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1 **A pragmatic approach for human risk assessment of chemical mixtures**

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13 **Keywords:** Risk assessment, mixtures, toxicology, hazard index.

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18 **Abstract**

19 Humans are continuously exposed to complex chemical mixtures from foods and the environment. Due to
20 our inadequate understanding of mixture effects, tools to assess the combined risk of mixed chemical
21 exposures have been difficult to develop. In recent years, regulatory authorities across the world have made
22 considerable progress towards developing pragmatic frameworks to deal with combined exposure to multiple
23 chemicals for risk assessment purposes. These approaches require a high level of information about chemical
24 exposures and toxicities, information that often is lacking. We see this data gap as delaying urgently needed
25 improvements in chemical safety. Herein, we present a pragmatic step-by-step procedure for mixture risk
26 assessment and propose tools for grouping of chemicals. Until we have a better understanding of adverse
27 outcome pathways, we suggest that grouping of chemicals for mixture risk assessment be based on integrated
28 in vivo and in vitro data, read-across as well as computational methods such as QSAR models or integrative
29 systems biology. These latter methods can be used to predict inherent hazards or modes/mechanisms of
30 action and to group the chemicals in cases, where no experimental data exist.

31 **1. Introduction**

32 Current regulatory approaches for assessing chemicals typically evaluate one chemical at a time, which fails
33 to take into account the human real-world scenario of low-dose exposures to multiple chemicals. Since a
34 growing body of evidence now suggests that simultaneous exposure to many chemicals at doses not
35 singularly causing any effects can add up to induce adverse outcomes [1,2], the current regulatory
36 approaches are inadequate. Due to the complexity of the issue, the implementation of methodologies for risk
37 assessment of chemical cocktails remains a challenge, hindering the urgent need to improve chemical risk
38 assessments. Here, we present a pragmatic step-by-step procedure for mixture risk assessment (MRA) and
39 propose approaches for grouping of chemicals.

40

41 **2. Current status of mixture risk assessment**

42 2.1 Additive mixture effects

43 Experiments have shown that chemicals, when exerting similar effects, typically act in a dose-additive
44 manner, and far less frequently in synergistic or antagonistic ways [3,4]. This means that the efficacies of
45 chemicals in a mixture can be added together, as long as the potency of the individual chemicals is
46 appropriately accounted for. As dose-response curves are usually sigmoidal, the combination of many
47 chemicals at low levels (located at the lowest horizontal part of the dose-response curve) can give rise to a
48 pronounced adverse mixture effect (corresponding to a move up to the steeper part of the dose-response
49 curve). This phenomenon has been observed in cellular systems [5] and animal studies [6-8].

50

51 Synergistic or antagonistic interactions are well-known occurrences in drug therapy, where it is common to
52 see drugs either enhancing or inhibiting each other's effect, primarily due to toxicokinetic mechanisms [9].
53 In contrast, kinetic interactions are much less likely with environmental chemicals, as human exposure levels
54 typically are much lower [10,11]. Thus, it seems reasonable to anticipate additive effects caused by real-life
55 exposures to environmental chemicals in most cases.

56

57 Over the last decade, public authorities such as the US-Environmental Protection Agency (US-EPA), the
58 Agency for Toxic Substances and Disease Registry (ATSDR), the World Health Organization (WHO), the
59 non-food Committees of the European Commission, and the European Food Safety Authority (EFSA) have
60 all made considerable progress towards developing pragmatic frameworks that are "fit for purpose" and
61 tiered, to deal with combined exposure to multiple chemicals for risk assessment purposes [12-16].

62

63 The assumption that chemicals act additively and behave as if they were a simple dilution of each other has
64 resulted in the development of methods for cumulative risk assessment using various approaches: Hazard
65 Index (HI), point of departure index (PODI), relative potency factors (RPF) and toxicity equivalency factors
66 (TEF). These relatively simple tools form a good basis for development of the future paradigm for MRA.

