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Report on approaches to identify mixture health effects

Deliverable Report

D15.4

WP15 - Mixtures, HBM and human health risk

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2 Glossary

ADI Acceptable Daily Intake

AF Assessment Factor

AOP Adverse Outcome Pathway

BMD Benchmark Dose

CA Concentration Addition

DA Dose Addition

DNT Developmental Neurotoxicants

HBGV Health-based Guidance Values

HI Hazard Index

IA Independent Action

LOEL Lowest Observed Effect Level

LOAEL Lowest Observed Adverse Effect Level

MCR Maximum Cumulative Ratio

MOE Margin of Exposure

MRA Mixture Risk Assessment

NOEL No Observed Effect Level

NOAEC No Observed Adverse Effect Concentration

NOAEL No Observed Adverse Effect Level

PAH Polycyclic Aromatic Hydrocarbon

PBDE Poly -Brominated Diphenyl Ethers

PNEC Predicted No Effect Concentration

POD Point of Departure

PODI Point-of-Departure Index

RPF Relative Potency Factor

RV Regulatory Value

TDI Tolerable Daily Intake

TEF Toxicity Equivalency Factor

TTC Threshold of Toxicological Concern

TUS Toxic Unit Summation

TWI Tolerable Weekly Intake

UF Uncertainty Factor

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3 Abstract/Summary

We define the purpose of mixture risk assessment case studies in HBM4EU and developed and agreed on a common consistent framework for all case studies. This was achieved by elaborating clear rules for a tiered analysis, developing decision rules to define when analyses should be refined or discontinued, and adopting clear criteria for the grouping of chemicals to be subjected to mixture risk analyses. These grouping criteria are based on Adverse Outcome Pathway considerations.

We develop an advanced decision tree and workflow scheme for the hazard assessment arm of mixture risk assessment. This adopts the principle of advancing mixture risk assessment in a step-wise manner to approximate the scientific principles of experimental mixture assessments based on Dose Addition or Independent Action. We newly introduce the concept of drivers of mixture effects and the exploitation of the Maximum Cumulative Ratio as a tool for elaborating risk assessment options.

All 5 case studies have assembled a great deal of the necessary input data (exposure estimates, toxicity values). Screening analyses have revealed exceedances of acceptable combined exposures in all cases, highlighting a need to refine the analyses by adopting endpoint-specific HBGV.

Mixture studies aiming at identifying chemicals influencing health outcomes and relying on spot exposure biospecimens suffer from decreased performances which can be improved by collecting repeated biospecimens per subject, especially for non-persistent chemicals. The within-subject biospecimens pooling approach appears as an efficient way to limit bias without increasing assay cost.

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4   Introduction

Human populations are exposed to rather large numbers of chemicals simultaneously and it is increasingly realised that chemical risk assessment approaches should take account of this reality. As much as possible, human biomonitoring should also integrate knowledge about mixture effects and begin to develop strategies that can cope with the reality of multi-chemical, multi-pathway exposures of humans.

This Deliverable describes viable approaches to describe and assess mixture health effects for relevant mixtures identified under Tasks 15.1 and 15.2 and proposes topical case studies as proof of concept.

The case studies will deal with the question: Do predicted mixture risks from combined chemical exposures exceed the levels regarded as acceptable for humans? The following specific aspects will be addressed:

- Are there specific human health endpoints for which acceptable risks are exceeded?
- Are there pollutants that contribute most to defined health endpoints of interest and are therefore drivers of mixture risks?
- Is it possible to devise human biomonitoring strategies that capture those pollutants?

The case studies are intended to structure and organise the work in WP15 in a logical and consistent manner. They should also crystallise questions and issues that are not sufficiently resolved, for debate within the HBM4EU project. Especially, the case studies should:

- Identify methods for the prediction of mixture effects that can be used consistently for human risk assessments and can inform biomonitoring strategies in terms of chemicals to be monitored together.
- Define properties of data required as input for mixture effect predictions and define the nature of data requirements.

Accordingly, this Deliverable is structured as follows:

We will first provide an overview of general mixture risk assessment concepts and principles. This is followed by setting out in detail selection criteria for the case studies which provides the foundations for detailed descriptions of proposed case studies.

We will then describe an advanced decision tree and workflow for the hazard assessment arm of mixture risk assessments (MRA). This decision tree was developed as part of the EU-funded SOLUTIONS project and is shown here, adapted for the purposes of HBM4EU.

We elaborate the concept of drivers of mixture effects as an organising principle of decision trees and workflow schemes. We apply the concept of Maximum Cumulative Ratio (MCR) as a tool for analysing whether exceedances of acceptable combined exposures are attributable to multiple chemicals or whether they are due to single or a small number of chemicals.

In the interest of coherence, we incorporate into Deliverable D15.4 the previous Additional Deliverables AD15.6 and AD15.4.

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5 Mixture risk assessment (MRA): General considerations, approaches and methods

5.1 General considerations on combined toxicity and its assessment

The combined effects of several chemicals are often referenced in terms of two fundamentally different modes: similar action and dissimilar action (sometimes also called independent joint action). These distinctions were first introduced by Bliss (1939) and Hewlett and Plackett (1952) on the basis of statistical principles. They have gained wide acceptance for the interpretation of mixture effects and are allied to the assessment concepts of dose addition (DA, linked with similar action) and independent action or response addition (IA, RA, linked with dissimilar action). Many regulatory bodies and competent authorities have used these terms in broadly identical ways, although there are differences in the level of detail specified for each of the two modes (summarised in Kortenkamp *et al.* 2012). There is a consensus that similar action "occurs when chemicals in a mixture act in the same way, by the same mechanism/mode of action, and differ only in their potencies" (EFSA 2008). Conversely, "dissimilar action" occurs with combinations of chemicals that produce a common effect by action through different modes of action, or at different sites.

While these definitions are clear-cut in principle, in practice it is often not straight-forward to distinguish between dissimilar action and similar action. In many cases, the mechanistic information needed to differentiate between the two types of combination effect is not available. Clear distinctions are further complicated by ambiguities concerning the precise meaning of the terms "mode of action" and "site of action" and its implications for assessments of combination effects. For example, two chemicals might affect different sites of an effector chain leading to an adverse outcome, in agreement with a key feature of simple dissimilar action. However, if the same key metabolite or intermediate is affected, the toxicological consequences could be better described in terms of simple similar action.

Dose addition is based on the idea that all components in a mixture behave as if they were dilutions of one another (Loewe and Muischnek 1926). Examples would be combinations of chemicals that all exert their toxicity by binding to the same receptor, e.g. the Ah receptor (polychlorinated dioxins) or the active centre of acetylcholinesterase (organophosphates, carbamates). In these cases, similar action applies because one chemical can be replaced by an equal fraction of an equi-effective concentration (e.g. an EC_{50}) of another, without diminishing the overall combined effect. In mathematical terms, this concept can be formulated as:

$$ECx_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} = \left(\sum_{i=1}^{n} \frac{p_i}{F_i^{-1}(x_i)} \right)^{-1}$$

with n denoting the number of mixture components, p_i the relative fraction of chemical i in the mixture (percentage of the total dose or concentration), x a common effect level (e.g. 10%), ECx_{Mix} the total dose or concentration causing the effect x, ECx_i the equi-effective doses or concentrations of the individual chemicals, F_i the individual dose or concentration response functions, and F_i^{-1} the corresponding inverse functions.

Dose addition implies that every toxicant in the mixture contributes to the combination effect in proportion to its dose and individual potency. Whether the individual doses are also effective on their own does not matter. Thus, under dose addition, combination effects can be expected when toxicants are present at levels below effect thresholds, but only if the number of components sums up to a total mixture dose sufficiently large to produce effects. For example, two chemicals

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combined at 1/10 of their threshold concentration are not expected to produce a combination effect according to dose addition.

Independent action (response addition) conceptualises mixture effects in a different way. It assumes that a combination effect can be calculated from the responses of the individual mixture components by following the statistical concept of independent random events (Bliss 1939). This can be mathematically expressed by the equation

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)]$$

which can be transformed into

$$x\% = 1 - \prod_{i=1}^{n} \left(1 - F_i(p_i \bullet (ECx_{Mix}))\right)$$

with $E(c_i)$ denoting the fractional effects (0-100%) that the individual mixture components would cause if applied singly at that dose or concentration at which they are present in the mixture, and $E(c_{Mix})$ the effect provoked by the total mixture (other parameters as defined for DA above).

On a population level, the idea of independent random events may also apply to one and the same chemical administered in a sequential fashion if non-reversible events such as mortality are investigated within time frames where recovery of the population does not occur. In this situation, the mode of action is identical, but the randomness of events is introduced by exposures that occur in sequence. The overall effect is then accessible by multiplication of the likelihood of independent events (administrations of the chemical). In the case of simultaneous exposure of an individual to several chemicals, the principle of independence of effects is only applicable when all the chemicals in the mixture act through dissimilar modes by affecting different target sites (dissimilar action). Examples would be combinations of chemicals that affect algal reproduction by disrupting photosynthesis, DNA synthesis and multiple other sub-systems. The principles of independence of effects also imply that components present at doses below thresholds and thus associated with zero effects will not contribute to the joint effect of the mixture. If this condition is fulfilled for all mixture components, combination effects are not expected under independent action.

Mixed Models (MM) combine both concepts, DA and IA, in cases where the components of a mixture can be clustered into groups of strictly similar acting substances while the groups cause a common effect by strictly dissimilar modes of action. The MM approach includes completely similar action (DA) and completely dissimilar action of all components (IA) as the two possible extreme situations. The approach presumes that all mixture components can be exclusively assigned to one out of a number of different MoA groups. Where mixture components are multi-site inhibitors contributing to different MoAs or where adverse outcome pathways (AOP) do not lead to a common endpoint in completely independent ways, but are interlinked at some point along the route, the MM approach may not be applicable. In such a situation, an exact prediction of the mixture toxicity is not possible on the basis of the concepts of DA and IA, but it can be expected to lie somewhere in the prediction window between the extremes defined by DA and IA. In practice, this prediction window may be rather small, as further detailed in section 5.4 below.

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5.2 The additivity expectation

By making the assumption that each mixture component exerts its effects in the same way as it would do when present on its own, i.e. without diminishing or enhancing the toxicity of other components, it becomes possible to formulate a quantitative expectation of the joint effects of multiple chemicals. These so-called additivity expectations are calculated on the basis of the effects of each individual chemical in the mixture. Additivity expectations can be derived from independent action or from dose addition or from a mixed model and serve as points of reference for the identification of synergisms or antagonisms. This idea is only workable when ***both the mixture and all its components are able to produce the same toxic effect*** (when present at sufficiently high concentrations or doses). In cases when some chemicals in the mixture do not affect the endpoint under consideration, it is not possible to predict a joint effect from the effects of mixture components. Similar difficulties also arise if some or all components interact with each other, e.g. by induction of toxifying or detoxifying metabolic steps. In such situations, the observed mixture effects will differ from the expected additive effects which were calculated on the basis of the effects of its individual components. With reference to the expected additive effects, deviations in the direction of stronger responses can then be classed as synergism or as antagonisms if the observed effects fall short of those predicted. However, the magnitude of deviations from expected additivity cannot be calculated from information about the individual effects of mixture components.

