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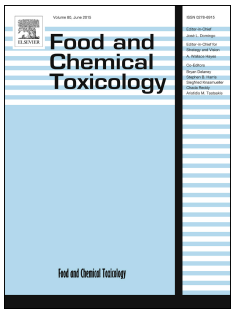
Published in:
Food and Chemical Toxicology

Link to article, DOI:
10.1016/j.fct.2021.112167

Publication date:
2021

Document Version
Peer reviewed version

Journal Pre-proof



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PII: S0278-6915(21)00200-3

DOI: https://doi.org/10.1016/j.fct.2021.112167

Reference: FCT 112167

To appear in: *Food and Chemical Toxicology*

Received Date: 10 November 2020

Revised Date: 26 March 2021

Accepted Date: 29 March 2021

Please cite this article as: Boberg, J., Bredsdorff, L., Petersen, A., Löbl, N., Jensen, B.H., Vinggaard, A.M., Nielsen, E., Chemical Mixture Calculator - A novel tool for mixture risk assessment, *Food and Chemical Toxicology*, https://doi.org/10.1016/j.fct.2021.112167.

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Nielsen E: Conceptualization; Investigation; Data curation; Methodology; Visualization; Writing - original draft; Writing - review & editing.

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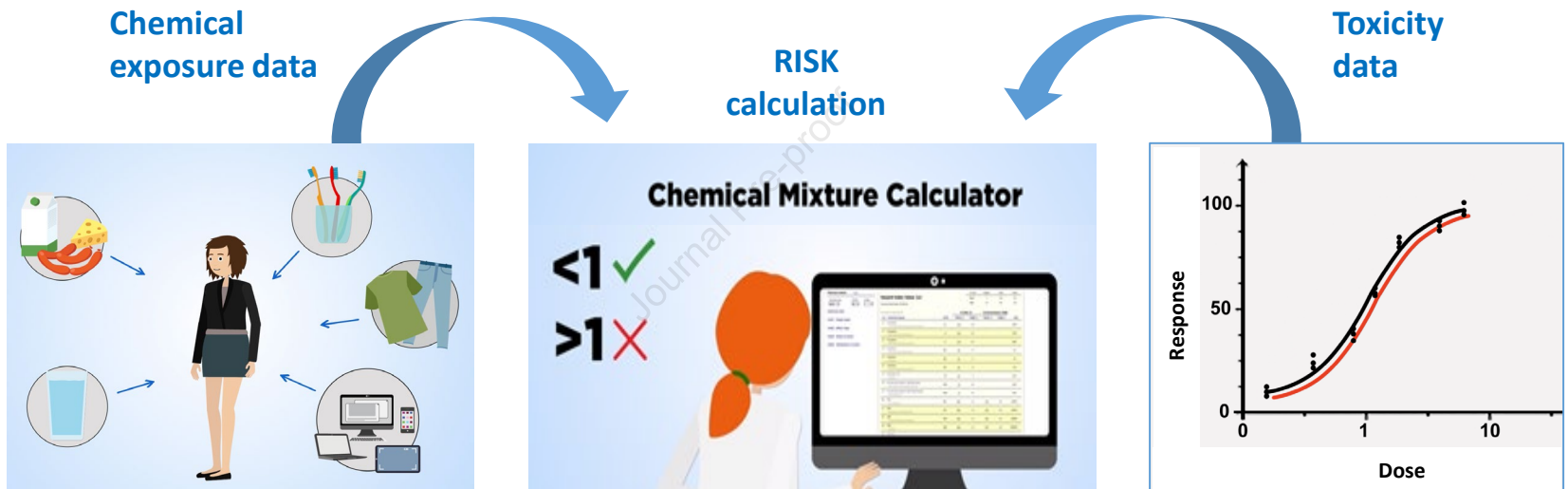
Abstract

Humans are continuously exposed to complex chemical mixtures from foods and the environment. Experimental models *in vivo* and *in vitro* have increased our knowledge on how we can predict mixture effects. To accommodate a need for tools for efficient mixture risk assessment across different chemical classes and exposure sources, we have developed fit-for-purpose criteria for grouping of chemicals and a web-based tool for mixture risk assessment. The Chemical Mixture Calculator can be used for mixture risk assessment or identification of main drivers of risk. The underlying database includes hazard and exposure estimates for more than 200 chemicals in foods and environment.

We present a range of cumulative assessment groups for effects on haematological system, kidney, liver, nervous system, developmental and reproductive system, and thyroid. These cumulative assessment groups are useful for grouping of chemicals at several levels of refinement depending on the question addressed. We present a mixture risk assessment case for phthalates, evaluated with and without contributions from other chemicals with similar effects. This case study shows the usefulness of the tool as a starting point for mixture risk assessment by the risk assessor, and emphasizes that solid scientific insight regarding underlying assumptions and uncertainties is crucial for result interpretation.



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Highlights (3-5 bullets, 85 characters)

- Chemical mixture risk assessment made possible using novel online tool
- Suggestions for grouping based on toxic effects on six target organs or systems
- Case: Mixture risk assessment for phthalates and chemicals with similar effects

1. Introduction

Humans are continuously exposed to complex chemical mixtures from foods and the environment. Current regulatory approaches for assessing chemicals typically evaluate one chemical at a time, an approach that fails to take into account the human real-world scenario of low-dose combined exposures to multiple chemicals. A growing body of evidence suggests that simultaneous exposure to many chemicals at doses not individually causing any effects can add up to induce adverse outcomes (Kortenkamp and Faust, 2018). Over the last decade, authorities and scientific bodies have all made considerable progress towards developing pragmatic frameworks that are “fit for purpose” and tiered to deal with combined exposure to multiple chemicals for risk assessment purposes (EFSA 2013a, EFSA 2019a, Joint Research Centre 2018, ECHA 2017, US EPA 2008, WHO/IPCS 2009). Experimental models in vivo and in vitro have provided us with extensive knowledge on how chemicals interact or cause combination effects, and how we can predict mixture effects. The assumption that chemicals act additively and behave as if they were a simple dilution of each other has resulted in the development of methods for mixture risk assessment using various approaches (EFSA, 2019a; Boberg et al., 2019). With these principles of risk assessment at hand, there is now a need for tools for efficiently performing mixture risk assessment using the available data. Importantly, mixture risk assessment should be carried out across various chemical classes, regulatory sectors and exposure sources as demonstrated in several cases (ECHA 2016; Andersen et al., 2012; Larsen et al., 2017, Adams et al. 2017, Vejdovszky et al. 2019, Bopp et al. 2019). So far, however, pragmatic tools for mixture risk assessment across chemical classes are few and some require detailed exposure information, thus limiting the number of substances evaluated (Boon et al. 2015; Sprong et al. 2020). A simple and more pragmatic tool would be useful for low-tier mixture risk assessment.