67 Currently, EFSA is compiling the available knowledge on methodologies used to perform mixture risk
68 assessments for human health, animal health and ecological mixture risk assessment [17]. They present a
69 tiered approach to a component based mixture risk assessment using principles of dose addition. HI
70 calculation is considered a first tier summing hazard quotients (HQ) for all components regardless of
71 toxicological endpoint. In the next tier, a refined HI approach takes into consideration that not all the
72 components have the same adverse effect/target organ. A target organ toxicity dose is derived for each end-
73 point to estimate an end-point-specific Hazard Index [17].

74

75 However, as we see data gaps on single chemicals as the major bottleneck for MRA, we suggest a more
76 specific workflow that allows the use of predicted or estimated hazard and exposure data for data-poor
77 chemicals. The philosophy behind this is that risk assessment of chemicals would be greatly improved by
78 taking mixture effects into account, even if great uncertainties are introduced for data-poor chemicals, and
79 that this is better than doing nothing.

80

81

82 2.2 Probabilistic models for exposure and hazard

83 Real-life human exposure is highly variable and temporally dynamic, both at the individual and population
84 levels. In spite of this, human exposures via the food is traditionally estimated using average intake levels of
85 the chemicals, which is a deterministic method that does not incorporate information about the variability of
86 real exposures. In order to reflect a more realistic exposure scenario, probabilistic exposure models are
87 currently being developed and used for MRA [3,18,19]. In the same way, new tools for probabilistic hazard
88 assessment are also being developed, which can be very useful for data-rich chemicals such as pesticides.
89 However, for the vast majority of environmental or industrial chemicals, there is a lack of toxicity and
90 exposure data; and since it is unrealistic to obtain ‘perfect data’ for most chemicals within the foreseeable
91 future, probabilistic models may not be the best way forward for a pragmatic MRA of real-world mixtures

within foreseeable future. This means that we currently are forced to perform MRA based on ‘average’ exposure and hazard data.

2.3 Bottlenecks for mixture risk assessment

One major challenge for MRA is that exposure to multiple chemicals currently is considered under different pieces of legislation, also known as regulatory silos, such as REACH or the EU pesticides Regulation [20,21]. Typically, humans are exposed to multiple chemicals from multiple sources, which can give rise to mixture effects, i.e. they can elicit similar effects or exhibit the same mode of action [22]. However, these chemicals may be risk assessed, risk managed or regulated by different authorities based on the legislations to which the chemicals belong. Thus, legal mandates for performing MRA across regulatory silos and legislations are needed, as are pragmatic MRAs allowing for cross-silos assessments.

Data gaps for individual chemicals seem to be a major issue when it comes to dealing with MRA, both regarding hazard and exposure data. In an attempt to address the lack of toxicity data, we suggest a pragmatic approach with a focus on how to derive information on hazards in cases where data are scarce and on methodologies for grouping of chemicals for MRA.

3. A pragmatic approach for mixture risk assessment

We have constructed a decision tree outlining a step-by-step procedure for MRA based on the use of Hazard Index (HI) (Fig. 1). This approach can be used to obtain a conservative (cautious) starting level for overall conclusions, and only progress to more refined methods including effect-based grouping of chemicals in cases where an initial concern for human toxicity is identified. This approach differs from previously suggested ‘tiered’ approaches [14,23], in which crude estimates with a high degree of caution are performed at early tiers in data poor situations, whereas higher tiers use more refined approaches with increasing data demand and higher certainty. However, since mixtures will usually contain both data-rich and data-poor

117 chemicals, we need to be able to derive a HI for mixtures of chemicals with information of various refined
118 levels.

119 Our suggestion for a pragmatic MRA approach is shown in Figure 1 and described below. The numbers refer
120 to the steps outlined in the figure.

121 Before starting the MRA, the challenge of the specific mixture is investigated and relevant questions relating
122 to for instance the exact composition of the mixture and the potential for concomitant human exposure is
123 addressed.