5.3 Dose addition as a default assumption

In the light of the practical difficulties encountered when using considerations of similar or dissimilar action as the starting point for MRA (lack of mechanistic data, ambiguities associated with key terms such as mode of action, mechanism of action), the dichotomous approach to MRA is increasingly considered as problematic. To deal with this problem, there is now a growing consensus that MRAs should start from the default assumption of DA for all mixture components, regardless of MoAs, as a reasonable worst-case estimate. If this indicates a significant risk, refined MoA-based assessments may be conducted where the necessary data are available. Alternatively, precautionary measures may be taken. This way of thinking has guided ecotoxicological MRAs for quite some time already, but is now also gaining increased acceptance in the human arena (Meek at al. 2011, EFSA 2013). This development opened the way for using consistent and coherent approaches across the disciplinary borders.

The use of DA as a pragmatic and precautionary default assumption can be justified by a combination of four arguments:

- Data requirements for a proper application of DA are much easier to fulfil than for IA or MM.

- Usually, the assumption of DA provides a more conservative estimate of mixture toxicity than the alternative assumption of IA. Theoretically, the reverse situation is possible but the practical relevance of such a situation has a yet not been demonstrated.

- Synergistic effects that significantly exceed the DA expectation are exceptions and not the rule, at least for multi-component mixtures.

- The DA assumption is conservative, but not vastly over-conservative. Typically, the prediction window between DA and IA is not very wide. For realistic assessment situations it will rarely exceed an order of magnitude on the concentration axis. Typically, it is much smaller. Even with mixtures composed of up to 100 chemicals, predicted effect concentrations of the mixture derived from CA and IA differing by a factor of less than 5, as explained theoretically and demonstrated practically in Chapter 13.4 of Kortenkamp et al. (2012).

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5.4 Data requirements and the distinction between scientific concepts of additivity and pragmatic simplifications for regulatory MRAs

As mentioned before, the data requirements for DA-based MRAs are easier to meet than for IA or MM. Proper application of IA requires knowledge about the slope of dose response functions F of the individual mixture components (see the equation above), MM additionally requires knowledge of MoAs. In contrast, under the assumption of DA, the prediction of effect concentrations (or effect doses) of mixtures only requires that equivalent effect concentrations of single substances are available. Furthermore, the formula for DA can be directly transformed into a risk quotient for mixtures. The algebraic equivalent of the DA formula usually used for this purpose is the so-called toxic unit summation (TUS) as explained in more detail below.

DA and TUS require ECx or EDx values that refer to the same endpoint in the same species under comparable conditions. In a regulatory context, even this relatively simple data requirement may be impossible to meet. As a result, a number of pragmatic approaches have been derived from the original DA concept. Partly they have already become established procedures under specific pieces of legislation in the EU or in the US. Some prominent examples are the Hazard Index (HI), Point of Departure Index (PODI), Relative Potency Factors (RPF) and the concept of Toxic Equivalency Factors (TEF). All these MRA methods are simplifications of the DA concept. As a common feature, they make use of the DA formula as a calculation rule, but either they use input data that deviate more or less from the strict requirements of the original concept, or they make additional simplifying assumptions about the individual dose response curves. Below, we will first introduce these MRA methods, and then spell out their simplifying assumptions.

Simplifying regulatory approaches explicitly derived from IA are not developed. In human toxicology, an implicit application of IA as a MRA method is the assumption that mixture effects will not arise when all chemicals in question are present at levels below their ADIs, with the additional tacit assumption that ADIs represent true zero effect levels. It should be emphasised that the implicit application of independent action can only be used for chemicals for which ADIs have been derived and is thus applicable only to the minority of chemicals for which ADIs have been formally established.

5.5 Mixture risk assessment methods in practical use

5.5.1 Toxic unit summation

The method of Toxic Unit Summation (TUS) (Sprague 1970) is a direct application of the DA concept and defined by the formula

$$ TUS = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} $$

where c_i are the actual concentrations (or doses) of the individual substances in a mixture and ECx_i denote equi-effective concentrations (or doses) of these substances if present singly (e.g. $EC50_i$). The quotients c_i / ECx_i are termed Toxic Units (TU). Toxic Units rescale absolute concentrations (or doses) of substances to their different individual toxic potencies. They express the concentrations (or doses) of mixture components as fractions of equi-effective individual concentrations (or doses) ECx_i. Typically, $x = 50\%$ ($EC50_i$) is chosen as the reference level, but TUS can also be calculated for any other effect level x. If TUS = 1, the mixture is expected to elicit the total effect x. If the Sum of Toxic Units ($\sum TU$) is smaller or larger than 1, the mixture is expected to elicit effects smaller or larger than x, respectively.

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5.5.2 The Hazard Index

The Hazard Index (HI) (Teuschler and Hertzberg 1995) is a regulatory approach to component-based mixture risk assessment derived from DA and which can be generally defined by the formula

$$HI = \sum_{i=1}^{n} \frac{EL_i}{AL_i}$$

where EL is the exposure level, AL is the acceptable level, and *n* is the number of chemicals in the mixture. The ratio of EL to AL is called the Hazard Quotient (HQ).

Various measures for exposure levels and expectable levels may be applied; the only constraint is that EL and AL must be expressed in the same unit. Input values for AL can be ADIs or reference doses (RfD) for specific endpoints.

If HI > 1, the total concentration (or dose) of mixture components exceeds the level considered to be acceptable. The method offers flexibility in applying different assessment factors (AF) when defining AL for the individual substances.

An assumption implicit in the use of the HI approach, and one that derives from the principles of the DA concept, is that the acceptable levels AL for each individual chemical represent exposures associated with the same (small or negligible) effect. In most cases, this is not proven in practice, and will remain unproven in the foreseeable future. For most practical applications, however, the error in making this assumption can be considered small.

5.5.3 The Point-of-departure Index

The Point of Departure Index (PODI) is an approach to component-based mixture risk assessment which is similar to the HI and TUS. In contrast to the HI, however, exposure levels (EL) of chemicals in a mixture are not expressed as fractions of individually acceptable levels (AL) but as fractions of their respective points of departure (PODs) such as NOAELs or benchmark concentrations or doses (BML). In this way, different uncertainty factors that may be included in AL values (see HI) are removed from the calculation (Wilkinson et al. 2000):

$$PODI = \sum_{i=1}^{n} \frac{EL_i}{POD_i}$$

A PODI lends itself to the estimation of margins of exposure for the mixture of interest. Similar to the HI, there is the implicit assumption that all PODs are associated with the same effect magnitude, a principle derived from the features of DA.

As argued by Wilkinson and colleagues (Wilkinson *et al.*, 2000), the outcome of MRAs using the HI approach may be biased through the use of ALs that were derived by application of differing uncertainty factors (UF). UFs between 10 and 1000 are commonly used to AL for chemicals. These UFs are the composite of two different kinds of sub-factors: Those for the adjustment of differences in the data quality of experimental values (e.g. LOAEL to NOAEL extrapolations) and those necessary for the realisation of protection goals enshrined in various chemical legislations (e.g. animal to human extrapolation, increased protection against carcinogens). The mixing of these different UFs may then yield AL that are a poor reflection of the potency of the various chemicals considered together. However, in mixture toxicology, data that strictly reflect the toxic potency of mixture components must to be used for the evaluation of experimentally observed combined effects. Although the problem does not arise when using the HI with AL based on the same UFs, it may be more appropriate to use mixture risk assessment methods that implement

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strict separations of the subclasses of UFs. The Point-of Departure-Index (PODI) is one such method that fulfills this goal.

5.5.4 Relative potency factors

The Relative Potency Factor (RPF) approach is another application of the DA concept for mixtures of chemical substances that are assumed to be toxicologically similar (US EPA 2000). The effective concentrations (or doses) of mixture components are scaled relatively to the effective concentration (or dose) of an index compound, and then summed up. The scaling factor is called RPF. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of the index compound. In general, the mixture concentration C_m expressed in terms of the index compound for n compounds is

$$C_m = \sum_{i=1}^{n} (c_i * RPF_i)$$

where c_i is the concentration of the ith mixture component, and $RPF_1 = 1$, as $i = 1$ indicates the index chemical.

5.5.5 The toxic equivalence factor concept

The Toxic Equivalence Factor (TEF) is a specific type of RPF formed through a scientific consensus procedure (USEPA 2000). Based on the assumptions of a similar mechanism of action of structurally related chemicals and parallel concentration (or dose) response curves, they were first developed for dioxins. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of an index compound. The total equivalent quantity TEQ is estimated by summation of the concentrations (or doses) of mixture components c_i multiplied by the respective TEF_i:

$$TEQ = \sum_{i=1}^{n} (c_i * TEF_i)$$

5.5.6 Data requirements and applicability of simplified mixture risk assessment methods

All of the above cumulative risk assessment methods require at least rudimentary dose-response information of individual mixture components which is used to derive the input values, be they ADIs, RfDs, POD or information about relative potencies such as RPF or TEF. Information about exposures must also be available.

The HI sums up ratios of exposure levels and ADIs or RfDs of chemicals. These estimates can be arrived at by utilizing different assessment factors (AF) for each mixture component, in order to deal with differences in data quality and sources of uncertainty.

If this is perceived to be inadequate, the PODI method can be used. PODI is based not on reference doses, but on points of departure (NOAELs, benchmark doses). Extrapolation issues (e.g. animal to human) are dealt with either by using one overall AF, or by estimating margins of exposure (MOE).

The TEQ concept is predicated on the choice of a reference chemical and requires parallel dose-response curves for all components. Both these requirements are often not met by chemicals, but the method has been validated for dioxins and dioxin-like substances.

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5.6 The principal assumptions and simplifications behind mixture risk assessment methods

In common with the MRA approaches applied in regulatory practice and the WHO/IPCS approach (Meek et al. 2011), the practical application of mixture risk assessment approaches is based on a number of assumptions and simplifications which must be made explicit, as follows:

- **The possibility of synergisms or antagonisms is disregarded**. This assumption is the direct consequence of the fact that the degree of synergism or antagonism cannot be predicted quantitatively on the basis of the toxicity of the mixture components. All mixture effect prediction methods and accordingly, all MRA methods, assume additivity. Considering that the likelihood of synergisms is relatively small when multiple toxicants are present at low regulatory acceptable levels (Boobis et al. 2011; Kortenkamp et al. 2009), the disregard for toxic interactions may be regarded as sufficiently protective.