We have developed fit-for-purpose criteria for grouping of chemicals and a publicly available web-based tool for mixture risk assessment. Grouping of chemicals is based on similar effects in a specific target

organ or system, as experimental evidence shows that dose-additive effects are often observed following exposure to chemicals exerting the same type of effect, even when they act via different modes and mechanisms of action (Christiansen et al., 2020; Christiansen et al., 2009; Conley et al., 2018). This approach has the advantage that mixture risk assessment can be performed based on available data and that data on mode of action are not required at first tiers (Nielsen et al., 2012).

If mode of action data are available, refined grouping can be made at a next tier. Here, toxicological data for health effects of chemicals are applied for grouping of substances into cumulative assessment groups (CAGs) at different levels as presented by Nielsen et al. (2012) and EFSA (2013b). This approach is in line with a recently published EFSA guidance document (EFSA, 2019a). EFSA proposed a tiered approach for component-based mixture risk assessment, in which the first tier is mixture risk assessment of all components regardless of toxicological endpoint. At the next tier, grouping is based on specific effects, and a target organ toxicity dose is derived for each endpoint (EFSA, 2019a, Vejdovszky et al. 2019). Likewise, ECHA has presented a tiered approach in their guidance document for mixture risk assessment of active substances in biocidal products, where the lowest tiers involve mixture risk assessment without consideration of target organs or systems, while higher tiers consider target organ specific effects and mode of action for subgrouping (ECHA, 2017).

The presented tool includes a database on chronic exposure and toxicity data for a number of chemicals present in food and/or the environment such as e.g. environmental and process contaminants, food additives, and selected groups of pesticides. Flexibility of the tool ensures that the user can include their own exposure data, if for example a national exposure data set is available, or a specific mixture is investigated. In addition, exposure data from probabilistic modelling can be included, if available. A publicly available user interface is presented here for mixture risk assessment and includes a step-by-step guide for the risk assessor. In order to illustrate the use of this tool, we present a mixture risk assessment case for a group of phthalates, evaluated with and without contributions from other chemicals exerting similar effects. This example was selected as humans are exposed to the same phthalates from various sources and because of their potential to affect several organs and systems. Furthermore, several researchers as well as EFSA and ECHA (EFSA 2019d, ECHA 2016, Christensen et al. 2014;

Kortenkamp and Koch, 2020) have recently carried out mixture risk assessment of phthalates, and our results are discussed and compared to these evaluations.

2. Methodology

The web-based tool, which is designated the “Chemical Mixture Calculator” facilitates a tiered approach for performing mixture risk assessment for effects on six different target organs / systems. The tool includes a database on chronic dietary and non-dietary exposure estimates and toxicity data for more than 200 chemicals present in food and/or the environment such as environmental and process contaminants, food additives, contaminants migrating from food packaging materials and selected groups of pesticides. As a first tier, it is possible to perform mixture risk assessment without consideration of different effect types. At following tiers, grouping of chemicals is based on similar target organs / systems, similar effects in a specific target organ or system, or similar mode or mechanism of action. The risk assessors can choose to use all chemicals included in the Chemical Mixture Calculator for their mixture risk assessment, or to select a subset of chemicals, e.g. a chemical class or any other group of chemicals identified as relevant with respect to co-exposure.

The Chemical Mixture Calculator is accessible through [Insert link for webpage] and a user manual is included in supplementary materials.

2.1 Principles of mixture risk assessment

The Chemical Mixture Calculator applies principles of dose-addition in a tiered approach for mixture risk assessment, which is the recommended model for component-based approaches by EFSA (EFSA 2019a). The equation below (Eq. 1) is applied to derive the hazard index (HI) for a mixture (EFSA 2019a, US EPA 2008; Reffstrup et al., 2010, Kortenkamp and Koch, 2020). For each component of the mixture, the ratio between an exposure estimate and a health-based guidance value (HBGV) is established for each chemical and designated the hazard quotient (HQ). The HI is the sum of the HQs of several

104 compounds, and if a HI is above 1, this indicates that a potential risk is identified using the applied data.

105 In case of conservative assumptions on e.g. exposure, further refinement can be considered.

reference doses have been applied in setting the DTDs (ECHA 2012, EFSA 2012, WHO/IPCS 2009). Toxicity data were mainly collected from EFSA and ECHA scientific opinions, which contain the most detailed information on effects seen at the lowest dose level, i.e. the critical effects. Further data were therefore scrutinized to identify effects observed at higher dose levels for the selected target organs / systems (see Supplementary file S1 regarding data collection principles). Each compound can thus have several DTDs, as a specific DTD is designated for each CAG to which the compound is allocated. At CAG level 2, the DTD corresponds to the term Target organ toxicity dose (TTD) applied by EFSA and others (EFSA 2013a, EFSA 2019a, Adams et al. 2017).

For a specific CAG, a HI can be determined using the different DTDs for this CAG.

class or any other group of chemicals identified as relevant with respect to co-exposure. Abbreviations: ADI: acceptable daily intake, CAG: cumulative assessment group, DTD: derived tolerable dose, HI: hazard index, HQ: hazard quotient, MRA: mixture risk assessment, TDI: tolerable daily intake.