124

125 1. First, an evaluation of data availability is made for individual chemicals from the mixture and, if possible,
126 for the whole mixture, i.e., is relevant hazard information on the individual chemicals available (1A? If
127 possible, NOAELs or similar hazard measures from in vivo experiments are used to set a reference value
128 (RfV) for each chemical. If not possible, toxicity may be estimated by using relevant in vitro information in
129 combination with a human exposure level (see example in [24, this issue]). If no experimental data exists,
130 read-across data or quantitative QSAR data may be applied and toxicity values for similar chemicals can be
131 used as a surrogate. Next, do human exposure data exist (1B)? Do reliable human biomonitoring data exist?
132 Have the sources been identified? Can exposure via the food be estimated from contaminant data for specific
133 food items? Furthermore, it needs to be considered whether mean or median exposure values, or 95%
134 percentiles should be applied in the given situation.

135

136 2. Hazard quotients (HQs) are calculated as the ratio between exposure estimate and RfV for a given
137 substance based on the collected data in step 1. We suggest allowing the use of HQs calculated from relevant
138 in vitro data and for instance human biomonitoring data. An example of this can be found in Johansson et al.
139 (2019) in this issue. If the HQ is above or close to 1 for any single chemical, the decision to regulate is
140 handed over to the regulatory bodies. If all HQs are less than 1, proceed to step 3.

141

142 3. At this step, it is considered whether deviations from dose additivity can be expected. Are there reasons to
143 suspect that interaction between the chemicals will occur by toxicodynamic or toxicokinetic interactions?

144 Are CYP450 interactions likely at the present exposure levels e.g. due to CYP450 induction? If no deviations
145 from dose-addition are expected, proceed to step 4. However, if deviations can be expected, a case-by-case
146 MRA should be applied.

147

148 4. Assuming dose additivity, a calculation of a rough and non-refined HI is performed. At this step, all
149 chemicals are added in the same formula, irrespective of the type of toxicity they are causing. The rationale
150 for summing up all hazards irrespective of effects is based on considering the human body as one complex
151 organism, as well as the hypothesis that various toxicities are often interrelated. A major limitation of this
152 step is a high degree of inherent uncertainties when setting RfV and exposure estimates for each chemical in
153 the mixture. In addition, it is acknowledged that use of the dose-addition principle is not precise for chemical
154 mixtures for which different endpoints have been used for deriving RfVs. If the calculated HI exceeds 1, it
155 will be necessary to group the chemicals according to their specific toxicities (step 5). In cases where the HI
156 becomes less than 1, proceed to step 6.

157

158 5. For mixtures with a non-refined HI higher than 1, it is necessary to group the chemicals according to effect
159 or mode-/mechanism-of-action using available in vivo, in vitro or in silico data (see below). For each group
160 of chemicals, a more specific and refined HI is calculated. If any of these HI's exceeds 1, regulatory action
161 should be decided upon by the authorities. For those HI's that are less than 1, proceed to step 6.

162

163 6. For HIs less than 1, with or without grouping, it should be considered whether other sources of the same
164 chemicals exist or if other chemicals are present that are likely to contribute to the mixture effect. If so, it
165 should be accounted for as described in step 7. If not, no human risk is anticipated for the mixture.

166

167 7. If other chemicals or sources are expected to contribute to the mixture effect, the HI cut-off of 1 should be
168 adjusted to take this extra contribution (X) into account, i.e. to a cut-off of (1-X). For example, for a specific
169 mixture obtained via the diet, it may be estimated that humans are exposed to the same chemicals from other
170 sources such as via inhalation or via dermal absorption and that these would contribute with 30% of the
171 acceptable risk. Then, the cut-off for acceptable risk for the evaluated mixture is adjusted to 70% (0.7)
172 instead of 1. If the $HI < (1-X)$ where X designates the extra contribution, then no human risk is anticipated.
173 In this example, a HI below 70% would be deemed acceptable. If the calculated HI exceeds (1-X), it will be
174 necessary to group the chemicals according to their specific toxicities as described in step 5. Thus, using this
175 example a HI above 70% would lead to (re-) evaluation of the possibility of grouping mixture components
176 according to shared effects or modes of action (step 5). If any of the HI's exceeds the adjusted cut-off value
177 after grouping of the chemicals, regulatory action should be decided upon by the authorities. If all HI's are
178 still below the adjusted cut-off value after grouping, then no human risk is anticipated for the mixture.