- **Simultaneous exposure to multiple chemicals is assumed.** In numerous settings encountered by humans and species assemblages there is simultaneous exposure to multiple chemicals. For example, there is consumption of single food items that contain multiple chemicals and, even when food items are consumed sequentially, the subsequent exposure of body tissues to the chemicals contained within the items may be simultaneous. Strictly sequential exposures are also a reality, but the risk assessment methods available for MRA are not applicable to sequential exposure to multiple chemicals. In theory and concept, the development of methods for the assessment of sequential exposures is still in its infancy (Altenburger and Greco 2009).

- **Potency estimates for mixture components may be derived from different endpoints.** Application of DA requires the use of potency estimates for the same adverse outcome as input values. However, such input values are often not available because chemical safety testing is geared towards identifying critical toxic effects which can be used for the establishment of reference doses (ADI, TDI). In practice, this means that toxicity information for chemicals that occur together in mixtures often derives from disparate endpoints. To enable assessments of cumulative risks, the demand for potency estimates for the same endpoints is therefore relaxed, especially for simplified analyses at lower tiers. This simplification is in line with the principles of the framework analysis suggested by WHO/IPCS (Meek et al. 2011).

- **It is assumed that the potency estimates entered into MRA methods (e.g. ADIs, POD) describe doses associated with the same effect magnitude.** The equations for DA are based on single chemical effect doses for identical effect magnitudes. When applied to the PODs that enter the mathematical expressions used in MRA methods such as HI or PODI, this means that all PODs should describe effect doses for the same effect levels. In practice however, this demand cannot always be met, except in the case of benchmark doses which are defined in relation to the same effect levels. In human toxicology, the effects associated with NOAELs that form the basis for ADIs by combination with AFs are normally not known. To make cumulative risk assessment methods workable despite these knowledge gaps, ADIs and PODs are taken as if they described effect doses for the same effect magnitude. Similar considerations apply to ecotoxicology with PNECs.

- **Potency estimates can be derived from different tests, performed under different conditions.** In the interest of consistency, the evaluation of experimental mixture effects by using the concept of DA should utilise effect data for all the mixture components that were gathered under the same experimental conditions, with the same test organisms. If this condition is not fulfilled, a bias may be introduced into the analysis, leading to erroneous determinations of mixture effects in terms of additivity, synergy or antagonism. MRA

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however has to rely on data that were produced in the context of single chemical testing, under widely varying experimental conditions, even when the same organisms were used, so that the demand of consistency of data cannot be realised in practice. To allow continuation of MRA, this demand therefore has to be relaxed.

- **Data on exposures and potency must be recorded by using the same dose metric.** To allow utilization of the formula for HI or PODI, for all mixture components both exposure and toxicity data must be expressed in the same unit and must refer to the same route or matrix, either in terms of an external concentration in an environmental medium such water or food, or in terms of an amount taken up per unit of time and biomass via a defined route such as oral, dermal, inhalative etc., or in terms of an aggregated dose via different routes, or in terms of an internal concentration in defined tissue or body fluids such fat or blood, or in terms of a total body burden.

5.7 The distorting influence of pragmatic simplifications of dose addition used in regulatory practice

The use of dose addition for calculations of the expected toxicity of combinations of chemicals requires that toxicity data for all single components are available. Ideally, the toxicity has to be determined for the same toxicity endpoint and effect magnitude, in the same test under identical experimental conditions. To achieve reliable predictions of mixture effects, well characterised dose-response relationships should be available for all mixture components.

Although these stringent data requirements can be realised in experimental studies, they are practically never met in regulatory practice. Simplifications of the scientific Concentraton Addition (CA) concept are intended to overcome this problem, such as the Point-of-Departure-Index (PODI, Wilkinson et al. 2000), and the Hazard Index (HI, Teuschler and Hertzberg 1995). These approaches make use of the CA formula as a calculation rule, but they use input which does not fully comply with the strict requirements of the original concept but which may be easier available for regulators. This includes the use of values which (i) may have been derived by using different AFs, (ii) merge data for different endpoints or species, or (iii) are estimates of not exactly defined effect levels such as LOEL or NOAEL, as further detailed in the following.

Instead of effect doses associated with the same effect magnitude, the PODI can also be based on estimates of so-called points of departure (PODs) as inputs, including no-observed-effect-concentrations (NOEC), no-observed-adverse effect-levels (NOAEL) or lowest observed effect levels (LOEL). The simplifying pragmatic assumption is that all these PODs refer to the same (low) effect magnitude, when in fact most NOAELs or NOECs correspond to effects of between 10 and 30% (Moore and Caux 1997). To blur matters further, PODs may refer to different toxicity endpoints determined in different test species from the same species group. Thus, in comparison to the use of DA in experimental studies, the PODI introduces two sources of uncertainty for the prediction of mixture risks: it mixes inexactly defined effect sizes and different toxicity endpoints as well as species (although from similar species groups).

By using regulatory values or reference values such as ADI or DNEL as input, the HI introduces a further source of uncertainty. These reference values are derived from PODs by using assessment factors (AFs) intended to accommodate various extrapolations steps and to compensate for differences in data quality. Since AFs can vary from 10 to 10,000, chemicals with regulatory values of the same magnitude may not always have comparable potency. The size of regulatory values may simply reflect vastly differing uncertainties inherent in their derivation.

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5.8 Principles of a step-wise (tiered) analysis in mixture risk assessment

Tiered approaches to MRA can avoid unnecessary expenditure of resources by offering the possibility of discontinuing the analysis on the basis of crude and simple assumptions about exposures and hazards when cumulative exposures are judged to be tolerable or acceptable. In this way, lengthy, but largely unproductive efforts of refining the analysis can be avoided.

This concept was adopted in the WHO/IPCS framework for assessing combined exposures to multiple chemicals (Meek et al. 2011). The framework is based on a series of four tiers that begins with simple and conservative screening assumptions and moves to higher tiers as necessary. The higher tiers adopt more refined, less conservative and more accurate assessments than the preceding tiers. This requires more resources in terms of additional exposure and toxicity data.

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6 Criteria for the selection of proposed case studies

6.1 Health endpoints of concern

In discussions among the partners contributing to WP 15, the following human health endpoints were selected as of concern:

- Developmental neurotoxicity
- Cancers, including e.g. lung cancer
- Nephrotoxicity
- Endocrine disruption, especially disruptions of male reproductive health

A key aspect for consideration included the interest to align the case studies as much as possible to the work ongoing in WP 14, with biomarkers of effect. As this focuses on using *in vitro* assays for the monitoring of total internal exposure to estrogen receptor agonists and androgen receptor antagonists, the choice of endocrine disruption (anti-androgens) as a health endpoint seemed especially appropriate.

6.2 Pollutants and chemicals

It was decided to align the pollutants and groups of chemicals to be selected for case studies to the priority pollutants chosen for HBM4EU. We realised this alignment by picking out the following pollutant groups and chemicals: PBDEs, chemicals with anti-androgenic properties, heavy metals with nephrotoxicity (lead, mercury, cadmium) and occupational carcinogens (chromium (VI), nickel and PAHs).

6.3 Cross-cutting issues

As an issue that applies to all health endpoints and all chemicals, it was decided to address exposure misclassification that can result from biomonitoring spot samples. When applied to multiple chemicals (exposures), this can seriously complicate exposure assessment, with the risk of bias towards the null (false negatives). Accordingly, this will be the topic of a separate case study.

6.4 Case studies

In the light of the above selection criteria, several case studies of mixture effects and mixture risk assessment were proposed and are actively pursued:

- Developmental neurotoxicity beyond PBDEs
- Heavy metals and nephrotoxicity
- Anti-androgenic chemicals and male reproductive health
- Chromium(VI), nickel and PAHs
- Exposure misclassification in mixture studies

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7 A common assessment framework and workflow for case studies

Based on the methodological considerations in Section 5 above, a framework and workflow for all case studies has been developed which includes the following:

- Clear **rules for tiering** of the exposure and hazard assessment arms of the framework

- Clear **decision rules** that define when further refinement of the analysis is to be conducted, and when the analysis can be stopped

- Consistent **application of assessment factors** in each of the tiers

- A concept that will remove in a step-wise fashion the distortions introduced through the use of different assessment factors in regulatory values for pollutants, with the aim of **approaching the scientific principles of Dose Addition** in higher tiers (points of departures for the same adverse outcome, representative of similar effect magnitudes, e.g. benchmark doses for 5% effects and based on the same test species)

- A consistent approach to **bridging toxicity data gaps** (e.g. by using the Thresholds of Toxicological Concern (TTC) concept)

- Clear **grouping criteria**, based on AOP considerations

In the **hazard assessment arm** of the mixture risk assessment, three Tiers will be used:

Tier 1 will check compliance with Health-based Guidance Values (HBGV) that have been defined for the chemicals to be subjected to mixture risk assessment. It is recognised that these HBGV can be for different critical toxicities; thus, at this stage the assessment mixes different health endpoints and assessment factors. The Hazard Index (HI) is the appropriate mixture risk assessment method for this Tier of the analysis. If non-compliance with the sum of risk quotients based on these HBGV is detected, the analysis is refined by moving to Tier 2.

Tier 2 improves the certainty of the assessment by removing the distortions that were introduced in Tier 1 through the mixing of HBGV based on different health endpoints, with different assessment factors. As much as possible, HBGV for common adverse outcomes are used. To deal with distortions arising from the use of different assessment factors behind these HBGV there are several options: Either the same assessment factors are employed for all chemicals, or, if this is not possible, the risk assessment will be conducted on the basis of points-of-departures.

Tier 3 introduces further refinements, if Tier 2 reveals unacceptable combined exposures. These refinements can focus on using potency values associated with identical effect magnitudes (as in benchmark modelling).

It is not necessary to carry the analysis through Tier 1 in all cases. If data of sufficient quality are available, Tier 1 can be skipped and the analysis begins with Tier 2.

The **exposure assessment arm** of the procedure can also be refined in a step-wise manner, as follows:

Tier 1 will normally use exposure data derived from census methods (combining e.g. food consumption data with levels of pollutants in food) for the population of relevance.

Tier 2 introduces refinements by utilising Human Biomonitoring data for the pollutants of interest.

Tier 3 uses Human Biomonitoring data for multiple pollutants measured in the same individuals.

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7 An advanced framework for the hazard assessment arm of human mixture risk assessment

Here, we extend the principles elaborated in Section 6. Our aim is to improve the reliability of mixture risk assessment, by introducing a tiering scheme for the hazard assessment arm of human mixture risk assessment. The scheme is based on concepts developed for the EU-funded SOLUTIONS project. It removes the distortions introduced by the use of HI or PODI in a step-wise manner, with the aim of realising the scientific principles of CA as much as is possible. Moreover, increasingly sophisticated assumptions about modes of action are introduced at higher tiers.

Accordingly, the decision tree is divided into 3 main tiers.

In **Tier 1**, regulatory or reference values (RV) such as ADI or TDI are used together with modelled or measured data about exposures. Expected mixture risks are calculated by using the HI approach.