2.2 Establishing cumulative assessment groups

To enable the grouping of compounds, we selected six main target organs / systems: haematological system, kidney, liver, nervous system, developmental and reproductive system, and thyroid gland. Table 2 presents the main CAGs within each of these target organs / systems, including a description of the specific effects within each organ/system. Overall, the CAGs reflect the grouping previously described by Nielsen et al. (2012), although in some cases less refined CAGs were established here by grouping different types of effects, as described below.

For each target organ / system (CAG 1), it can be relevant to group based on specific effects, and for this purpose CAGs at level 2 (specific effects) are defined. To give an example, the CAG “Haematological system” at level 1 is divided into three CAGs for specific effects “Anaemia”, “Thrombocytosis”, and “Thrombocytopenia” at level 2 (Table 2). Likewise, several CAGs at level 3 and at level 4 may be relevant, as a specific effect can be induced by different modes or mechanisms of actions for the respective substances. This means that the CAG at level 2 “Anaemia” can be further refined to the CAG “Haemolysis” at level 3 (Table 2). Other modes or mechanisms may be identified in the future when sufficient knowledge becomes available.

In some CAGs, effects are included that may not be considered adverse in traditional hazard assessment of single compounds. For example, an increase in plasma cholesterol under a specified level is often considered non-adverse, but in mixture risk assessment such an effect may become relevant, as the accumulated increase caused by several substances each contributing with a small increase may result in exceedance of the specified level. Likewise, some CAGs include effects that may be considered adaptive in traditional hazard assessment of single compounds. In toxicological experiments, an effect is considered adaptive if the effect is observed after a relatively short time of exposure, but vanishes after prolonged exposure. However, the organism clearly uses some of its “reserve capacity” to handle this single substance. As we cannot rule out that an organism will be unable to adapt to several substances simultaneously, we have included such effects (e.g. changes in red blood cell parameters) even if the effect seems to vanish after prolonged exposure.

A number of potential CAGs were not included for various reasons. Some effects may be considered adverse and relevant for human risk assessment, but are not considered relevant for inclusion in a CAG. Examples include non-specific or indirect effects that can occur either secondary to a specific (direct) effect, or as a consequence of high, massive (unrealistic) exposure to a substance. For some target organs / systems (e.g. for adrenal gland, bone marrow, spleen and urinary bladder), effects are generally considered as unspecific or it is not clear whether the identified effects are direct or indirect (Nielsen et al., 2012). For other target organs / systems (e.g. for bones/skeleton, cardiovascular system, gallbladder, muscles, parathyroid gland) the contribution to the mixture risk is considered to be minor, as we only identified such effects for a few pesticides in Nielsen et al. (2012). For the eye, effects seen for pesticides were generally considered to be age-related changes that may be incidental and not well understood, and therefore the relevance for mixture risk assessment is unknown (Nielsen et al., 2012).

If in the future further information becomes available for organs / tissues not included in the current version of Chemical Mixture Calculator that allows grouping, the tool can be updated to also include CAGs for these organs / tissues.

2.3 Data collection and tool

The Chemical Mixture Calculator is available online and a password can be obtained on request (see contact information for corresponding author of this paper). The user interface is designed to calculate mixture effects of chemicals at various levels of detail and based on the assumption that chemicals are acting additively. The Chemical Mixture Calculator enables grouping of chemicals into different CAGs by filtering functions. Additionally, exposure data can be filtered to focus on specific age groups. In practice, drop-down menus are applied to select a) a specific age group (toddler, child or adult) and b) a specific CAG. This filtering of data enables HI calculations for each CAG, and results are presented as a table showing HI values calculated for mean as well as high exposure values for each selected age group.

For a number of chemicals present in food and/or the environment we have collected data on 1) dietary and non-dietary exposure, and 2) toxicity including information applicable for grouping purposes. The tool includes an option to transfer output data to Excel for further processing, e.g. substitution of the available exposure data with own exposure data. This way, the primary use of the tool would be the grouping of chemicals, whereas HI calculations can be made in the resulting Excel sheet.

In brief, the chemical exposure estimates for children and adults was mainly based on Danish data pertaining to content in food, and consumption data from the Danish Dietary Survey (Petersen et al., 2013; Jensen et. al, 2019). For chemicals for which Danish dietary data were not available, exposure data were collected from published reports and risk assessments from e.g. EFSA and ECHA. Non-dietary exposure data were also collected from published reports. When possible, a mean and a high value for each chemical were selected for three specific age groups: toddlers, children and adults. High values were generally defined as 95^{th} percentiles or the 97.5^{th} percentile, if 95^{th} percentiles were not available. Further details on exposure data collection are found in Supplementary material S1 and S2.

As noted in the introduction, the user can choose to include own exposure data, which could be derived from a national exposure data set or from a specific mixture.

Toxicity data were mainly collected from scientific opinions and reports published by EFSA and ECHA. In order to enable first-tier mixture risk assessment, HBGVs (mainly TDIs and ADIs) were listed when available. To enable refined mixture risk assessment, each compound was allocated to specific CAGs, and a DTD (defined as in section 2.2) applicable for risk assessment was derived for each CAG. Further details on collection of toxicity data are found in Supplementary material S1 and S3. Relevant target populations were indicated for each DTD. For example, the CAG “prenatal death” is relevant for pregnant women, but not for children or toddlers. This means that no HI values are calculated for children and toddlers, when this CAG is selected. In other cases such as for substances that accumulate in the kidney (e.g. cadmium) the effect is relevant for adults only, as long-term exposure is needed for the effects to arise and thus they do not occur in toddlers or children.

2.4 Use of the tool: Case study on mixture risk assessment of phthalates

To evaluate the use of the Chemical Mixture Calculator, we focused on a specific group of chemicals, the phthalates, which are found in food, dust and various consumer products. While previous mixture risk assessments of phthalates have focused on this chemical class alone, we have performed a mixture risk assessment that included not only the phthalates, but also other substances that exert similar specific effects in different organs / tissues. In addition, the use of the Chemical Mixture Calculator enabled mixture risk assessment at different levels of refinement.

