e. synergistic or antagonistic) at high and low dosage level. The choice of the best fitting model could not be made objectively based on similar or dissimilar mode of action.

In summary, to date the major benefit of applying omics in the context of mixtures is to use them for unravelling MoAs of the individual components in order to group them and facilitate appropriate predictions of mixture toxicity.

3.4. Quantitative Structure-Activity Relationships (QSARs)

Quantitative Structure-Activity Relationship (QSAR) models can be used to obtain information on the properties and activities of substances based on their chemical structure alone, and can thus be used to fill data gaps in the safety assessment of chemicals. Predictive approaches, such as QSARs, are essential for estimating mixture toxicity as the number of possible mixtures is extremely large (Kim and Kim, 2015).

There are three main ways in which QSARs can be applied for the assessment of mixtures: (1) for predicting (missing) information on individual compounds (physico-chemical properties, toxicological effects) (2) for predicting directly or stepwise the combined effects and interactions of chemicals in a mixture (3) for assessing whether chemicals will act in a similar or dissimilar way to perform their grouping.

Altenburger et al. (2003) outlined how QSARs could support mixture toxicity evaluations. All organic compounds (also those with specific MoA), will exert baseline toxicity resulting from non-specific effects, related to partitioning into membranes and adsorption to macromolecules. Thus all organic chemicals will contribute in an additive way to baseline effects of a mixture also at very low concentrations. Thus a mixture will be at least as toxic as corresponds to the sum of the fractional baseline toxic concentrations of the components. This holds true however only in the absence of antagonism that could result from metabolic detoxification of some components induced by other components of the mixture. In a mixture with differently acting chemicals, specific effects might not be triggered due to very low concentrations of the individual components. However, their contributions to baseline toxicity will remain and might add up to overall significant effects of the mixture. QSARs can therefore be used to predict

the baseline toxicity of a mixture and when comparing to measured toxicity of a mixture help identifying if specific effects were occurring in addition.

Altenburger et al. (2003) also found that it is sometimes assumed that if one QSAR can be applied to predict the toxicity of all mixture components, the compounds will by default follow CA. However, this is not always the case since compounds may contribute to different extent to narcotic and specific toxicity in a QSAR. Therefore, this assumption is not generally valid.

When comparing experimentally measured mixture toxicities with those predicted by QSARs, deviations observed were bigger for binary mixtures than for multiple mixtures (Altenburger et al., 2003). This might be due to greater experimental variability for more complex mixtures as well as due to an increasing degree of compensation of deviations with increasing number of components in the mixture.

Direct prediction of mixture toxicity by QSARs is rather rare. It is only possible if the detailed mixture composition is known. In some cases (Altenburger et al 2003), predicted mixture toxicity is higher than experimentally determined, mainly due to inadequate predictions for some individual components. If predictions are based on observations at higher concentrations, this can lead to overestimations of toxicity, since at low concentrations the specific effects of some compounds may not be triggered yet (e.g. in the case of pesticides) and hence they act as nonspecific toxicants. If the mixtures are composed of compounds that are predicted adequately individually, also the joint toxicity is mostly predicted with a sufficient degree of accuracy.

QSARs can be used to discriminate classes of toxicants, i.e. to assess mixture components for similar or dissimilar mode of action. QSAR-based tools to look for functional similarities comprise molecular indices, topological indices and atom pairs, physicochemical and quantum chemically derived stereoelectronic descriptors, together with subsequent discriminant analysis. A sequential analysis applying the different approaches can enhance the predictive capacity when dealing with several different classes of chemicals (Altenburger et al., 2003). For example, Mwense et al. (2006) use a set of molecular descriptors to determine the overall structural similarity and dissimilarity within a mixture based on all the pairwise similarities and dissimilarities between the constituent molecules. Then the degree of similarity vs dissimilarity is used to weight the relative contributions of concentration addition and independent action in a mathematical model based on both approaches.

QSARs can be used for modelling exposure concentrations and have been proposed for calculating internal exposure concentrations by modelling internal distribution and metabolism (Altenburger et al., 2003). This is especially important when chemicals are reactive or interacting with protein macromolecules. For example, Verhaar et al (1997) illustrated an approach to assess the toxicity of complex multi-component mixtures, where QSARs can provide input parameters for PBPK models and a lumping analysis to reduce mixture complexity to a limited number of compound categories.

Kim and Kim (2015) reviewed recently developed computational methods based on QSARs for predicting mixture toxicity in environmental risk assessment (ERA). They searched for related peer-reviewed articles published 2011-2013 in the fields of toxicology, environmental science and ecology and engineering. They identified empirical QSARs developed mainly based on partition coefficients. In the case of single substance QSARs these are usually based on K_{ow}, in the case of mixtures it is proposed to base them on K_{md} (i.e. the C_{18} EmporeDisk/water partition coefficient of the mixture). K_{md} was found to be promising for predicting the mixture toxicity of some chemicals (halogenated benzenes, phenols, petroleum, PCBs, organochlorines, herbicides). However, these QSARs can only assess EC_{50}s of non-interacting mixtures ignoring synergistic effects. Tang et al. (2013) proposed an approach for deriving effect based water quality trigger

values. Trigger values are derived in two steps, firstly estimating the individual chemicals EC_{50} for non-specific MoA/baseline toxicity by QSARs and secondly calculating mixture toxicity by CA. This represents a strategic approach to quantitatively derive reference concentrations of mixture toxicity of different pollutants regulated by water quality guidelines. Also non-empirical QSARs based on quantum chemistry and molecular docking processes were identified. Quantum-based QSARs based on atomic charges were developed that allow the prediction of mixtures at non-equitoxic concentrations of the components. Two-step models are available that first assign chemicals via structural similarity to relevant MoA and then calculate the toxicity for similar chemicals based on CA and in a second step for the dissimilar groups based on IA.

In summary, QSARs can provide valuable input to assessing the toxicity of mixtures. Some general challenges and limitations for application of QSARs to predict mixture toxicities remain:

1. The principal difficulties in dealing with mixtures, limit the quantitative application of QSARs in environmental field research (Altenburger et al., 2003). The restrictions mostly relate to the characterization of proper QSAR input parameters, since so far the impact of molecular properties on the mode of interaction in mixtures is essentially unknown.
2. QSARs to date mainly use CA for mixture toxicity prediction; interactions (especially synergisms) need to be further addressed. The lack of quality data (on molecular and biological mechanisms) to increase understanding of synergism is essentially the most critical challenge in modelling it. Molecular docking based QSAR models have the potential to contribute to the prediction of synergisms. The main influencing factors for synergisms are bioavailability, internal transportation, metabolization, binding at the target site and excretion. Thus molecular docking theory seems most promising to address this (Kim and Kim, 2013).
3. Most current QSAR developments focus on binary mixtures, QSARs enabling the assessment of multi-component mixtures need to be further developed. Only two out of eleven reviewed QSAR models were able to address multi-component mixtures (Kim and Kim (2015).
4. It is important to acknowledge that the combined effect can be rather different when considering predictions based on EC_{50} values instead of considering low dose effects at concentrations below the NOEC (Kim and Kim, 2015). This is supported by the observation that at low concentrations, the specific effects of these compounds may not be triggered yet and hence they act as nonspecific toxicants (Altenburger et al. 2003). Most QSAR models for mixtures predict an EC_{50} for the mixture, which is probably not relevant for environmental exposures. QSAR models that are able to predict multi-point estimates around threshold effect levels should be developed (Kim and Kim, 2015).
5. Most current QSARs for mixtures focus on acute rather than chronic toxicity. QSARs based on molecular docking might help to improve chronic predictions, but at the moment are only able to predict toxicity for binary mixtures (Kim and Kim, 2015).

Nevertheless, descriptive QSARs can be very useful for deriving basic understanding of relevant interactions and molecular mechanisms. They can help in designing and interpreting studies to link biological effects with chemical analysis (Altenburger et al 2003).

3.5. Read-across

In the following some general principles of read-across and specific issues to consider when applying read-across to mixtures will be briefly discussed. For more detailed general considerations please refer to the OECD Guidance on Grouping of Chemicals (OECD, 2014) or to the ECHA Read-Across Assessment Framework (ECHA, 2015).

Read-across is a technique that allows predicting endpoint or test information for a chemical (target chemical) based on the information available on the same endpoint or test for one or more similar chemicals (source chemical(s)) (OECD, 2014). Two main approaches for read-across are usually distinguished, i.e. the analogue and the category approach. The analogue approach is usually used to read-across between a small number of similar chemicals, in a simplest case from a single source chemical to a target chemical. The category approach is usually applied to read-across between/within whole groups of similar chemicals, mostly used for structurally similar groups of chemicals and chemical families (ECHA, 2015).

Read-across can be applied for the prediction of various properties in order to fill data gaps on e.g. physico-chemical properties, environmental fate, human health effects and ecotoxicity. Read-across can be performed in a qualitative or quantitative way. Qualitative predictions usually address the absence or presence of a certain property or activity. Quantitative read-across instead predicts a value for a certain property or endpoint, e.g. a dose-response relationship and effect concentrations (such as NO(A)EL, LO(A)EL) (OECD, 2014).

The basis for read-across from one or more chemicals to another chemical or a group of chemicals is the similarity in structure, properties and activities of the involved chemicals. Structural similarity provides a convenient means of identifying likely analogues. Similarity may be based on common functional groups, common chemical class, or common precursor or breakdown products (i.e. similar metabolic or degradation pathway). In addition to forming groups based on structural similarity, groups can be further developed based on biological information. The adverse outcome pathway (AOP) framework (see section 3.1) can help to group chemicals according to the molecular initiating events (MIEs) or key events (KE) that they trigger. For this purpose it is not needed that the whole sequence of events of an AOP is known, however, a reasonable link needs to be made with the adverse outcome that is to be predicted by read-across (OECD, 2014).

Read-across can be of value in the assessment of mixtures mainly in two ways:

- Read-across for untested constituents of a mixture in a component based approach.
- Read-across for similar mixtures in a whole mixture approach.

For the first case, approaches as described above can be followed for the individual mixture components. In mixtures of structurally diverse compounds, read-across for several constituents might be an option. In cases of mixtures of substances of one chemical class, a category approach might be followed to read-across among different components of the class for which less information is available. An example is presented for phthalates by Health Canada/Environment Canada (2015).

An example for the second case is the application of read-across to complex substances such as MCS (Multi-Constituent Substances) and UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological material). However, read-across is limited to substances with sufficient knowledge about the composition (identity and properties of constituents) and understanding of key structures that are determining the mixture's behaviour. Category members are often grouped based on how these are manufactured, defined and used, which can provide boundaries for the constituents chemical space (OECD, 2014).

The OECD QSAR toolbox is a software application that allows identifying and filling of data gaps for chemical hazard assessment. It comprises (eco)toxicological experimental data and prediction tools which can be used for grouping of chemicals and data gap filling (for details see OECD, 2009 and webpage with related tutorials). The OECD

toolbox allows also the assessment and prediction of mixtures if the chemical components are known. One can enter the individual components, gather the available experimental data on certain endpoints for the individual components and the toolbox can provide a prediction of the endpoint for the mixture giving the choice of similar or different MoA consideration. Thus this tool allows performing data gap filling by read-across and mixture endpoint prediction in one workflow.

3.6. Toxicokinetic and toxicodynamic modelling

Toxicokinetics (TK) describe the concentration and time-dependent fate of a substance within an organism whereas toxicodynamics (TD) describe the subsequent interaction with biological targets and how this may lead to adverse health effects (Bessems et al., 2015). In the context of human health and environmental risk assessment usually the terminology of toxicokinetics and toxicodynamics is used, however, sometimes the terms pharmacokinetics (PK) and pharmacodynamics (PD) are used synonymously depending on the origin of the models and underlying data.

TK/TD considerations can support the assessment of chemical mixtures in several ways with the main areas of application being:

- Determination of internal exposure concentrations, e.g. enabling a relation between body concentrations and *in vitro* experiments (i.e. IVIVE, *in vitro* to *in vivo* extrapolations), of relevance for single chemicals as well as for chemical mixtures.
- Considering the simultaneous or sequential exposure to different mixture components, assessing the probability that those reach the same target.
- Predicting interactions among mixture components on TK and TD level.

The classically applied methods based on CA and IA lack a mechanistic basis and are thus of limited utility for high-to-low dose or animal-to-human extrapolations. PBPK models allow the extrapolation between doses, routes and species. PBPK/PD models can also help in detecting shifts in the mechanism of action at varying doses and in predicting interactions and their respective thresholds (El-Masri, 2007).

Most of the identified relevant studies available in the literature, investigate the utility of physiologically based (PB) PK/PD models to assess and predict interactions of chemicals in a mixture, i.e. looking at deviations from strict additivity. Tan et al. (2011) reviewed PBPK/PD modelling efforts to investigate the chemical interactions at the PK and PD levels. Most interactions studied to date focus on PK interactions. PK interactions mean that mixture components influence each other's target tissue dose. This can occur if one chemical in a mixture affects the absorption, distribution, metabolism or excretion (ADME) of other components of the mixture, e.g. by inducing or inhibiting metabolising enzymes, competing for transporters etc. PD interactions mean that mixture components influence each other's target tissue response based on one unit of target tissue dose, e.g. if one chemical impairs repair or homeostasis mechanisms. Examples can be found in Tan et al. (2011); however, most examples address higher (occupational) exposure where interactions are more likely to occur than at lower (environmental) exposure levels.