In **Tier 2**, the distorting influence of assessment factors of differing magnitude is removed. As much as possible, PODs for common toxicities and adverse outcomes relevant for risk assessment are introduced. The mixture risk assessment method of choice for these input data is the PODI.

In **Tier 3**, we institute sub-groups of mixture components that affect a common endpoint through a similar mode of action. We introduce a hybrid approach (so-called mixed models) that allows us to aggregate mixture effects for a common endpoint resulting from sub-groups with similar modes of action. This aggregation is achieved by using IA.

Tiers 2 and 3 are further divided into **sub-tiers** designed to evaluate whether definitive conclusions about expected mixture risks can be reached without meeting all the data requirements defined for the respective main tiers 2 and 3.

7.1 Assessment rules for the main tiers

If the use of regulatory values in conjunction with the HI in Tier 1 yields indices < 1, no significant mixture risks are expected and the analysis can be stopped. The assessment is moved to Tier 2 only in cases where a significant risk cannot be ruled out under reasonable worst-case assumptions (regarding both exposures and toxicity), i.e. when the HI > 1. If there is pre-existing information about significant mixture risks and if the necessary input data are readily available, Tier 1 may be skipped. At any time, the analysis can begin at appropriate higher tiers.

The PODI to be used in Tier 2 assessments is normally applied in connection with margin-of-exposure (MoE) considerations. For example, a PODI of 0.1 indicates that the components in the mixture jointly reach concentrations (doses) 10-fold below PODs, with a margin-of-exposure (MoE) of 10. The assessment requires decisions about acceptable MoEs. Following the principle that MRA should neither be more, nor less stringent than single chemical risk assessments, a default MoE of 100 suggests itself for human MRA. Accordingly, a PODI of smaller than 0.01 (or a PODI x 100 < 1) signals that combined exposures of concern are not to be expected and that the analysis can be discontinued. In all other cases, the assessment is moved to Tier 3.

In Tier 3, the refined CA-based worst-case estimates from the previous Tier are complemented by IA-based best-case estimates. This extends the conservative, dose addition-based MRA to the minimum risk of the "prediction window" delimited by predictions derived from IA. The assessment is stopped, if a significant risk is detected on the basis of a reasonable best-case estimate of the lowest expectable risk and if this cannot to be ruled out by any further refinement (Tier 3A). This is the case when the ratio of exposure to POD of one single component in the mixture exceeds its permissible value (0.01 for human MRA, 0.1 for ecotoxicological MRA) for a reasonable best-case

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scenario. This is analogous to the "gatekeeper step" proposed by Moretto et al. 2017 as a filtering step at the beginning of the assessment.

If an exceedance of exposure standards for a single component is not apparent, the above exercise is repeated for groups of mixture components that contribute to a common adverse outcome through similar modes of action, by calculating PODIs for these sub-groups (Tier 3B). If any of these PODIs exceed the critical value under reasonable best-case assumptions, we conclude that the mixture exposure exceeds the standard and stop the assessment.

In the remaining cases, Tier 3 considers scenarios where the maximum expectable risk may be of concern, while the minimum expectable risk is not. Only these cases are taken forward to the final sub-tier (Tier 3C), which means a full application of mixed-modelling approaches. Where the high data demands and the conceptual premises of this approach can be met, a definite final assessment of the significance or insignificance of the mixture risks can be derived. Otherwise the evidence remains inconclusive, because dose addition-based concerns cannot be ruled out by advanced modelling due to missing data or knowledge.

7.2 New criteria for identifying drivers of mixture effects

The identification of drivers of mixture effects is central to our framework, and directly helps to inform risk management to focus on compounds that apparently matter most. It will allow us to simplify the analysis by avoiding to carry those chemicals forward into higher tiers that clearly make only a marginal contribution to mixture effects. To identify such drivers of mixture risk, quantitative criteria are needed. Price et al. (2012) proposed to define drivers as chemicals that contribute at least 50% to the overall mixture exposure, i.e. when the ratio $\sum RQ/RQ_{max} > 2$. However, this criterion might be too restrictive when dealing with scenarios where several chemicals contribute to mixture toxicity.

An alternative would be to define drivers as those mixture components that do not fit the case where all components contribute equally to the combined effect. In such artificial "balanced" mixtures, the hazard quotients of all components are equal to the quantity $\sum RQ/n$, where n is the number of components. Drivers can then be defined as those mixture components whose RQ is larger than the average RQ, i.e. $RQ_i > 1/n \sum RQ$.

However, especially with large $\sum RQ$ and highly skewed RQ distributions, this approach may underestimate the number of drivers: Consider the case of $\sum RQ = 2$. To achieve acceptable mixture exposures, assume that the $\sum RQ$ has to reduce to 1 or smaller. This can be realised by a 50% decrease of $\sum RQ$. If mixture components are ranked according to the numerical value of their RQ, from large to small, drivers would be those chemicals whose RQ together make up 50% of $\sum RQ$. With $\sum RQ = 10$, this expands to 90%, with $\sum RQ = 100$ to 99%, and so on (**Fig 1**). According to this approach, a driver belongs to the group of chemicals whose RQs cumulatively exceed acceptable mixture exposures, i.e. $\sum RQ_{drivers} = \sum RQ_{mixture} - \sum RQ_{acceptable}$. To capture those chemicals that contribute the largest RQs, it is additionally required that $\sum RQ_{drivers}$ is a rank order of individual RQs from largest to smallest.

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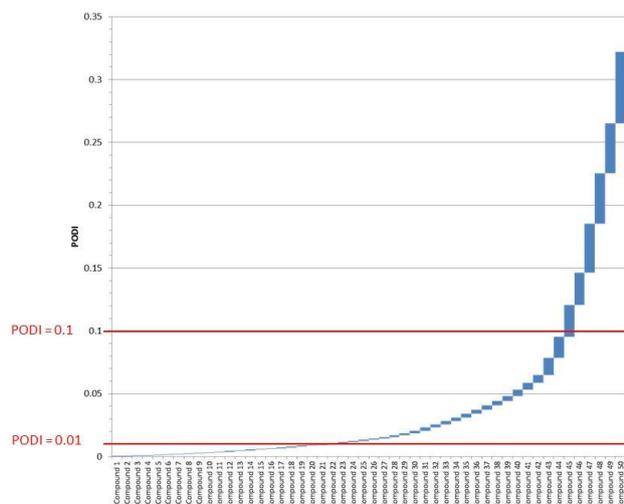
Figure 1: The principle of driver identification. Chemicals 1 – 50 are arrayed in ascending order according to the size of their RQ (here demonstrated for the Point of Departure Index, PODI, where RQ = exposure/POD). If PODI = 0.01 is used as the limit of acceptable exposures, chemicals 1 – 22 comply with this standard, but jointly, chemicals 23 – 50 exceed it and will be identified as drivers of a PODI. Accordingly, if PODI = 0.1 is deemed acceptable, the group of drivers reduces to chemicals 45 - 50.

The identification of a chemical as „driver" depends on the limit of acceptable combined exposures. All chemicals that exceed such a standard, will be identified as drivers.

7.3 Dealing with missing toxicity data and implications for identifying mixture components as drivers

As a minimum requirement for starting the MRA in Tier 1, regulatory maximum acceptable doses or protective concentrations, so-called regulatory or reference values (RV) must be available for all mixture components, and for all receptors. This will include ADI and TDI, as appropriate. In Tier 2 of the analysis, this will be replaced by POD (NOECs, NOAELs, BMD and equivalent). The required data may not be available for all chemicals that make up mixture exposure scenarios of interest. In principle, there are two options for dealing with such data gaps: (a) chemicals without RV shall be excluded from analysis – this will lead to underestimations of risks associated with combined exposures; (b) missing RV will be bridged by using methods that estimate RV by various methods, but this may imply overestimations of risks. To avoid a bias towards the null, it is proposed to follow option b.

Mixture components whose potency had to be estimated due to missing toxicity data may in some cases make a disproportionately large contribution to the overall mixture risk. These chemicals cannot be said to drive a mixture risk, but instead signal a high priority for further testing with the aim of filling the respective toxicity data gaps. It is suggested to identify these chemicals and to collect them in a list termed "Pool I" (**Fig 2**). If the remainder of the chemicals exceeds acceptable mixture exposures, the analysis moves to the next Tier, but chemicals with unknown toxicity are omitted from further consideration. This filtering step ensures that the analysis is not stalled by insurmountable data gaps. However, it should be noted that only a sub-set of chemicals is taken forward into the next Tiers of the assessment. The advantage is that the analysis is put on a secure footing in that it allows assessments of partial risks on the basis on sound data. The mixture risk associated with the entire ensemble of chemicals originally entered into the analysis cannot be smaller than the risk identified for a sub-sets for which toxicity data are available.

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7.4 Decision tree and workflow for advanced human mixture risk assessment

For modelled exposure scenarios, the scheme starts with defining the mixture of concern in Step 1 of Tier 1 (**Fig 2**), which is assumed to be fixed and not subject to further refinements; only the hazard assessment aspect of the MRA is refined in a stepwise fashion.

For exposure scenarios derived from monitored (measured) values, it is necessary to also refine the exposure assessment arm by dealing with analytical non-detects and by making adjustments for bioavailability. We propose to deal with these issues in higher Tiers of the analysis, after identification of drivers of mixture risks.

The scheme is divided into three major tiers, with tiers 2 and 3 each being sub-structured into a number of sub-tiers (2A-C and 3A-D):

- Tier 1 MRA (**Fig 2**), assuming dose addition and using the HI method with RV (or equivalents)

- Tier 2 MRA (**Fig 3-5**) assuming dose addition and using

    (A)   the PODI method based on species group specific NOAELs or NOECs, for different toxicity endpoints

    (B)   the PODI method based on endpoint specific species group NOAELs or NOECs; if available, NOAELs or NOECs can be replaced by BMD or EC10

    thereby removing distorting factors of the CA calculation in a stepwise fashion.

- Tier 3 MRA (**Fig 6-7**), taking a Mixed Model (MM) approach, i.e. assuming dose addition (DA) for groups of similarly acting mixture components, and independent action (IA) between the groups, structured into

    (A-C) limit value calculations, defining a "prediction window" between minimum and maximum expectable risk on the basis of incomplete specifications of IA model parameters and/or insufficient knowledge of MoAs, and finally

    (D)   exact MM-based MRA, based on knowledge of DRCs and MoAs for all (relevant) mixture components.

At every tier and sub-tier, similar tasks are performed, including:

- compilation of the necessary input data,

- bridging of data gaps,

- assessment of the mixture risk,

- identification of drivers of the overall risk,

- identification of single chemicals with a high priority for testing, i.e. suspected drivers with missing test data (Pool I),

- identification of single chemicals with a high priority for risk management, i.e. confirmed drivers that exceed their permissible exposure levels (Pool II).