First, grouping of the six phthalates for which toxicity and exposure information was available (DEHP, DBP, DIBP, BBP, DINP, DIDP) was performed at four levels of refinement. Then the same procedure was carried out for all chemicals included in the Chemical Mixture Calculator:

i) using lowest available DTD (including ADI/TDI if these HBGVs provide the lowest reference point) for HI calculation,
ii) subgrouping into phthalate-relevant CAGs at level 1, i.e. haematological system, kidney, liver, developmental and reproductive system, thyroid,
iii) subgrouping into phthalate-relevant CAGs at level 2, i.e. specific effects, see Table 3 (phthalates) and Table 4 (all chemicals),
iv) subgrouping into phthalate-relevant CAGs at level 3, i.e. mode of action, see Table 3 (phthalates) and Table 4 (all chemicals).

In practice, grouping of chemicals into these five levels was selected by using the filtering functions in the Chemical Mixture Calculator. By filtering for each specific CAG (Groups ii-iv), the number of compounds was reduced to substances allocated to that specific CAG. For each CAG, HIs were calculated specifically for toddlers, children and adults at mean and high exposure values.

3. Results and discussion

We have developed fit-for-purpose criteria for grouping of chemicals and a web-based tool for mixture risk assessment, the Chemical Mixture Calculator. To illustrate the use of the Chemical Mixture Calculator, we present a mixture risk assessment for a group of phthalates, evaluated with and without contributions of other chemicals that exert similar specific effects in different organs / tissues.

3.1 Cumulative assessment groups

The ability to perform mixture risk assessment using existing data on numerous substances is an important step in meeting future requirements of performing mixture risk assessment across different chemical classes, different target organs / systems and exposure sources. We propose here CAGs for six different target organs / systems: haematological system, kidney, liver, nervous system, developmental and reproductive system, and thyroid (Table 2).

The CAGs at level 2 are very diverse, some very broad and others very narrow. In some cases, CAGs are broader than previously proposed (Nielsen et al., 2012). One reason is that we now acknowledge that some effect types might be correlated, i.e. one type of effect may be secondary to another direct effect in the same target organ / system or in a different target organ / system. Another reason is that the CAGs proposed by Nielsen et al. (2012) were based on pesticide active substances only for which the data package generally is more comprehensive compared to many other substances included in the Chemical Mixture Calculator.

Grouping at CAG level 2 (specific effect in a target organ / system) may be considered conservative in cases when substances in the same CAG at level 2 do not present additive effects. On the other hand, there may also be cases of synergism where this approach may underestimate the risk. In a systematic review, Martin et al. (2021) confirmed the utility of default application of the dose addition concept for predictive assessments of simultaneous exposures to multiple chemical substances, unless there is specific evidence that interactions might be relevant.

Below, the CAGs at level 2 to 4 for each target organ / system are discussed in relation to the CAG proposals by Nielsen et al. (2012), EFSA (EFSA 2019b, EFSA 2019c) and Foster et al. (2020).

3.1.1 Thyroid

Thyroid toxicity includes effects on follicular cells and parafollicular cells.

Regarding toxicity to follicular cells, various effect patterns may be correlated, and therefore we have identified a combined CAG at level 2 and 3 “Effects on follicular cells and/or the thyroid hormone system“. This CAG builds on a combined mode of action model for specific effects on the follicular cells and/or on the thyroid hormone levels, although effects on the thyroid hormone system could be considered as a mode of action rather than a specific effect (Nielsen et al., 2012). The same underlying principles were applied by EFSA in relation to the CAG at level 2 “Hypothyroidism” (EFSA 2019c). EFSA defined hypothyroidism as an altered function of the thyroid gland resulting in follicular cell hypertrophy, hyperplasia and neoplasia. Furthermore, EFSA noted that when low thyroid hormone levels lead to increased thyroid stimulating hormone levels as a compensatory response of the hypothalamic-pituitary-thyroid axis, this condition is also referred to as ‘Hypothyroidism’, i.e. as a specific effect and not a mode of action. In agreement with EFSA, we include substances in this CAG, which affect the thyroid hormone system indirectly via effects on the liver, i.e. increased elimination of thyroid hormones that could be considered a mode of action rather than a specific effect. We also propose subgrouping at CAG level 4 (mechanism of action) for effects on follicular cells refinement (Table 2).

Our CAG at level 2 “Effects on parafollicular cells and/or calcitonin system” is broader than EFSA’s CAG at level 2 “C-cell hypertrophy, hyperplasia and neoplasia” as our CAG at level 2 also includes C-cell atrophy, necrosis and cell death, as well as changes in serum calcitonin concentrations. This decision was based on the assumption that various effect patterns may be correlated in the parafollicular cells, in consistency with the approach made for follicular cells.

3.1.2 Nervous system

We have identified five different CAGs at level 2 for the nervous system (Table 2). Three of the CAGs at level 2 “Functional alteration related to the motor division of the nervous system”, “Functional alteration related to the sensory division of the nervous system”, and “Effects on cognition” are in accordance with the three CAGs at level 2 recommended for the nervous system in Nielsen et al. (2012). The first two of our CAGs at level 2 are also in accordance with two CAGs at level 2 presented by EFSA “Functional alteration of the motor division” and “Functional alteration of the sensory division” (EFSA 2019b). Cognitive effects were not addressed by EFSA (2019b), as EFSA considered the information insufficient to address the combined effects of pesticides.

Our CAG 2 “Histopathological changes” corresponds in some respects to the CAG at level 2 “Neuropathological effects” presented by EFSA (2019b). Two of the three indicators identified by EFSA for this CAG (myelin degeneration and neuronal degeneration/necrosis) are similar to the two CAGs at level 3 (“Demyelination” and “Neuronal degeneration”) identified in the current project as well as in Nielsen et al. (2012). Nielsen et al. (2012) did not define a CAG at level 2 for histopathological changes.

Developmental neurotoxicity was not addressed by EFSA (2019b) as EFSA considered the information insufficient to address the combined effects of pesticides.