179

180 **4. Grouping of chemicals**

181 An essential step in MRA is the grouping of chemicals according to their effects or mode/mechanism of
182 action (step 5) using relevant animal and non-animal data. A standardized methodology for grouping of
183 chemicals based on target organ toxicities has been proposed by Adams et al. [25]. This corresponds to the
184 second tier proposed for component based mixture risk assessment in EFSA's draft guidance document [17].
185 Furthermore, EFSA has presented cumulative assessment groups (CAGs) for pesticides with effects on either
186 the nervous system or the thyroid hormone system [26], and CAGs for several other target organs have been
187 proposed by Nielsen et al. 2012 [27]. This approach is limited to chemicals for which in vivo data exist. As
188 an alternative, grouping based on common modes of action may include grouping of compounds that act on
189 the same adverse outcome pathways (AOPs). An AOP describes a sequence of events from initial
190 interaction(s) of a stressor with a biomolecule (molecular initiating event), causing perturbations which can
191 progress through a series of intermediate key events and culminate in an adverse outcome [28]. In the future,
192 complex AOP networks may be a preferred methodology for grouping of chemicals, but requires the

development of a more densely populated AOP wiki (<https://aopwiki.org/>). Until such time, we suggest to use empirical in vivo and in vitro data, read-across as well as computational methods such as QSAR models or integrative systems biology. These latter non-test methods can be used to predict inherent hazards or modes/mechanisms of action and to group the chemicals in cases where no experimental data exist.

4.1 Grouping according to experimental data

Toxicological signatures (or profiles) of chemicals obtained from in vitro studies can be used for grouping. Profiling of chemicals across a panel of in vitro tests was previously applied to explore whether structurally related chemicals share the same mechanism of action. As shown in Fig. 2A, a range of bisphenols had a qualitative similar profile across a panel of assays for endocrine activity [29] and should therefore be grouped together, whereas a number of fluorinated chemicals should be divided into different subgroups based on their specific in vitro signatures [30].

Toxicological signatures based on omics data such as metabolomics, transcriptomics or proteomics can be established from either in vivo or in vitro studies. By using omics data, chemicals with specific modes/mechanisms of action can be identified based on the signature of affected metabolites, genes or proteins [31,32] and this can be used to decide on grouping of chemicals or not.

4.2 Grouping based on computational tools

Another grouping approach is the use of computer models known as QSARs (Quantitative Structure-Activity Relationships). QSARs estimate the relationship between the structure of chemical substances and another property such as the ability to cause toxic effects. QSAR models can therefore be used to predict hazards of chemicals with no or insufficient test data. Figure 2B depicts an example of a QSAR screening performed on 2,076 chemicals from food contact materials, including predictions for genotoxic carcinogenicity, mutagenicity, and developmental toxicity [33]. Detailed information on the applied decision-algorithms and the performance of the individual models has been described previously [34]. The figure shows the number of chemicals predicted to be active for each hazard as well as the overlap between the three hazards, which