As pointed out earlier, it is not necessary to take all analyses through every proposed step; if there are indications of unacceptable mixture risks on the basis of simple worst-case assumptions, the MRA can commence at a higher tier.

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Where the core task of assessing the mixture risks reveals indications of absence or presence of a significant risk, this is highlighted in the schemes by **green** and **red** colouring, respectively. Assignments to Pools I and II are highlighted in **blue**.

As the MRA becomes more certain in higher Tiers, so does the identification of drivers. This means that driver identification in higher tiers overrules lower tiers and consequently the substances assigned to Pool II may be sub-grouped accordingly.

The scheme is designed to provide clear decisions about the absence or presence of exceedances of combined exposures, wherever possible. In Tiers 1 and 2, DA-based worst case estimates are generated and refined step by step. The assessment is discontinued, if there is no indication for a significant risk. Otherwise it is moved forward to the next Tier.

Tier 3 MRA is based on the conceptual assumptions of a hybrid DA/IA model, or Mixed Model (MM). The data and knowledge requirements for correct application of the MM approach are hard to fulfil and the conceptual premises may not be exactly met. To deal with these issues, the proposed scheme includes three pre-evaluation steps (Tier 3A –C), which provide best-case estimates of minimum expectable risks that can be calculated with limited data.

In Tier 3A the RQ of individual mixture components are assessed. The mixture toxicity predicted by the MM can never be lower than the toxicity of the most toxic mixture component alone (at the dose or concentration present in the mixture). Where a risk is estimated for one or more individual mixture components for a relevant endpoint, the evaluation is stopped. Performing an MM-based assessment is not worth the considerable effort this will entail: significant risks from single substances cannot be ruled out by assuming IA for dissimilarly acting (groups of) mixture components.

Tier 3B is an assessment of the sums of RQ for sub-groups of similarly acting mixture components, where the available knowledge allows to identify such groups. The mixture toxicity predicted by the MM can never be lower than the joint toxicity of the chemicals in the most toxic sub-group, as calculated by assuming DA within the groups. Where a significant risk can be demonstrated for one or more sub-groups of similarly acting mixture components for one or more endpoints relevant in fish, the evaluation stops. The conclusion is essentially the same as in Tier 3A: performing an MM-based assessment is not worth the considerable necessary effort. Significant risks from a common-MoA-group cannot be ruled out by assuming IA for dissimilarly acting (groups of) mixture components.

Tier 3C assumes completely independent action for all mixture components as an extreme case for the MM approach. In Tiers 1 and 2 we assumed completely similar action as the other extreme. If this extreme assumption of IA in Tier 3C indicates a significant risk, the conclusion is essentially the same as in Tiers 3A and 3B: completing the MM-based assessment by introducing MoA-based grouping of mixture components is not worth the effort. Significant risks from a completely independent action of all mixture components cannot be ruled out by assuming DA (or CA) for similarly acting groups of mixture components.

Finally, in Tier 3D we apply the MM approach as the final step in our proposed scheme. It is conducted in situations where the maximum expectable risk is significant (as calculated in Tier 2C on the basis of the CA assumption), while the best case estimate of the minimum expectable risks is not (as calculated in Tier 3C on the basis of the IA assumption). Where all data requirements and conceptual assumptions can be met, Tier 3D will provide a definite assessments in terms of combined risks. Otherwise, the assessment will end with the finding that all available data and information provide "inconclusive evidence". In cases where Tier 3D assessments provide "inconclusive evidence" for unacceptable mixture risks, the logic of the proposed decision tree

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means that the problem cannot simply be solved by additional standard testing of single substances, but only by further research of MoAs and/or by experimental testing of the whole mixture. Alternatively, precautionary risk reduction measures may be taken into consideration.

Where the procedure finally results in the indication of a significant risk, validation of predictions by experiments or field observations may be taken into consideration. Alternatively, options for risk reduction should be explored. Where the procedures provide inconclusive evidence, dedicated follow-up studies on MoAs or experimental mixture toxicity testing may provide the missing evidence. Alternatively, precautionary risk reduction measures may be considered.

Figure 2: Tier 1 decision tree and workflow scheme

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Tier 2A MRA

11. Compile (lowest) POD values for relevant test species(-groups) (**NOAEL**$_{i, RAT}$**; NOEC**$_{i, FISH}$**; or equivalents**)

12. Are **POD**s available for all mixture components?

no → **13**. Bridge gaps by using TTC, read-across etc.

yes

14. Determine **PODI** for rats (or other human toxicity models) and for (tested) fish species.

15. Assess mixture risks for humans by applying an appropriate (default) AF or a corresponding MOE approach

(PODI x AF) ≤ 1 → **No significant risk**

(PODI x AF) > 1

16. Identify **drivers** of **PODI**

17. Are any of the drivers chemicals where PODs were estimated by using TTC, read-across etc. ?

yes → **18**. **Remove** these chemicals from analysis, add to **Pool I**, and re-determine PODI and mixture risk for the rest

no

(PODI x AF) > 1 → **20**. Identify **test-based drivers of high PODIs** > (1/AF) and add them to **Pool II**

(PODI x AF) ≤ 1

19. Repeat Tier 2A MRA when single substance data gaps have been closed

Pool I: Single chemicals with high priority for **testing**

Pool II: Single chemicals with high priority for **risk management**

21. Move to **Tier 2B MRA**

Figure 3: Tier 2A decision tree and workflow

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Tier 2B MRA

22. Compile (lowest) benchmark doses or low-effect concentrations for relevant test species(-groups) ($BMD_{i, RAT}$; $EC10_{i, FISH}$; or equivalents)

23. Are BMDs and low ECx values available for all mixture components ?

no → **24**. Bridge gaps by using read-across. QSAR etc.

yes

25. Determine **BMD- or ECx-based PODI** for rats (or other human toxicity models)

26. Assess mixture risks for humans and for wildlife fish by applying an appropriate (default) AF or a corresponding MOE approach

(BMD- or ECx-based PODI x AF) ≤ 1 → **No significant risk**

(BMD- or ECx-based PODI x AF) > 1

27. Identify **drivers** of **BMD- or ECx-based PODI**

28. Are any of the drivers chemicals where BMD- or ECx-based PODs were estimated by using read-across etc. ?

yes

29. **Remove** these chemicals from analysis, add to **Pool I**, and re-determine BMD- or ECx-based PODI and mixture risk for the rest

no

(BMD- or ECx-based PODI x AF) > 1

(BMD- or ECx-based PODI x AF) ≤ 1

31. Identify **test-based drivers of high BMD- or ECx-based PODIs** > (1/AF) and add them to **Pool II**

30. Repeat Tier 2B MRA when single substance data gaps have been closed

Pool I: Single chemicals with high priority for **testing**

Pool II: Single chemicals with high priority for **risk management**

32. Move to **Tier 2C MRA**

Figure 4: Tier 2B decision tree and workflow

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Tier 2C MRA

Figure 5: Tier 2C decision tress and workflow

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Tier 3A MRA

44. Do any mixture components singly pose a concern for one or more relevant endpoints (adverse outcomes) in humans, i.e. $TUx_i > (1/AF)$?

yes

no

45. Begin **Tier 3B MRA**

Tier 3B MRA

46. Collect information on **MoA** (AOPs) of all mixture components for all relevant endpoints.

47. Is sufficient information on MoA available?

no

48. Where possible, bridge gaps by using read-across, SAR etc.

yes

49. Build **groups** of mixture components contributing to a common endpoint via a **common MoA**. (Several groups may be applicable for the same chemical.) For chemicals that cannot be assigned, assume a dissimilar MoA at this stage.

50. Determine ∑**TUs** separately for every group j and endpoint k

51. Does any of the individual groups pose a concern (disregarding other mixture components), i.e. $\sum TUx_{j,k} > (1/AF)$?

no

yes

52. Identify **drivers of high ∑TUs within MoA groups** and add them to **Pool II**

Pool II: Single chemicals with high priority for **risk management**

STOP the model-based MRA for humans and/or fish. **Further refinement is pointless**. Significant risks from a single substance or from a common-MoA-group cannot be ruled out by assuming IA for dissimilarly acting (groups of) mixture components.

Significant risk.
- Consider validation of predictions by experiments or field observations.
- Identify options for risk reduction.

53. Begin **Tier 3C MRA**

Figure 6: Tier 3A B decision tree and workflow

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Tier 3C MRA

Starting situation: endpoint-specific TU-calculations do not indicate a significant risk for any individual mixture component and not for any group of components with a known common MoA, but for the whole mixture of concern.

54. Collect information on the **slopes of DRCs** (or CRCs) for all mixture components and for all endpoints of concern.

55. Is **information on slopes** of DRCs **available** for all components or at least for all components driving the overall ∑TUs (as identified in step 31)**?**

no

yes

56. Is the **data quality sufficient** for IA-based predictions of low-effect doses (BMD) or concentrations (EC10) for mixtures**?**

no

yes

57. Calculate the expectable mixture toxicity under the **extreme assumption of completely independent action** of all mixture components: BMD_{mix}^{IA}, $EC10_{mix}^{IA}$.

58. Assess the **minimum mixture risks** for humans and for wildlife fish by applying an appropriate (default) AF: $BMD_{mix}^{IA} < [(\sum EL_{i,HUMAN}) \times AF]$, and/or $EC10_{mix}^{IA} < [(\sum EL_{i,FISH}) \times AF]$?

yes

no

Inconclusive evidence.

DA- or CA-based concerns cannot be ruled by advanced modelling due to insufficient information about single substance DRCs.

- Consider advanced experimental and/or statistical dose response analyses for single substances, and/or experimental mixture toxicity testing, and/or precautionary

STOP the model-based MRA for humans and/or fish. **Further refinement is pointless.** Significant risks from a completely independent action of all mixture components cannot be ruled out by assuming DA (or CA) for similarly acting groups of mixture components; significance can only be

Significant risk.
- Consider validation of predictions by experiments or field observations.
- Identify options for risk reduction

59. Perform final **Tier 3D MRA**

Figure 7: Tier 3C decision tree and workflow

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7.5 Advanced analyses of the outcome of mixture risk assessments: The Maximum Cumulative Ratio (MCR)

It may be of interest to establish whether a subject's combined exposure is dominated by one chemical or whether it is influenced by several chemicals simultaneously. This will show the extent of cumulative risks that is missed in traditional single chemical risk assessments. A convenient way of addressing this issue is to calculate the ratio of the HI and the maximum RQ among the RQs that sum up to the HI, called Maximum Cumulative Ratio (Price and Han 2011). By definition, the MCR cannot exceed the number of mixture components, n. The MCR equals n when the RQs of all chemicals contribute equally to the HI. The value of MCR approaches 1 where only one substance makes up virtually 100% of the HI.