In contrast to the current project, EFSA has identified a specific CAG at level 2 “Functional alteration of the autonomic division” (EFSA 2019b). EFSA identified six indicators for functional alteration of the autonomic division, i.e. miosis, mydriasis, increased salivation, lacrimation, piloerection, and urination. Such acute clinical signs were not considered of relevance for mixture risk assessment of chronic exposure in Nielsen et al. (2012) and also not in the current project. A CAG at level 2 “Functional alteration of the autonomic division” could be considered relevant if the Chemical Mixture Calculator is updated to include data for mixture risk assessment of acute exposure.

EFSA (2019b) has also identified a specific CAG at level 2 “Brain and/or erythrocyte acetylcholinesterase inhibition“ as being relevant for combined effects of pesticides when the inhibition leads to a statistically significant decrease of the AChE activity of 20% or more compared to concurrent control groups. In accordance with Nielsen et al. (2012), we consider such effects to be evidence of a specific mechanism

of action, and we therefore defined “Acetylcholinesterase inhibition” as a CAG at level 4 for the specific CAG at level 2 “Functional alteration related to the motor division of the nervous system“.

In addition to the two CAGs at level 3 mentioned above we have defined three additional CAGs at level 3, as well as three CAGs at level 4, see Table 2. These CAGs were also recommended for the nervous system in Nielsen et al. (2012). Additional CAGs at level 3 and at level 4 in Nielsen et al. (2012) were not considered in the current project, but could be relevant to include in the future if more data become available for the substances included in the Chemical Mixture Calculator.

3.1.3 Liver

We have identified five different CAGs at level 2 for the liver (Table 2). This is different from the approach taken in Nielsen et al. (2012) where 11 CAGs at level 2 were recommended for the liver. We have combined previously suggested specific CAGs at level 2 as detailed information necessary for more specific CAGs was not available for most of the substances included in the Chemical Mixture Calculator. To give an example, the current CAG 2 “Hepatocellular degeneration” also covers the previously recommended CAGs at level 2 “Inflammation” and “Cholestasis”.

EFSA has not developed CAGs for the liver so far. However, an EFSA supporting publication published by RIVM, IPCS and ANSES (2016) identified 15 CAGs at level 2 for the liver, including the 11 CAGs at level 2 originally identified in Nielsen et al. (2012).

Foster et al. (2020) critically assessed the basis for deriving the liver CAGs at level 2 in the report by RIVM, IPCS and ANSES (2016) and proposed a subcategorization and simplification to more accurately reflect the known pathophysiology of the toxic lesions to the liver. The authors suggested six CAGs at level 2 based upon primary hepatic pathology and relevance, i.e. “Hepatocellular necrosis”, ”Hepatic hypertrophy”, ”Cholestasis”, ”Porphyrin pigmentation”, ”Fatty change” and ”Hepatocellular neoplasms” (Foster et al. 2020). These six CAGs were all among the recommended 11 CAGs at level 2 in Nielsen et al. (2012) and three of them are similar to those proposed here (“Hepatocellular necrosis”, “Hepatic hypertrophy” and “Fatty change”). The three other CAGs at level 2 suggested by Foster et al. are either covered here by another CAG at level 2 (“Cholestasis”) or not included because sufficient information

were not available for most of the substances included in the Chemical Mixture Calculator ("Porphyrin pigmentation" and "Hepatocellular neoplasms").

In the current project, we have identified one CAG at level 3 for the liver and six CAGs at level 4 related to this CAG 3. Additional CAGs at level 3 and at level 4 were also recommended in Nielsen et al. (2012), but not addressed here because sufficient information was not available for most of the substances included in the Chemical Mixture Calculator.

3.1.4 Kidney

Neither EFSA nor other bodies have developed CAGs for the kidney so far. We suggest three different CAGs at level 2 for the kidney and one CAG at level 3 (Table 2). The CAGs at level 2 are in accordance with those recommended for the kidney by Nielsen et al. (2012). The CAG at level 3 ("Calculi / Crystals") identified here is the result of merging two different CAGs at level 3 ("Calculi" and "Crystals") originally identified by Nielsen et al. (2012) for the specific effects at CAG level 2 'Pelvic hyperplasia'. As a conservative approach these two CAGs at level 3 were not recommended for cumulative risk assessment by Nielsen et al. (2012) because calculi and/or crystals are not necessarily the cause of pelvic hyperplasia.

3.1.5 Haematological system

Neither EFSA nor other bodies have developed CAGs for the haematological system so far. We suggest three different CAGs at level 2 for the haematological system and one CAG at level 3 (Table 2). This is in accordance with the CAGs recommended for the haematological system in Nielsen et al. (2012).

3.1.6 Developmental and reproductive system

We have identified seven different CAGs at level 2 for the developmental and reproductive system (Table 2). This is a simplified and conservative approach compared to the approach presented in Nielsen et al. (2012). Some CAGs at level 2 for the developmental and reproductive system are very broad, reflecting many different effects that may be related to similar toxic mechanisms or may lead to common effect patterns. For example, effects on reproductive organs of male and female offspring may be induced by

various endocrine disrupting modes of action and induce different effect patterns (Christiansen et al., 2020, Conley et al., 2018). However, only a limited number of endocrine sensitive targets have been investigated for each of the evaluated substances. Therefore, we consider all effects in reproductive organs of male and female offspring, respectively, to be potentially related and potentially able to induce cumulative effects, regardless of the mode of action of the evaluated compound. This is a conservative choice considered relevant for grouping at CAG level 2. Further refinement with regard to mode of action and mechanism of action will be possible at CAG level 3 and 4, respectively (Table 2).

Other CAGs at level 2 comprise toxicity endpoints that are expected not to induce combination effects. An example is the CAG 2 “Malformations and variations”, which comprises a number of adverse effects with complex modes of action that may not lead to combination effects. Using such a CAG for mixture risk assessment can be considered a conservative choice that is useful as an early tier, but will need further refinement using subgroups that were not defined in this project. Such subgroups at CAG level 2 reflecting specific skeletal and visceral malformations and variations were proposed by Nielsen et al., 2012.

