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Methodology for health risk assessment of combined exposures to multiple chemicals

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ABSTRACT

Focus on risks to human health and the environment from combined exposure to multiple chemicals ("mixture risk assessment") has increased in the last couple of decades. There has been a rise in awareness and concern in the community, especially concerning unintentional environmental exposure to unknown chemical mixtures. The Horizon 2020 project EuroMix has developed methodologies and tools for mixture risk assessment with a focus on component-based approach where substances are grouped based on toxicological considerations. Dose addition is used as the model for calculating the combined toxicity of mixture components. The methodology is anchored in the Adverse Outcome Pathway (AOP) concept, which provides a structured basis for e.g. grouping substances into assessment groups and identifying and collecting relevant toxicity data. The aim of this paper is to describes development of the methodology for mixture risk assessment and to provide detailed methodology for problem formulation, use of AOP networks for development of tiered testing strategies and grouping of substances, as well as considerations for use of dose addition methodology.

1. Introduction

Focus on risks to human health and the environment from combined exposure to multiple chemicals ("mixture risk assessment") has increased in the last couple of decades. There has been a rise in awareness and concern in the community, especially concerning unintentional environmental exposure to unknown chemical mixtures. Requirements to assess risks from chemical mixtures (ranging from the simple to complex, intentional to unknown environmental mixtures) have been included in several regulatory frameworks in the EU, the US, as well as several other countries (Rotter et al., 2018). This increased focus has led to the generation of new knowledge and scientific advancements, driving the development and harmonisation of methodologies to assess exposure and hazards of chemical mixtures of different types. Several authorities and organisations have developed guidance documents for the application of such methods and different aspects of the risk assessment process for different purposes and under different jurisdictions. Several organisations, including the Organisation for Economic Co-operation and Development (OECD), the European Food Safety Authority (EFSA) and the World Health Organisation (WHO) have recently published guidance and considerations for mixture risk assessment that give an updated description of approaches, methods and specific challenges and research needs (EFSA, 2019a; OECD, 2018a; Meek et al., 2011; WHO, 2017). Specific challenges for the risk assessment and management of chemical mixtures were recently summarized in a publication from the EU Commission Joint Research Center (JRC) (Bopp et al., 2019). This report also proposes ways forward based on novel methodologies.

While harmonisation of methods and approaches for risk assessment of chemical mixtures is desirable, for example to promote consistency and stakeholder confidence, strict alignment of the risk assessment process may not be feasible due to the different contexts in which risk assessment of chemical mixtures have to be conducted and the different legislated requirements that may apply. However, there are many similarities in the approaches and methodologies described for mixture risk assessment.

The recent OECD report "Considerations for Assessing the Risks of Combined Exposure to Multiple Chemicals" presents elements to recognise when conducting health or environmental risk assessment of chemical mixtures (OECD, 2018a). In order to be applicable for risk

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assessments within different contexts and legal frameworks, the OECD recommends a general framework starting with problem formulation and scoping, and continuing with considerations for hazard and co-exposure characterisation and the application of risk characterisation through a tiered approach. Both whole mixture approaches and component-based approaches are considered.

EFSA published their "Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals" in March 2019 (EFSA, 2019a). The EFSA guidance describes harmonised risk assessment methodologies for mixture risk assessment for all relevant areas within EFSA's remit, i.e. human health, animal health and ecological areas. Similar to the OECD guidance, EFSA proposes a framework for mixture risk assessment that includes problem formulation, as well as tiered approaches for hazard identification and characterisation, exposure assessment and risk characterisation. Both whole mixture approaches and component-based approaches are considered.

The overall aim of the Horizon 2020 funded EuroMix project (https://cordis.europa.eu/project/id/633172) was to develop methodology and tools for mixture risk assessment. The aim of this paper is to describes development of the methodology for mixture risk assessment and to provide detailed methodology for problem formulation, use of adverse outcome pathway (AOP) networks for development of tiered testing strategies and grouping of substances, as well as considerations for use of dose addition methodology.

2. Methods

The approach to develop methodology for mixture risk assessment in the EuroMix project was conducted in four steps. First, the current approaches for health risk assessment of chemical mixtures from different authorities, organisations and expert groups were reviewed. The following frameworks and methodologies were included in the review: ATSDR, 2004; ATSDR, 2018; Bopp et al., 2019; EFSA, 2008, 2013a, 2013b, 2013c, 2015, 2018a, 2019b; IGHRC, 2009; Meek et al., 2011; RIVM/ICPS/ANSES, 2013, 2016; SCHER/SCENIHR/SCCS, 2012; Solomon et al., 2016; Stein et al., 2014; Teeguarden et al., 2016; US EPA 2000, 2002, 2003, 2007, 2015, 2016; VKM, 2008. The analysis of the current approaches was reported in Rotter et al. (2017, 2018). In the second step, the outcome of the review formed the basis for development of EuroMix methodology in a series of workshops involving partners in the EuroMix project. The specific methodology and tools developed and applied by partners in the project were analysed and included. The recent documents on mixture risk assessment published by OECD and EFSA (OECD, 2018a; EFSA, 2019a) were also considered. In the third step, the draft description of the methodology was reviewed by all partners in the project and their input was included in a next version. In the fourth step, input was collected at stakeholder meetings, training events and webinars and the final description of the EuroMix methodology was developed.

In the development of the EuroMix methodology it was decided to focus on the component-based approach to mixture risk assessment where substances are grouped based on toxicological considerations. The methodology was anchored in the AOP concept, which provides a structured basis for e.g. grouping substances into assessment groups, identifying and collecting relevant toxicity data and identifying possible up-stream key events (KEs) that can be used to calculate relative potency factors (RPFs). One important focus of EuroMix was to develop methodology promoting and facilitating the use of mechanistic data from *in silico* models and *in vitro* assays to inform risk assessment and to prioritize substances (and mixtures) for further testing at higher tiers, e.g. using animal studies.

The EuroMix methodology was developed in close alignment with the development of the Monte Carlo Risk Assessment (MCRA) toolbox, also known as the EuroMix toolbox. The EuroMix toolbox is a web-based toolbox for mixture risk assessment that can be used for applying the EuroMix methodology (van der Voet, 2020).

3. Results

3.1. EuroMix methodology for mixture risk assessment

The methodology developed in the EuroMix project is consistent with and expands upon the recent guidance for mixture risk assessment published by OECD and EFSA (EFSA, 2019a; OECD, 2018a). The aim is to provide practical support for mixture risk assessment. The EuroMix methodology focuses on component-based mixture risk assessment where substances are grouped based on toxicological considerations. Toxicity and exposure information for each substance in the assessment group is used for estimation of the combined risk using the dose-addition hypothesis and RPFs. The exposure assessment of mixtures is based on probabilistic methodology considering the individual consumption and concentration data allowing estimation of a range of percentiles of exposure to the mixture. The focus is on dietary exposure. The EuroMix methodology is flexible, enabling assessment of both data-rich and data-poor substances. The methodology and the EuroMix toolbox can also be applied for substances grouped based on other than toxicological considerations, e.g. structure or exposure.

The methodology and tools address the key elements in the framework for mixture risk assessment: problem formulation, hazard assessment, exposure assessment and risk characterisation. General issues of tiering approaches and uncertainty analysis are described (Fig. 1). This paper provides detailed methodology for problem formulation, use of AOP networks for development of tiered testing strategies and grouping of substances as well as considerations for use of dose addition methodology. Hypothetical examples are included in the Supplementary material to illustrate the methodology. The EuroMix handbook for mixture risk assessment, a deliverable from the EuroMix project, provides additional methodology, examples and templates for mixture risk assessment (Zilliacus et al., 2019).

3.2. EuroMix toolbox

The EuroMix toolbox, also referred to as MCRA 9, is a web-based toolbox for mixture risk assessment developed in the EuroMix project (https://mcra.rivm.nl). It provides a range of tools for application in hazard, exposure and risk assessment of data-rich, as well as data-poor substances. Exposure and toxicity data can be uploaded and used for calculation of e.g. exposure levels, RPFs and risk levels. For a description of the EuroMix toolbox we refer to van der Voet et al. (2020).

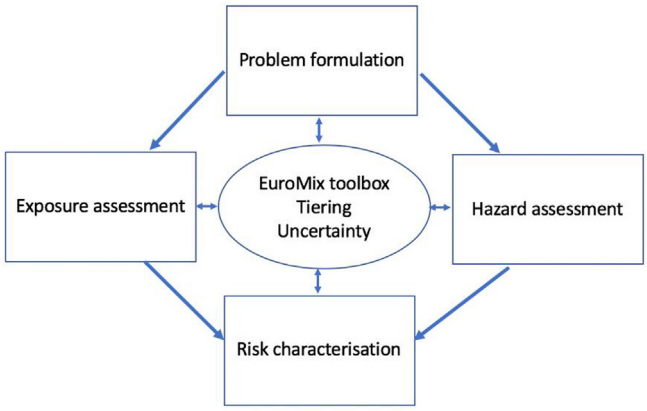

Fig. 1. The EuroMix methodology and tools address key elements in the framework for mixture risk assessment, i.e. problem formulation, hazard assessment, exposure assessment and risk characterisation. Furthermore, the EuroMix toolbox MCRA 9, tiering approaches and uncertainty analysis are described.

3.3. Problem formulation

Problem formulation is a systematic and often iterative process of defining the purpose and scope of a risk assessment, such as appropriate population groups to be evaluated, relevant substances to be considered, the regulatory goal and intended outcome/use of the assessment, as well as the boundaries of the analysis. In application of the EuroMix methodology, the outcome of the problem formulation is an analysis plan, which provides details for how the assessment will be carried out. The problem formulation is often based on a request from risk managers and is developed in dialogue between the risk managers and risk assessors. As such, the problem formulation may include considerations of policy issues, in addition to scientific considerations. Importantly, the terms of reference or mandate for the assessment, should be explicit and clearly understandable to all relevant parties. Several organisations have recently discussed and provided guidance for problem formulation in mixture risk assessment (ATSDR, 2018; EFSA, 2019a; Meek et al., 2011; OECD, 2018a; Solomon et al., 2016). The methodology for the problem formulation described here is based on this work.

The scope of mixture risk assessments may vary greatly depending on the specific focus and risk assessment question. The assessment may for example be based or focused on a specific endpoint, chemical class, exposure source or population, and the problem formulation process will not be the same in each case (OECD, 2018a). The development of the EuroMix methodology has mainly focused on exposure to substances with a common toxicological effect via food. Case studies and method development have thus been endpoint-based to a large degree. However, the EuroMix problem formulation methodology may be adapted and applied to any type of question or scope.

The EuroMix methodology for problem formulation includes considerations for formulating the risk assessment question, description of the mixture, conceptual model and methodological approach, which includes considerations for e.g. tiering, uncertainty analysis, timeframe and resources. The outcome of the problem formulation is an analysis plan, for which a template has been developed. For an example of a hypothetical analysis plan, see Supplementary materials, Appendix 1.

3.3.1. Risk assessment question

The risk assessment question is often received from the risk managers. It may include descriptors for the (sub-)population for which the assessment has to be conducted, the type of substances that are of interest, the sources and/or routes of exposure, as well as the type of effect that should be assessed. The initial risk assessment question does not have to be very detailed and can be refined as needed as the assessment progresses.

3.3.2. Description of the mixture

The aim of this step is to identify whether a mixture risk assessment is required, and if so, which substances should be considered. Description of the mixture should firstly include a description of the concern with regard to the adverse health effect in the population i.e. that there is sufficient evidence (or suspicion) for a common target organ, effect or mode of action that is of relevance for human health. Secondly, it should address if there is sufficient evidence of co-exposure to or co-occurrence of the substances identified in the assessment group. An example of co-occurrence are multiple substances on certain food (e.g. eight pesticides on a monitoring sample of strawberries). Co-occurrence or co-exposure may also occur as a consequence of exposure to single or multiple substances in the several food items consumed by the same individual during one day. Ideally, it can be noted that co-exposure refers to the internal exposure to the substances at the time scale relevant for the adverse effect. In other words, there can be co-exposure even if external exposure does not occur at the same time. Toxicokinetic properties of substances will also influence the potential for co-exposure. This step requires (preliminary) investigation of available data for both exposure and toxicity. In cases the internal exposure is of relevance, the EuroMix toolbox can be used to investigate which substances link to *in silico*, *in vitro* or *in vivo* hazard data in any step of the relevant AOP network, to investigate co-exposure patterns, and to take account of toxicokinetics.

3.3.3. Conceptual model

The conceptual model aims to define the boundaries for answering the risk assessment question. It provides the basis and rationale for the methodologies applied in the assessment. The description of the conceptual model includes:

- The regulatory framework and remit under which the assessment is being conducted.
- Substance categories included.
- The relevant exposure sources and routes.
- The appropriate population (sub)group(s), e.g. described by sex, age, occupation, country.
- The toxicological effect being assessed and the level for grouping, i.e. common target organ, common effect/adverse outcome (AO) or common specific mode of action/AOP.

3.3.4. Methodological approach

In this step, the applied methodology for e.g. collection and generation of toxicity data, grouping of substances, calculation of RPFs, collection and generation of exposure data, models for exposure assessment, choice of risk metrics should be explicitly considered and reported. For details of methodological aspects see Supplementary materials, Appendix 1.

The problem formulation and resulting analysis plan will also depend on the amount and quality of data available ("data rich" vs "data poor" situations). The EuroMix template for an analysis plan includes a section to specify the level/tier of the different parts of the assessments and the level of complexity of the methods and models to be used. The choice of tier is also influenced by the purpose of the assessment, i.e. in certain cases it is sufficient to conduct a low-tier assessment. Nevertheless, if the available data allow for a refined high-tier assessment, e.g. individual consumption data, such data can be routinely used.

A description of the method for uncertainty analysis and planned modelling of the uncertainty can be included in the problem formulation. The EuroMix toolbox allows for quantifying several sources of uncertainty in exposure and hazard data. An estimation of the type of expertise, number of persons and other resources needed as well as an estimated timeframe can be described. Plan for stakeholder consultation and peer review can be included.

3.3.5. Analysis plan

The outcome of the steps above is summarized in an analysis plan that describes the planned mixture risk assessment. The problem formulation should be viewed as an iterative process and refinements of the analysis plan may become necessary as the risk assessment progresses and information is gathered.

3.4. Hazard assessment

The EuroMix methodology for hazard assessment is anchored in the AOP concept, which provides a structured basis e.g. for grouping substances into assessment groups, identifying and collecting relevant toxicity data and identifying possible up-stream KEs that can be used to calculate RPFs. In the following sections, the methodology for identification and assessment of AOP networks for use is mixture risk assessment is first described. Then, the methodology for grouping of substances into assessment groups is covered, followed by an outline of the development of tiered testing strategies based on the AOP networks. In the last section, considerations when using RPFs are discussed.

3.5. Identification and assessment of AOP networks for mixture risk assessment

The concept of AOP networks can be useful for mixture risk assessment to support grouping of substances into assessment groups and to identify upstream KEs that can provide toxicity data for RPFs (EFSA, 2019a; OECD, 2018a).

An AOP describes the pathway from a molecular initiating event (MIE), i.e. the interaction between the substance and biological target (e.g. receptor, enzyme), via subsequent KEs at molecular, cellular, tissue and organ levels to the AO in an individual organism. Multiple AOP can form an AOP network by converging at the same AO, and/or sharing MIEs or other KEs. The methodology to develop and assess AOP is described in detail in the OECD Users' handbook supplement to the guidance document for developing and assessing AOP (OECD, 2018b).

Which AOP to include in an AOP network will depend on the problem formulation in the specific case and on current mechanistic knowledge and understanding. It may, for example, be the intention to describe all possible pathways resulting in a specific AO. However, it should not be assumed that all pathways are known or have been investigated or described. Therefore, potential omission of pathways that could be relevant should be acknowledged as an uncertainty. The consequences of this uncertainty for the outcome of the risk assessment will depend on the problem formulation. In the EuroMix project the following methodology was developed to identify and assess AOP networks for use in mixture risk assessment. For an illustrative example from the EuroMix project see Supplementary materials, Appendix 2.

- First, for the AO of interest any existing AOP published in the AOP wiki (https://aopwiki.org) or literature should be identified and used as basis for any further development. In case none are available, the development of a new AOP can start by identifying the AO and thereafter identifying KEs leading to the AO.
- KEs leading to the AO are identified by searching the literature for evidence linking the KEs to each other and to the AO, using the methodology described in OECD 2018b. It is most useful to identify KEs that can be easily measured to inform grouping and provide toxicity data for RPFs.
- It is not necessary to develop a complete AOP. Even an AOP with only a single KE in addition to the AO may be useful initially. However, further development to include additional KEs will increase confidence in the single AOP as a whole. The level of confidence required from the AOP included in the AOP network will depend on the problem formulation and intended use of the risk assessment by risk managers.
- When the AOP has been postulated, it should be assessed, as described in the OECD AOP handbook (OECD, 2018b). The assessment includes evaluation of the biological plausibility and empirical support for the key event relationships (KERs) linking the KEs, as well as evidence supporting the essentiality of the KEs. Ideally, any new AOP that are developed should be submitted to the OECD AOP Development Programme for review and inclusion in the AOP Wiki (https://aopwiki.org/). It is acknowledged that AOP that are endorsed by the OECD and published in the OECD Series on Adverse Outcome Pathways (https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x) have a level of acceptance that increases utility for regulatory purposes.
- Multiple AOP for the same AO should be combined in an AOP network. The EuroMix toolbox allows to specify an AOP network in terms of effects (AO, KEs and MIEs) and their causal relations.

3.6. Grouping of substances based on toxicological considerations

The process and principles for grouping substances into assessment groups will be determined by the problem formulation. Substances can be grouped into relevant assessment groups using different approaches. The EuroMix methodology for grouping is based on toxicological considerations. Dose addition is the default recommended model for mixture risk assessment (EFSA, 2019a; OECD, 2018a) and is used in the EuroMix methodology for substances grouped into the same assessment group.

AOP networks can provide a framework for grouping of substances. Grouping based on toxicity can be performed at different levels of biological organisation, i.e. common target organ, common effect/AO or common specific mode of action/AOP. The EuroMix toolbox can be used for any of these levels by specifying the appropriate effects. Grouping at the level of a common target organ may be necessary for some data-poor substances for which no information on specific effects/AO in the target organ is available. Grouping at the level of common effect/AO will probably be useful in most cases. Even if empirical evidence is lacking, a common assumption is that substances that act via different AOP leading to the same AO (i.e. different modes of action) can be grouped together at the level of common effect/AO and dose addition then applies (EFSA, 2013c). In certain cases, evidence may indicate that the substances cause the AO via separate independent AOP and the model for dose addition does not appropriately describe the combined effect of the separate AOP. In such cases, the substances would be grouped based on the specific mode of action/AOP and the model for response addition could potentially be used.

The decision whether a substance should or should not belong to an assessment group should be made based on all available relevant evidence. However, in many cases it is uncertain which substances should belong to a specific assessment group. In such cases, the uncertainty about assessment group allocation can be expressed as a probability (between 0 and 1). The EuroMix toolbox includes a function to consider probabilities of allocation to a specific assessment group, which is referred to as "group membership" in the EuroMix methodology.

Specific criteria related to exposure and toxicity can also be used to decide on assessment group membership, such as exclusion of substances below a specified exposure level or exclusion of substances for which the point of departure (POD) of the critical effect, that is the basis for setting the ADI/TDI, is lower than the POD of the specific effect that is the focus of the mixture risk assessment. Such criteria should be clearly described and justified in the problem formulation, but note that such criteria typically depend on assessment results, e.g. exposure or POD, that should already be available. This illustrates the iterative nature of many risk assessments.

The following methodology can be applied for grouping at any level, i.e. common target organ, common effect/AO or common specific mode of action/AOP, and irrespective of whether the group membership is expressed as a probability or as "included/not included". How to group substances into relevant assessment groups is inherently reliant on the problem formulation and assessment groups could vary depending on problem formulation. The EuroMix methodology is intended to promote structured and transparent process for grouping. The described methodology is based on EuroMix case studies (Kyriakopoulou et al., 2016) and is consistent with and expands upon methodology described by EFSA (EFSA, 2018a; 2019b). For a hypothetical example, see Supplementary materials, Appendix 3.

- The level of grouping, e.g. common target organ, common effect/AO or common specific mode of action/AOP, is first decided.
- An AOP network for the AO is identified, when needed. In cases where grouping is done at the level of common effect/AO and toxicity data are available for the AO for all substances in the assessment group, information on the AOP network is not necessary to decide on grouping. However, in cases where toxicity data on the AO are missing for some or all substances, toxicity data for KEs in the AOP network can be used to inform the grouping. The EuroMix toolbox can be used to find these linkages.
- Substance category to be assessed is identified in the problem formulation, e.g. pesticides approved in Europe or contaminants

identified by human biomonitoring.

- Toxicological data for the substances are collected from scientific literature and relevant databases. The data can be from *in silico*, *in vitro*, *in vivo* or human studies and can be related to the AO or any KEs in the AOP network. In case data from *in vivo* studies for the AO are available, additional data might not be needed. Data collection can be done in a tiered manner, and additional data are only required when the uncertainty of group membership is high. In the special case where only *in silico* data are available, grouping can be done based on the results from the *in silico* models only. Uncertainties concerning group membership, e.g. due to lack of data, should be clearly expressed. The EuroMix toolbox allows to logically organise *in silico* data (predictions from QSAR models, molecular docking model binding energies), *in vitro* and/or *in vivo* dose-response data, *in vitro* and/or *in vivo* POD data, and human reference value data.
- The data are organised into lines of evidence. For example, data can be arranged for each KE and for the AO and can be further organised according to data from *in silico*, *in vitro*, *in vivo* or human studies.
- The reliability (quality) of the data is evaluated. The relevance of the data for grouping into assessment groups is also evaluated. For example, data from *in silico* and from *in vitro* studies for up-stream KEs might be considered as less relevant than *in vivo* studies measuring the AO. Scoring systems for reliability and relevance can be used if they facilitate the assessment and the following steps. In the EuroMix toolbox, *in silico* memberships can be provided or calculated as probabilities, in order to express the uncertainty. The limited precision of dose-response data can be quantified using benchmark dose (BMD) modelling.
- Decision of group membership is done based on the data, considering their reliability and relevance in a weight-of-evidence approach. Well-organised data, including information and justification of the approach used for evaluation of reliability and relevance, facilitates the decision-making. The decision on group membership should be done by at least two experts and a pre-defined process should be in place to resolve any disagreements between experts. Formal expert knowledge elicitation can be used, and is preferable when quantifying probabilities of group membership (EFSA, 2014; 2018a, 2019b).
- The group membership for each substance is expressed as 0 (not included) or 1 (included) or as a value between 0 and 1 indicating the probability for belonging to the assessment group. The EuroMix toolbox can be used by assigning a 0 or 1 for group membership even in cases when a thorough process for grouping has not been performed due to lack of information or for the purpose of a low tier assessment.

3.7. Tiered testing strategies

One of the challenges of mixture risk assessment is availability of toxicity data for all substances that are included in the assessment group. In case there are no existing data it may become necessary to generate data specifically for the assessment at hand. A tiered testing strategy can be to first use data from *in silico* modelling (typically available for almost all substances), to set priorities for *in vitro* testing. The mechanistic *in silico* and *in vitro* data may then further inform and set priorities for *in vivo* testing (OECD, 2018a). For missing *in vivo* data, it is also possible to impute a POD, such as a BMD from benchmark dose modelling or a No Observed Adverse Effect Level (NOAEL), by read-across using either a fixed value obtained externally or a distribution of PODs in the same class of chemicals. The Threshold of Toxicological Concern (TTC) approach originally based on the NOAEL data from Munro et al. (1996) can be used to impute the missing POD. The TTC imputation method and generalisations thereof are available in the EuroMix toolbox. The use of *in silico* and *in vitro* data to the maximum extent possible supports the 3R principles (replacement, reduction and refinement) and helps to avoid animal testing.

In silico data from quantitative structure activity relationship (QSAR) models can predict toxicity at organ level (e.g. hepatotoxicity) or at the level of an effect/AO (e.g. liver steatosis). QSAR models can also predict activation of MIEs, such as nuclear receptor activation. The QSAR data can be used for grouping of substances and for prioritisation of testing, however, the grouping might include false positives and/or false negatives depending on the accuracy, sensitivity and specificity of the QSAR model used. *In silico* data from molecular docking, using either experimental three-dimensional (3D) structures, when available, or comparative 3D models, can be used to estimate binding energies to receptors and enzymes and thereby provide low tier toxicity data for RPFs (Cotterill et al., 2016). *In vitro* data from e.g. cell lines, organ cultures or zebrafish embryos can be useful for grouping, to derive potency information and for prioritisation of further testing *in vivo* (Luckert et al., 2017, 2018a, 2018b). Examples of QSAR, molecular docking and *in vitro* data have been organised in the data platform of the EuroMix toolbox.

AOP, networks can provide the basis for planning strategic testing at different levels of biological organisation (OECD, 2016). The AOP network makes it possible to identify effects/KEs that can be tested using *in silico* models, *in vitro* and *in vivo* assays. EuroMix has developed the following methodology that can be used to develop a tiered testing strategy to generate further data needed for mixture risk assessment based on AOP networks. A similar strategy may be used for risk assessment of single substances, however, for mixture risk assessment selected KEs (first point) and assays (sixth point) should specifically provide relevant information for grouping and for determining RPFs.

- Identification of the KEs in the AOP network that can provide information for grouping and/or RPFs.
- Identification of *in silico* models (e.g. QSAR or molecular docking models), *in vitro* and *in vivo* assays for measurement the KEs.
- Assessment and description of the relevance of the *in silico* models and *in vitro* and *in vivo* assays used for measuring the KEs. The assessment should take into consideration e.g. the applicability domain of the *in silico* model, the relevance of the specific measured response, as well as the relevance of the *in vitro* and *in vivo* test system.
- Assessment and description of the reliability of the outcome from the *in silico* models and *in vitro* and *in vivo* assays. The assessment should take into consideration e.g. accuracy, sensitivity and specificity of the *in silico* model and *in vitro* and *in vivo* assay.
- Assessment of the availability and feasibility, in terms of costs and other resources, for the *in silico* models and *in vitro* and *in vivo* assays.
- Assessment and description of the information provided by the *in silico* models and *in vitro* and *in vivo* assays to support the mixture risk assessment, i.e. for grouping, RPFs and/or prioritisation for further testing.
- Selection of the final *in silico* models and *in vitro* and *in vivo* assays to be included in the tiered testing strategy based on the assessments above.
- The tiered testing strategy can include recommendations on a stepwise approach for the testing, e.g. which models/assays to use first and how to proceed dependent on positive or negative results in the previous model/assay.

3.8. Relative potency factors

Dose addition using RPFs is the primary method for modelling the risk of mixtures in the EuroMix methodology. The exposure of each substance is multiplied with the RPF of the substance and the potency-scaled exposures are summed. Often, the RPF of each specific substance is derived by dividing the POD of the index substance with the POD of the specific substance. The POD value can be a BMD from benchmark dose modelling or a NOAEL. The method is flexible; the RPFs can be

based on the POD for the critical effect of the substance that is the basis for setting the ADI/TDI or the POD for the specific effect that is the focus of the mixture risk assessment. Alternatively, ADI/TDI can be used instead of POD, i.e. the POD divided by the assessment factors. Note that this approach will lead to the same RPFs if the assessment factors are equal for all substances, but to different RPFs if different assessment factors are specified. The type of RPFs should be taken into consideration when interpreting the margin of exposure (MOE). The choice of using POD from a critical or specific effect or a ADI/TDI instead of POD depends on the tier of the mixture risk assessment and data availability. Note that PODs derived from *in vitro* data may typically represent internal dose levels rather than the usual external (often oral) dose levels from *in vivo* studies.

The EuroMix toolbox allows multiple types of RPF specification or calculation, for example external RPFs based on ADI/TDI or internal RPFs based on *in vitro* PODs. The uncertainty of dose-response modelling underlying the PODs used can be used to specify a part of the uncertainty in the RPFs based on such PODs.

3.8.1. Index substance

The index substance is important in the RPF approach and should be chosen considering the following criteria:

- confidence that the substance is representative for the specific assessment group
- confidence that the substance causes the effect that is the basis for the risk assessment
- the POD is derived from an *in vivo* study for the effect in focus for the mixture risk assessment
- quality and quantity of toxicity data, resulting in a high confidence in the POD

It should be noted that the index substance does not have to be the most toxic substance (i.e. lowest POD) in the assessment group.

3.8.2. Selection of POD

There might be several different PODs available from different studies measuring the same response or different responses. The selection of POD to be used for the mixture risk assessment should consider the following.

- Comparability within the assessment group. The selected PODs for the substances within the assessment group should be comparable. Therefore, the PODs should be for the same response for all substances, i.e. the same outcome measured using the same study design. In cases where this is not possible, similar responses should be selected.
- Responses from different KEs in the AOP network. PODs might be available for all substances in the assessment group from different responses measuring either upstream or downstream KEs in the AOP network. In these cases, the relevance of the responses, including the study design, for the mixture risk assessment should be considered. Responses measuring KEs close to the AO are probably more relevant and closer to the *in vivo* POD than responses measuring the MIEs or upstream KEs.
- Several PODs for same response. In the case that several PODs are available for a substance for the same response but from different studies, either the most reliable or the most conservative POD can be selected. An overall POD can also be chosen by considering the studies together and choosing the highest NOAEL that provides a reasonable margin to the lowest LOAEL (IPCS, 2009). Alternatively, the available values could be aggregated into one POD value, under consideration of their respective uncertainties. However, models for such aggregation are not yet sufficiently developed. The EuroMix toolbox also allows for automatic selection of the POD for a substance in case several PODs have been uploaded into the toolbox. The lowest POD (conservative) or the mean POD can be chosen, or, as a tentative aggregation model, the harmonic mean value can be calculated.

3.9. Exposure assessment

The EuroMix methodology focuses on dietary exposures. However, the EuroMix toolbox also provides the possibility to model the risk from exposure to a combination of dietary and non-dietary sources. In the EuroMix toolbox probabilistic modelling of the dietary exposure is commonly used. Probabilistic exposure assessment can provide a distribution of the exposure, quantified by estimated percentiles. Quantification of uncertainty is also performed. The EuroMix handbook for mixture risk assessment also describes methods for exposure assessment in cases when exposure data are missing for a one or several substances in a mixture (Zilliacus et al., 2019).

3.10. Risk characterization

In the EuroMix approach, the default assumption is that the model of dose addition applies to all substances that cause the same AO, even if they act via dissimilar modes of action. This is in line with current opinions and approaches (e.g. EFSA, 2013c, 2019a; OECD, 2018a). It is acknowledged that this is a conservative approach to mixture risk assessment. The assumption of dose-additivity can be checked in the Euromix toolbox by visual inspection of a graph where dose-response curves of multiple substances expressed as equivalents of the index substance are super-imposed. The dose addition model may be refined by adding chemical- and effect-specific POD data. In cases where there are sufficient data to support a deviation from the dose addition model, for example convincing evidence that mixture components are only acting via dissimilar modes of action, other models can be applied.

Risk characterisation in the EuroMix toolbox is conducted by calculating the MOE using RPFs to scale the exposure of the individual substances. The methodology is flexible and can apply different levels of refinement, i.e. using ADI/TDI values at lower tiers or a POD for critical or specific effect for the hazard at higher tiers. Depending on the chosen approach, MOEs should be evaluated relative to some threshold, e.g. 100 for the common case when exposure is compared to a POD and assessment factors 10 are used for inter- and intra-species safety factors, or 1, when a comparison to a ADI/TDI is made. In the EuroMix toolbox several options are possible, and the user can specify a threshold for interpreting MOEs. The EuroMix toolbox can also calculate a more refined risk assessment using both probabilistic exposure assessment and probabilistic hazard characterisation according to the Integrated Probabilistic Risk Assessment (IPRA) model (van der Voet 2007, 2009).

3.11. Tiering principles

Tiering in mixture risk assessment refers to the process in which different steps of the assessment can be performed using simple, conservative approaches at lower tiers and more advanced approaches requiring more data at higher tiers (EFSA, 2019a; OECD, 2018a). If a conservative lower tier assessment indicates that the MOE is sufficiently protective, the assessment does not have to be refined and proceed to a higher tier. Tiering approaches apply to all the different steps in the mixture risk assessment, including grouping of substances into assessment groups, hazard assessment, exposure assessment and risk characterisation. Different tiers can be used at different steps in the same assessment, e.g. a low tier approach for hazard and a high tier approach for exposure, dependent on the need for refinement and the data availability. In principle, the EuroMix methodology and tools provide possibilities to perform the assessment at different tiers.

3.12. Uncertainty analysis

Many of the uncertainties in a mixture risk assessment are comparable to those in risk assessment of single substances (EFSA, 2018b). Mixture-specific uncertainty analysis is described in EFSA guidance (EFSA, 2019a). Uncertainties are related to e.g. the grouping of substances into assessment groups, estimation of RPFs, missing toxicity data for included substances, missing exposure data for included substances, left-censored data below detection, quantification or other reporting limits for concentration data of substances, choice of dose addition model and potential interactions (synergism/antagonism).

The uncertainty analysis should clearly identify and describe the uncertainties in the different steps in a mixture risk assessment. The identified uncertainties should be quantified if possible. In the EuroMix toolbox, uncertainties related to data or other types of input for the assessment can be modelled in probabilistic 2D Monte Carlo simulations. For left-censored data (concentrations not reported below certain limits) the toolbox offers various options of modelling, from simple imputation by 0 (optimistic) or the limit value (pessimistic) in line with the EFSA guidance on probabilistic modelling (EFSA, 2012) to more advanced options like fitting a censored lognormal model or imputing the censored values partly with the limit value (possibly multiplied by a factor, e.g. 0.5) and partly by zeroes, based on estimated use frequencies from observed occurrence patterns (van Klaveren et al., 2019a, 2019b). Uncertainties can be modelled in several steps in the assessment and can be propagated to the final risk characterisation. The overall quantified uncertainty is visualised in plots.

4. Discussion

The purpose of this paper is to describe methodologies developed in the EuroMix project. Additional methodologies, examples and templates are included in the EuroMix handbook for mixture risk assessment (Zilliacus et al., 2019).

Although use of AOP networks in mixture risk assessment is a promising approach, the EuroMix methodology and toolbox can also be applied without this information. For example, the EuroMix toolbox was applied in a pilot project of EFSA within the scope of the problem formulation set by the European Commission. Pesticides were assigned to assessment groups by experts and the probabilistic exposure assessment was performed using the EuroMix toolbox (EFSA, 2019c). The EuroMix toolbox has also been used for assessing the risk of co-occurrence of the residues of all pesticides grouped by EFSA in two cumulative assessment groups as part of the work EFSA performed on request of the European Commission (van Klaveren et al., 2019a, 2019b). Note that co-occurrence is based on the assumptions that exposure occurs on one day for acute toxic pesticides. Co-exposure to pesticides based on the assumption of chronic life-long exposure might be refined if toxicokinetic information is available. Based on the outcome of this step the decision is made to perform a mixture risk assessment or not.

There is a need for assessment of the health risks of chemical mixtures that is scientifically robust and sufficiently protective of human health. However, mixtures may contain a large number of possible chemicals in many different combinations, which, under the current risk assessment paradigm, could increase the need and requirements for toxicity testing in animals. At the same time, there is a general desire in society to move away from toxicity testing in animals, which stems from ethical considerations to reduce animal suffering, development of novel methods that are better models for human health effects, as well as a need for resource-efficient methods to screen large amounts of chemicals for different health effects (high-throughput screening).

There is currently a rapid development of non-testing and *in vitro* methods driven by stakeholder needs, academic research interests and increased regulatory focus on the 3R concept. Recent reports from e.g. the OECD emphasize the importance of such novel approaches for research and development, as well as in the regulatory context, e.g. for providing mechanistic understanding and potential for high-throughput screening of chemicals (OECD, 2016, 2017, 2018c). The EuroMix methodology promotes the use of data from such novel methods in mixture risk assessment. However, regulatory use of novel methods is often hampered by a lack of method validation and standardization. Thus, the extent to which such data can be relied on in the specific case will depend on the problem formulation and intended use of the assessment outcome, including considerations of whether methodologies are fit-for-purpose in the specific case.

Another important factor limiting the regulatory use of non-testing and *in vitro* methods for health risk assessment of chemicals in general is a lack of understanding of the relationship between what is tested and the adverse effect that is being predicted. AOP are promoted as a means to connect *in vitro* and other non-animal mechanistic data to health effects on the individual level (OECD, 2016). AOP and AOP networks can thus provide the mechanistic understanding needed to integrate data from e.g. *in silico* and *in vitro* methods to support conclusions about health effects, as well as for developing test methods that target certain KEs.

In the EuroMix project, AOP networks have been used as a basis for identifying KEs that are specifically relevant and feasible to target for testing, e.g. to inform grouping or to generate data for determining RPFs. The focus has been specifically on the integration of *in silico* and *in vitro* models and assays to support hazard and risk assessment. A long-term goal was also that such methods could be used with increasing confidence in the future to predict adverse effects at the organism level, thus contributing to the reduction of animal testing for hazard and risk assessment.

Extrapolation from *in vitro* to *in vivo* is still hampered by uncertainties that prevent reliable predictions of apical health effects from *in vitro* mechanistic data. Non-animal data can be integrated with *in vivo* data to fill information gaps and provide a robust scientific basis for these purposes, e.g. for deriving RPFs and health-based guidance values, but non-animal data cannot completely replace animal testing as the basis for mixture risk assessment at this time.

In terms of uncertainty in mixture risk assessment, the integration of mechanistic data can be discussed from two perspectives. First, *in silico* and *in vitro* data may contribute to reducing uncertainty in risk assessment conclusions by providing information that can be integrated together with *in vivo* data, and thus fill information gaps. Secondly however, the uncertainties concerning the risks for complex health effects will likely be increased if animal testing is completely replaced with non-animal models and assays. Therefore the emphasis needs to be on reduction and refinement, not just replacement.

The EuroMix toolbox was developed to make potentially useful methods and data available for risk assessors and researchers. The various modules of the EuroMix toolbox have been described in more detail in van der Voet et al. (2020). The toolbox includes methods with a strong regulatory acceptance, such as probabilistic exposure assessment methods developed in collaboration with EFSA (van Klaveren et al., 2019a, 2019b; EFSA et al., 2020a, 2020b) and benchmark dose response models (EFSA, 2017). But the toolbox also includes newer and more experimental methods, such as a method deriving RPFs from dose response data obtained for KEs at different levels of biological organisation (van der Voet et al., 2020).

The methodology and tools developed within EuroMix provide a basis for future research and development in the area of mixture risk assessment that are in line with identified needs, for example by the OECD, EFSA and JRC (OECD, 2018a; EFSA, 2019a; Bopp et al., 2019). Nevertheless, there are still gaps in the methodology, for example regarding mixtures of substances from different regulatory domains, optimal integration of data, formal checks on the appropriateness of the dose addition model and full specification of uncertainties. Before the methodology for mixture risk assessment described here can be put to use, additional case studies that include the application and scientific verification of different aspects of the EuroMix toolbox and

methodology are needed to illustrate their practical applicability, as well as to identify future development needs. Importantly, applications in the regulatory setting need to be evaluated and validated through processes at regulatory authorities and organisations, such as EFSA and the OECD. One example is the development and assessment of AOP and AOP networks, which may be time- and resource demanding but is crucial for application in regulatory risk assessment.

In addition, there is a need for development and assessment of additional AOP networks that can be useful for mixture risk assessment and to further explore and gain more experience in EuroMix methodology for grouping of substances based on toxicological considerations, including the use of expert knowledge elicitation to quantify probabilities for group membership. Based on the AOP concept, and especially by constructing and assessing AOP-networks depicting different pathways to a specific outcome, it is also possible to explore whether interactions between mixture components invalidate the dose addition model. For example, if synergistic or antagonistic effects are likely to be substantial, alternative models should be further investigated. In principle, when interactions are absent or have minor impact, the AOP concept makes it possible to derive RPFs of mixture components based on non-animal data anchored to KEs in an AOP network.

In conclusion, the EuroMix methodologies and toolbox provide practical steps forward to address the challenges of mixture risk assessment in line with the recent guidance from OECD and EFSA.

CRediT authorship contribution statement

Anna Beronius: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Johanna Zilliacus:** Methodology, Investigation, Writing - original draft, Writing - review & editing. **Annika Hanberg:** Methodology, Investigation, Writing - review & editing. **Mirjam Luijten:** Methodology, Investigation, Writing - review & editing. **Hilko van der Voet:** Methodology, Investigation, Writing - review & editing. **Jacob van Klaveren:** Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interest

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

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Appendix A. Supplementary data

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References

AOP wiki https://aopwiki.org.

ATSDR, 2004. Agency for Toxic Substances and Disease Registry. Guidance Manual for the Assessment of Joint Toxic Action of Chemical Mixtures. US Agency for Toxic Substances and Disease Registry. Division of Toxicology May 2004. Available from: http://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf.

ATSDR, 2018. Framework for Assessing Health Impacts of Multiple Chemicals and Other Stressors (Update). US agency for toxic substances and disease registry. Division of toxicology Available from: http://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf.

Bopp, S., Kienzler, A., Richarz, A.N., van der Linden, S., Paini, A., Parissis, N., Worth, A., 2019. Regulatory assessment and risk management of chemical mixtures: challenges and ways forward. Crit. Rev. Toxicol. 49, 174–189. https://doi.org/10.1080/10408444.2019.1579169.

Cotterill, J., Eberini, I., Rorije, E., Peijnenburg, A., Price, N., Ridgway, C., 2016. Report on the Use of In-Silico Methods for the Prioritisation of Substances and Mixtures Thereof. https://doi.org/10.5281/zenodo.3465632.

EFSA, 2008. European Food Safety Authority. Opinion of the Scientific Panel on Plant Protection Products and their Residues to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to human health with a view to set MRLs for those pesticides in the frame of Regulation (EC) 396/2005. EFSA J. 704, 1–84. https://doi.org/10.2903/j.efsa.2008.705.

EFSA, 2012. European Food Safety Authority. Guidance on the use of probabilistic methodology for modelling dietary exposure to pesticide residues. EFSA J. 10, 2839. https://doi.org/10.2903/j.efsa.2012.2839. 2012.

EFSA, 2013a. European Food Safety Authority. Scientific report of EFSA - international Frameworks Dealing with human risk assessment of combined exposure to multiple chemicals. EFSA J. 11, 3313. https://doi.org/10.2903/j.efsa.2013.3313.

EFSA, 2013b. Scientific Opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile. EFSA J. 11, 3293. https://doi.org/10.2903/j.efsa.2013.3293.

EFSA, 2013c. Scientific opinion on the relevance of dissimilar mode of action and its appropriate application for cumulative risk assessment of pesticides residues in food. EFSA J. 11, 3472. https://doi.org/10.2903/j.efsa.2013.3472.

EFSA, 2014. Guidance on expert knowledge elicitation in food and feed safety risk assessment. EFSA J. 12, 3734. https://doi.org/10.2903/j.efsa.2014.3734.

EFSA, 2015. Summary Report - EFSA Scientific Colloquium 21: Harmonisation of Human and Ecological Risk Assessment of Combined Exposure to Multiple Chemicals. Scientific Colloquium Series. EFSA Supporting Publicationhttps://doi.org/10.2903/sp.efsa.2015.EN-784. EN-784.

EFSA, 2017. European Food Safety Authority. Update: use of the benchmark dose approach in risk assessment. EFSA J. 15, 4658. https://doi.org/10.2903/j.efsa.2017.4658.

EFSA, 2019a. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA J. 17, 5634. https://doi.org/10.2903/j.efsa.2019.5634.

EFSA, 2018a. Draft EFSA Scientific Report Establishment of Cumulative Assessment Groups of Pesticides for their Effects on the Nervous System. Available from: http://www.efsa.europa.eu/en/consultations/call/180508-0.

EFSA, 2018b. Guidance on uncertainty analysis in scientific assessments. EFSA J. 16, 5123. https://doi.org/10.2903/j.efsa.2018.5123.

EFSA, 2019b. Draft EFSA Scientific Report Establishment of Cumulative Assessment Groups of Pesticides for Their Effects on the Thyroid. Available from: https://www.efsa.europa.eu/sites/default/files/consultation/consultation/190213-ag.pdf.

EFSA, 2019c. Public Consultation on the Draft EFSA Scientific Reports on a Cumulative Dietary Risk Characterisation of Pesticides that have Acute Effects on the Nervous System and Chronic Effects on the Thyroid. Available from: http://www.efsa.europa.eu/en/consultations/call/public-consultation-draft-efsa-scientific-reports.

EFSA, Craig, P.S., Dujardin, B., Hart, A., Hernandez-Jerez, A.F., Hougaard Bennekou, S., Kneuer, C., Ossendorp, B., Pedersen, R., Wolterink, G., Mohimont, L., 2020a. Cumulative Dietary Risk Characterisation of Pesticides that have Acute Effects on the Nervous System. https://doi.org/10.2903/j.efsa.2020.6087.

EFSA, Craig, P.S., Dujardin, B., Hart, A., Hernandez-Jerez, A.F., Hougaard Bennekou, S., Kneuer, C., Ossendorp, B., Pedersen, R., Wolterink, G., Mohimont, L., 2020b. Cumulative Dietary Risk Characterisation of Pesticides that have Chronic Effects on the Thyroid. https://doi.org/10.2903/j.efsa.2020.6088.

EuroMix project https://cordis.europa.eu/project/id/633172.

IGHRC, 2009. Chemical Mixtures: a Framework for Assessing Risks to Human Health. Report from the UK Interdepartmental Group on Health Risks from Chemicals. Available from: https://osha.europa.eu/sites/default/files/seminars/documents/Risk-Assessment-Procedures_Levy_9.pdf.

IPCS, 2009. Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food. Chapter 5, p21. Available from: https://www.who.int/foodsafety/publications/chemical-food/en/.

Kyriakopoulou, K., Nikolopoulou, D., Machera, K., Peijnenburg, A., Louca-Christodoulou, D., Hadjiloizou, P., Beronius, A., Håkansson, H., Hanberg, A., Eberini, I., Rorije, E., 2016. Report Describing Cumulative Assessment Groups for a Broad Range of Chemicals, Based on Information Extracted from (Literature) Databases. https://doi.org/10.5281/zenodo.1065327.

Luckert, C., Braeuning, A., Lampen, A., Bovee, T., van der Ven, L., 2017. Development of CAG-specific PCR Arrays. https://doi.org/10.5281/zenodo.3371504.

Luckert, C., Braeuning, A., de Sousa, G., Durinck, S., Katsanou, E.S., Konstantinidou, P., Machera, K., Milani, E.S., Peijnenburg, A., Rahmani, R., Rajkovic, A., Rijkers, D., Spyropoulou, A., Stamou, M., Stoopen, G., Sturla, S.J., Wollscheid, B., Zucchini-Pascal, N., Lampen, A., 2018a. Adverse Outcome Pathway-Driven Analysis of Liver Steatosis In Vitro: a Case Study with Cyproconazole. Available from: https://cordis.europa.eu/project/rcn/193181/results/en.

Luckert, C., Braeuning, A., de Sousa, G., Durinck, S., Katsanou, E.S., Konstantinidou, P., Machera, K., Milani, E.S., Peijnenburg, A., Rahmani, R., Rajkovic, A., Rijkers, D., Spyropoulou, A., Stamou, M., Stoopen, G., Sturla, S.J., Wollscheid, B., Zucchini-Pascal, N., Lampen, A., 2018b. Adverse outcome pathway-driven analysis of liver steatosis in vitro: a case study with cyproconazole. Chem. Res. Toxicol. 31, 784–798. https://doi.org/10.1021/acs.chemrestox.8b00112.

Meek, M.E., Boobis, A.R., Crofton, K.M., Heinemeyer, G., Raaij, M.V., Vickers, C., 2011. Risk assessment of combined exposure to multiple chemicals: a WHO/IPCS framework. Regul. Toxicol. Pharmacol. 60, S1–S14. https://doi.org/10.1016/j.yrtph.2011.03.010.

Munro, I.C., Ford, R.A., Kennepohl, E., Sprenger, J.G., 1996. Correlation of structural class with no-observed-effect levels: a proposal for establishing a threshold of concern. Food Chem. Toxicol. 34, 829–867. https://doi.org/10.1016/s0278-6915(96)00049-x.

OECD, 2016. Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrated Approaches to Testing and Assessment (IATA). ENV/JM/MONO(2016)67 Available from: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2016)67/&doclanguage=en.

OECD, 2017. Guidance Document for Describing Non-guideline in Vitro Test Methods, OECD Series on Testing and Assessment, No. 211. OECD Publishing, Paris. https://doi.org/10.1787/9789264274730-en. Available from:.

OECD, 2018a. Considerations for Assessing the Risks of Combined Exposure to Multiple Chemicals, Series on Testing and Assessment No. 296. Environment, Health and Safety Division, Environment Directorate Available from: http://www.oecd.org/chemicalsafety/risk-assessment/considerations-for-assessing-the-risks-of-combined-exposure-to-multiple-chemicals.pdf.

OECD, 2018b. OECD Users' Handbook Supplement to the Guidance Document for Developing and Assessing AOPs. Available from: https://www.oecd-ilibrary.org/environment/users-handbook-supplement-to-the-guidance-document-for-developing-and-assessing-adverse-outcome-pathways_5jlv1m9d1g32-en.

OECD, 2018c. Guidance Document on Good in Vitro Method Practices (GIVIMP), OECD Series on Testing and Assessment, No. 286. OECD Publishing, Paris. https://doi.org/10.1787/9789264304796-en. Available from:.

RIVM/ICPS/ANSES, 2013. Toxicological Data Analysis to Support Grouping of Pesticide Active Substances for Cumulative Risk Assessment of Effects on Liver, on the Nervous System and on Reproduction and Development. EFSA Supporting Publicationshttps://doi.org/10.2903/sp.efsa.2013.EN-392. 2013:EN-392.

RIVM/ICPS/ANSES, 2016. Toxicological Data Collection and Analysis to Support Grouping of Pesticide Active Substances for Cumulative Risk Assessment of Effects on the Nervous System, Liver, Adrenal, Eye, Reproduction and Development and Thyroid System. EFSA supporting publicationhttps://doi.org/10.2903/sp.efsa.2016.EN-999. 2016:EN-999.

Rotter, S., Beronius, A., Hanberg, A., Zilliacus, J., Nikolopoulou, D., Machera, K., Solecki, R., 2017. Report on Mixtures and Implementation Strategy in Europe – Assessment of Chemical Mixtures under Consideration of Current and Future Regulatory Requirements and Scientific Approaches. https://doi.org/10.5281/zenodo.1067586.

Rotter, S., Beronius, A., Boobis, A.R., Hanberg, A., van Klaveren, J., Luijten, M., Machera, K., Nikolopoulou, D., van der Voet, H., Zilliacus, J., Solecki, R., 2018. Overview on legislation and scientific approaches for risk assessment of combined exposure to multiple chemicals: the potential Euromix contribution. Crit. Rev. Toxicol. 1–19. https://doi.org/10.1080/10408444.2018.1541964.

SCHER/SCENIHR/SCCS, 2012. Scientific Committee on Health and Environmental Risks (SCHER), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and Scientific Committee on Consumer Safety (SCCS): Toxicity and Assessment of Chemical Mixtures. Directorate-General for Health and Consumers 2011. Available from: http://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf.

Solomon, K.R., Wilks, M.F., Bachman, A., Boobis, A., Moretto, A., Pastoor, T.P., Phillips, R., Embry, M.R., 2016. Problem formulation for risk assessment of combined exposures to chemicals and other stressors in humans. Crit. Rev. Toxicol. 46, 835–844. https://doi.org/10.1080/10408444.2016.1211617.

Stein, B., Michalski, B., Martin, S., Pfeil, R., Ritz, V., Solecki, R., 2014. Human health risk assessment from combined exposure in the framework of plant protection products and biocidal products. J. Consum. Protect. Food Saf. 9, 367–376. https://doi.org/10.1007/s00003-014-0915-7.

Teeguarden, J.G., Tan, Y.M., Edwards, S.W., Leonard, J.A., Anderson, K.A., Corley, R.A., Kile, M.L., Simonich, S.M., Stone, D., Tanguay, R.L., Waters, K.M., Harper, S.L., Williams, D.E., 2016. Completing the link between exposure science and toxicology for improved environmental health decision making: the aggregate exposure pathway framework. Environ. Sci. Technol. 50, 4579–4586. https://doi.org/10.1021/acs.est.5b05311.

US EPA, 2000. US Environmental Protection Agency. Supplementary Guidance for Conducting Health Risk Assessment of Mixtures. Available from: https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=20533.

US EPA, 2002. US Environmental Protection Agency. Guidance on Cumulative Risk Assessment of Pesticide Chemicals that have a Common Mechanism of Toxicity. Office of Pesticide Programs Available from: https://www.epa.gov/sites/production/files/2015-07/documents/guidance_on_common_mechanism.pdf.

US EPA, 2003. US Environmental Protection Agency. Framework for Cumulative Risk Assessment. Available from: https://www.epa.gov/risk/framework-cumulative-risk-assessment.

US EPA, 2007. US Environmental Protection Agency. Concepts, Methods and Data Sources for Cumulative Health Risk Assessment of Multiple Chemicals, Exposures and Effects: a Resource Document. Available from: https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=190187.

US EPA, 2015. US Environmental Protection Agency. Human Health Risk Assessment. Strategic Research Action Plan 2016-2019. Office of Research and Development, Human Health Risk Assessment. EPA 601/K-15/002. Available from: http://www.epa.gov/sites/production/files/2015-10/documents/strap_2016_hhra_508.pdf.

US EPA, 2016. US Environmental Protection Agency. Pesticide Cumulative Risk Assessment: Framework for Screening Analysis Purpose. Office of Pesticide Programs Office of Chemical Safety and Pollution Prevention. Available from: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/pesticide-cumulative-risk-assessment-framework.

van der Voet, H., Slob, W., 2007. Integration of probabilistic exposure assessment and probabilistic hazard characterization. Risk Anal. 27, 351–371. https://doi.org/10.1111/j.1539-6924.2007.00887.x.

van der Voet, H., van der Heijden, G.W.A.M., Bos, P.M.J., Bosgra, S., Boon, P.E., Muri, S.D., Brüschweiler, B.J., 2009. A model for probabilistic health impact assessment of exposure to food chemicals. Food Chem. Toxicol. 47, 2926–2940. https://doi.org/10.1016/j.fct.2008.12.027.

van der Voet, H., Kruisselbrink, J.W., de Boer, W.J., van Lenthe, M.S., van den Heuvel JJB, Crépet, A., Kennedy, M.C., Zilliacus, J., Beronius, A., Tebby, C., Brochot, C., Luckert, C., Lampen, A., Rorije, E., Sprong, C., van Klaveren, J.D., 2020. The MCRA toolbox of models and data to support chemical mixture risk assessment. Food Chem. Toxicol. 138, 111185. https://doi.org/10.1016/j.fct.2020.111185.

van Klaveren, J.D., Kruisselbrink, J.W., de Boer, W.J., van Donkersgoed, G., te Biesebeek, J.D., Sam, M., van der Voet, H., 2019a. Cumulative Dietary Exposure Assessment of Pesticides that Have Chronic Effects on the Thyroid Using MCRA Software. EFSA supporting publicationhttps://doi.org/10.2903/sp.efsa.2019.EN-1707. 2019:EN-1707.

van Klaveren, J.D., Kruisselbrink, J.W., de Boer, W.J., van Donkersgoed, G., te Biesebeek, J.D., Sam, M., van der Voet, H., 2019b. Cumulative Dietary Exposure Assessment of Pesticides that Have Acute Effects on the Nervous System Using MCRA Software. EFSA supporting publicationhttps://doi.org/10.2903/sp.efsa.2019.EN-1708. 2019:EN-1708.

VKM, 2008. Combined Toxic Effects of Multiple Chemical Exposures. Report from the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety (VKM). Available from: http://www.vkm.no/dav/15ec300082.pdf.

WHO, 2017. Chemical Mixtures in Source Water and Drinking-Water. World Health Organization, Geneva 2017., Licence: CC BY-NC-SA 3.0 IGO. https://apps.who.int/iris/bitstream/handle/10665/255543/9789241512374-eng.pdf.

Zilliacus, J., Beronius, A., Hanberg, A., van der Voet, H., Luijten, M., van Klaveren, J., 2019. EuroMix Handbook for Mixture Risk Assessment. https://doi.org/10.5281/zenodo.3560719.