The ratio of the total impact of a combined exposure (equivalent to the HI) to the largest single-chemical impact (equivalent to RQ $_{max}$) was originally used by Könemann (1981) to distinguish types of joint action of chemicals in fish, and by Junghans et al. (2006) to establish the factors that determine the degree of divergent predictions of mixture effects derived from the assessment concepts of dose addition and independent action. Price et al. (2011, 2012) employed the MCR to group exposure scenarios into categories that can support risk management decisions. By creating scatter plots of MCR_p versus log HI_p different groupings can be distinguished (Apel et al., 2020, **Figure 8**), as follows, here discussed by using the example of phthalates and their anti-androgenic effects:

Exceedances of acceptable combined and single exposures (category 3 in Figure 8), exceedances of acceptable combined exposures (category 2) and combined exposures where multiple phthalates contribute to the HI (categories 2b and 3b).

Data points to the left of a vertical line demarcating an acceptable HI (commonly 1) depict individuals who experienced combined exposures that do not present concerns (category 1). Conversely, data points to the right of that line show subjects who exceeded their acceptable combined exposures. These individuals fall into two separate categories: Data points that sit in the segment defined by the vertical line for acceptable HIs and the curved line depicting MCR = HI signify individuals that have experienced unacceptable combined exposures without exceeding acceptable levels for any single phthalate (category 2). Conversely, data points to the right of the MCR = HI line show study participants with combined exposures above acceptable levels for at least one chemical (category 3). In turn, each of these three categories can be divided into two subgroups according to their MCR. Data points below the horizontal line corresponding to MCR = 2 show individuals where one phthalate contributed 50% or more to the HI (categories 1a, 2a 3a). Above this line are subjects who experienced combined exposures where multiple phthalates contributed to the HI (categories 1b, 2b, 3b).

With these groupings, Price et al. (2012) identified the following risk management options: Categories 2 and 3 signal concerns as acceptable combined exposures are exceeded. For categories 2a and 3a these issues can be mitigated by targeting one phthalate with exposure reduction measures. The unacceptable exposures to several phthalates experienced by Category 3b subjects can be addressed by reducing exposures to several phthalates. In contrast, Category 2 represents subjects with unacceptable risks which would have gone unnoticed during conventional single phthalate risk assessment and can only be addressed through cumulative risk assessment.

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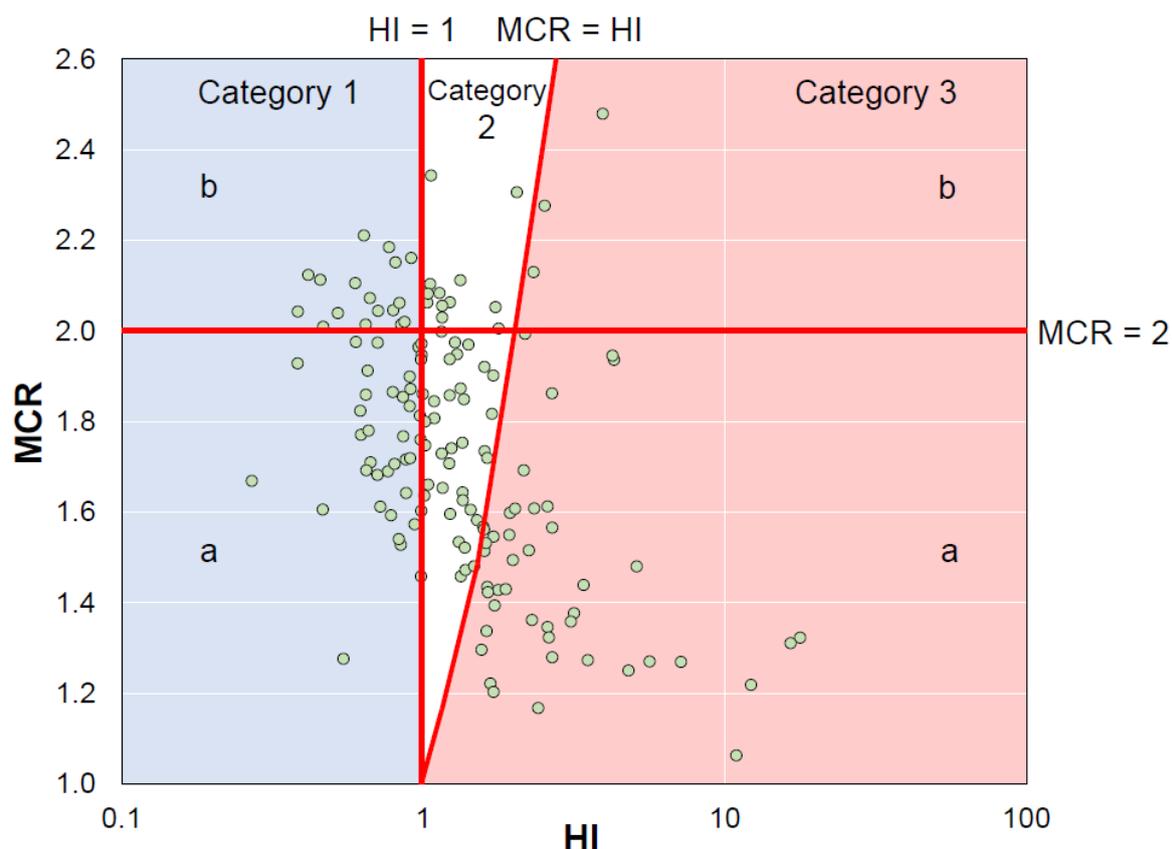
Figure 8: Example scatter plot of Maximum Cumulative Ratio (MCR) versus Hazard Index (HI) with categories for risk management. For further details, see section 7.6 (Apel et al. 2020.

7.6 Time and changes in usage patterns of chemicals as an aspect in mixture risk assessments

In several human biomonitoring surveys, changes in the usage patterns of chemicals have come to light (cited in Apel et al. 2020), but their influence on the risks associated with combined exposures is insufficiently understood. We have recently conducted an analysis of changing phthalate exposures and the impact on cumulative risks, based on the largest study to date, the 27-year survey of urinary phthalate metabolite levels in 24-hour urine samples from the German Environmental Specimen Bank (Apel et al. 2020). The analysis adopted the Hazard Index (HI) approach based on the five phthalates DBP, DIBP, BBP, DEHP and DINP. Calculations of the hazard index for each study participant included updated phthalate reference doses for anti-androgenicity (RfD$_{AA}$s) that take account of new evidence of phthalates' developmental toxicity (Kortenkamp and Koch, 2020).

The Maximum Cumulative Ratio (MCR) approach was used to establish whether a subject's combined exposure was dominated by one phthalate or was influenced by several phthalates simultaneously. Generally, over the years there was a shift towards lower HIs and higher MCRs, reflecting an increased complexity of the combined exposures. The decade from 1988 to about 1999 was characterised by rather high HIs of between 3 and 7 (95th percentile) which were driven by exposure to DBP and DEHP, often exceeding their single acceptable exposures. From 2006 onwards, no study participant experienced exposures above acceptable levels for a single phthalate, but combined exposures were still in excess of HI = 1. From 2011 onwards most individuals stayed below HI = 1 (Apel et al., 2020, **Figure 9**).

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The analysis highlights that mixture risk assessments must also ensure consistency in the selection of exposure data and make sure they originate from similar study years. Otherwise, the results will be confounded by mixing incompatible exposure data.
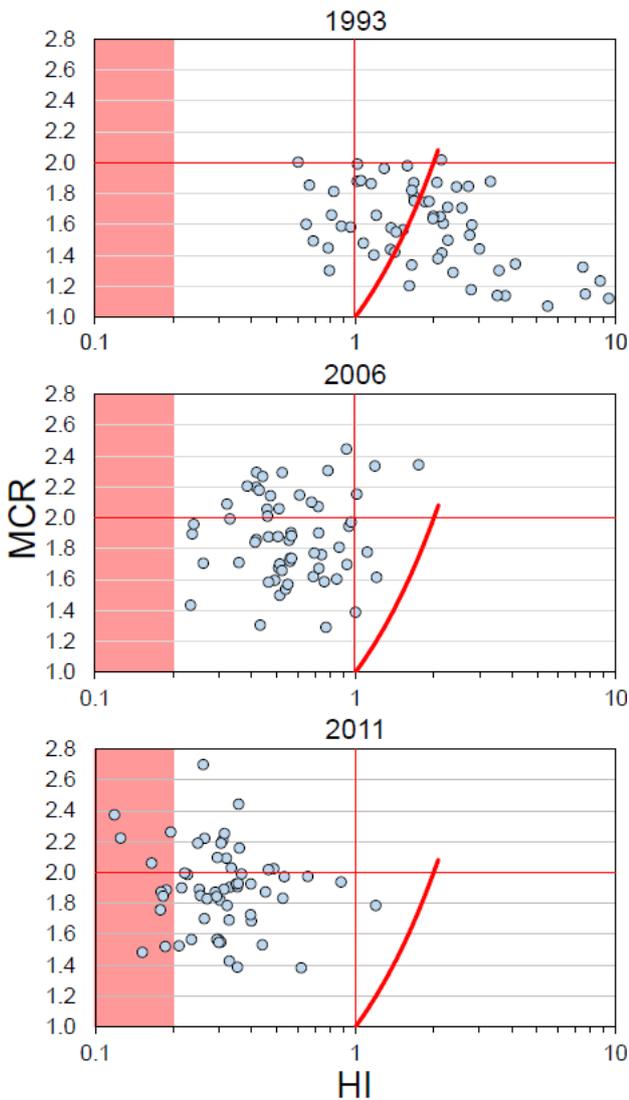
Figure 9: Analysis of combined phthalate exposures with MCR versus HI scatter plots for 1993, 2006 and 2011.

Individual study participants are shown as blue dots. The vertical red line shows HI = 1, the horizontal red line is for MCR = 2. The curved red dotted line depicts MCR = HI. The red box in each plot shows the range of HI between 0.1 and 0.2 as suggested values for evaluation of combined phthalate exposures that can accommodate substances also contributing to male reproductive risks (Apel et al. 2020).

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8 Progress reports for case studies

8.1 Developmental neurotoxicity beyond PBDEs

Adopting the analysis by Landrigan and Grandjean (2006), 12 developmental neurotoxicants (DNT) will be considered jointly in this case study. Apart from PBDEs, these chemicals include arsenic, chlorpyrifos, PCBs, methylmercury, lead, fluoride, manganese, DDT, tetrachloroethylene, alcohol and toluene. Recent evidence shows that phthalates are emerging DNTs, and accordingly, this group of chemicals will be considered at a later stage.

A Tier 1 scoping level analysis for the 12 DNTs was conducted by BRUNEL by employing the Hazard Index (HI) method. This analysis revealed considerable exceedances of combined acceptable levels. PBDEs, toluene, methyl-mercury, arsenic, fluoride, tetrachloroethylene, PCBs and manganese were drivers of non-compliance with acceptable joint exposures.