There may be cases when different CAGs at level 2 may need to be combined for mixture risk assessment. To give an example, it is not always possible to determine whether an observed effect is a sign of impaired fertility or developmental toxicity or both. One such example is decreased litter size observed in two-generation animal studies as this may be due to e.g. decreased fertilization (fertility effect) or foetal death (developmental toxicity). Another example is perinatal mortality, which may be due to impaired parturition in the dams (fertility effect) or effects on the foetus/new born offspring (developmental toxicity). In those cases, combining two related CAGs at level 2 may be relevant, for example combining the CAG for “Fertility and/or reproductive organs of males/females” with the CAG for “Prenatal death” or “Postnatal death”.

3.2 Data collection and uncertainties

The data in the Chemical Mixture Calculator on exposure and toxicity build on information in scientific opinions and reports on chemical risk assessment published by national and international bodies. The

information on exposure as well as on toxicity is associated with some uncertainty, as is always the case in risk assessment of individual chemicals, and in mixture risk assessment such uncertainties may be amplified.

Here, exposure data mainly originate from Danish data pertaining to content in food and consumption data from the Danish Dietary Survey (Pedersen et al., 2015, Petersen et al., 2013; Jensen et al., 2019). In some cases, exposure data were collected from published reports from e.g. EFSA and ECHA. We decided to include only one “mean/median” and one “high” exposure value per substance and per age group, whereas other current mixture risk assessment initiatives aim to include more refined exposure assessment by using e.g. probabilistic methods (Sprong et al., 2020, Crépet et al. 2019, EFSA 2020a, 2020b). Such valuable attempts are currently focused on pesticides, as detailed exposure data are scarce for many other chemicals including environmental contaminants, mycotoxins and process contaminants. Thus, our approach using available data is considered relevant despite uncertainties.

Toxicity data were mainly collected from scientific opinions and reports published by EFSA and ECHA. In order to enable refined mixture risk assessment, each compound was allocated to specific CAGs, and a DTD (defined in section 2.2) applicable for risk assessment was derived for each CAG. The decision to allocate a substance into a specific CAG is primarily dependent on the interpretation of the descriptions and evaluations of the toxicological studies presented in the individual opinions and reports. There are several causes of uncertainty: 1) The inherent degree of uncertainties in the measure of effects in toxicological studies. In many cases, specific endpoints were observed in only one or a few studies, and/or the findings were not consistent across studies, sex and/or species; 2) The inconsistencies between descriptions and interpretations of the same toxicological effect in the individual opinions and reports; 3) A general uncertainty regarding species differences between experimental animals and humans.

There are also uncertainties related to grouping principles. As stated by EFSA (2013b), grouping based on effect rather than mode of action will lead to more uncertainties in whether the compounds indeed induce a common effect, and using effect-based grouping can be considered a conservative approach. On the other hand, there will also be an uncertainty if substances with limited information on mode of

action are excluded. Generally, the current approach is considered useful for enabling mixture risk assessment of multiple chemicals based on the available information, particularly if combined with an evaluation of the size and direction of related uncertainties (over- or underestimation of risk). Recently, EFSA has provided substantial guidance for analysing uncertainty in risk assessments (EFSA 2018) as well as for specific cases of cumulative risk assessment of pesticides, presenting an extensive quantitative analysis of the uncertainties related to cumulative risk assessment for pesticides having chronic effects on the thyroid (EFSA 2020a) and acute effects on the nervous system (EFSA 2020b). These principles could be applied by the risk assessor as a final step following the use of the Chemical Mixture Calculator.

3.3 Case study on mixture risk assessment of phthalates

Using the Chemical Mixture Calculator we performed a mixture risk assessment for a group of phthalates with and without contributions of other chemicals with similar effects. The Chemical Mixture Calculator presents a range of HIs for phthalates only (Table 3) and for phthalates and other chemicals (Table 4) at different levels of refinement of mixture risk assessment. Both mean and high exposure values are included.

3.3.1 Choice of level of refinement

When looking at phthalate data only (Table 3), the first row (A) shows that without any grouping, the HI exceeds 1 for toddlers with high exposures only, whereas all other HIs are below 1. The HI above 1 indicates a possible risk for highly exposed toddlers, while HI below 1 indicates a low risk for all other population groups. The following rows show that grouping based on effects on similar organ system (CAG level 1) or similar specific effect (CAG level 2) reduces the HI as expected. For toddlers with high exposures, HIs are still above 1 for some target organ systems even with refinement of mixture risk assessment. Table 3 allows comparison of HIs for different effect types. For the liver and the developmental and reproductive system, the HIs are still above 1 for highly exposed toddlers and for some effects even when refining mixture risk assessment at CAG level 2 and 3 (Row F-M). This indicates a possible risk at high exposure levels. An overestimation of the risk may occur as exposures are not

necessarily correlated and only a few individuals will be exposed to such high levels of all included substances. The risk of effects on the haematological system, kidney and thyroid can be considered low, as HIs are below 0.1 for most population groups even at CAG level 1, when considering phthalate exposure only (Row B-D, E-F and O-P).

3.3.2 Including other substances

When evaluating HIs for all chemicals including phthalates (Table 4), it is clear that there are large differences in HI when going from the ungrouped mixture risk assessment (Row A) to grouping based on target organ systems or specific effects. Clearly, the number of substances in each group also declines with subgrouping. HIs above 1 are seen for certain subgroups even at CAG level 2 indicating a possible risk of exposure to these substances.

When comparing HIs for phthalates only with HIs for all substances (comparing Table 3 with Table 4) it is clear that the phthalates contribute only to a minor degree to the total HIs for some target organ systems. Specifically, phthalates do not contribute markedly to effects on the haematological system or thyroid (HIs in rows B-D and O-P are lower in Table 3 than in Table 4). For kidney, the phthalates contribute markedly to the total HI for toddlers and children, but not for adults (HIs in rows E-F of Table 3 and 4). This difference between age groups occurs because there are more substances included in the HI calculation for adults than for toddlers and children. Other substances, particularly metals, contribute markedly to the HI for adults (Table 5).