PBPK/PD modelling is often hampered by the limited availability of input parameters. Verhaar et al. (1997) proposed an integrated approach using PBPK/PD modelling with QSAR analysis and lumping analysis to predict mixture toxicity. QSARs were used to derive needed input parameters for unknown chemicals, e.g. partition coefficients, metabolic rate constants, and pharmacodynamics parameters. Since with a higher number of components in a mixture this becomes rather cumbersome, they proposed to combine it with a lumping analysis to build categories to reduce the number of components of the mixture to be addressed.

PBPK modelling can also support cumulative risk assessment including both, exposure to multiple chemicals and non-chemical stressors. Wason et al. (2012) investigated the cumulative risk for children exposed to multiple pesticides and non-chemical stressors, such as dietary factors. The study developed a general framework for such approaches, is however, not further discussed here since the focus is on chemical stressors.

In summary, the integration of TK in the assessment of mixture hazards is of value in order to generate a better mechanistic understanding and currently mostly used to predict and interpret interactions between mixture components.

3.7. Dynamic Energy Budget (DEB) models

The approach of dynamic energy budgets (DEBs) is applied in the ecotoxicology area. It considers toxicity as a process over time that depends firstly on the build-up of the internal concentration, and secondly on a hazard model that describes the adverse effect as a chance process, e.g. using a killing rate (Løkke et al., 2013). Compared to the classical approaches based on CA and IA that interpret effects on different endpoints separately, DEB models have the advantage that by their mechanistic basis they allow extrapolating experimental results to e.g. other time points, time-varying exposures, other mixtures, other organisms, and other (non-chemical) stressors, such as e.g. food limitation (Baas et al., 2010).

DEB models mostly use few parameters which have a clear biological meaning (e.g. such as killing or elimination rates). In order to develop DEB models, the results of a time series of toxicity endpoints (e.g. data on survival) are needed, which might add extra costs; however, this allows some increased understanding of the underlying mechanisms and better predictions of mixture effects.

The first step in the DEB model addresses toxicokinetics, to determine the internal concentration looking at uptake and elimination in a one compartment model. Then a model description of the processes in the organism follows looking at assimilation, maintenance, growth, reproduction, death. Resulting effects on the organism can lead to changes in toxicokinetics for other mixture components and are considered in a feedback loop. The advantage of this modelling framework is that it can be applied to a large variety of different species, in contrast to e.g. PBPK/PD models which are very species specific (Baas et al., 2010). For each component in the mixture, three toxicity parameters are needed: the no effect concentration, the killing rate and the elimination rate. In addition one extra parameter to correct for the mortality in unexposed controls is needed. Among different examples of applying the DEB model to binary mixtures, it was also proven to work for more complex mixtures. Baas et al. (2009) addressed effects of mixtures of 80 different compounds in surface water on Daphnia, where 92% of the cases were correctly predicted. Using this approach the chemicals driving the effects could also be identified.

Most examples in the literature are dedicated to predicting mortality and similar endpoints. Studies by Jager et al. (2010, 2014) showed however that DEB modelling can be also applied for sub-lethal endpoints elicited by mixtures.

In summary, for the time being DEB models are not yet regularly used in the assessment of mixtures. They are however a promising tool, since they look at effects in a more integrated and mechanistic way. However, more work has to be done to specify what type of information is needed to identify the various mechanisms of action, and to quantify the importance of a correct choice for the population effects (Jager et al., 2014).

3.8. Threshold of Toxicological Concern (TTC)

The Threshold of Toxicological Concern (TTC) is a methodology that can be applied to assess potential human health concerns for chemicals based on their chemical characteristics and estimated exposure. It is applicable to substances for which the chemical structure is known but for which there are few or no relevant toxicity data. The classification of chemicals according to their chemical structure is an essential component of the current TTC approach (Cramer classes, Cramer et al., 1978, Munro et al., 1996). It was first developed for substances in food contact materials. EFSA's Scientific Committee (2012) investigated the applicability of the TTC approach for its own work (i.e. food-related risk assessments) and recommended a revision and refinement of the Cramer Classes and underlying database. Nevertheless, the Cramer classification scheme is considered conservative and protective of human health (EFSA's Scientific Committee, 2012). EFSA's Scientific Committee has identified several cases where the TTC approach should not be applied, one of them being "mixtures of substances containing unknown chemical structures".

In their opinion on the "Toxicity and Assessment of Chemical Mixtures" (SCHER, SCCS, SCENIHR, 2012), the Scientific Committees discussed the application of the TTC approach in the assessment of mixtures. They recommend the TTC approach could be used at a screening level for comparing first estimates of combined exposure to the TTC. For representative substances in assessment groups where data on limit values are missing, QSAR predictions, read-across, or the TTC approach could be used to fill the data gaps. A TTC-like approach can be used to eliminate combinations that are of no concern, if conservative exposure concentrations are used.

Exemplary case studies exist in the literature where the TTC approach was used to assess human health risk from chemical mixtures in surface water. Price et al. (2009) explored the use of the TTC approach for the evaluation of the chronic non-carcinogenic effects of hypothetical and actual examples of chemical mixtures and it proved to provide conservative estimates of mixture toxicity. They therefore propose to use the TTC in screening assessments of mixtures where compound specific data for components of a mixture are missing. Along these lines, also Boobis et al. (2011) showed the application of the TTC approach in a Tier 0 risk assessment with the intention to prioritize the need for further evaluation of chemical mixtures. Data were based on surface water monitoring data in order to create a hypothetical mixture of 10 compounds from different classes (fragrances, pesticides, surfactants, personal care products, solvents, petrochemicals). They applied some worst case assumptions (i.e. direct consumption of surface water without treatment and investigating lifelong chronic exposure at maximum detected levels). The TTC using ToxTree and the concentration addition based Hazard Index were applied. In the specific case, no risk was identified. The case study confirmed the utility of using the TTC approach at Tier 0 as suitable tool for mixture assessments. Terry et al. (2015) showed the application of the TTC approach for facilitating the risk assessment of parent substances and their environmental metabolites using a pesticide as an example. The TTC approach was used for some metabolites with low predicted concentrations. Utilizing information on mode of action, relative potency, hazard characterisation, read across, predicted exposure and TTC provided a robust database minimizing animal use for the assessment.

In summary, the TTC approach provides a useful tool to be used at lower tiers in the assessment of mixtures, providing limit values for mixture components with missing information. It is currently limited to its application in the area of human health. However, an international activity is currently ongoing to develop the ecoTTC approach for environmental hazard assessment, which is based mainly on aquatic toxicity data (Belanger et al. 2015).

3.9. Integrated Approach to Testing and Assessment (IATA)

With the more regular use of new techniques like e.g. *in vitro* testing, omics approaches, computational methods, there is a need to develop strategies for evaluating the data generated and interpret them in a joint approach. There is also a need to strategically direct testing efforts in order to save resources. Efforts for developing such strategies are ongoing in various fora and usually include a framework to integrate test and non-test information in a weight of evidence approach. Under the OECD, these approaches are called Integrated Approaches to Testing and Assessment (IATA). They "integrate existing knowledge based on classes of chemicals with the results of biochemical and cellular assays, computational predictive methods, exposure studies, and other sources of information to identify requirements for targeted testing or develop assessment conclusions. In some cases, the application of IATA could lead to the refinement, reduction, and/or replacement of selected conventional tests (e.g., animal toxicity tests)." (NAFTA, 2012).

The development of IATA is nowadays strongly linked to the development and availability of AOPs. The AOPs offer the biological framework to build around the testing and assessment strategy (Tollefsen et al., 2014). A testing and data interpretation strategy can be developed by addressing MIEs and KEs in an AOP.

Related testing strategies can follow a battery approach (all tests performed and results collected), sequential or tiered approaches (results are collected in a given sequence and further testing is stopped when sufficient information is available), or result-driven further testing approach (depending on results next most valuable testing is decided). The integration of results from the different information sources can occur at different levels, i.e. form raw data to summary/category level. Different approaches (deterministic, decision trees, scoring approaches etc.) can be applied (Tollefsen et al., 2014).

IATA provide another framework to collect information on individual mixture components as well as on whole mixtures, allowing a more structured (and if AOP based more mechanistically relevant) way of data generation and interpretation.

4. Status of current mixture risk assessment based on a dedicated expert survey

In order to gain an overview of the current practices and experiences with assessing effects and risks from combined exposure, a survey among experts in authorities, academia and industry was performed. The online survey was published in the EU survey platform (https://ec.europa.eu/eusurvey/) on 23/01/2015 and closed on 23/03/2015. The link to the survey was sent to experts in the field that were identified in the following ways: (1) from scientific literature, (2) experts involved in developing the WHO/ICPS framework for combined exposure to chemical mixtures, (3) experts involved in developing the Opinion of the SCHER/SCHENIHR/SCCS on "Toxicity and Assessment of Chemical Mixtures", (4) participants of the recent EFSA Scientific Colloquium N°21 on "Harmonisation of human and ecological risk assessment of combined exposure to multiple chemicals", (5) representatives of the ongoing OECD project on combined exposure to multiple chemicals, (6) members of the EURL ECVAM Stakeholder Forum (ESTAF) and the PARERE expert network dedicated to the Preliminary Assessment of Regulatory Relevance of alternative test methods proposed for validation.

The questionnaire contained the following sections:

- General information on the respondent
- Experience with different approaches for the assessment of mixture toxicity
- Expert opinions regarding the use of specific approaches for mixture toxicity assessment
- Views on the use of novel tools for the assessment of mixture toxicity
- Comments on existing frameworks for the risk assessment of combined exposure to multiple chemicals
- Possibility to provide references or files regarding relevant projects, publications, case studies.

4.1. Information on respondents

Fifty-eight valid responses were received and evaluated. Responses were received from 48 experts from 16 different EU countries and 10 experts from 5 non-EU countries (Figure 1). Survey participants represented experts from academia, authorities, and industry in nearly equal parts (Figure 2). Participants had experience in the following sectors: chemicals (multiconstituent and UVCB substances under REACH), plant protection products, biocides, pharmaceuticals, cosmetics, food or feed additives, food or feed contaminants, surface water, drinking water, waste streams, soil, air, medical devices, alloys, botanicals, cigarette smoke, landfill leachate, solid waste from industrial combustion processes, tobacco toxicants, jewellery, toys, food contact materials, and sewage sludge.
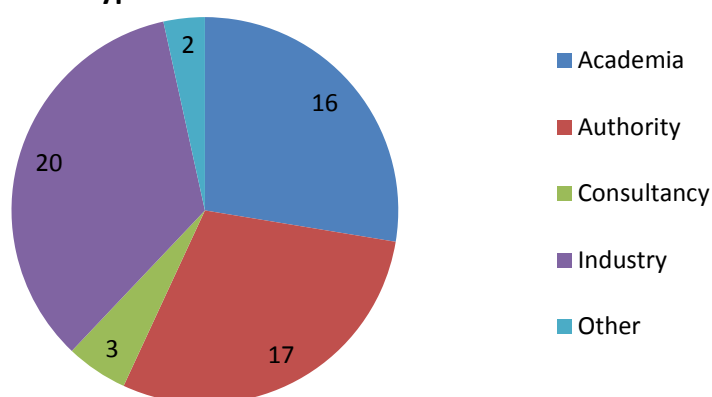
Country of respondent
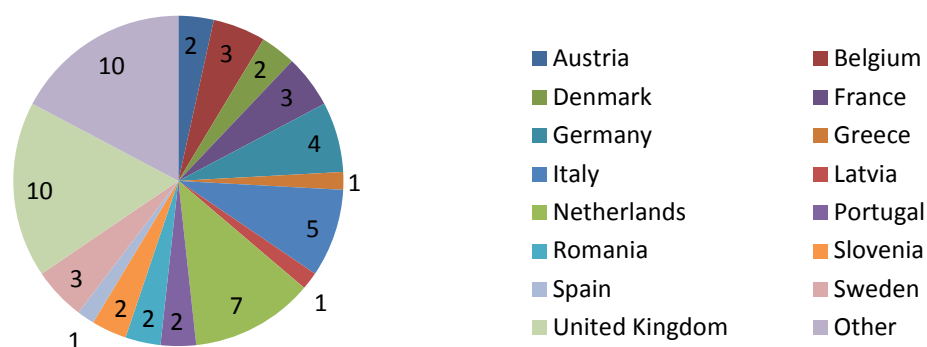
Figure 1 Country of Respondent. Survey participants were from 16 different EU countries and 5 non-EU countries (indicated as "Other": Canada, Japan, Norway, Serbia, Switzerland, US).