Developmental neurotoxicants: scoping-level HI

PBDEs	5.46
Toluene	2.30
Methylmercury	1.00
Arsenic	0.90
Fluoride	0.57
Tetrachloroethylene	0.50
PCBs	0.40
Manganese	0.34
Lead	0.01
DDT	0.00
Chlorpyrifos	0.00
Ethanol-------x	
HI	11.48
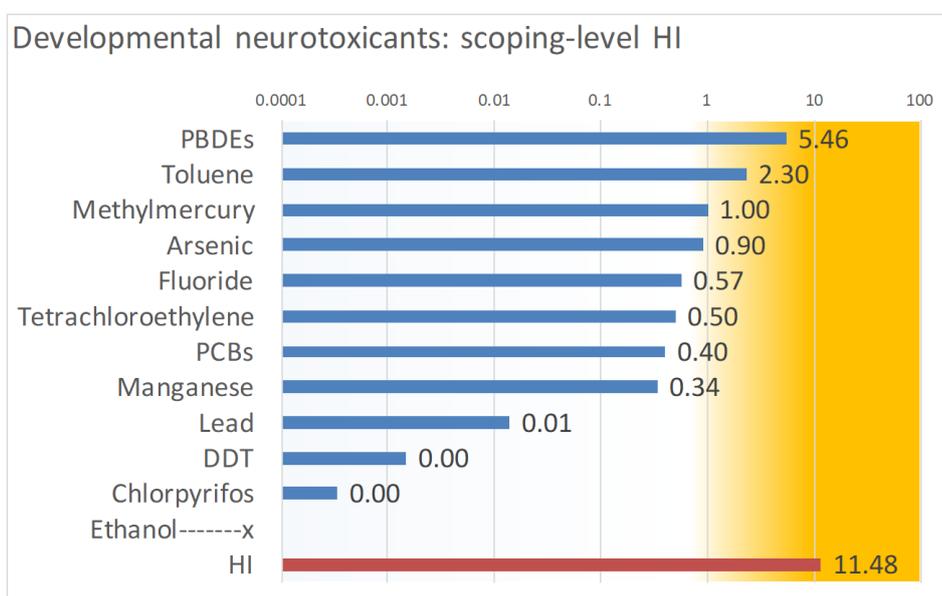
Due to the high HI values, the analysis has now proceeded to Tier 2. To make the mixture risk assessment consistent, potency values (ideally benchmark doses) for mixture components will be based on human epidemiological studies as much as possible. Such benchmark doses should be derived for health endpoints relevant to DNT, such as declines in IQ. Quantitative exposure-risk assessments on the basis of human epidemiological data are possible for lead, PCBs and methyl-mercury. HBGV for these pollutants have been located from EFSA CONTAM reports.

However, human epidemiological data that allow benchmark dose modelling are currently not available for PBDEs. Efforts of locating equivalent data for the other selected DNT are ongoing.

In parallel, we are assembling exposure data for all chosen DNTs. This process is complete for PBDEs, lead, PCBs and methyl-mercury.

8.2 Heavy metals and nephrotoxicity

It is clear that the general population is exposed, through the diet, to a mixture of heavy metals on a daily basis. In addition to the dietary exposure, the plant material in cigarettes may contain heavy metals and a well-known exposure route of Cd is inhalation of cigarette smoke. Several heavy metals may have nephrotoxic effects and e.g. Cd, Pb and Hg may cause some kind of kidney dysfunction like decreased glomerular filtration, proximal tubular damage or CKD. Due to the fact that these heavy metals may accumulate in kidneys during life, these effects are more prominent in

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the elderly. The accumulation of these heavy metals is due to a continuous (almost daily) exposure in combination with a slow elimination profile (no metabolism and slow excretion) and, in some cases, specific binding (Cd in bones).

This case study, led by RIVM, focuses on the nephrotoxic effect of a mixture of four heavy metals, namely As, Cd, Hg and Pb. Only the inorganic forms of these heavy metals will be studied, thereby excluding the organic forms of As and Hg.

In accordance with the guidance of EFSA for risk assessment of combined exposure to multiple chemicals three issues will be addressed in this case study: 1) calculation of the Hazard Index (HI), 2) calculation of the Point of Departure Index (PODI) and 3) determination of an adverse outcome pathway (AOP). In this case study, the HI and PODI calculations were performed parallel to the development of the AOP.

As a first step, we focused on dietary exposures and adults. For dietary risk assessment, we used data on Cd and Pb available for adults in the Netherlands. Because data for the Dutch dietary exposure to As and Hg were lacking, As and Hg data for adults in Europe were taken from two EFSA opinions. The data for the Dutch population were merely used as an example. As a next step, we will calculate the HI and PODI for dietary exposure using only data for the European population. Where possible, the dietary risk assessment will be expanded to other population groups.

We constructed an AOP with the aim to gain insight into the molecular mechanisms by which heavy metals cause nephrotoxicity, and to identify common toxicity pathways as well as biomarkers that could be used for risk assessment purposes. An AOP is a linear representation of a sequence of biological events, starting with a molecular initiating event (MIE) followed by several key events (KEs), that lead towards an adverse outcome (AO). Several sources, such as the OECD AOP User's handbook, the AOP-Wiki and scientific publications were used to establish the correct approach to build an AOP. Subsequently, online available literature from scientific literature databases such as Pubmed and Web of Science was used to build the AOP.

HBGVs have been established only for Cd and Hg. In both cases a TWI, based on BMD modelling has been derived by EFSA. Therefore, these two metals were used for determining a HI (see section 3.1.1). By contrast, a POD has been derived for all four metals. These PODs are BMD confidence intervals, including lower (BMDL) and upper (BMDU) bounds [6,7,10]. Here, the lower bounds, *i.e.* BMDL values, were used to determine the PODI.

While using the HBGVs of Cd and Hg for the determination of a HI, two matters are important to note: the uncertainty factors used for the derivation of the TWI are significantly different (4 for Cd and 100 for Hg) and the points of departure are different. For Cd the POD is (proximal) tubular damage and for Hg the POD is kidney weight, although the latter is (in)directly related to proximal tubular damage.

The HI for the dietary exposure of Cd and Hg is approximately 1.5 and consequently larger than 1. Additional dietary exposure of As and Pb will only increase this HI. Therefore, combined dietary exposure to the four heavy metals is assumed to exceed the level that is considered acceptable.

While using the BMDLs for the determination of a PODI, two matters are important to note: the points of departure are different and the studied species are different (humans in case of As, Cd and Pb; rats for Hg). For As the critical effect is bladder/urinary tract cancer, for Cd (proximal) tubular damage, for Pb CKD (decreased GFR) and for Hg kidney weight. Furthermore, it is unclear if and what (overall) uncertainty factor needs to be used.

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The PODI for a mixture of four heavy metals is larger than 1, namely 1.7. This PODI should be multiplied by an uncertainty factor (UF) which can be a default UF or a chemical specific adjustment factor (CSAF) depending on the data available. Although it is not sure what (overall) UF should be used (due to the different species that were used) it is already clear that the total dietary exposure of a mixture of four heavy metals exceeds the level considered to be acceptable without the application of an UF.

In addition to the results achieved so far, a couple of extra steps are foreseen. Regarding dietary risk assessment, we will calculate the HI and PODI using data relevant for European (sub)populations. Where possible, other population groups besides adults will be included. In addition to the deterministic approaches used, we will also refine the dietary risk assessment. To this end, we will employ probabilistic approaches by making use of a data platform developed in the Horizon2020 project 'EuroMix', the so-called 'EuroMix Toolbox'.

Another highly important next step for this case study is to assess the risk related to occupational exposure. For this, we are liaising with HBM4EU partner FIOH, to obtain relevant exposure data.

Regarding the AOP, we aim to evaluate the WoE for each of the specific KEs included in the 'main' AOP for each of the individual heavy metals. This will enable us to assess the contribution of each of the heavy metals to the perturbation of each of the KEs. This approach will also make it possible to indicate which new effect biomarkers can qualify for biomonitoring purposes. Moreover, this will allow us to identify one or multiple KEs to calculate RPFs, which may lead to a refined risk assessment of combined exposure to these four heavy metals.

8.3 Anti-androgenic chemicals and male reproductive health

In joint discussions DTU and BRUNEL agreed to offer **two approaches** in this case study which will differ in terms of the dose metrics employed:

The **first approach**, pursued by BRUNEL, utilises exposure data and reference doses with the classical metric dose/kg/d.

First, criteria for the inclusion of chemicals in mixture risks assessments for male reproductive health were developed by examining the mechanisms of action of various chemicals capable of disrupting male sexual differentiation. BRUNEL constructed an Adverse Outcome Pathway (AOP) network for malformations of the male reproductive system that includes new findings about the role of disruptions of prostaglandin signalling. This network was used to identify pathways that converge at critical nodal points to produce down-stream adverse effects. From this knowledge, combinations of chemicals with different mechanisms of action were predicted that should result in cumulative effects. These predictions were mapped against evidence from experimental mixture studies with relevant combinations. This analysis showed that cumulative assessment groups for male reproductive health risks should not only include phthalates but also comprise androgen receptor (AR) antagonists, chemicals capable of disrupting steroid synthesis, InsL3 production, prostaglandin signalling and co-planar polychlorinated dibenzo-dioxins together with other dioxin-like compounds. A minimum set of chemicals to be assessed together with phthalates includes pesticides such as vinclozolin, prochloraz, procymidone, linuron, the pain killers paracetamol, aspirin and ibuprofen, pharmaceuticals such as finasteride, ketoconazole, and the lipid-lowering drug simvastin, polychlorinated dibenzo-dioxins and other dioxin-like pollutants and phenolics such as bisphenol A and butylparaben (see figure below).

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Phthalates
Diethyl-hexyl phthalate (DEHP)
Di-n-butyl phthalate (DBP)
Butyl-benzyl phthalate (BBP)
Di-iso-nonyl phthalate (DINP)
Di-n-pentyl phthalate (DPP)
Di-iso-pentyl phthalate (DIPP)
Di-iso-butyl phthalate (DIBP)
Di-cyclo-hexyl phthalate (DCHP)
Di-n-hexyl phthalate (DNHP)
Di-iso-heptyl phthalate (DIHP)

AR antagonists and inhibitors of steroidogenic enzymes
Vinclozolin
Procymidone
Prochloraz
Bisphenol A
Linuron
Butylparaben

Pain killers
Paracetamol
Aspirin
Ibuprofen

Dioxin-like pollutants
2,3,7,8 TCDD
PCB 169
Other congeners, including furans?

Other pharmaceuticals
Finasteride
Ketoconazole
Simvastin

Second, BRUNEL developed a set of new reference doses for phthalates that can be used in mixture risk assessments. These reference doses focus on anti-androgenic effects of phthalates.

Third, utilising these new anti-androgenicity reference doses, BRUNEL, in collaboration with UBA and IPA (Bochum, Germany), conducted a mixture risk assessment based on a survey of urinary phthalate metabolite levels in 24-hour urine samples from the German Environmental Specimen Bank. For every study year and study participant, hazard indices (HIs) for the 5 phthalates DBP, DIBP, BBzP, DEHP and DINP were calculated. The Maximum Cumulative Ratio (MCR) approach was used to establish whether a subject's combined exposures were dominated by one phthalate or were influenced by several phthalates simultaneously. Generally, over the years there was a shift towards lower HIs and higher MCRs reflecting an increased complexity of the combined exposures (Apel et al. 2020).

The scene is now set to begin the assembly of similar reference doses for anti-androgenicity for the other chemicals to be subjected to mixture risk assessment in this case study. This process is well underway.

The **second approach**, led by DTU, utilises internal tissue concentrations as the dose metric for mixture risk assessments, with a focus on internal anti-androgenic load. Human Biomonitoring data that are actually measured exposure data independent of the source of exposure will be used. For the hazard assessment, human data such as in vitro anti-androgenic activities and relevant human epidemiological data will be applied rather than rodent-based hazard values. This approach offers the advantage of being independent of exposure sources and species differences and is able to link to effect-based Human Biomonitoring that has utilised *in vitro* androgen receptor reporter gene assays for the identification of anti-androgenicity in human tissue samples. This approach starts directly at tier 2 of the suggested procedure.

The group of anti-androgenic chemicals is very diverse, including phthalates, bisphenols and other phenolic substances, certain pesticides, PAHs, PCBs, dioxin and pharmaceuticals such as analgesics. Thus, several of the prioritised chemical groups in HBM4EU belong to the 'anti-androgenic' chemicals. This case study can be linked to the work on biomarkers of effect that is ongoing in WP14 and WP16.

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These biomarkers focus among other things on responses to anti-androgens in cell-based bioassays of human placenta tissue. So far, the following work has been performed:

1. Gathered existing literature on 'antiandrogenic' mixtures (DTU).

   Identified the known and widespread 'antiandrogenic' chemicals and drugs for which data is available. Priority was given to human-relevant experimental and epidemiological data and a list of ~200 compounds have been identified (DTU)

2. Agreed on a step-wise plan for data collection together with UGR and UBA

3. Collected hazard data from available sources for a selected subset of chemicals (DTU)

Future work includes:

1. Collect relevant exposure data from available sources (human exposure levels, Cmax values for drugs, internal exposure levels). UGR and UBA have access to human exposure data and will gather information from Human Biomonitoring studies.

2. Calculate Hazard Quotients and Hazard Index. Consideration of inclusion of uncertainty factors to correct for in vitro to in vivo extrapolations.

3. Integrating of outcome with other WPs. In WP14 we investigate anti-androgenic effects of the placenta extracts from 24 normal women as a biomarker of effect. This biomarker activity will be linked to HBM data obtained for the extracts, and in WP16 emerging chemicals are identified in the extracts. The sources of the integrated mixture effects in the placentas will be investigated and may inform the overall mixture evaluation.

8.4 Chromium (VI), nickel and PAHs

Occupational exposure limits (OEL) have been applied to prevent morbidity and mortality arising from exposure to single chemicals in occupational settings but do not account for all of the complexities of the work environment. Thus, there is a need for OELs and other risk management tools to integrate wider consideration of risks from multiple exposure pathways and routes as well as the combined risk from exposure to several chemicals in the workplace.

To develop the case study on occupational exposure to Cr (VI), Ni and PAHs and lung cancer a tiered framework has been applied by INSA. The Tier 1 is intended to answer the following questions: i) Do the predicted mixture risks from combined occupational exposure to Cr (VI), Ni and PAHs exceed the levels regarded as acceptable? ii) Are there drivers of mixture risks, i.e., substances that contribute most to lung cancer?

The first step included the collection of relevant exposure information on occupational cohorts gathered from literature to derive the Hazard Index (HI). Considering the chromate occupational study that is underway within the HBM4EU (task 8.5) and from which real exposure data is expected, we selected an exposure scenario consisting of a workplace with electroplating baths where all the coating process [commonly using Cr (VI) and Ni], for an aerospace company is performed. Additionally, exposure to PAHs occurs because this workplace is located nearby the area where aircrafts' engines are being repaired and tested, thereby generating large quantities of diesel exhaust.

The information collected from two published studies (Jeffrey et al., 2000; Health and Safety Executive, 2013) was used to start building hazard quotients (HQ) derived from relevant occupational exposure limits for Cr (VI), Ni and PAHs, followed by the determination of the HI. The results are presented in Table 1.

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Table 1: HQ and HI calculations for the occupational exposure to Cr (VI), Ni and PAHs.

Substance	TLV-TWA* (mg/m³)	Exposure data TWA (mg/m³)	HQ	HI
Cr VI	0.005 [Carcinogenic and Mutagenic Directive]	0. 011 (HSE, 2013)	**2.2**	
Soluble nickel compounds	0.5 [Chemical Agents Directive (98/24/EC)]	0.077 (HSE, 2013)	0.154	**2.369** [>1]
PAHs PAHs mixtures containing benzo[a]pyrene	0.00007 [proposal for Carcinogenic and Mutagenic Directive]	1.03×10^{-6}** benzo[a]pyrene (Jeffrey et al., 2000)	0.015	

*Threshold limit value − time-weighted average (TLV-TWA): average exposure on the basis of an 8h/day, 40h/week work schedule; ** TWA calculated based on the data available (considered the benzo[a]pyrene concentration/2 hours sampling during the engine run-up and the rest of 6 hours without exposure); HQ – Hazard Quotient; HI – Hazard Index.

The first attempt to derive a HI for the target mixture shows that cumulative exposures pose a risk that deserves to be further analysed (HI > 1) and that the driver substance is Cr (VI). Data from other studies is being collected in order to simulate other exposure scenarios and estimate the correspondent HQ and HI.

In Tier 2, the biomonitoring levels of Cr (VI) and Ni and the data on levels of PAHs (occupational hygiene data or biomonitoring data, if available) produced in the chromate occupational exposure study, will allow a refinement of the exposure assessment, comparatively to the literature-based results obtained. In addition, the characterisation of early genotoxic effects (e.g. through the micronucleus assay in peripheral blood lymphocytes) in a subset of the exposed and control individuals may help refining the hazard assessment component within the risk assessment framework.

This work was presented as an oral communication titled "A case study on occupational exposure to chromium (VI), nickel, PAH mixtures and lung cancer" by Maria João Silva, H. Louro, S. Viegas, B.C. Gomes, T. Santonen, A. Kortenkamp and E. Lebret, at the International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11), 28-30 August 2019, Leuven, Belgium.

8.5 Exposure misclassification in mixture studies

Background: The concept of the exposome was defined as encompassing the totality of environmental exposures from the prenatal period onwards.1 Efficiently identifying the exposures affecting health (i.e. with good sensitivity and low false detection proportion, or FDP) in this context is generally a challenge, in particular when exposures show some correlation, which is a realistic assumption. Exposome studies typically rely on biomarkers to assess exposures. Often, a single biospecimen is collected in each subject, which, whatever the accuracy of the measurement

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technique used, will induce measurement error for the least persistent compounds (those with the lowest intraclass coefficient of correlation, or ICC), for which a spot biospecimen will provide a poor estimate of exposure over long time windows.

In a single exposure context, exposure measurement error can strongly impact the estimation of exposure-health relationships, as we and others previously showed. In the case of classical-type error, i.e. when the exposure is measured with independent additive error (so that the within-subject average of repeated measurements is an unbiased estimate of exposure), naïve models not accounting for measurement error provide regression estimates that are attenuated (or biased towards the null), and have decreased statistical power. The exposome concept calls for assessing numerous exposures, typically using biomarkers. The amount of measurement error may differ between exposures, possibly influencing the performance of models used in exposome-health studies. In this case study, we aimed to evaluate this impact, and the efficiency of two measurement error correction methods relying on the collection of repeated biospecimens per subject.

Methods: Our approach is that of a simulation study. We generated 237 exposures with different amounts of measurement error, assuming that 1 to 10 exposures linearly influenced a continuous health outcome and that up to 10 biospecimens were available per subject. We applied within-subject biospecimens pooling or regression calibration (RC), both followed by the deletion/substitution/addition (DSA) algorithm to estimate the exposome-health associations, and compared their performances.

Results: In the absence of measurement error, the average sensitivity to identify exposures influencing health was 75% and false detection proportion (FDP) was 26%. Measurement error decreased sensitivity to 46%, increased FDP to 49% and caused 66% attenuation bias in dose-response functions. When repeated biospecimens were available, within-subject pooling and RC improved sensitivity (average, 63%), FDP (average, 37%) and attenuation bias (average, 49%); performances increased with the number of available biospecimens. Relying on repeated biospecimens only for the exposures with the largest amount of measurement error provided similar performance improvement. The sensitivity and FDP estimates under the assumption of a simulation in which a single exposure among 237 biomarkers truly affected the considered health outcome is shown in the figure below.
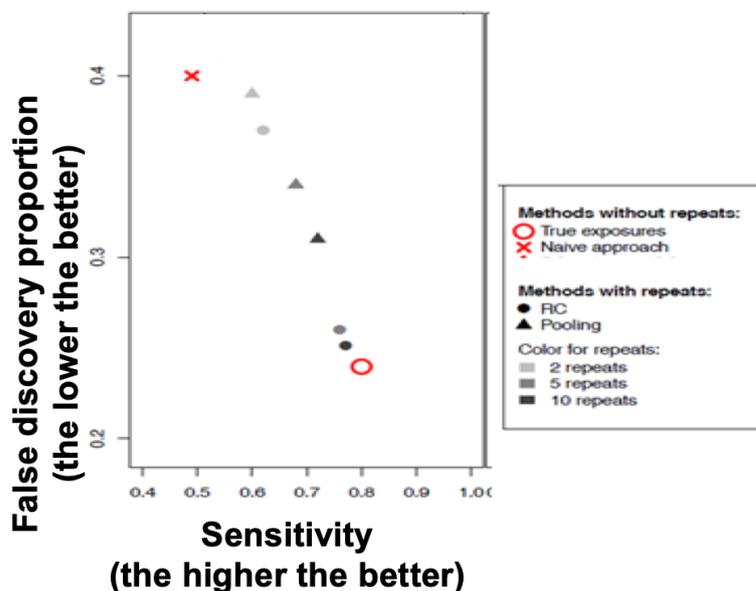
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Conclusions

Mixture studies aiming at identifying chemicals influencing health outcomes and relying on spot exposure biospecimens suffer from decreased performances, of greater amplitude for the exposures with the largest amount of measurement error. Study performances can be improved by collecting repeated biospecimens per subject, more specifically for non-persistent chemicals. In particular, the within subject biospecimens pooling approach (Perrier et al., Epidemiology, 2016) appears as an efficient way to limit bias without increasing assay cost.

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