219 provides a basis for grouping of the chemicals. The free Danish QSAR database can be searched for
220 estimates related to e.g. metabolism and toxicity for more than 600,000 chemicals (<http://qsar.food.dtu.dk/>).
221 This database is part of the OECD QSAR Toolbox, a software application which incorporates information
222 and tools from various sources (<https://www.qsartoolbox.org/home>).
223 ‘Read across’ is one of the most commonly used alternative approaches for filling data gaps in registrations
224 for REACH and entails the use of relevant information from analogous chemicals to predict for instance
225 hazards for the chemical in question [35,36]. Thus, chemicals whose toxicological properties are likely to be
226 similar or follow a regular pattern as a result of structural similarity may be considered a category [36]). The
227 read-across hypothesis needs to be justified by scientifically credible explanations and sufficient supporting
228 information such as information from QSARs or experimental data addressing specific aspects of the read-
229 across hypothesis [36]. Many tools for read-across assessment are available, and in this regard the OECD
230 Toolbox is most advanced in terms of addressing current regulatory needs [37].

231

232 4.3 Grouping based on a systems biology approach

233 A third approach for grouping chemicals is by use of systems biology approaches (Fig 2C). Advanced
234 bioinformatics tools can be used to gain mechanistic knowledge by taking advantage of already existing
235 information about associations between human targets and chemicals. For example, Kongsbak et al [38,39],
236 retrieved information for known chemical-protein associations from two publicly available databases, the
237 Comparative Toxicogenomics Database (CTD) [40] and ChemProt 2.0 [41]. This type of analysis gives an
238 overview of known and potential human targets for the chemicals of interest. If chemicals in a mixture then
239 turn out to target the same molecules or are significantly associated with the same disease, or affect the same
240 signaling pathway, it is likely that these chemicals can be allocated to the same group for MRA. In Figure
241 2C, it is illustrated how mancozeb can be excluded from the group, as this chemical belongs to its own
242 network.

243 In conclusion, there are several ways of grouping chemicals, which should be used and explored in more
244 detail for MRA of chemicals.

245

246 **5. Perspectives**

247 Although several proposals have been published for how to handle risk assessments of chemical mixtures, no
248 consensus has been reached. Given the complexity of MRA and the potential human health risk not
249 accounted for, we propose a pragmatic approach. On the one hand this approach will improve on chemical
250 risk assessment, but on the other require that we accept some uncertainties in the evaluations. The HI is not
251 necessarily an accurate indicator of risk as it is based on hazard and exposure values that are subject to
252 uncertainty. Nevertheless, it provides a low-input, straightforward risk assessment tool that is an
253 improvement compared to the alternative of performing single-chemical risk assessments.

254

255 An important limitation of current MRA is the lack of data on individual chemicals, both with regard to
256 human exposure and toxicity data. To date, only a minor fraction of the many thousands of chemicals in
257 current use have been evaluated and allocated an ADI, TDI, NOAEL, or similar. There is thus an urgent need
258 to address this lack of toxicity data and a way forward could be to develop alternative ways of risk assessing
259 chemicals based on for instance defined panels of in vitro models, or computational tools such as QSARs
260 and physiologically-based kinetic modelling.

261 Our proposal is designed to prevent bottlenecks such as lack of data on individual chemicals or complex real-
262 life exposures stopping us from performing a pragmatic MRA of chemicals.

263

264 **Conflicts of interest**

265 All authors declare no conflicts of interest.

266 **References**

267 Papers of particular interest have been highlighted as:

268 * of special interest

269 ** of outstanding interest

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Figure legends

Figure 1. The proposed decision tree based on the use of the hazard index. The step-by-step procedure is described in detail in the text.

Figure 2. Examples of approaches to group chemicals. A) Profiling of chemicals using defined panels of in vitro tests. As shown, a range of bisphenols had a qualitative similar profile in a panel of assays for endocrine activity, while fluorinated chemicals could be divided into different groups based on the in vitro signature [27,28]. B) Venn diagram showing the number of chemicals predicted by QSAR to be active for each hazard as well as the overlap between the three hazards. C) Chemical-protein association network of five chemicals and their associated proteins. The colors of the proteins/genes indicate the primary function of the encoded protein. As seen from the figure, mancozeb does not share any associations with the four other chemicals, which means that this compound can be excluded from the mixture in a grouping exercise [34].