Type of Affiliation

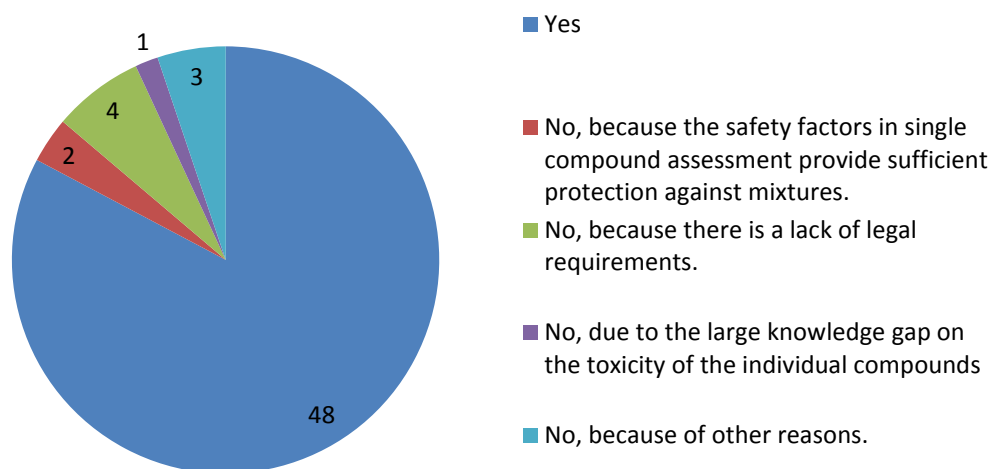**Figure 2** Survey respondents' affiliation

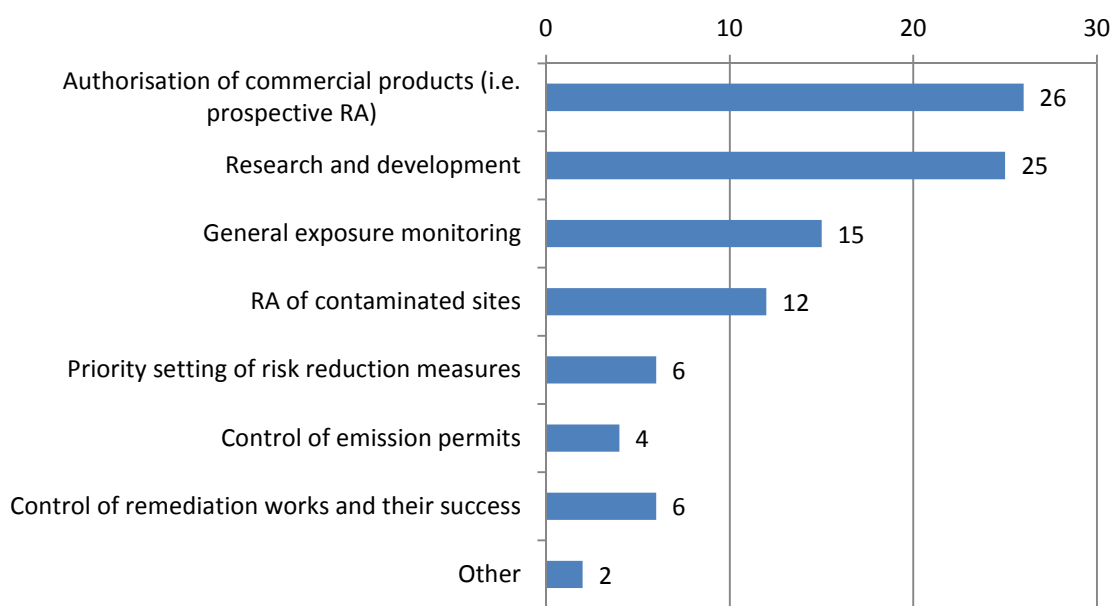## 4.2. General experience with mixture toxicity/risk assessment

The majority of the respondents (48) had performed risk assessment(s) for chemical mixtures in the area of human health risk assessment (HRA) or environmental risk assessment (ERA) (Figure 3). Those that replied yes (i.e. had already performed mixture risk assessment) indicated that they had mainly assessed chemical mixtures in the context of the authorisation of commercial products and research & development (Figure 4).

Did you ever need to perform a RA of chemical mixtures for HRA or ERA?

Legend:
- Yes
- No, because the safety factors in single compound assessment provide sufficient protection against mixtures.
- No, because there is a lack of legal requirements.
- No, due to the large knowledge gap on the toxicity of the individual compounds
- No, because of other reasons.

Figure 3 Replies to the question "Did you ever need to perform a risk assessment of chemical mixtures for HRA or ERA?"

For which purpose(s) did you assess the overall toxicity of chemical mixtures?

Purpose	Value
Authorisation of commercial products (i.e. prospective RA)	26
Research and development	25
General exposure monitoring	15
RA of contaminated sites	12
Priority setting of risk reduction measures	6
Control of emission permits	4
Control of remediation works and their success	6
Other	2

Figure 4 Replies to the question "For which purpose(s) did you assess the overall toxicity of chemical mixtures?" Other purposes indicated were 1) development of CEFIC MIAT conceptual framework and CLP workplace monitoring.

4.3. Experience with the whole-mixture and component-based approaches

Those participants having experience in performing mixture risk assessments (i.e. that answered "yes" to the question in Figure 3) were asked about their experience in applying whole mixture and component-based approaches for assessing intentional and unintentional mixtures (Figure 5). They further specified for which type of commercial product (intentional mixture) or sample (unintentional mixture) they applied such approaches (Figure 6) and which kind of component-based approaches they are mostly using (Figure 7).

Have you ever applied a mixture assessement using whole mixutre/component based approaches for un/intentional mixtures?
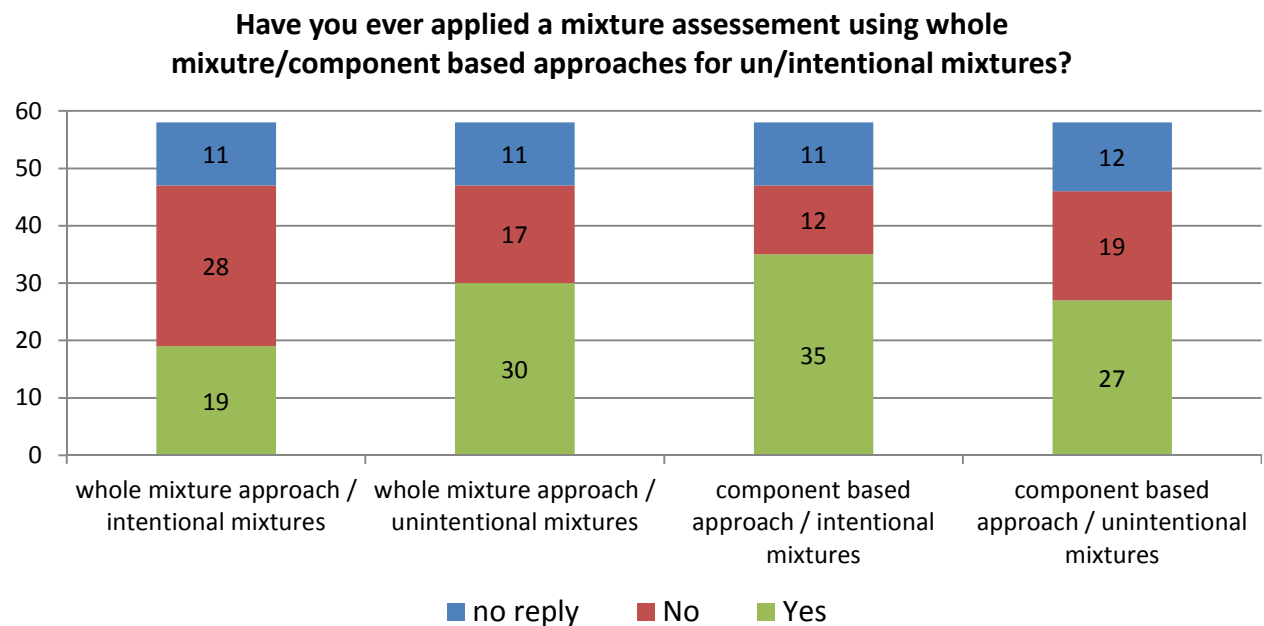
Figure 5 Experience in performing mixture risk assessments using whole mixture or component-based approaches applied to intentional or unintentional mixtures.

Intentional mixtures

Category	whole-mixture approach	component-based approach
Chemicals	13	12
PPPs	14	16
Cosmetics	6	3
Biocides	5	5
Food / feed additives	2	1
Pharmaceuticals	1	2
Others	3	3

Unintentional mixtures

Category	whole-mixture approach	component-based approach
Sediment	5	3
Soil	5	8
Food contaminants	4	9
Surface Water	4	9
Air	2	5
Biota	1	3
Drinking Water	1	5
Ground Water	1	3
Industrial Waste Water	1	1
Municipal Waste Water	1	2
Feed contaminants	0	0
Marine Water	0	1
Other	6	6

Figure 6 Replies to the question "For which type of mixture(s) have you applied a whole mixture or component-based approach?" Chemicals were further specified in the survey as "multiconstituent or UVCB substances under REACH". Other types of mixtures mentioned were medical devices, alloys, botanicals, food ware materials, jewellery, toys, cigarette smoke/tobacco toxicants, human tissue extracts, landfill leachates, solid waste from industrial combustion processes, sewage sludge.

Respondents were asked which kind of tests they used for the whole-mixture and component based approaches for assessing intentional and unintentional mixtures. A wide range of tests reflecting all major tests usually used in single substance testing was mentioned.

Experts were further asked about their experiences with different component-based approaches. The approaches most frequently used by the participating experts are the direct application of the Concentration Addition (CA) equation, the Hazard Index (HI) as well as Toxic Equivalency Factor (TEF) (Figure 7). Concentration Addition based approaches seem much more wide spread than independent action (IA) based predictions.

How often have you used the following component-based approaches?

Legend: ■ Never+no reply ■ Occassionally ■ Frequently

Category	Never+no reply	Occassionally	Frequently
Direct Application of CA formula	34	9	15
TUS	46	6	6
TEF	37	15	6
RPF	43	10	5
PODI	53	4	1
HI	34	12	12
HI(Int)	54	2	2
MCR	50	5	3
Other CA based approaches	50	6	2
Direct application of the IA formula	47	6	5
Other approaches	50	6	2

Figure 7 Replies to question "How often have you used the following component-based approaches?" (CA=concentration addition, TUS=Toxic Unit Summation, TEF=Toxic Equivalency Factor, RPF=Relative Potency Factor, PODI=Point of Departure Index, HI=Hazard Index, HI(int)=Hazard Index including interactions, MCR=Maximum Cumulative Ratio, IA=Independent Action)

Experts could also provide information on additional component-based approaches they are using. For example, ADIadjusted, AOELadjusted, ARfDadjusted (based on common target organ/common toxicity; use of biotic ligand models (BLM); use of TTC and Margin of Safety (MoS) were mentioned.

Experts were then asked about their experience with other approaches not fitting into the CA or IA category. Some examples mentioned were that mixtures were assessed and toxicity related to the main two constituents; effect-directed fractionation (e.g. toxicity identification evaluation TIE) for identifying most potent components in a mixture; approaches based on TKTD modelling, to assess dynamic effects of mixtures over time; history of safe use via well-established dietary intakes. These additional approaches were mainly used for research and development purposes.

4.4. Expert opinions on mixture toxicity assessment

Experts were asked about their opinions on mixture toxicity assessment. Experts gave their view on priority mixtures (Figure 8). Chemicals under REACH and plant protection products were mentioned most often. Experts were further asked whether they see a need to address interactions (e.g. synergisms/antagonisms) in the mixture risk assessment. Multiple options with possible reasons to take combination effects into account or not were given in the survey. Interestingly, several experts selected yes and no options together. The overall picture can be found in Figure 9. Among the experts that selected the "other" option, the main reasoning provided in the free text option was that interactions are considered rare, especially at low/environmental concentrations. However, most experts agreed that interactions should be taken into account on a case-by-case basis and one expert proposed to look at interactions especially in the case of active substances like plant protection products, biocides, pharmaceuticals.
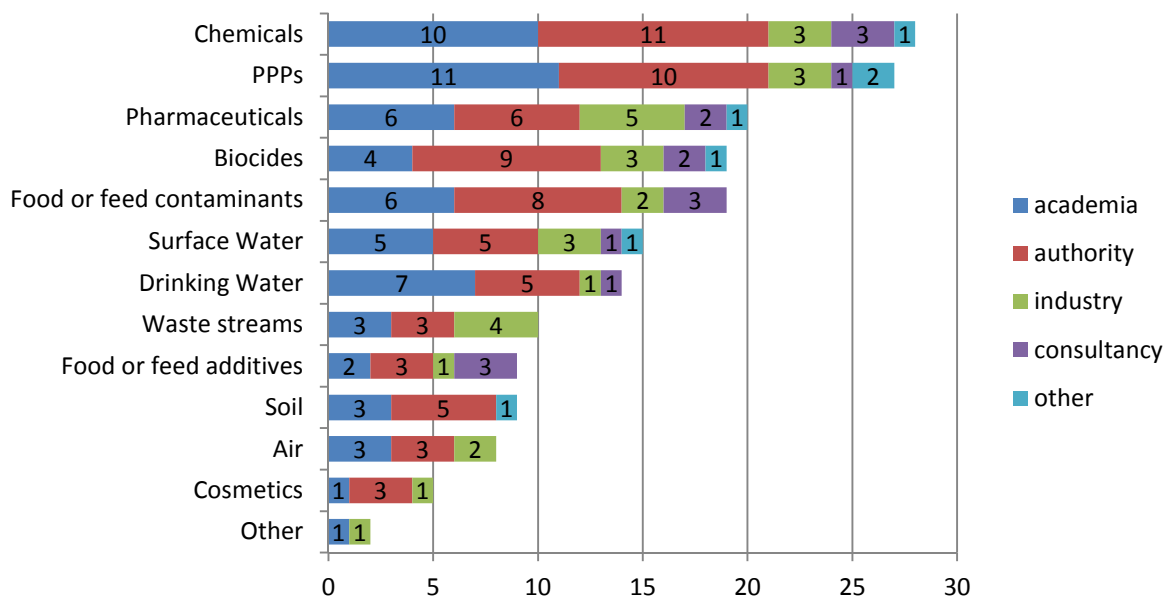
Figure 8 Replies to the question "Which type of mixture(s) or samples would you identify as highest priority for risk assessment that needs to take mixture effects into account?" divided by stakeholder group. Chemicals were further specified in the survey as "multiconstituent or UVCB substances under REACH". Other mixtures of importance mentioned were those present in human tissues and container systems.
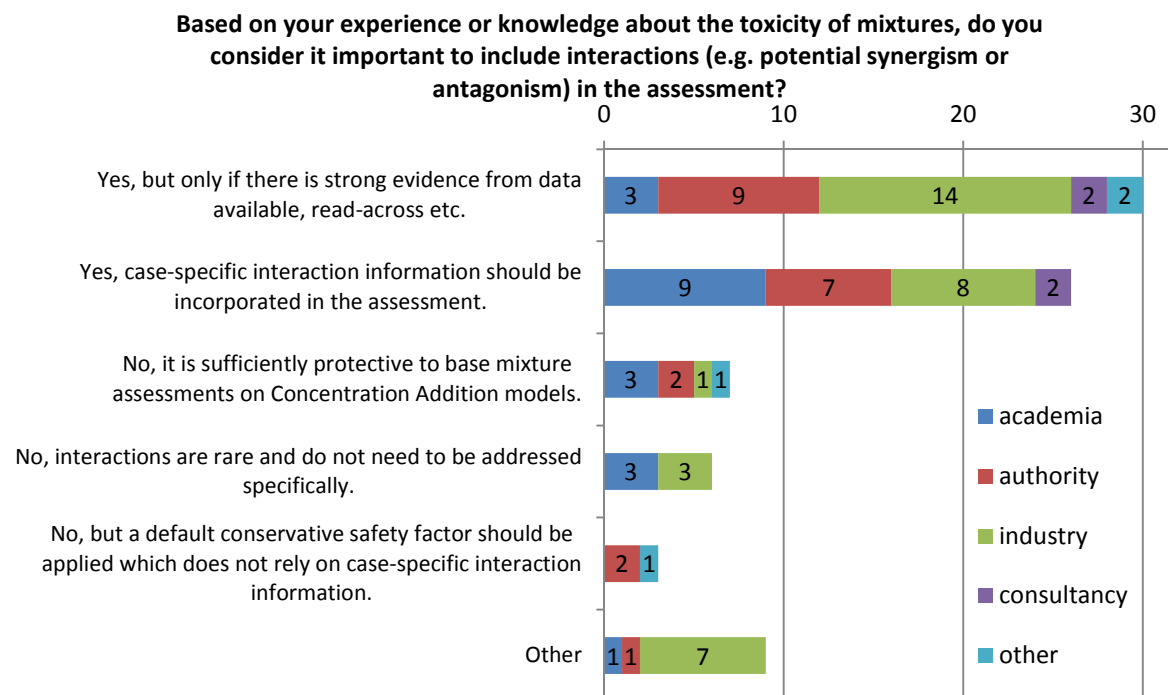
Figure 9 Replies to the question "Based on your experience or knowledge about the toxicity of mixtures, do you consider it important to include interactions (e.g. potential synergism or antagonism) in the assessment?" divided by stakeholder group. Among the experts that selected the "other" option, the main reasoning provided in the free text field was that interactions are considered rare, especially at low/environmental concentrations. However, most experts agreed that interactions should be taken into account on a case-by-case basis and one expert proposed to look at interactions especially in the case of active substances like plant protection products, biocides, pharmaceuticals.

Experts were asked about approaches that they consider particularly valuable or have abandoned based on their experience as well as information on software tools and databases relevant to assessing mixture effects (Figure 10).
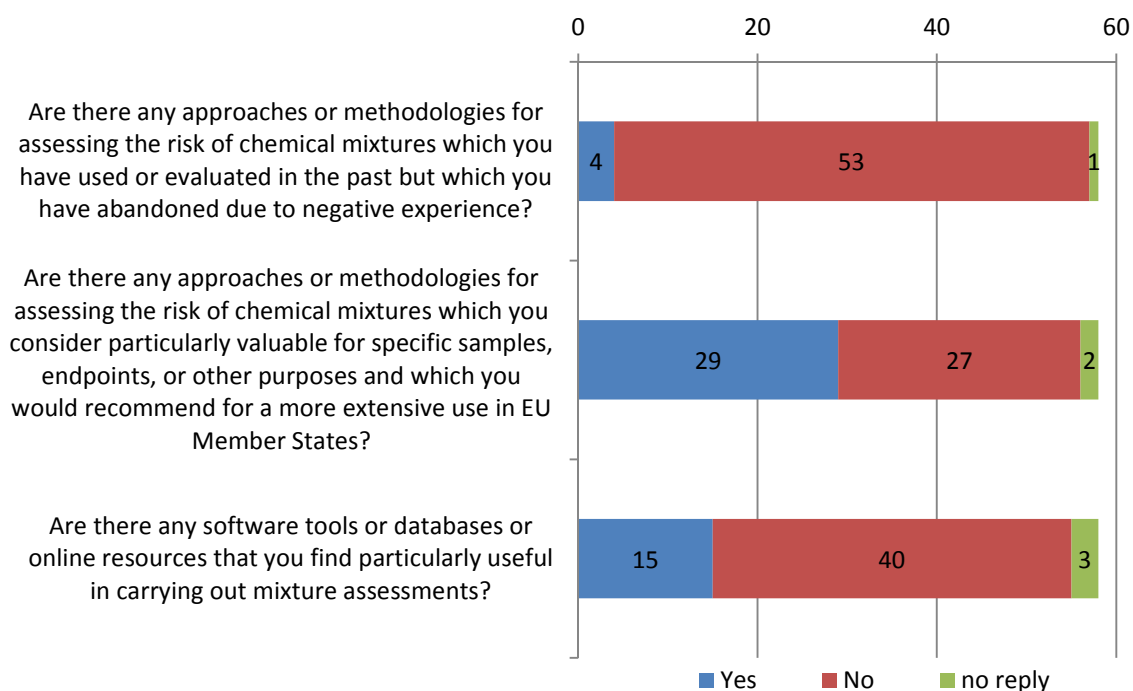
Figure 10 Replies to the questions on approaches the experts have abandoned due to negative experience, specific approaches that the experts would recommend for further considerations, and on use of relevant software tools and databases.

The approach being mentioned most often as being abandoned was mainly Independent Action with the general reason that it is too data demanding. Specific approaches that were recommended for more extensive use were the MCR approach (Price and Han, 2011), mixture toxic pressure approach (e.g. De Zwart and Posthuma, 2006), whole mixture testing at human relevant levels, use of TTC, *in vitro* methods, TKTD and DEB modelling. Software tools and databases mentioned of use for carrying out mixture assessments were CREME, CARES, MIXTOX, DEBtox, ToxTree, DEREK, Acropolis tool MCRA, US EPA BMDS, ToxCalcMix, Metals Classification Tool MeClas.

4.5. Use of novel tools in mixture toxicity assessment

Experts were asked about the use of novel tools in the assessment of mixtures. An overview of the general responses regarding their use can be found in Figure 11.

Do you apply novel tools in the RA of mixtures?
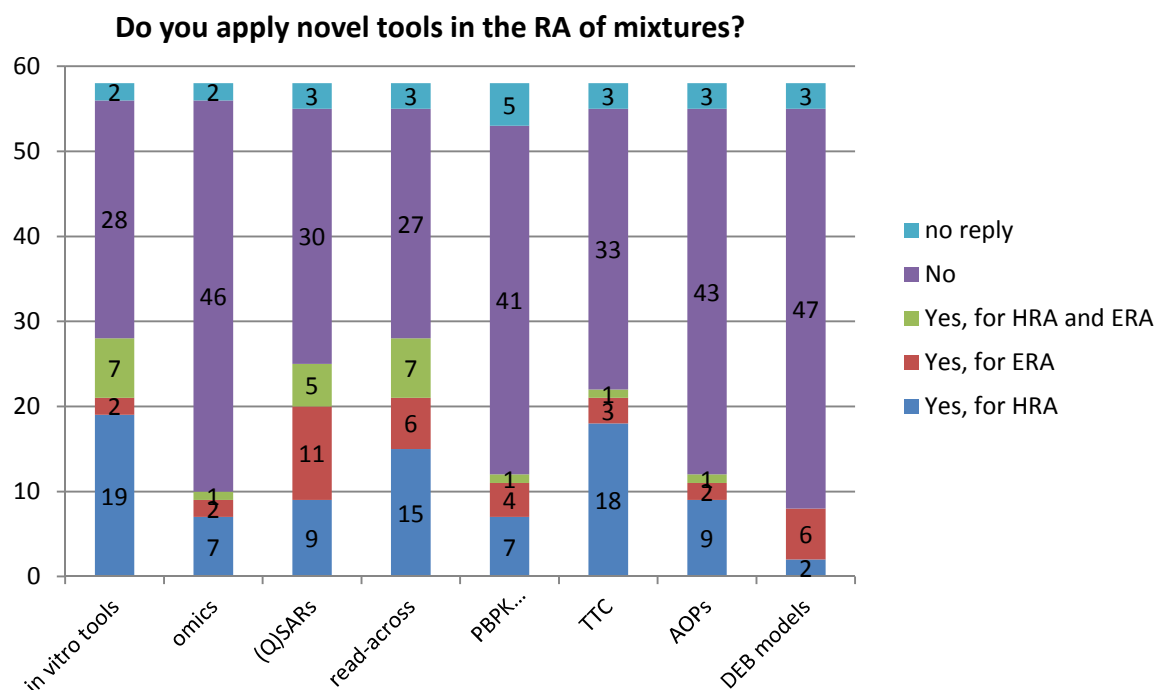
Figure 11 Replies to the question "Do you apply *in vitro* tools/omics approaches/ (quantitative) structure activity relationships ((Q)SARs)/ read-across/ physiologically based pharmacokinetic (PBPK) modelling/ the toxicological threshold of concern (TTC) concept/ Adverse Outcome pathways (AOPs)/ dynamic energy budget (DEB) models for human health risk assessment (HRA), environmental risk assessment (ERA) or both?"

Experts were then asked further on their reasons for using specific tools or not using them. Results are presented in the following sections 4.5.1-4.5.9.

4.5.1. Use of *in vitro* assays in mixture toxicity assessment

In vitro tools are increasingly applied, both in human and environmental risk assessment. Not only because they are powerful tools to investigate TK processes, but also because they can provide key information on the mechanism of action. Especially with complex mixtures like environmental samples, they might provide insight in toxicity beyond the traditional chemical analysis.

28 experts replied that they use *in vitro* assays in the assessment of mixtures. The tests mentioned were tests from the OECD endocrine disruptor testing framework, cell-based transactivation assays, irritation/sensitisation assays, epiocular assay, dermal absorption, Ames test, micronucleus assay, genotoxicity, mutagenicity, comet assay, ROS production, cytotoxicity, microtox, algae assays, zebrafish embryo tests, bacterial reporters, rodent and human cell lines and human tissue cultures.

The reasons for using *in vitro* assays are presented in Figure 12. Among the other reasons, one expert replied that the use of *in vitro* tools combined with PBPK modelling is *the* way forward for chemical risk assessment. Other 28 experts replied that they are not using *in vitro* assays and were asked for the reason(s) (Figure 13). The main reasons

for not using *in vitro* methods are the lack of legal drivers and lack of guidance and validation.

What are the main reasons you are using these *in vitro* assays?

Reason	Value
The results are quickly available	15
The mechanism of action tested has a clear in vivo relevance	15
The method is cost efficient	11
The method allows for whole mixture testing	10
The results are easy to interpret	8
In vivo method should be the last resort	8
The assay(s) can provide key TK information	2
Other reason(s)	4

Figure 12 Replies to the question "What are the main reasons you are using these *in vitro* assays?" Other reasons mentioned were for research purpose, Ames test because it is a data requirement in some regions, and combination of *in vitro* testing with PBPK modelling is seen as the way forward for chemical risk assessment.

What are the main reason(s) you are not using *in vitro* assays?

Reason	Value
The method lacks ecological relevance	7
There are no legal drivers for this method	7
The method is not validated	6
The method lacks (in vivo) predictability (in vitro to in vivo extrapolation)	4
The method lacks the proper metabolisation steps	4
There is no generally accepted protocol (e.g. OECD) for this test	4
The method is too costly	3
The results from the test are difficult to interpret	2
The method is too complicated	1
The samples are generally too complex to test reliably	1
There are no suitable reference matierals available	1
There is a lack of proper QA/QC for this method	1
The method is too labour intensive	0
The method is not reliable enough	0
The method takes too much time to produce results	0
Other reason(s)	11

Figure 13 Replies to the question "What are the main reasons you are not using *in vitro* assays? The main "other reasons" given were lack of EU guidance and validation, and in the case of cosmetics that *in vitro* tests are less relevant for the final products as clinical tests are performed with cosmetic formulations.

4.5.2.   Use of omics approaches in mixture toxicity assessment

Omics technologies are increasingly applied to gain insight in the mechanism of action of compounds and mixtures, at the transcription level (transcriptomics), the protein level (proteomics) or even the whole metabolome (metabolomics). Genomic tools can potentially be used also to investigate differences in responses between individuals and species.

10 experts had experience with using omics tools in mixture risk assessment. Most experience is available for transcriptomics, and to a lesser extent with proteomics and metabolomics (Figure 14). The main reasons for using omics technologies are presented in Figure 15. The main advantages mentioned were the possibility to study overall effects and ability to gain mechanistic information as well as the sensitivity of the methods. 46 experts replied they were not using omics technologies for mixture risk

assessment. The main reasons for not using omics are presented in Figure 16, with one of the main problems identified being the lack of clear guidance and protocols.

Which type of omics technologies are you using?
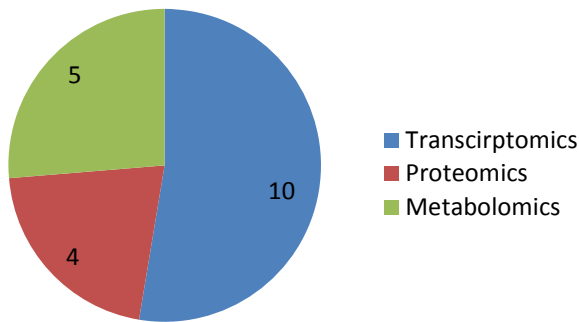
Figure 14 Replies to the question "Which type of omics technologies are you using?"

What are the main reasons you are using omics based approaches?
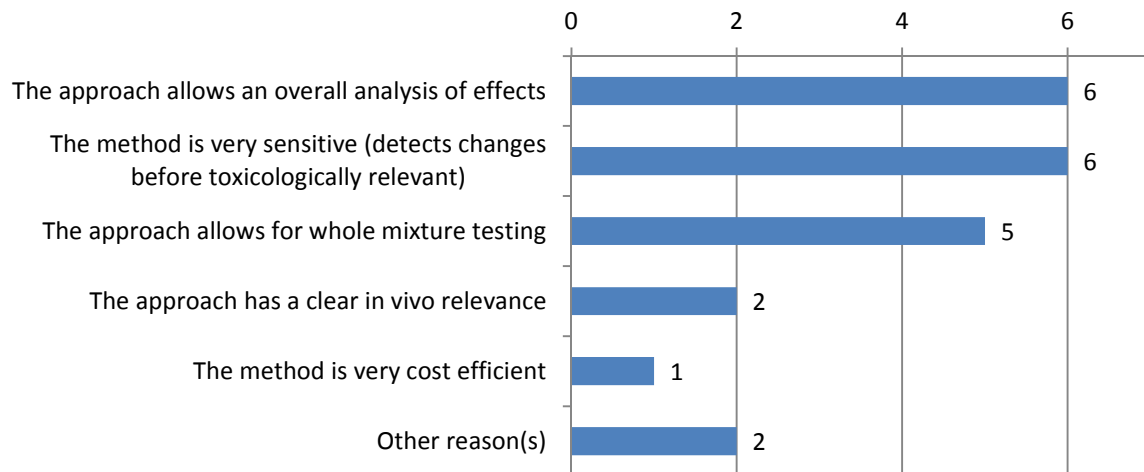
Figure 15 Replies to the question "What are the main reasons you are using omics based approaches?" The other reasons given were the possibility to study mechanisms of action.

What are the main reason(s) you are not using omics based assays?

Reason	Value
There are no legal drivers for this method	13
There is no generally accepted protocol (e.g. OECD) for this method	13
The method is not validated	10
The data analysis is too complex	9
The method lacks (in vivo) predictability	9
The method is too complicated	7
The method lacks ecological relevance	6
There are no suitable reference materials available	3
The method is too costly	3
The method is too labour intensive	2
The samples are generally too complex to test reliably	2
The method is not robust enough	1
There is a lack of proper QA/QC for this method	1
The method takes too much time to result	0
Other reason(s)	12

Figure 16 Replies to the question "What are the main reasons you are not using omics based assays?" The main "other reason" provided was the unavailability of the relevant practical tools in the facilities of the responding experts, as well as the lack of clear guidance.

4.5.3. Use of (Q)SAR models in mixture toxicity assessment

(Quantitative) structure activity relationship ((Q)SAR) models are mathematical models that have been developed to predict a number of physico-chemical and (eco)toxicological properties of chemicals without performing *in vitro* or *in vivo* tests. The models use information on the structure of the chemical, and combine this with a database with toxicological information on related/comparable chemicals.

25 of the participating experts are using (Q)SAR in the mixture risk assessment. They consider (Q)SARs very useful because they represent an alternative to *in vivo* studies to some extent and they can serve for prioritising experimental testing, and results are quickly available (Figure 17). The purposes for which the experts apply (Q)SAR methods are shown in Figure 18 with the main purpose being hazard identification. The endpoints for which (Q)SARs are mainly used are endocrine activity in cell based transactivation assays, mortality, reprotoxicity, acute (eco)toxicity, mutagenicity, genotoxicity, cancer

alerts, skin sensitisation, toxicokinetics, secondary poisoning, $logK_{ow}$, $logK_{oc}$, BCF, biodegradation, fate and behaviour. Experts were also asked to provide information on the QSAR tools they are using (Figure 19).

30 experts replied that they are not using QSARs for mixture assessments. The main reasons for not using (Q)SARs are presented in Figure 20.

What are the main reasons you are using (Q)SARs?
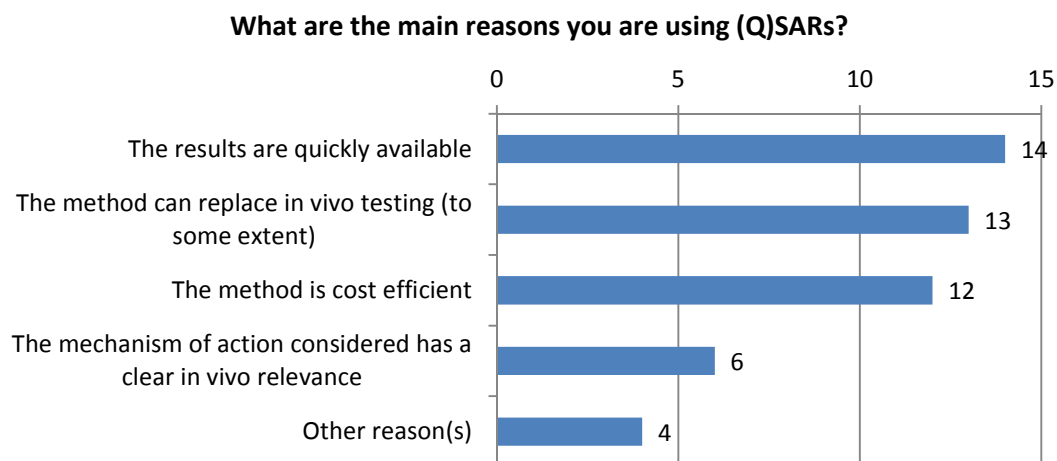
Figure 17 Replies to the question "What are the main reasons you are using (Q)SARs?" "Other reasons" mentioned were the application to metabolites and impurities where no data are available, and the possibility to identify substances of concern and drive further experimental testing. Also the use for chemical fate aspects was mentioned.

For what purpose(s) are you applying (Q)SARs?
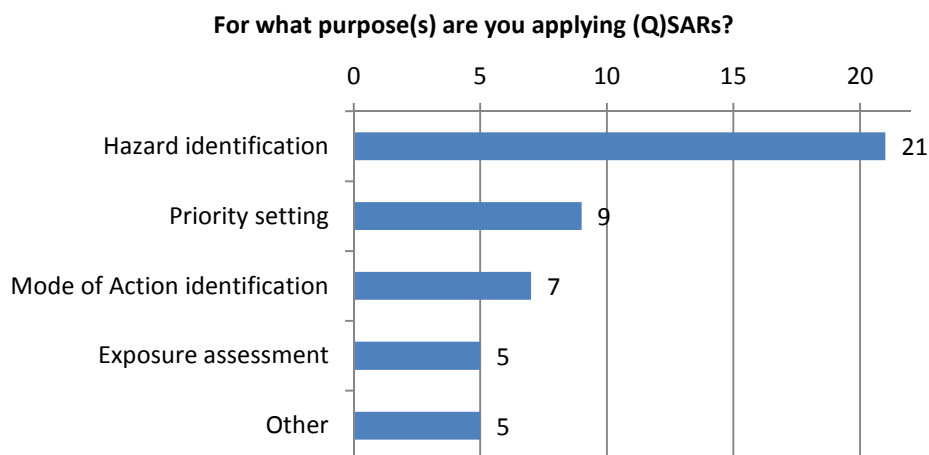
Figure 18 Replies to the question "For what purpose are you applying (Q)SARs? "Other reasons" mentioned were data gap filling, prediction of physic-chemical properties, grouping of metabolites and impurities, and fate predictions.

Which (Q)SAR models do you apply?

Model	Count
OECD Toolbox	14
Derek (Nexus)	11
ECOSAR	8
Toxtree	7
TOPKAT	5
CAESAR	5
Danish EPA QSAR database	5
Lazar	2
OASIS TIMES	2
MultiCASE	1
DEMETRA	1
OncoLogic	0
HazardExpert	0
Meteor (Nexus)	0
Metabolexpert	0
CHEMPROP	0
Other(s)	7
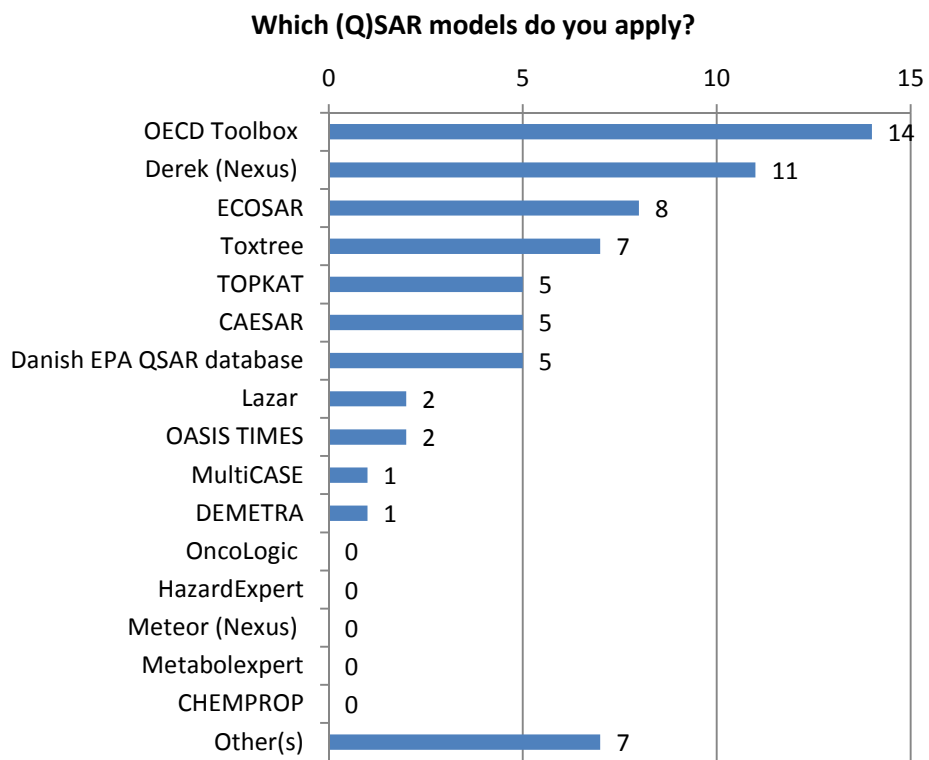
Figure 19 Replies to the question "Which (Q)SAR models do you apply?" Other models mentioned were DEBtox, VEGA platform models, ACD Labs Percepta, Episuite, SarPy.

What are the main reasons you are not using (Q)SARs?

The method is not properly validated — 9
No good models are available for the inteded application — 8
Too much expert judgement is needed to run the method and interpret results — 7
The method lacks (in vivo) predictability — 5
There are no legal drivers for this method — 5
Appropriate software is not available to the user — 4
Competing models show conflicting results — 2
There is a lack of information regarding the identity of the compounds — 2
The method lacks ecological relevance — 0
There is a lack of proper QA/Q for this method — 0
The method is too costly — 0
Other reason(s) — 11

Figure 20 Replies to the question "What are the main reasons you are not using (Q)SARs?". The main "other reasons" given were that databases are not validated, lack of guidance, (Q)SARs are applied to single substances but not to mixtures because only qualitative QSAR information is used, (Q)SARs are not relevant because of available testing information (e.g. for PPPs), too many uncertainties associated with (Q)SARs, lack of knowledge/training.

4.5.4. Use of read-across approaches in mixture toxicity assessment

Read-across is a technique for predicting endpoint information for one substance by using data on the same endpoint from (an)other substance(s). This can be performed with a limited set of substances (analogue approach) or within a large group of substances (category approach).

28 experts replied that they are using read-across in the assessment of mixtures. The main reason given for using read-across was to replace *in vivo* testing to some extent (Figure 21). Regarding the endpoints for which read-across is applied, experts were mostly answering that they use read-across for any kind of endpoint where needed/information is suitable and where read-across is accepted by ECHA for REACH assessments. Endpoints mentioned include the whole spectrum of short and long-term animal study endpoints, as well as ecotoxicity. 27 experts replied they are not using read-across approaches, mainly because of lack of expert knowledge (Figure 22).

What are the main reasons you are using a read-across approach?
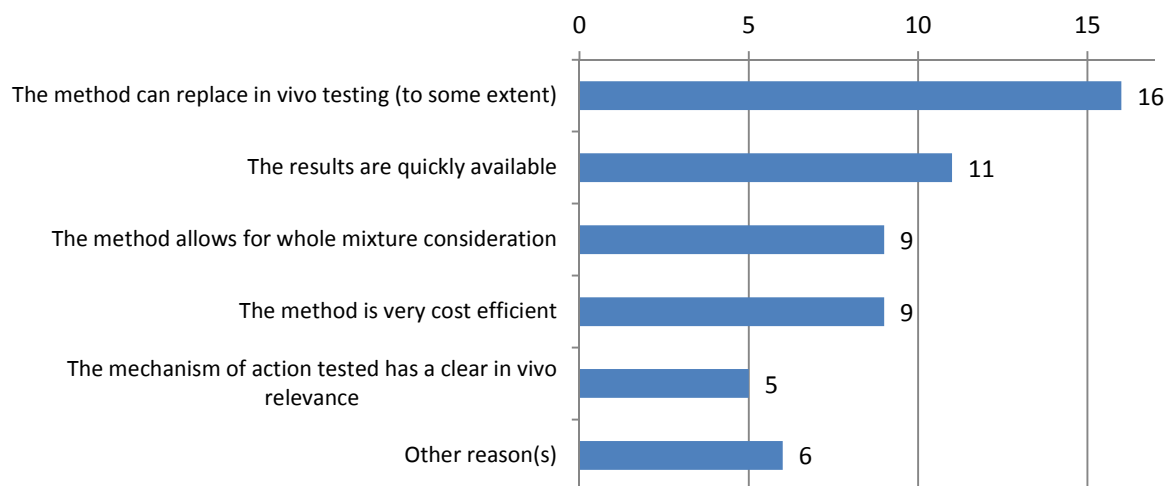
Figure 21 Replies to the question "What are the main reasons you are using a read-across approach?" Main "other reasons" were that read-across is a useful tool for the use for metabolites and impurities, where toxicity information is limited, it is encouraged under REACH, can be used to support *in vitro* test results and refine experimental design.

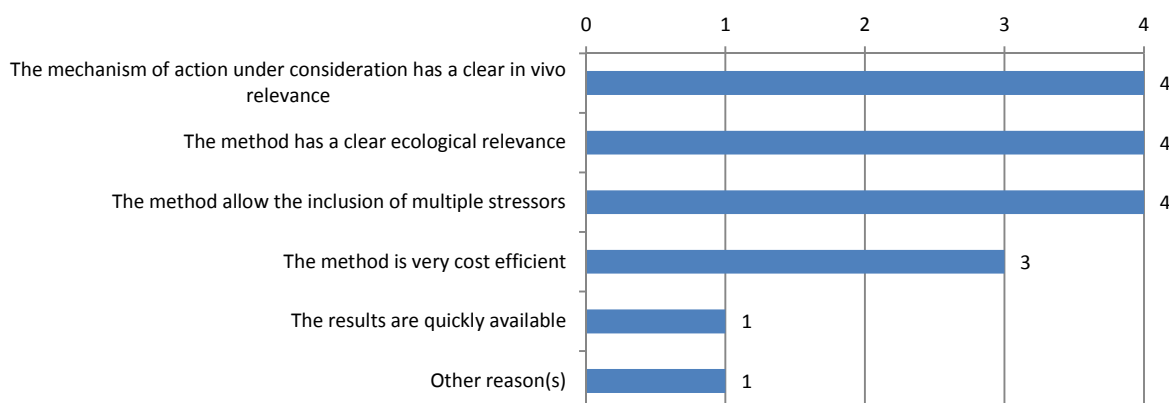**What are the main reasons you are not using a read-across approach?**

Figure 22 Replies to the question "What are the main reasons you are not using a read-across approach?" Main "other reasons" given were a lack of guidance and validation.

4.5.5. Use of PBTK modelling in mixture toxicity assessment

Physiologically based (PB) models are used for modelling toxicokinetic (TK) processes (PBTK) or TK and toxicodynamic (TD) processes (PBTKTD). The PBTK models are especially useful to assess hazard, as they provide a quantitative means to address TK processes. PBTKTD models link the TK and TD dimension and therefore are generally more complex.

12 experts replied that they are using PBTK modelling in the assessment of mixtures. The main reasons for using PBTK models were the replacement of *in vivo* testing to a certain extent, since PBTK models allow relating *in vitro* experiments to *in vivo* internal exposures (Figure 23). The main purpose for which PBTK modelling is used in mixture assessment is to correlate *in vitro* concentrations to *in vivo* scenarios/to link internal and external dose, to identify TK interactions, to understand metabolite generation, species comparison, route of exposure, assess effects of intermittent exposure often mentioned in the context of PPPs.

What are the main reasons you are using a PBTK model?
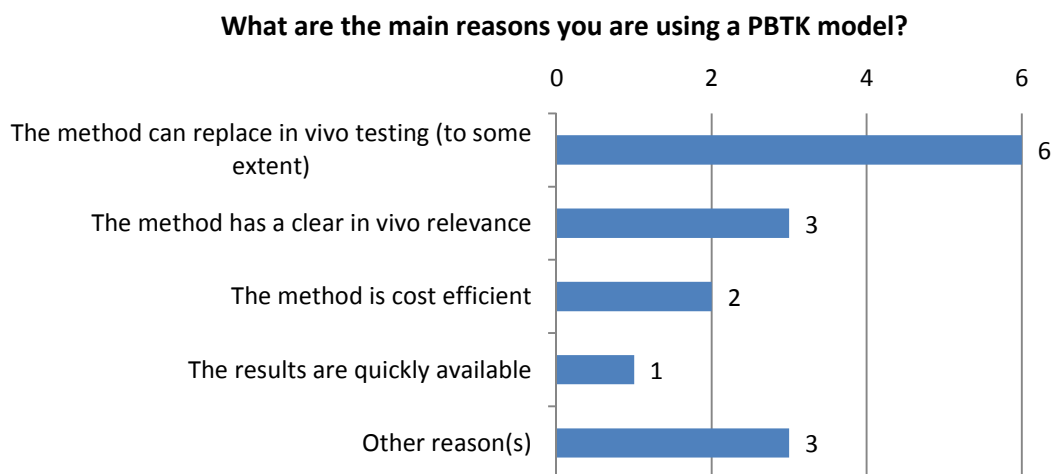
Figure 23 Replies to the question "What are the main reasons you are using a PBTK model?" The main "other reasons" were to study mechanisms, study mechanisms by which mixtures affect life-history traits, assess intermittent exposures

What are the main reasons you are not using PBTK models?
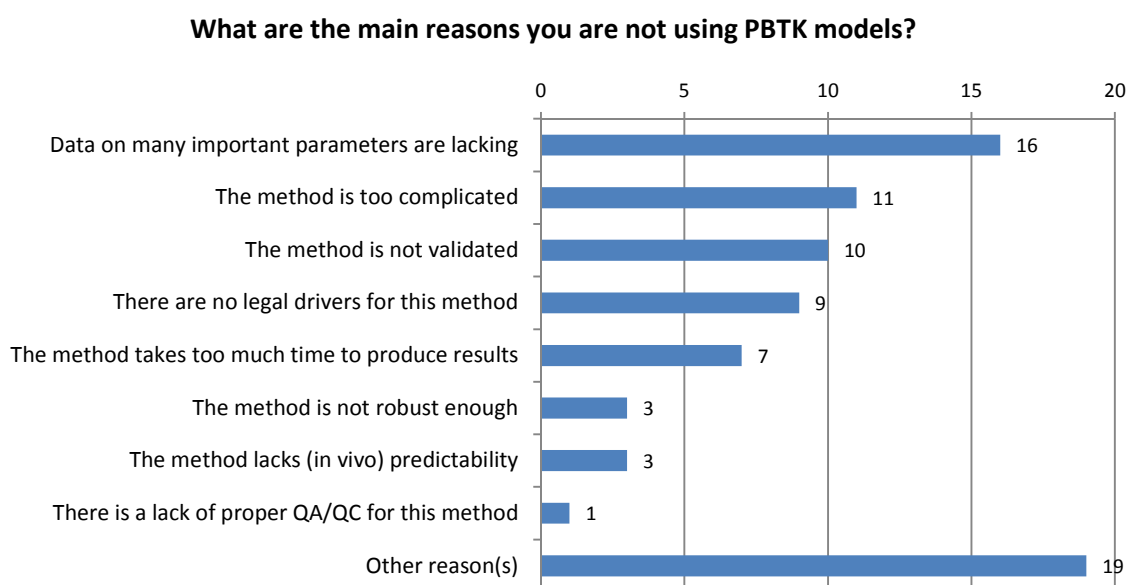
Figure 24 Replies to the question "What are the main reasons you are not using a PBTK model?" The main "other reasons" given were lack of knowledge/expertise/training, lack of guidance and tackling related uncertainties.

41 experts are not using PBTK modelling for mixture assessments. The main reason for not using PBTK modelling is a lack of knowledge/training as well as the lack of many input parameters (Figure 24).

4.5.6. Use of the TTC approach in mixture toxicity assessment

The approach of the threshold of toxicological concern (TTC) is based on historical toxicological data, that show empirically that there is a threshold below which toxicity does not occur (for non-cancer effects) or likelihood of tumour incidence is negligible (cancer effects). There are thresholds derived for different classes of compounds (Cramer classes).

22 experts replied that they apply the TTC approach in the assessment of mixtures. The main reason is that it can allow the waiving of additional testing (Figure 25). 33 experts are not using the TTC approach with the main reason being that it was developed for human health assessments and not for environmental assessments (Figure 26).

What are the main reasons you are using the TTC approach?

Reason	Value
The method can waive additional testing	14
The results are quickly available	13
The method is very cost efficient	10
Other reason(s)	5
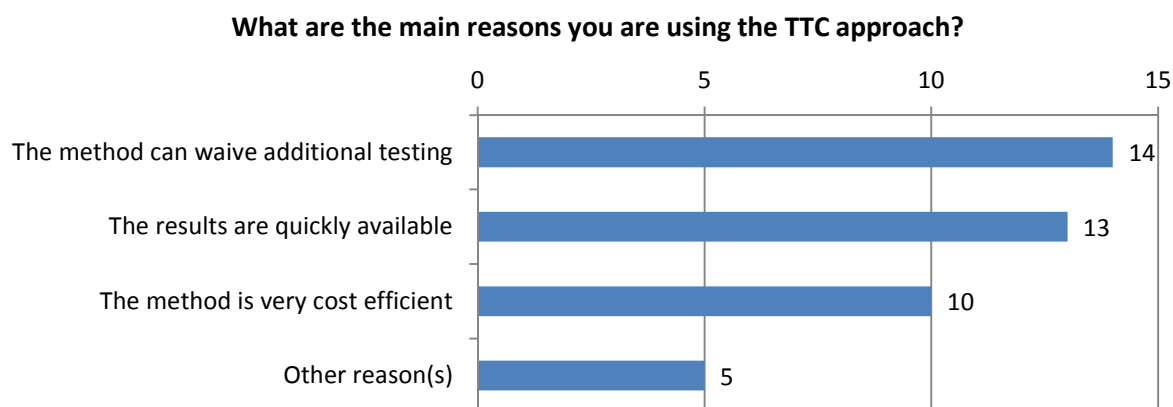
Figure 25 Replies to the question "What are the main reasons you are using the TTC approach?" Main "other reasons" were the availability for chemicals without chronic data, the use to identify and prioritise testing needs, avoiding testing, and application to ingredients of biological origin and residual impurities.

What are the main reasons you are not using the TTC approach?

The method was developed for human health only, not for environmental assessments — 11

Difficulties in assigning compound to the right class — 7

There are no legal drivers for this method — 7

The method lacks (in vivo) predictability — 5

The method is not applicable to the compounds under consideration, e.g. hormones — 3

There is a lack of information on the identity of the compounds — 3
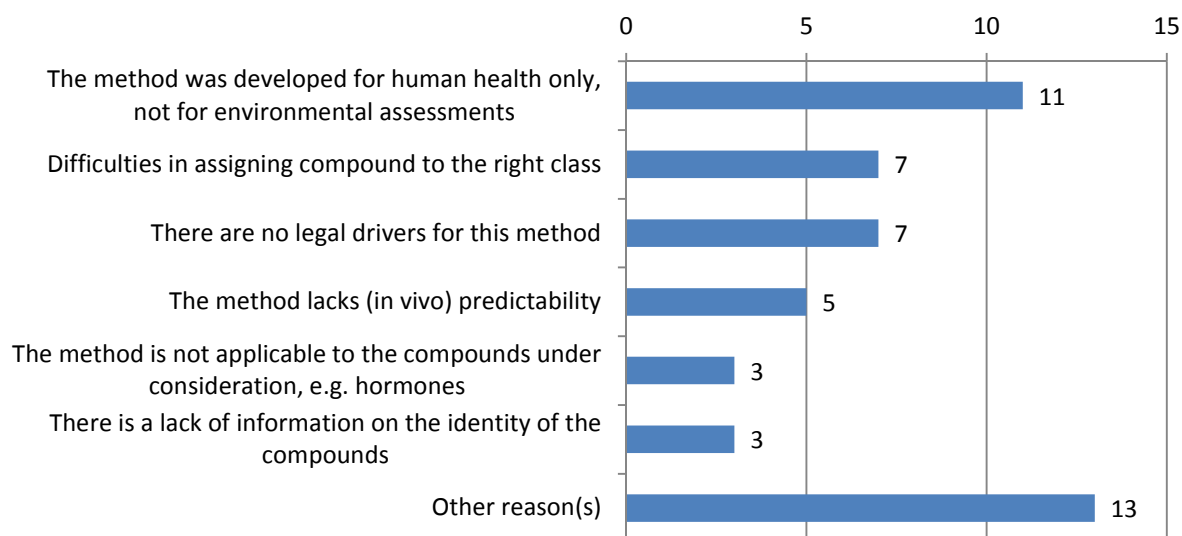
Other reason(s) — 13

Figure 26 Replies to the question "What are the main reasons you are not using the TTC approach?" Main "other reasons" were the need of updating the TTC approach, the lack of experience, and dealing with higher substance concentrations.

4.5.7. Use of AOPs in mixture toxicity assessment

An Adverse Outcome Pathway (AOP) is an analytical construct, describing a sequential chain of causally linked events at different levels of biological organisation that lead to an adverse health or ecotoxicological effect. AOPs might provide insight into the relevance of combinational effects when assessing the toxicity of mixtures.

12 experts replied that they are using AOPs in the assessment of mixtures. The main reasons for applying AOPs were that the method shows insight into potential interactions and that the considered mechanisms have a clear *in vivo* relevance (Figure 27). 43 experts are not applying AOPs with the main reason being the limited availability of relevant, sufficiently developed AOPs (Figure 28).

What are the main reasons you are using an AOP approach?

The method shows insight in potential interactions — 9

The mechanism of action considered has a clear in vivo relevance — 9

The approach has clear ecological relevance — 2
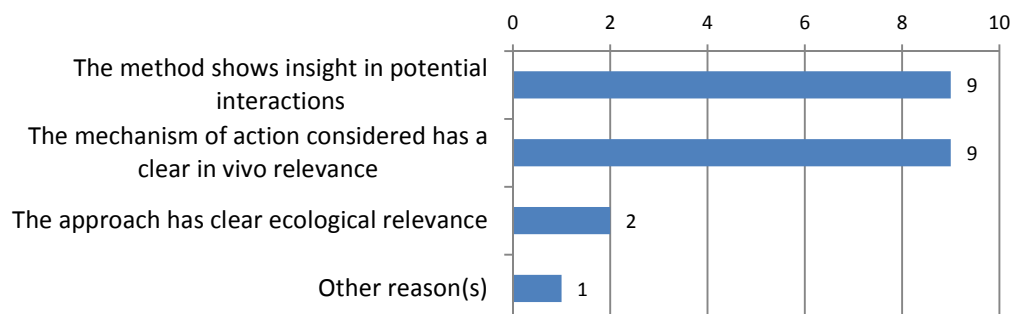
Other reason(s) — 1

Figure 27 Replies to the question "What are the main reasons you are using an AOP approach?" The "other reason" mentioned was to explore its potential for risk assessment.

What are the main reasons you are not using an AOP approach?

	0	5	10	15	20

Relevant AOPs are not available or not sufficiently developed — 20

The approach is non-formalised — 10

There are no legal drivers for this method — 8

The results are difficult to interpret — 5
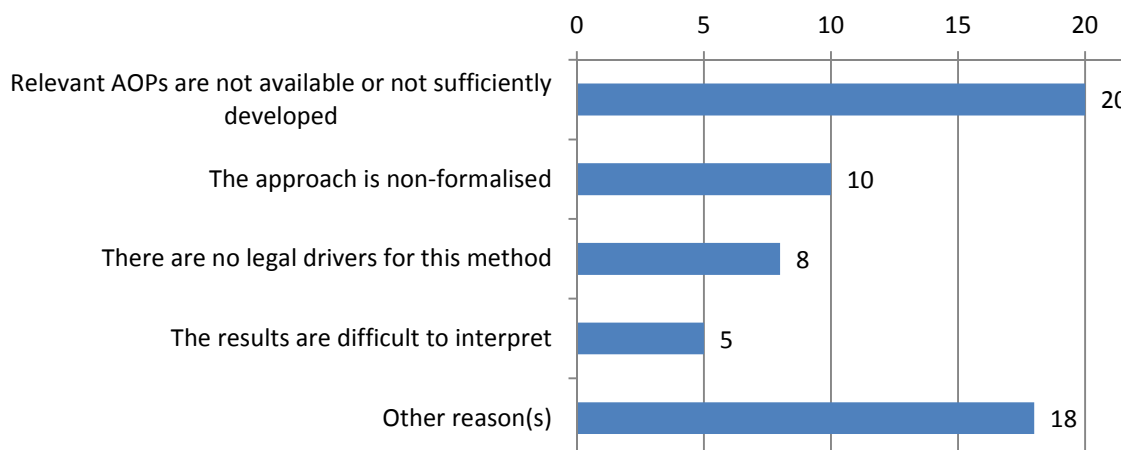
Other reason(s) — 18

Figure 28 Replies to the question "What are the main reasons you are not using an AOP approach?" The "other reasons" mentioned are mainly the lack of expertise/experience, and difficulties to implement this new concept in risk assessment.

4.5.8.   Use of DEB models in mixture toxicity assessment

Dynamic Energy Budget (DEB) models aim to identify (simple) quantitative rules for the processes of uptake of substrate by organisms and the use for maintenance, growth, maturation and reproduction. By linking a TK model to the DEB, effects of chemicals can be incorporated as well.

8 experts replied they are using DEB models in the assessment of mixtures with the main reason being the inclusion of multiple stressors, the ecological relevance, and the *in vivo* relevance (Figure 29). 47 experts were not using DEB models with the main reason being on the one hand that it is a concept usually applied in ecotoxicology rather than for human health assessments and on the other hand the lack of expertise and the lack of validation (Figure 30).

What are the main reasons you are using DEB models?

	0	1	2	3	4

The mechanism of action under consideration has a clear in vivo relevance — 4

The method has a clear ecological relevance — 4

The method allow the inclusion of multiple stressors — 4

The method is very cost efficient — 3

The results are quickly available — 1

Other reason(s) — 1

Figure 29 Replies to the question "What are the main reasons you are using DEB models?" The "other reason" was stating that DEB models are the most promising approach to interpret and predict effects of stressors on growth and reproduction and to learn from experimental testing.

What are the main reasons you are not using DEB models?

Reason	Value
There are no legal drivers for this method	9
Much of the required information for the model is lacking	7
The method is too complex	6
The method is not robust enough	5
The method lacks (in vivo) predictability	1
The method lacks ecological relevance	1
Other reason(s)	24
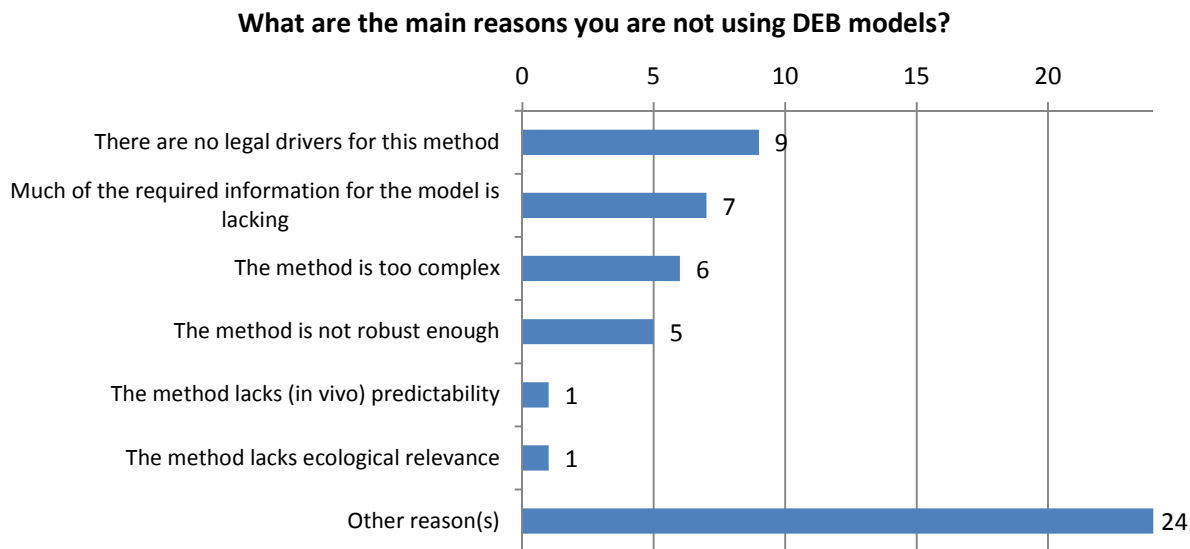
Figure 30 Replies to the question "What are the main reasons you are not using DEB models?" Main "other reasons" were a general lack of expertise/experience, for experts in human RA its focus on environmental RA, and a lack of validation.

4.5.9. Use of IATA frameworks in mixture toxicity assessment

IATA provide a framework to integrate existing knowledge based on classes of chemicals with the results of biochemical and cellular assays, computational predictive methods, exposure studies, and other sources of information to identify requirements for targeted testing or develop assessment conclusions.

8 experts had experience in applying IATA frameworks to the assessment of mixtures (Figure 31). The IATA mentioned are skin and eye irritation, integrated testing strategies (ITS) described in the ECHA guidance and some tailored customised approaches.

Do you use any IATA framework for the assessment of mixtures?

- Yes: 8
- No: 47
- no reply: 3
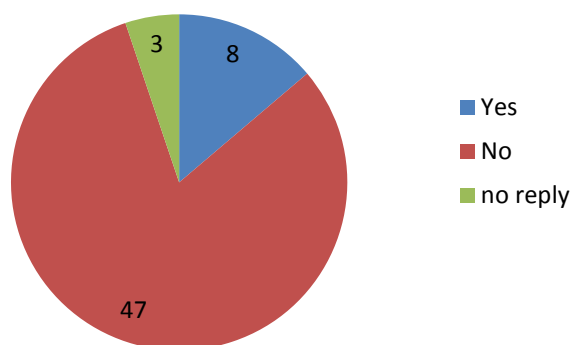
Figure 31 Replies to the question "Do you use any IATA framework for the assessment of mixtures?"

4.5.10. Summary on the use of novel tools in the assessment of mixtures

Many experts are already using several of the new tools. Some are still more frequently used as research activity, but start being applied also in regulatory context. Many tools are considered as promising but not yet ready for regulatory purposes. Often mentioned reasons for not applying novel tools are the lack of legal drivers, lack of standardisation / validation, lack of guidance, and a lack of expertise.

4.6. Frameworks for the risk assessment of combined exposure to multiple chemicals

Several international frameworks for addressing combined exposure to chemical mixtures were developed in recent years. Experts were asked in the survey about their experience with the most widespread three frameworks, i.e. the WHO/IPCS framework (WHO/IPCS 2009, Meek et al 2011), Proposal by the three non-food scientific committees of the European Commission (SCHER, SCENIR, SCCS, 2012), and the proposal by CEFIC MIAT (Price et al., 2012). 35 experts were familiar with at least one of the three frameworks. Most experience was shown for the WHO/IPCS framework (Figure 32).

Which framework(s) do you apply?

- ■ WHO/IPCS 2009, Meek et al 2011
- ■ Proposal by the three non-food Scientific Committees of the European Commission from 2012
- ■ Proposal by CEFIC Mixtures Industry Ad-Hoc team (MIAT) 2012
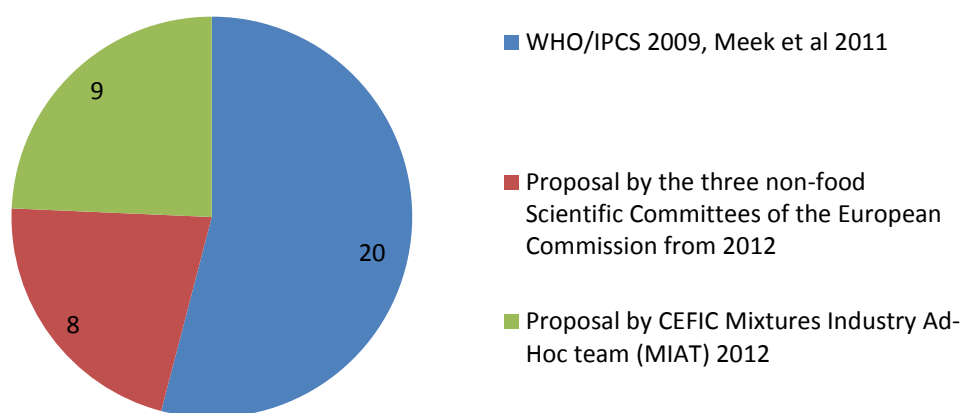
Figure 32 Replies to question "Which [international] framework(s) [for addressing combined exposure] do you apply?"

Experts were then asked to provide feedback on their experience with the above-mentioned frameworks. The experiences were generally positive. The main limitation mentioned especially for the WHO/IPCS and the SCHER/SCENIHR/SCCS framework is that they provide a more conceptual framework and less practical guidance.

The experts commented positively on the WHO/IPCS, rating it an easy and transparent approach. Critics were that it is rather general and lacks criteria when refinement should be stopped. The data available usually allow only to perform Tier 1 and 2 assessments and not to go to higher tiers. However, it was appreciated that Tier 0 and Tier1 can usually be performed with the data available.

The SCHER, SCENIHR, SCCS framework is considered useful for organising data and deciding how to perform the assessment, but it is more conceptual and provides limited guidance on refined assessments.

The CEFIC MIAT framework was judged as useful since it comprises practical tools. Most input received on this framework was from experts involved in its development.

Experts were then asked about other frameworks they are applying for assessing combined exposure. 15 experts replied they had experience with other frameworks, 40 replied to have no experience with other frameworks (Figure 33). The most often mentioned example was the cumulative assessment for plant protection products as developed by EFSA (EFSA PPR Panel, 2013). Furthermore, experts mentioned the US EPA, transitional guidance on mixture toxicity assessment of biocidal products (2014); EFSA guidance on Birds and Mammals (2009); Backhaus and Faust 2012 (Environ. Sci. Technol., 46 (5)); UK Combined toxicity assessment for plant protection products (non-dietary and dietary risk assessment); Methodology and the information to be specified for the assessment of pesticides used in tank-mixtures subject to prior assessment in accordance with the French Ministerial Order of 7 April 2010; Metals Classification tool (MeClas)/Transformation Dissolution Protocol.

Are you familiar with or do you apply any other framework to assess the toxicity of mixtures?
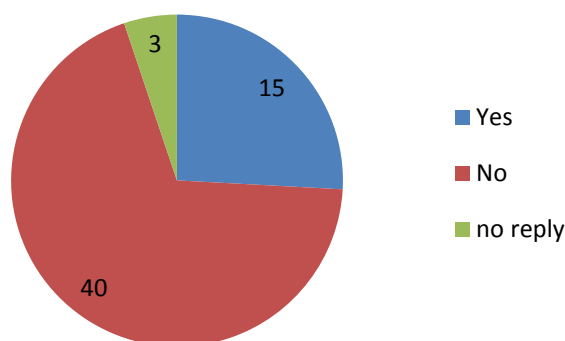
Figure 33 Replies to the question "Are you familiar with or do you apply any other framework to assess the toxicity of mixtures?"

4.7. General/additional remarks by experts in the survey

In the end of the questionnaire, experts had the possibility to add some general remarks or provide additional information in the form of references or uploading files. Experts were providing many useful references.

4.8. Conclusions from the expert survey

The expert survey was a success with 58 experts participating and providing extensive information in the free text fields. The results from the survey allow to derive a clear picture on the current status of assessing mixtures.

The main sectors where most experience is already gained in assessing mixtures are in the area of plant protection products and chemicals. These were also rated highest regarding the priority for performing mixture assessments. However, mixture assessments are also performed in many other areas.

Experts have experience with the whole mixture as well as the component-based approaches applying them to both, intentional and unintentional mixtures.

Mostly concentration addition (CA) based methods are used for predicting mixture effects. In contrast, several experts would not recommend the further use of independent action (IA) based approaches, mainly because of the higher need for input data for IA and considering the small differences in predictions by IA compared to CA. Experts were asked about their opinion on considering interactions. Most experts stated that interactions should be considered if there is specific evidence for interactions and on a case-by-case basis.

Regarding the use of novel tools in the risk assessment of mixtures (such as *in vitro* methods, omics, (Q)SARs, read-across, PBTK modelling, TTC approaches, AOPs, DEB models, IATA), expert opinions are split between those applying them (often more in a research context) and those that generally think these tools are valuable but their use is currently limited because of lack of guidance, lack of data, or lack of expertise.

Experts had experience with assessing mixtures, both in the context of human health and environmental risk assessment. Apart from some tools that were developed for a specific application in HRA (e.g. TTC approach) or ERA (e.g. DEB modelling), there seems to be no clear difference in the opinions/experiences provided.

A general need for clear and harmonised guidance for combined exposure assessments can be identified from the survey.

5. Conclusions

Humans and the environment are continuously exposed to a multitude of substances via different routes of exposure. However, the risk assessment of chemicals for regulatory purposes does not generally take into account the "real life" exposure to multiple substances, but mainly relies on the assessment of individual substances. A previous review on regulatory requirements for the assessment of mixtures shows that combined exposure is nowadays taken up in several pieces of legislation, however a harmonised consistent approach on performing mixture assessments across different regulatory sectors is still lacking (Kienzler et al., 2014).

Assessment in different sectors

Our expert survey showed that mixture risk assessment is taken up in various fields. The main sectors where most experience is already gained in assessing mixtures are in the area of plant protection products (PPPs) and chemicals falling under REACH, which is however also linked to the fields of work of the respondents. PPPs and chemicals under REACH were also rated highest regarding the priority for performing mixture assessments by the experts. The survey respondents seemed to rate active substances (such as biocides, plant protection products and pharmaceuticals) overall of higher concern, also regarding a potential presence of interactions (i.e. including the risk of synergistic effects). However, mixture assessments are also performed in many other areas. Looking at the available experience derived through the survey and at case studies in the literature (Kienzler et al., unpublished report), many examples can be found where environmental exposure or occupational exposure to mixtures were retrospectively assessed, based on monitored exposure data. Examples in the prospective risk assessment are rarer. One major achievement in this area is represented by the cumulative risk assessment and respective cumulative assessment groups as developed by EFSA's PPR Panel (EFSA PPR, 2014). Further development of consistent approaches for prospective risk assessment is still needed.

Component-based assessment approaches: concentration addition vs independent action

Currently, many experts have experience with the whole mixture as well as the component-based approaches applying them to both, intentional and unintentional mixtures, without a clear trend in their combination. Regarding component-based approaches, survey respondents mostly use concentration addition (CA) based methods for predicting mixture effects. Several experts would even not recommend the further use of independent action (IA) based approaches, mainly because of the higher need for input data for IA and considering the small differences in predictions by IA compared to CA. Overall, evidence in the literature supports the application of concentration addition as a first, protective approach. It is therefore also the default approach to start from in several international recommendations and frameworks, independent of components' similar or dissimilar mode of action. However, once a detailed risk assessment for a mixture is performed, relevant groupings will be based on common target organs and/or MoA (e.g. based on AOPs). The choice of the approach used depends strongly on the context of the risk assessment as well as on the information on which to base the grouping of components. Irrespective of the starting point for grouping, it is recommended to use all available information on the mixture and its components: physico-chemical properties, structural alerts, (Q)SAR and read-across information, evidence from omics, *in vitro* (high throughput screening or other) or *in vivo* experimental data, depending on availability.

Considering interactions in mixture assessments

Current evidence in the literature suggests that interactions (synergistic or antagonistic effects) at lower concentration levels such as environmental concentrations are rare and

if observed, leading to deviations from CA predictions that are relatively small. In the survey, experts were asked about their opinion on considering interactions. Most experts stated that interactions should be considered if there is specific evidence for interactions and on a case-by-case basis. Experts commented that the highest potential for interactions should be assumed for active substances (such as PPPs, biocides, pharmaceuticals). Some experts answered that they consider interactions rare and hazard sufficiently covered using CA based approaches. Only very few experts agreed to introduce a default safety factor to cover potential interactions, which is sometimes proposed in the literature (for overview see Backhaus, 2015). However, more knowledge could be gained from additional case studies covering different sectors to further underpin this.

To address and predict interactions, toxicokinetic and toxicodynamic modelling are valuable tools. PBTK and DEB modelling can support gaining further mechanistic understanding and looking at effects of individual chemicals in a mixture in an integrated approach. Toxicokinetic information to feed into these models, can be gained e.g. from *in vitro* studies. Also read-across information from similar mixtures can be used to identify mixtures where interactions could play a role and should be further investigated.

Application of "novel tools" in the assessment of mixtures

In this report the current state of the art of the application of alternative tools for assessing the hazard of chemical mixtures was briefly reviewed. The focus is hereby on the adverse outcome pathway (AOP) concept, *in vitro* methods, omics techniques, *in silico* approaches such as quantitative structure activity relationships (QSARs) and read-across, toxicokinetic and dynamic energy budget (DEB) modelling, and on integrated approaches to testing and assessment (IATA). A brief summary of the main possibilities for the application of each of these tools in the context of mixtures is given below:

- AOPs are already used for the grouping of chemicals according to their MoA, which is an important step in the assessment of mixtures. Apart from this, they can help in putting results from *in vitro* tests and computational modelling into context, e.g. in developing AOP based IATA, that allow for the integration of different data types.
- *In vitro* methods can support the assessment of mixtures in many ways, mainly in "whole-mixture testing" applied e.g. in effect-based environmental monitoring or in deriving relevant information on individual mixture components. Performing high-throughput screening of many chemicals in many different assays enables the characterisation of chemicals regarding their MoA which can help e.g. in the grouping of chemicals. AOPs can help in integrating data from diverse *in vitro* tests that address different steps in a chain of biological events. If *in vitro* test results are interpreted in connection with toxicokinetic information, even quantitative information on potency can be derived.
- The main potential of *omics* techniques (transcriptomics, proteomics, metabolomics) regarding the assessment of mixtures lies in the investigation of affected pathways for unravelling MoAs and investigating possible interactions, which can again support the grouping of chemicals.
- QSAR models can be used to obtain information on the properties and activities of substances from chemical structure alone, and can thus be used to fill data gaps in the safety assessment of chemicals. There are three main ways in which QSARs can be applied for the assessment of mixtures: (1) for predicting (missing) information on individual compounds (physico-chemical properties, toxicological effects) (2) for predicting directly or stepwise the combined effects and interactions of chemicals in a mixture (3) for assessing whether chemicals will act in a similar or dissimilar way.
- Read-across can be of value in the assessment of mixtures mainly in two ways, i.e. to predict missing information for untested constituents of a mixture in a component based approach, or to read-across for similar mixtures in a whole mixture approach.

- Toxicokinetics (TK) and toxicodynamics (TD) considerations can support the assessment of chemical mixtures in several ways with the main areas of application being (1) determination of internal exposure concentrations, e.g. enabling a relation between body concentrations and *in vitro* experiments (i.e. IVIVE, *in vitro* to *in vivo* extrapolations), of relevance for single chemicals as well as for chemical mixtures, (2) considering the simultaneous or sequential exposure to different mixture components, assessing the probability that those reach the same target, and (3) predicting interactions among mixture components on TK and TD level.
- The approach of dynamic energy budgets (DEBs) is applied in the ecotoxicology area. For the time being DEB models are not yet regularly used in the assessment of mixtures. They are however a promising tool, since they look at effects in a more integrated and mechanistic way, potentially integrating chemical and non-chemical stressors.
- The TTC approach is recommended in the literature for use at a screening level mixture assessment, for comparing first estimates of combined exposure to the TTC. It can serve eliminating combinations that are of low concern and to prioritise mixtures for further assessment. Currently, the TTC approach is limited to application in human health risk assessment; however, a corresponding ecoTTC approach is under development.
- In the context of the risk assessment from combined exposure, IATA provide another framework to collect information on individual mixture components as well as on whole mixtures, allowing a more structured (and if AOP based more mechanistically relevant) way of data generation and interpretation.

In the survey, expert opinions regarding these methodologies and tools were split between those applying them (often more in a research context) and those that generally think these tools are valuable but see their use as currently limited because of a lack of guidance, lack of data, or lack of expertise.

Overall, a high potential in applying novel tools and scientific methodologies for the assessment of chemical mixtures can be identified. They allow deriving meaningful information on individual mixture components or whole mixtures, enabling a better understanding of the underlying mechanisms of mixture effects. Their main strengths lie in their integrated use and smart combination to put different aspects regarding the hazard from combined exposure to multiple chemicals into context. In order to benefit from these tools in the hazard assessment of mixtures, more guidance on their use is needed to facilitate a more widespread application.

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List of abbreviations and definitions

AOP	Adverse Outcome Pathway
CA	Concentration Addition
CEFIC MIAT	European Chemical Industry Council Mixtures Industry Ad-hoc Team
CLP	Classification, labelling and packaging
DEB	Dynamic Energy Budget modelling
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ERA	Environmental Risk Assessment
ESTAF	EURL ECVAM Stakeholder Forum
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
HI	Hazard Index
HI_{int}	Hazard Index considering Interactions
HRA	Human Health Risk Assessment
IA	Independent Action
IATA	Integrated approaches to testing and assessment
MCR	Maximum Cumulative Ratio
MoA	Mode of Action
OECD	Organisation for Economic Co-operation and Development
PARERE	EURL ECVAM's Network for Preliminary Assessment of Regulatory Relevance
PBTK	Physiologically Based Toxicokinetic modelling
PODI	Point of Departure Index
PPP	Plant Protection Product
QA/QC	Quality Assurance/Quality Control
QSAR	Quantitative Structure Activity Relationship
RA	Risk Assessment
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RPF	Relative Potency Factor

SCCS	Scientific Committee on Consumer Safety
SCENHIR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
TEF	Toxic Equivalence Factor
TKTD	Toxicokinetic/Toxicodynamic modelling
TTC	Threshold of Toxicological Concern
TUS	Toxic Unit Summation
WHO/IPCS	World Health Organisation/ International Programme on Chemical Safety

List of figures

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