Interestingly, for the developmental and reproductive system, phthalates are not main contributors to HIs (lower HIs in rows J-N of Table 3 than J-N of Table 4). Sorting HQ values for each chemical in the Chemical Mixture Calculator can clarify which single chemicals or chemical groups that contribute most to the HI. Such an evaluation shows that exposure to dioxins and dioxin-like PCBs, nickel, lead, perfluorooctanesulfonic acid, bisphenol A, aluminium and perfluorooctanoic acid also contribute markedly to the overall HI for the developmental and reproductive system, even at CAG level 2 (Table 5). At CAG level 3, only phthalates and dioxins/dioxin-like PCBs are currently included in the CAG “Anti-androgenic mode of action” (row N of Table 3 and 4), but several other substances could be included (Kortenkamp

2020; Larsen et al., 2017). Overall, we conclude that it makes a considerable difference whether risks for health effects are evaluated for phthalates alone or in conjunction with the exposure from other chemicals with similar effects.

This illustration of the use of the Chemical Mixture Calculator shows that the selection of CAG level, i.e. refinement of mixture risk assessment, makes a large difference in the outcome of the assessment and therefore expert judgement is necessary. It is particularly important to note that going to a higher CAG level, e.g. from CAG at level 2 to CAG at level 3 and 4 means that substances for which mode/mechanism of action information is missing are omitted from the mixture risk assessment, thereby resulting in an underestimation of the combined risk. Therefore, we propose to select CAG at level 2 for mixture risk assessment. However, subgrouping at CAG level 3 is recommended, if specific mode/mechanism of action data are present for all substances to be included in the mixture risk assessment.

3.3.3 Influence of input variables

Several recent publications present mixture risk assessments for the phthalates in this case using different reference doses, mainly based on male reproductive toxicity (Table 6). The same data on exposure and toxicity were used in the current study and by ECHA (2016) (Table 3). In a recent risk assessment, EFSA applied a group-TDI set for DEHP to compare with potency-adjusted exposure estimates for DBP, BBP, DEHP, and DINP (EFSA 2019d). The toxicity data used for determining potency are more or less the same in the EFSA assessment as in our assessment (Table 3). Kortenkamp and Koch (2020) have suggested new reference doses for phthalates based on endpoints relevant to the phthalate syndrome assessed in rats. The phthalate syndrome here describes the collective pattern of effects of phthalates on the male reproductive system, and in practice is a part of our CAG for “Anti-androgenic activity”. For BBP, DEHP and DINP their suggested reference doses are 5 to 50 times lower than those defined as DTD for effects on reproductive organs of males in the Chemical Mixture Calculator. This is because Kortenkamp and Koch (2020) have applied data on changes in testosterone production *in vivo* to set the reference doses, whereas we and ECHA (2016) included only data on endpoints reflecting adverse effects such as altered anogenital distance and altered testicular

histopathology (ECHA 2016). For DIBP our reference dose is lower than set by Kortenkamp and Koch (2020) (Table 6). If we had used the reference doses suggested by Kortenkamp and Koch in our mixture risk assessment, this would have resulted in higher HIs for all age groups, and possibly an indication of risk of adverse health effects also for other population groups than toddlers. This illustrates how the choice of input variables is crucial for the results of any mixture risk assessment.

The benefit of using the Chemical Mixture Calculator in comparison with these previous mixture risk assessments is that we can perform mixture risk assessment including other substances with the same targets. Our approach is thus useful for identification of how much a certain chemical or chemical class contributes to the overall risk, and whether it makes a difference to include other chemicals in mixture risk assessment.

3.4 Perspectives

The Chemical Mixture Calculator can be used by risk assessors as a tool for mixture risk assessment or for identification of chemicals that are main contributors to the overall risk for adverse health effects under different conditions.

In the current form, the grouping principles and setting of DTDs are mainly based on experimental in vivo data summarized in scientific opinions by expert committees, predominantly EFSA.

Future development of the tool can include the use of in vitro or in silico data for chemicals for which no experimental animal data are available (Boberg et al., 2019). Combining the use of in vivo and in vitro data may be possible by e.g. combining knowledge on toxicokinetics and relative potencies in vitro of different substances.

The current version of the Chemical Mixture Calculator only includes a selection of substances in the chemical universe. The Chemical Mixture Calculator would benefit by inclusion of more substances for which an ADI/TDI have been set for performing mixture risk assessments at the first tier, or substances for which a DTD can be set for specific effects in target organs / systems for performing mixture risk assessments at a higher tier. Furthermore, the current version of the Chemical Mixture Calculator only

includes six target organs / systems and for these several CAGs at level 2 for specific effects are very broad. The tool would therefore benefit by inclusion of more target organs / systems as well as by defining more refined CAGs at level 2 when data are sufficient for this.

The current version of the Chemical Mixture Calculator only includes data for mixture risk assessment of chronic exposure. A future version could include data for mixture risk assessment of acute exposure. This would require development of CAGs of relevance for mixture risk assessment of acute exposure, which is considered of particular relevance for effects on the nervous system, as well as collection of acute exposure and toxicity data.

The Chemical Mixture Calculator includes some information on human exposure from other sources than food, but for most substances, this information is very limited. Future development of the tool may include the use of human biomonitoring data (e.g. from the IPCHEM database developed under the HBM4EU joint program: https://ipchem.jrc.ec.europa.eu/), which represent an integrated human exposure that could contribute to more realistic mixture risk assessments in some cases. However, in human biomonitoring the exposure sources of measured chemicals/metabolites are often unknown, and that may hamper regulatory actions.

In addition, the Chemical Mixture Calculator could be a useful research tool. The collected knowledge and the developed tool for grouping of chemicals may in the future be applicable for use in other fields such as risk-benefit analyses or life cycle assessment.

4. Conclusions

We developed a web-based tool, the Chemical Mixture Calculator that can be used by risk assessors for mixture risk assessment or for identification of chemicals that are main contributors to the overall risk for health effects, including in specific age groups and under varying exposure conditions. The tool can be

555 used for comparing risk in e.g. various population groups for which exposures are calculated using
556 deterministic or probabilistic models (Petersen et al. 2020; Boon et al. 2015). We present a range of
557 cumulative assessment groups (CAGs) for six target organ systems: haematological system, kidney, liver,
558 nervous system, developmental and reproductive system, and thyroid. These CAGs are useful for
559 grouping at several levels of mixture risk assessment refinement depending on the question addressed.

560 We consider it a strength of the Chemical Mixture Calculator that the underlying principles for mixture risk
561 assessment, definition of CAGs, and the type of exposure and hazard data, generally are in agreement
562 with principles suggested by, e.g. EFSA and ECHA (EFSA 2020a, EFSA 2020b, EFSA 2019a, EFSA
563 2013a, ECHA 2016, ECHA 2017). Even though the information on exposure and toxicity is associated
564 with uncertainty, as is also the case in risk assessment of individual chemicals, the use of this tool is
565 considered a useful and accessible starting point for mixture risk assessment for risk assessors and
566 researchers alike. The use of the tool is preferable in conjunction with expert judgement, as it is
567 necessary to have extensive understanding of underlying assumptions and uncertainties in mixture risk
568 assessments when using the tool. In addition, the selection of tier (level of refinement) requires scientific
569 insight and is crucial for interpretation of the results.

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6. Tables

Table 1. Terminology

Acceptable daily intake (ADI)	

mechanism of action	major steps leading to an adverse health effect following interaction of the compound with biological targets. It does not imply full understanding of mechanism of action at the molecular level, which is a detailed explanation of the individual biochemical and physiological events leading to a toxic effect (EFSA, 2019; EFSA, 2013a).
Reference dose	In this paper, used in a broad sense for any toxicological reference dose representing an estimate of the amount of a substance, expressed on a body weight basis that can be ingested daily in a period of 24 hours or less (acute) or over a lifetime (chronic) without an appreciable health risk. Covers acute reference dose (acute) and ADI, TDI, DTD (chronic).
Tolerable daily intake (TDI)	An estimate of the amount of a substance in food or drinking water which is not added deliberately (e.g. contaminants) and which can be consumed over a lifetime without presenting an appreciable risk to health. (EFSA Glossary)

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Table 2. Suggested Cumulative assessement groups (CAGs) for mixture risk assessment. To facilitate identification of CAGs, a systematic terminology is employed. A CAG at level 2 is described by a number followed by a letter (e.g. CAG 2a, CAG 2b etc.). A CAG at level 3 is described by a number followed by a letter and a number (e.g. CAG 3a1, CAG 3a2, CAG 3b1 etc.) and a CAG at level 4 by a number followed by a letter, a number and a letter (e.g. CAG 3a1a, CAG 3a1b, CAG 3a2a, CAG 3a2b, CAG 3b1a, CAG 3b1b etc.).

CAG level 1, Target organ/ system	**CAG level 2, Specific effect**	**CAG level 3, Mode of action**	**CAG level 4, Mechanism of action**
Haematological system	CAG 2a: Anaemia (decrease in number of red blood cells (RBCs), haemoglobin, haematocrit; increase in number of reticulocytes (immature RBCs))		

	inflammation, cholangiofibrosis (an inflammatory reaction involving bile duct epithelium in response to pronounced hepatic parenchymal necrosis))		
	CAG 2d: Bile duct hyperplasia (hypertrophy / proliferation)		
	CAG 2e: Karyocytomegaly (increase in the number of diploid nuclei per hepatocyte or an increase in the ploidy level of a single hepatocyte nucleus)		
Nervous system	CAG 2a: Functional alteration related to the motor division of the nervous system (muscle strength, locomotor activity, neuropathy)		

2f Effects on non-reproductive organs of offspring (e.g. histological or functional effects). Limited to effects that are due to exposure of developing animals and are not expected with adult exposure at the same doses. It is noted that effects on neurodevelopment are in the current project considered as neuroto xic and placed in CAGs for neurotoxicity.

			iodothyronine deiodinases; or induction of uridine diphosphate glucuronyl transferases (UDPGT) and/or sulphotransferases (SULT); or inhibition of SULT; or reduced re-absorption of T3 and T4 from the intestine)
	CAG 2b: Effects on parafollicular cells and/or calcitonin system (C cell atrophy, necrosis, cell death, hypertrophy / hyperplasia, changes in serum calcitonin)		

Table 3. Mixture risk assessment at different levels of refinement for a group of six phthalates (DEHP, DBP, DIBP, BBP, DINP, DIDP). Hazard Index (HI) values for CAG level 1 are in italics. NR: not relevant CAG for this subpopulation.

Table 4. Mixture risk assessment at different levels of refinement using data for all substances in the Chemical Mixture Calculator, including phthalates. Hazard Index (HI) values for CAG level 1 are in *italics*. NR: not relevant CAG for this subpopulation.

Level of refinement of mixture risk assessment	Number of substances

Table 5. Example of results output for phthalate case. The Hazard Index (HI) was calculated for each CAG and the chemicals contributing with more than 0.01 to the HI are listed together with their individual Hazard Quotients (HQ) for mean adult exposure. Note that some substances are present in several CAGs at level 1 and 2.

	Cumulative Assessment Group				
	Reproductive and developmental, CAG level 1	**Reproductive organs of male/female offspring, CAG level 2**	**Prenatal death, CAG level 2**	**Kidney, CAG level 1**	**Tubular cell degeneration, CAG level 2**
HI for mean adult exposure (sum of HQs for each substance)	4.3				

Table 6. Comparison of reference doses for phthalate effects on male reproductive endpoints in recent mixture risk assessment reports. Different terms are used for reference points (DTD, DNEL, TDI), see definitions in Table 1. Colour code indicates potency differences with the darkest red indicating highest potency. DIDP is not included as none of these reports considers this phthalate as a male reproductive toxicant.

- Chemical mixture risk assessment made possible using novel online tool
- Suggestions for grouping based on toxic effects on six target organs or systems
- Case: Mixture risk assessment for phthalates and chemicals with similar effects

Declaration of interests

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

□ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: