WaterTOP Training School: "Risk assessment approaches for water T&O
Rome October 16-18, 2023

Introduction to risk assessment procedure for combined exposures

The risk assessment of multiple chemicals contaminating drinking water: the harmonised procedures proposed by the EFSA guidance

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- ✓ The EU Chemicals legislation is based predominantly on risk assessments for individual substances.
- ✓ In reality humans are simoutaneously exposed to a wide variety of chemicals throughout their lives.
- ✓ There is increasing concern in the general public about the potential interactions between those substances

This is the case also of toxins/contaminants in drinking water.

What is a mixture ?





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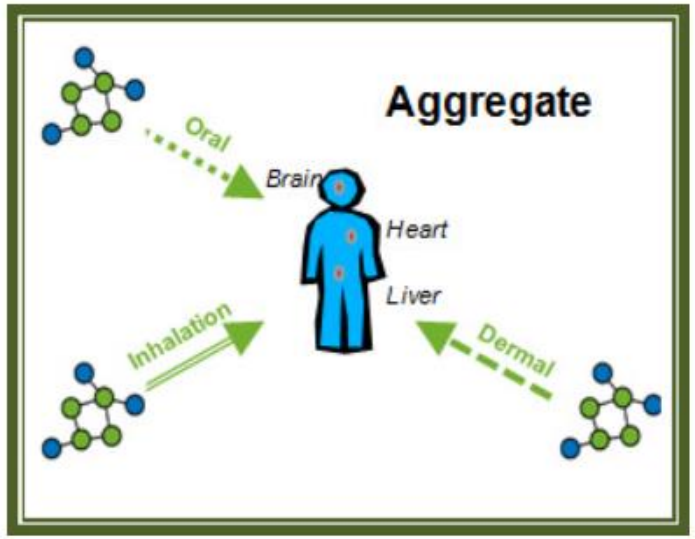
A mixture is defined as any **combination of two or more chemicals** that may contribute to effects

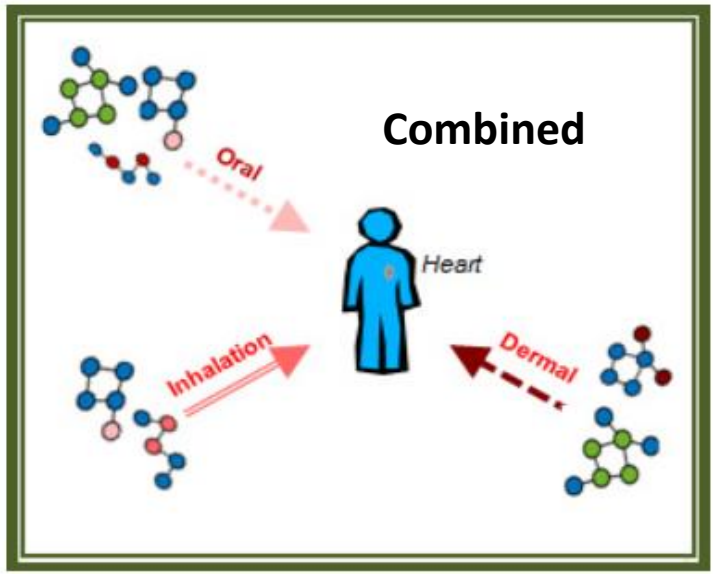
- **Intentional mixtures** are manufactured formulated products that are marketed as such, for example a formulated PPP or a flavouring agent used in food or feed.
- **Unintentional mixtures** originate from a single source, for example discharges to the environment during the production, transport, use or disposal of goods.
- **Coincidental mixtures** originate from multiple sources and through multiple pathways.

In each of these mixture types, the composition might be fully **chemically defined or chemically characterised to varying extents**.
The extent of characterisation of a mixture is an important factor in determining the approach to risk assessment.

- ✓ If a mixture is judged to be **fully chemically defined**, the preferred approach is generally component-based, i.e. the risk is assessed based on exposure and effect data of its individual components.
- ✓ If a mixture is **poorly defined**, then it may only be feasible to apply a whole mixture approach in which the mixture is treated as a single entity, similar to single chemicals. Examples of poorly defined mixtures include certain botanicals and novel foods.



An exposure is defined as **aggregated** when the individual can take the **single chemical substance through multiple sources** (food, drinking water, air), which therefore activate multiple pathways (oral, cutaneous, inhalation). If the exposure is not only oral and also dermal and/or via inhalation, the values obtained cannot simply be added together, but it is necessary to know the toxicokinetic behavior (for example the % of absorption for the various pathways that allow to estimate an internal dose) in order to do so.

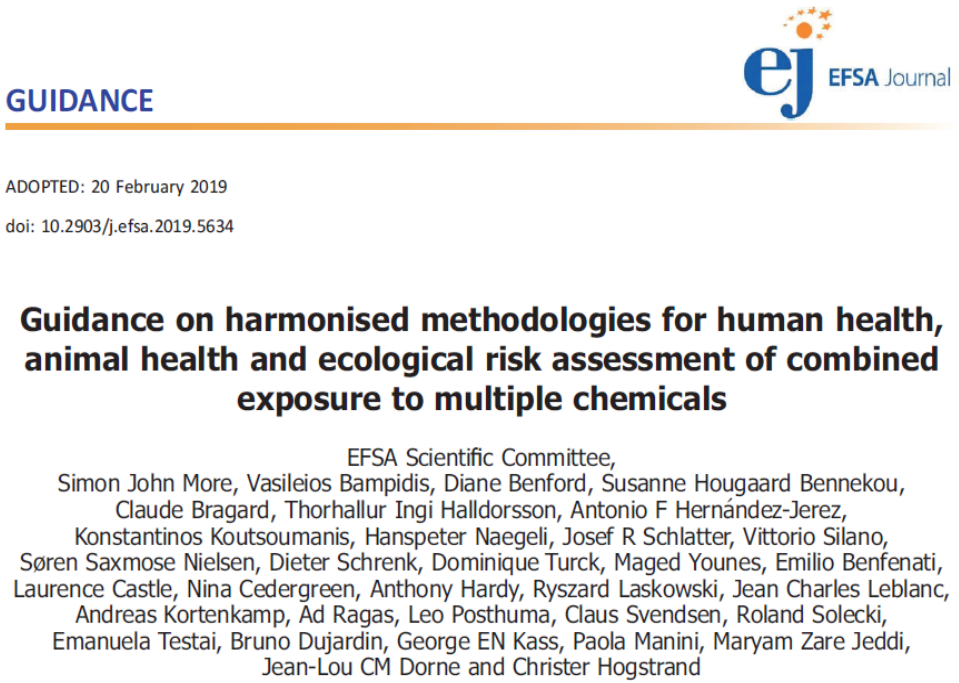


An exposure is defined as **combined** when the individual can be exposed to **multiple chemicals substance through one single or multiple sources** (food, drinking water, air), which therefore activate one or multiple pathways (oral, cutaneous, inhalation).

Many documents have been adopted by international Agencies dealing with human health risk assesment after combined exposure to multiple chemicals ('mixtures' or cocktail effects').

In its **Guidance document adopted in 2019, EFSA** describes harmonised framework based on the risk assessment steps, for both

- ✓ ***whole mixture approaches o WMA*** (when the mixture is treated as a single entity, similar to single chemicals) and
- ✓ ***component-based approaches or CBA*** (when the risk of combined exposure to multiple chemicals is assessed based on exposure and effect data of the individual components).



GUIDANCE

EFSA Journal

ADOPTED: 20 February 2019

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Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals

EFSA Scientific Committee,
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The harmonised framework consists of

- **problem formulation,**
- **exposure assessment,**
- **hazard identification and characterisation,**
- **risk characterisation**

The principles of **tiering** can be applied in both approaches

The tiered approach

Tiering principles allow for simple and conservative approaches/assumptions at lower tiers, avoiding unnecessary expenditure of resources.

An assessment can be concluded as soon as there is clarity on sufficient protection for the exposed population on the basis of simple assumptions on exposure and hazard estimates then resulting risk metrics do not flag potential risk (e.g. sufficient margins of exposure).

If clarity on sufficient protection is lacking, one progresses to risk management (e.g. introduction of risk mitigation measures) or to a higher tier.
At increasing tier levels (1, 2 and 3), more data should be available, allowing assessments to become more accurate, with a better characterisation and of uncertainties and eventually decreasing uncertainty.

the lower the uncertainty for risk assessment, the higher the tier

The tier applied is not necessarily symmetrical between exposure and hazard assessment or between the members of an assessment group, because availability of exposure and effect data may vary

The tiered approach

Tiering principles allow for simple and conservative approaches/assumptions at lower tiers, avoiding unnecessary expenditure of resources.

An assessmen for the exposed population on hen resulting risk metrics do not

If clarity on s .g. introduction of risk mitigation At increasing t ssments to become mo ventually decreasing un

tier

The tier applie ssment or between the m and effect data may vary)

Tier	Occurrence data	Consumption data	Exposure estimate
0	Default Values, Permitted Levels	Default Values, Portion Sizes	Semi-Quantitative Point estimates
1	Modelled and Experimental Data	Food Balance Sheet Food Basket	Deterministic
2	Monitoring Surveys	Summary Statistics	Semi-Probabilistic
3	Individual Co-Occurrence data	Individual data	Probabilistic

Note: Occurrence and consumption data ranges from default values (tier 0) to individual co-occurrence data and individual data, respectively (tier 3), and consequently, exposure estimates range from semi-quantitative point estimates (tier 0) to probabilistic (tier 3). Occurrence and consumption tiers do not necessarily match.

Figure 4: Examples of tiers in exposure assessments

Problem formulation

Problem formulation is **an iterative process** involving exchange and agreement between **risk assessors** and the originator of the request (in EFSA's context most often **risk managers**) during which the need for and the extent of a risk assessment are determined.

The problem formulation step takes on a particular importance in the context of combined exposure to multiple chemicals because **the demarcation of the problem generally is more complex than for single chemicals**. A dialogue between toxicologists and exposure assessors is recommended. This step results in an analysis plan.

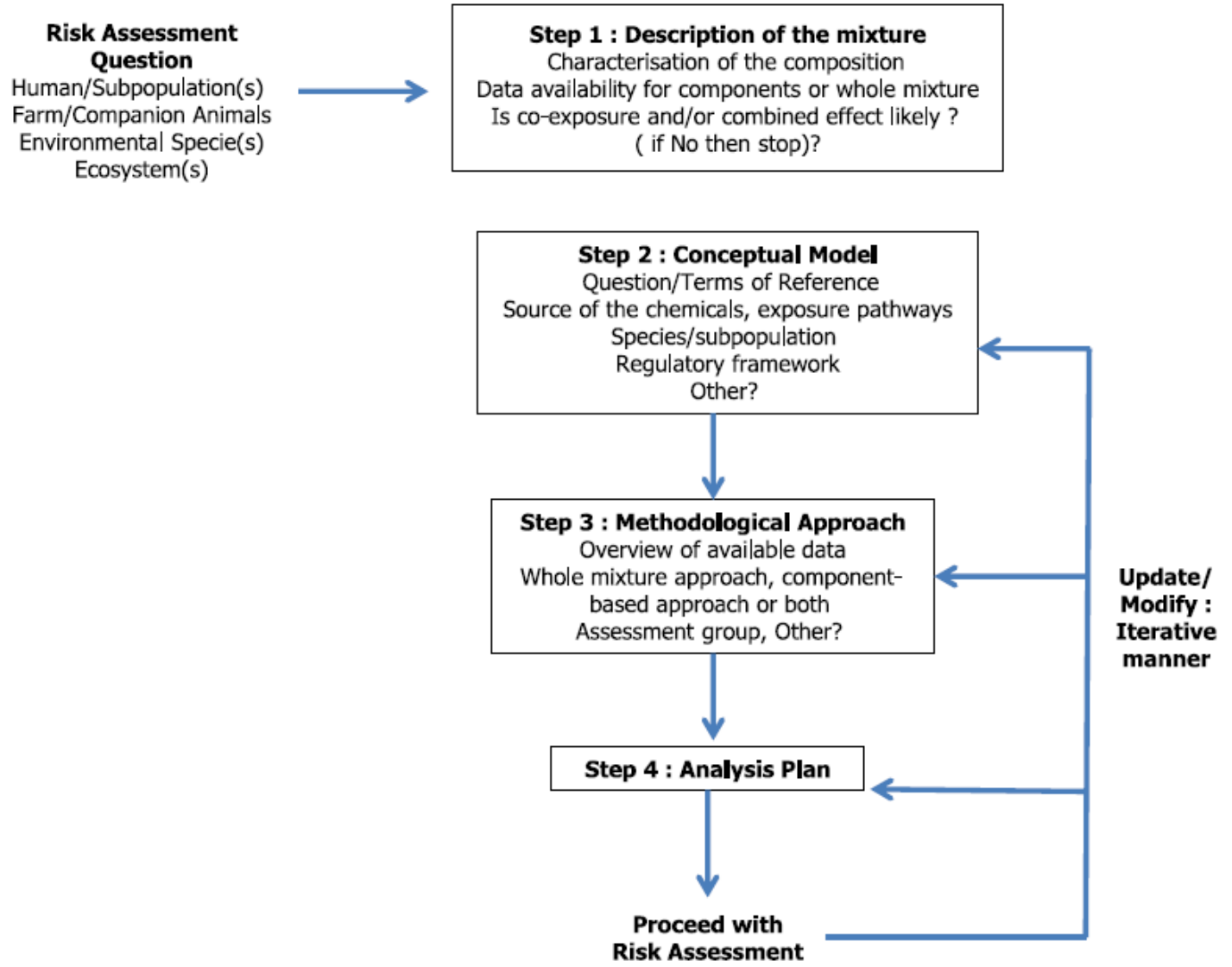


Figure 3: Problem formulation for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals

Table 2: Key issues to be considered in the problem formulation that are specific for risk assessment of combined exposure to multiple chemicals

Issues	Examples
On the basis of the assessment process:	
Is combined exposure assessment warranted?	Co-exposure and combined effects are likely based on available data. The regulatory context stipulates (or not) risk assessment of combined exposure to multiple chemicals
Characterisation of the mixture	Origin: e.g. regulatory product, contaminant, production process or emission sources Composition: e.g. chemical space, components, stability (does the composition of the mixture change over time), variability (batch-to-batch differences) Reactivity
Whole mixture and/or component-based approach?	*Whole mixture:* e.g. an essential oil, for which not all components have been chemically identified *Component-based*: e.g. pesticide residues with potential for co-exposure
On the conceptual model:	
Approach to exposure assessment	Availability of data on components of the mixture or on a marker substance for the whole mixture
On grouping of chemicals:	
Criteria for inclusion in the assessment group?	Similar origin, similar Mode of Action (MoA), same target organ, co-exposure
What to do with chemicals	Consider applying response addition

First issues to be addressed ('gatekeeper' step)

- Is a combined exposure assessment warranted?

and if so

- which chemicals should be considered together?

This step can be based on the likelihood that chemicals co-occur in the scenario that is the topic of the assessment.

If co-occurrence/co-exposure within a relevant time frame is unlikely, based on an initial assessment of the available data, a combined exposure assessment can be considered redundant.

The TK information can be used to investigate the likelihood of co-exposure within the organism. For example, **if the chemical is eliminated very fast (half-life of 1 h) likelihood of internal co-exposure may be less likely compared to a situation where the half-life is very long (years).**

For bioaccumulating compounds, simultaneous internal exposure can occurr also when external exposure events are not simultaneous

When no TK information is available :

(1)Chronic co-occurrence is assumed and combined toxicity using dose addition is applied.

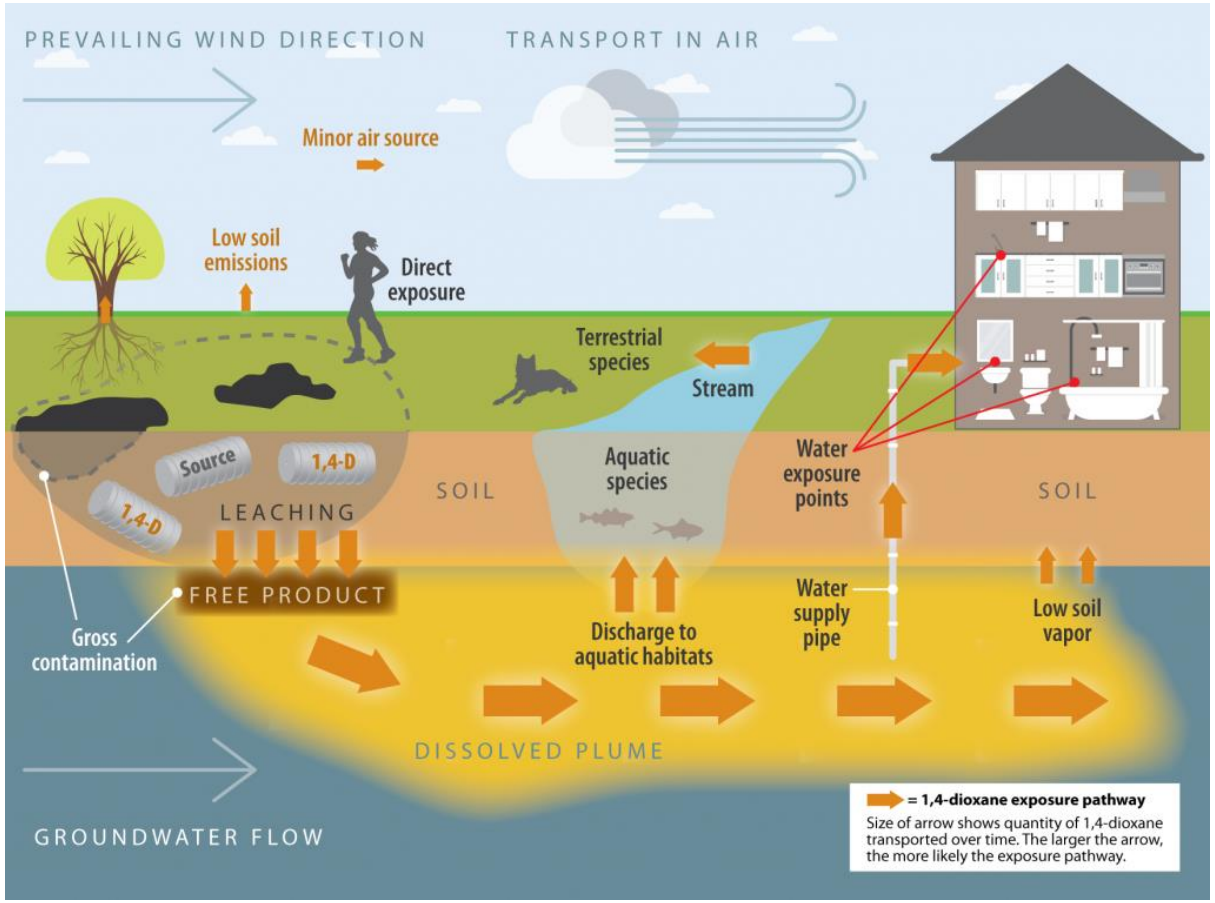
(2) For acute exposure, however, the relevant timescale required for two or more chemical substances to elicit combined toxicity may be as narrow as a single eating occasion for humans or animals, or a single environmental release of chemicals. Under these circumstances, detailed information on co-occurrence of the individual chemicals is required at sample level, and preferably potency-adjusted concentrations should also be calculated at the sample level before proceeding with the exposure calculations.

The whole mixture approach

In the whole mixture approach, the whole mixture is essentially **evaluated in the same way as for a single chemical substance** as the so-called UVCB substances (Substances of unknown variable composition, complex reaction products or biological materials) under REACH

EXPOSURE ASSESSMENT

Which is the level of exposure for the target population?

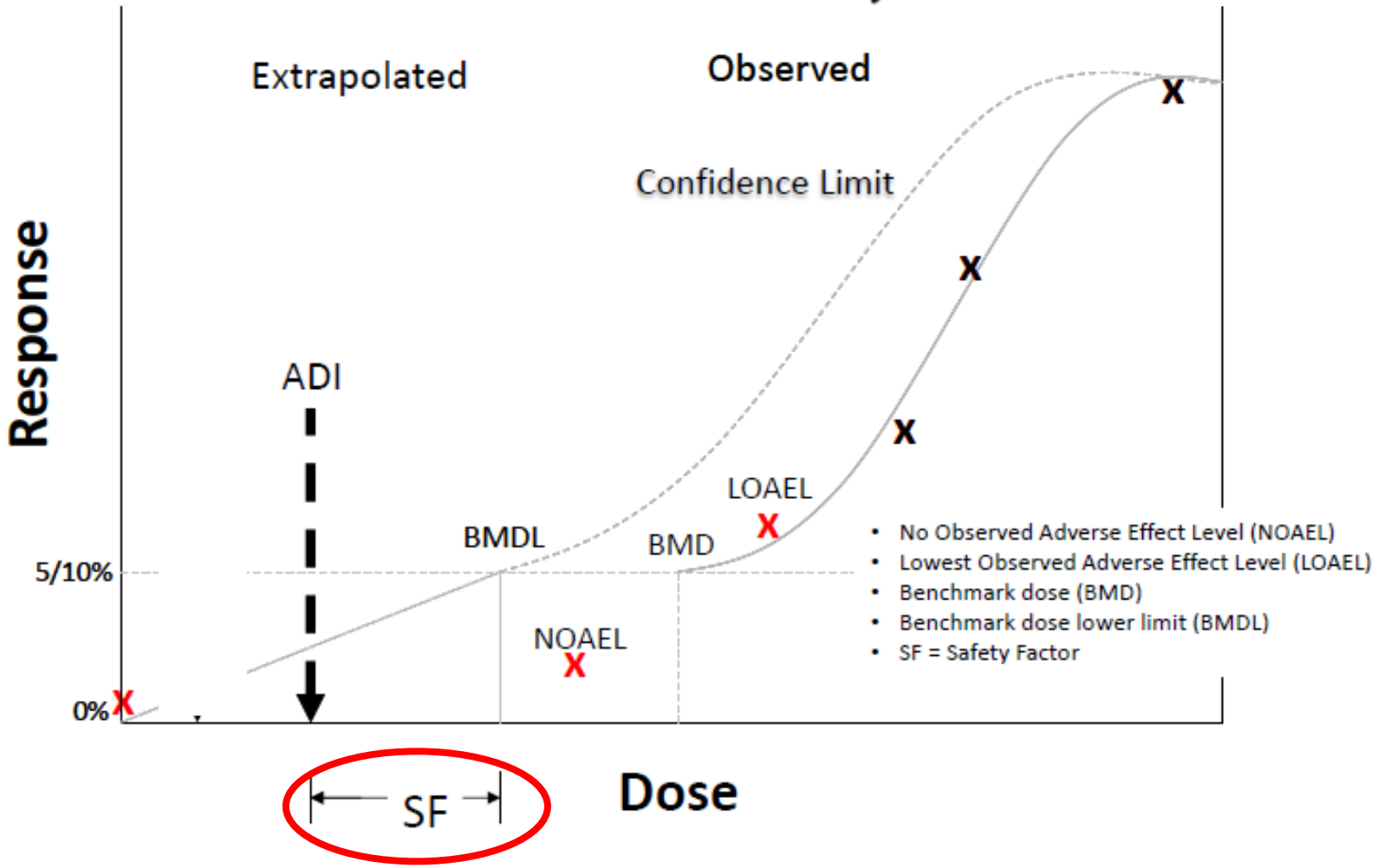


Assessment of combined exposure to multiple chemicals generally uses similar concepts and methods as for single chemicals, but can be more complex as chemical exposure may occur through multiple sources and sequential exposures.

Exposure is typically assessed by **combining occurrence data on chemicals with consumption data** for human and animal health

Questions related to the source(s), exposure pathway(s), exposed population(s), variation of doses over the exposed population(s) and time (**exposure scenarios**), and the uncertainty in the exposure estimates should be addressed.

HBV → ADI *(human dose)* = $\frac{\text{NOAEL } \textit{(experimental dose)}}{\text{Safety Factor(s)}}$



BMD=The dose associated with an effect in a certain percentage of animals (e.g.: 1, 5 or 10%) or with an effect equal to a % of the maximum effect (e.g. 0.1, 1, 5, 10%, depending on of the severity of the effect itself)

Advantages over NOAEL:

- Less dependence on the experimental design
- The entire dose response curve is used (also taking into account the slope)
- A measure of variability (biological and experimental) is included

HBV= Level of exposure below which adverse effects are assumed to have a near-zero probability of occurring in exposed populations

The whole mixture approach: hazard characterization

The whole mixture approach requires **dose–response information for the mixture** of concern.

When not available it may be obtained by **read-across from similar mixtures** having the same chemicals but in slightly different proportions or having most chemicals in common and in highly similar proportions.

For **poorly defined** whole mixtures, **options to generate information for hazard characterisation are extremely limited** as, in general, in silico and read-across methods require information on the chemical structures of components to establish the degree of similarity between these whole mixtures.

Application of the whole mixture approach can be facilitated by the identification of **marker substances**, which are readily measurable prevalent components of the mixture and therefore can be used in the exposure assessment and the dose–response analysis.

However, for human and animal hazard assessments, if information on the source of the mixture provides reassurance that certain types of chemicals (e.g. potent carcinogens or accumulating chemical substances) are not present, then it might be possible to use tools such as the **TTC approach**.

The whole mixture approach: pros and cons

The **advantage** of the WMA is its **holistic nature**, as the different components are taken into account as contributors to the overall toxicological activity of the mixture, including any potential synergistic or antagonistic interactions

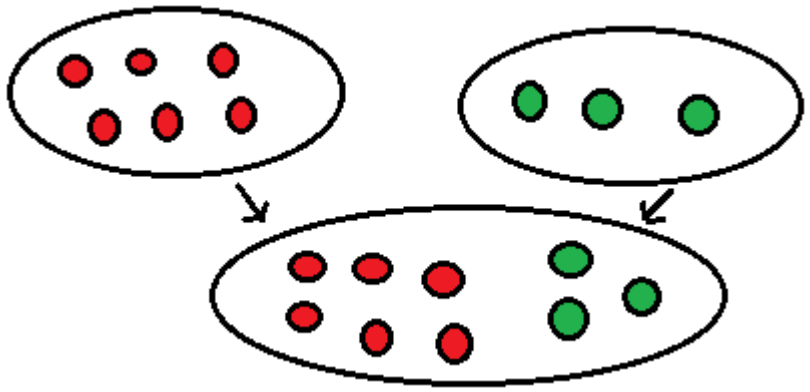
Limitations of the whole mixture approach include its applicability only to **mixtures** that that are **not variable** in composition and are not expected to change over time.

Testing of "real world" complex mixtures is rarely feasible (unless they are intentionally produced as for formulated products), due to the **huge number of possible combinations** and the variability of mixtures composition over time. Furthermore, such testing would require unnecessary animal testing and is scientifically unjustified.

The component based approach

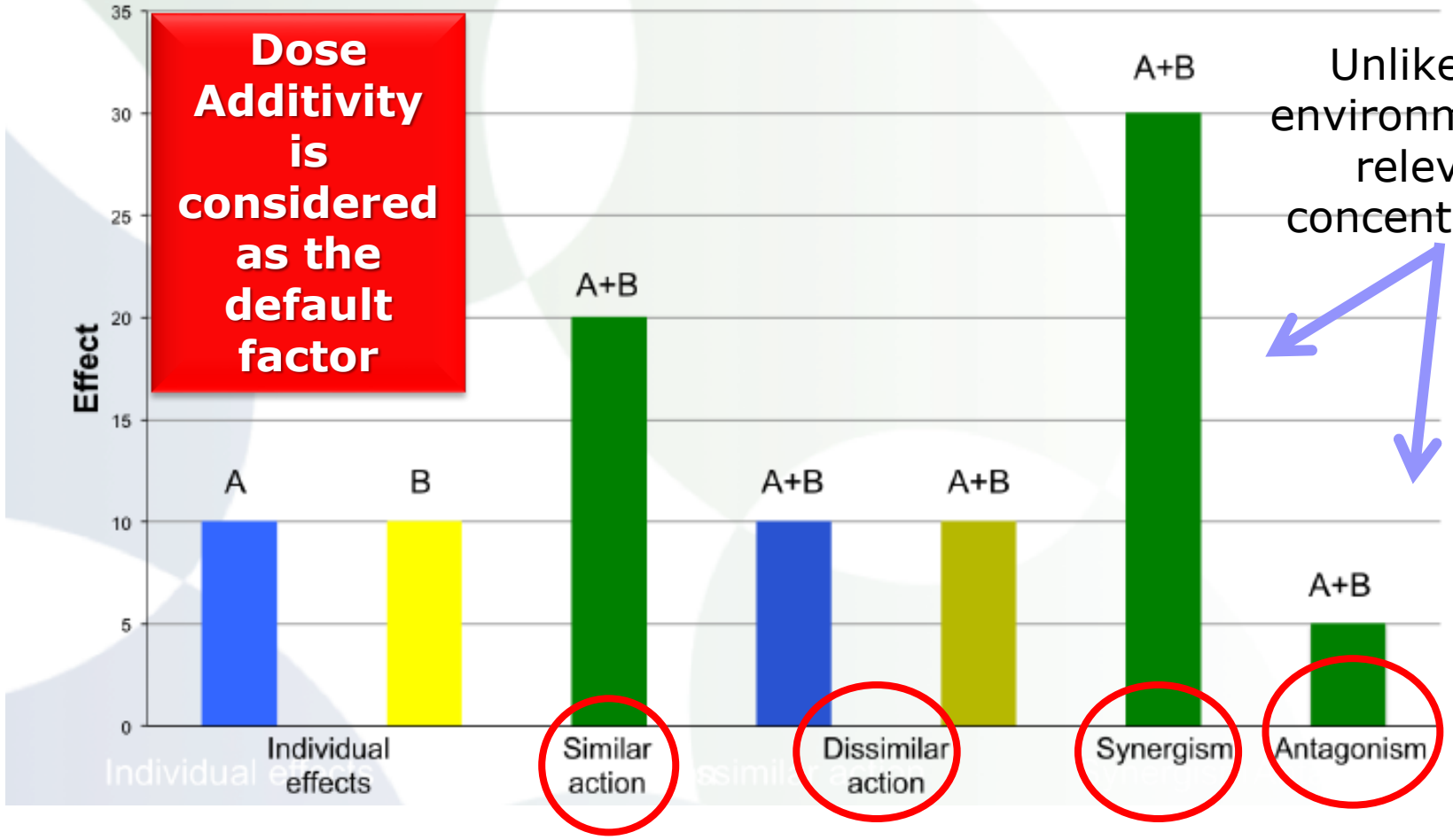
If the components of the mixture and their exposure levels are chemically defined, the CBA can be applied **using exposure and hazard data of the individual components**, often using the **dose addition model as a default**, unless it is known they act independently (dissimilar action)

It is assumed that the chemicals in the mixture act by exerting their effects without diminishing or enhancing each other's toxicity.



Dose addition has been shown to be applicable to a wide range of endpoints and provides sound approximations of observed combination effects

Possible combination effects of substances A and B



Unlikely at environmentally relevant concentrations

Combined effects can arise when each mixture component is present at doses around or above its no observed adverse effect level (NOAEL)

Dose addition: substances are assumed to behave as simple dilutions of each other.
The relative toxic potency is calculated with respect to the other components or a reference substance, added together and then treated as a single substance.
Possible interactions considered absent/negligible

Components are often organized in assessment groups

Setting up assessment groups can be based on the pragmatic aspects from the regulatory domain, from co-occurrence data or from common properties

The specific approach to be used for grouping will be determined by the context of the assessment and the problem formulation. The approach taken can also be a **combination of the different approaches**, for example grouping based on a MoA combined with kinetic considerations (grouping chemicals that affect a common target and that have similar kinetics).

Table 3: Examples of approaches for grouping chemicals into assessment groups

Grouping approach	Overarching common feature	Example	Comments
Common regulatory domain	Regulatory requirements	Biocides, pesticides, food additives, flavourings	
Common source	Exposure	Multiple biocidal and pesticidal active substances in a formulation, feed and drinking water contaminants	A lower-tier method when assessing the common occurrence for specific exposure scenarios
Environmental media	Exposure	Exposure through presence in common medium (e.g. river, soil)	Grouping driven by common exposure through a particular medium
Common functional group(s)	Common toxophore	Aldehyde, epoxide, ester, specific metal ion	
Common constituents or chemical classes, similar carbon range numbers	Physicochemical characteristics	Substances of unknown or variable composition, complex reaction products or biological material (UVCB substances)	Frequently used with poorly defined mixtures
Groups of chemicals with incremental or constant change across the category	Physicochemical characteristics	Mixtures of polyolefins	For example, a chain-length category or boiling point range
Common breakdown products	Physicochemical characteristics	Related chemicals such as acid/ester/salt	Likelihood of common bioactive breakdown products via physical or biological processes that result in structurally similar chemicals
Common 'critical' target organ(s)	Toxicological or biological properties	Cumulative assessment groups used for pesticides	EFSA, 2013 (EFSA PPR Panel, 2013b)
Common MoA or AOP	Toxicological or biological properties	Acetylcholine esterase inhibitors, AhR agonists, metabolism to similar bioactive metabolite(s)	Chemicals acting via same pathways that converge to common molecular target (US EPA 2007, 2016; OECD, 2017b)

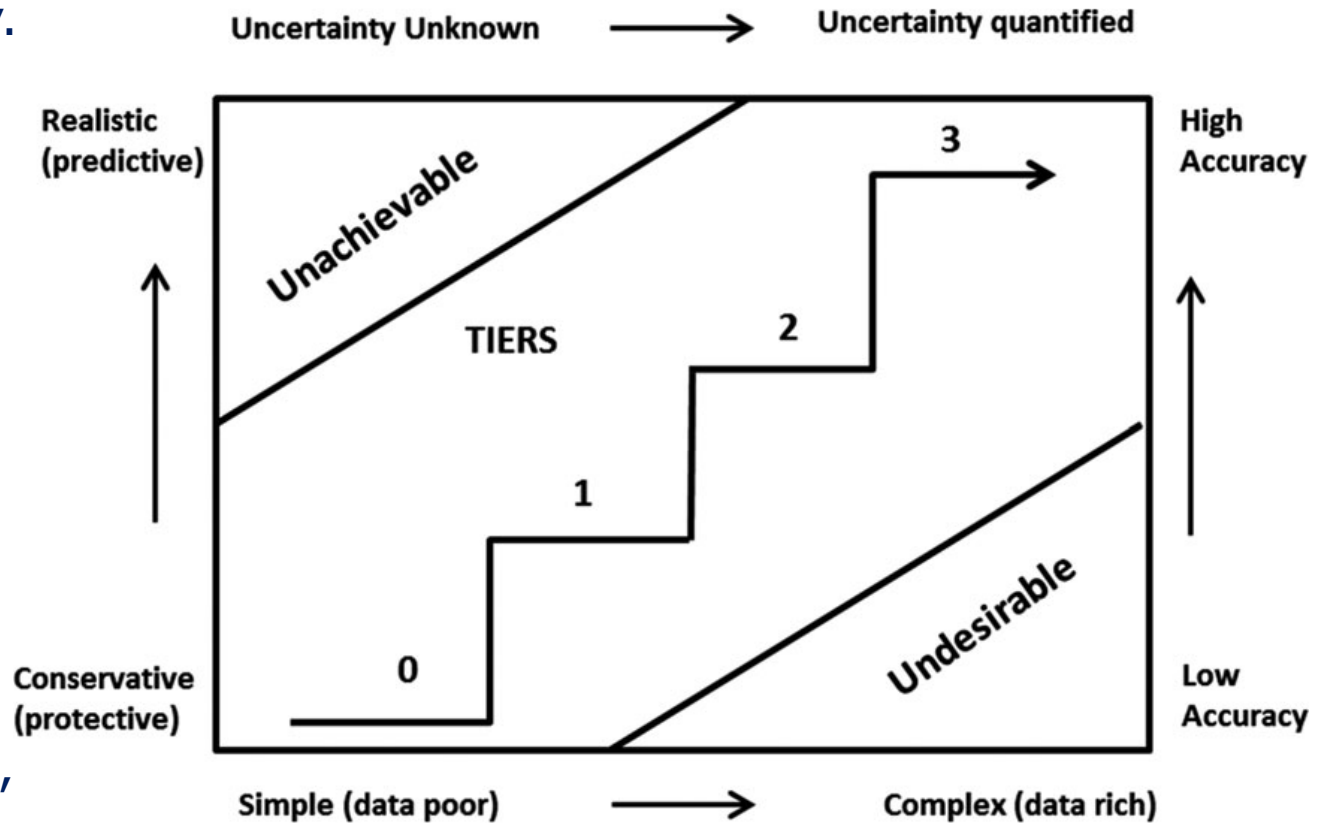
MoA: Mode of Action; AOP: Adverse Outcome Pathways; UVCB: Substances of unknown or variable composition, complex reaction products or biological materials; AhR: Aryl hydrocarbon.

Tiering and grouping relate in the following way.

At a lower tier, the analysis may begin with **all components being grouped together**, e.g. an exposure-driven grouping with neglect of modes of action.

This approach is **simple and conservative** (assuming all components having a common adverse outcome', which is unlikely)

If the outcome of the risk assessment shows **sufficient protection** for the exposed population, the simplified and conservative approach yields sufficient information to **stop the assessment**.



If not, it can be considered **a refinement is needed** (e.g. creating subgroups of chemicals based on hazard criteria, for example based on a common adverse outcome). Toxicokinetic data can also be useful for grouping, particularly when metabolism information is available for a class of compounds and common toxicologically relevant metabolites are shared.

- **Mechanistic concepts**, such as **mode of action, mechanism of action and the adverse outcome pathway**, can play an important role when grouping chemicals into assessment groups.

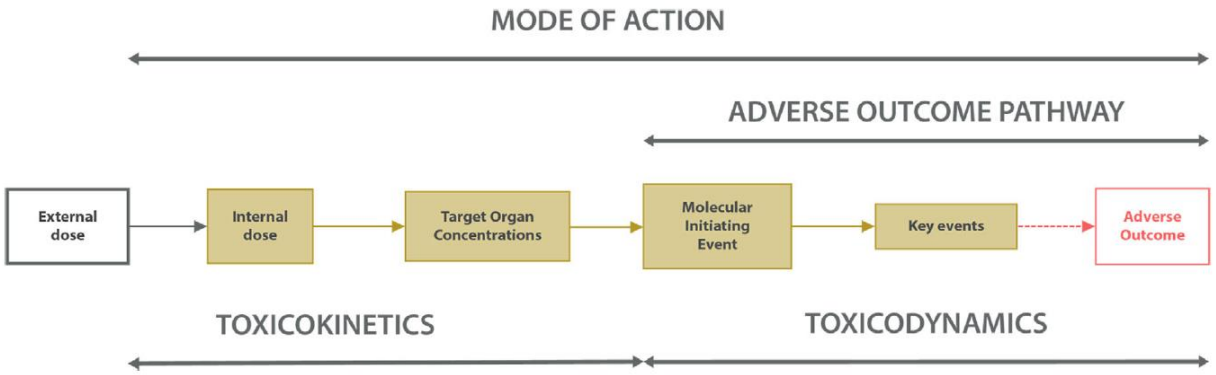


Figure 1: Conceptual representation of the mode of action and adverse outcome pathway frameworks under the exposure-response continuum

The EFSA guidance document on Grouping (2021) includes also **prioritisation methods** to be applied when the number of chemicals to be assessed is *a priori* vast and resources are limited.

These provide **means to reduce the number of chemicals to be considered for grouping or within an already formed assessment group**.

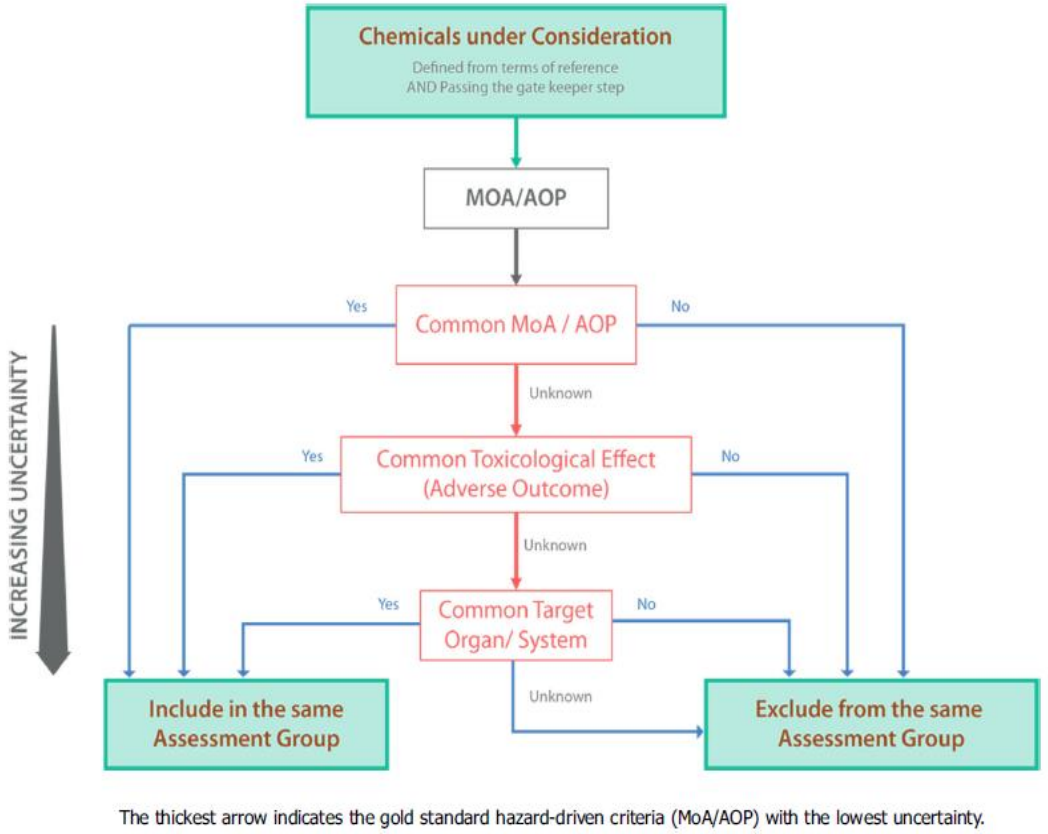


Figure 3: Top-down hierarchical process for grouping chemicals into assessment groups using hazard-driven criteria

Therefore, chemicals which are unlikely to co-occur in humans or otherwise would contribute only marginally to a combined risk can be considered of low-priority for grouping.

Risk characterisation of combined exposure to multiple chemicals aims to:
1) **Calculate the ratio of exposure to hazard or of hazard to exposure** to determine whether there is a possible concern.

2) **Identify the components in an assessment group that represent particularly important risk drivers for the component-based approach**.

Many combined exposure **risk characterisation methodologies** are available. They compare the sum of individual chemical exposures and the reference points or reference values to characterise the risk.

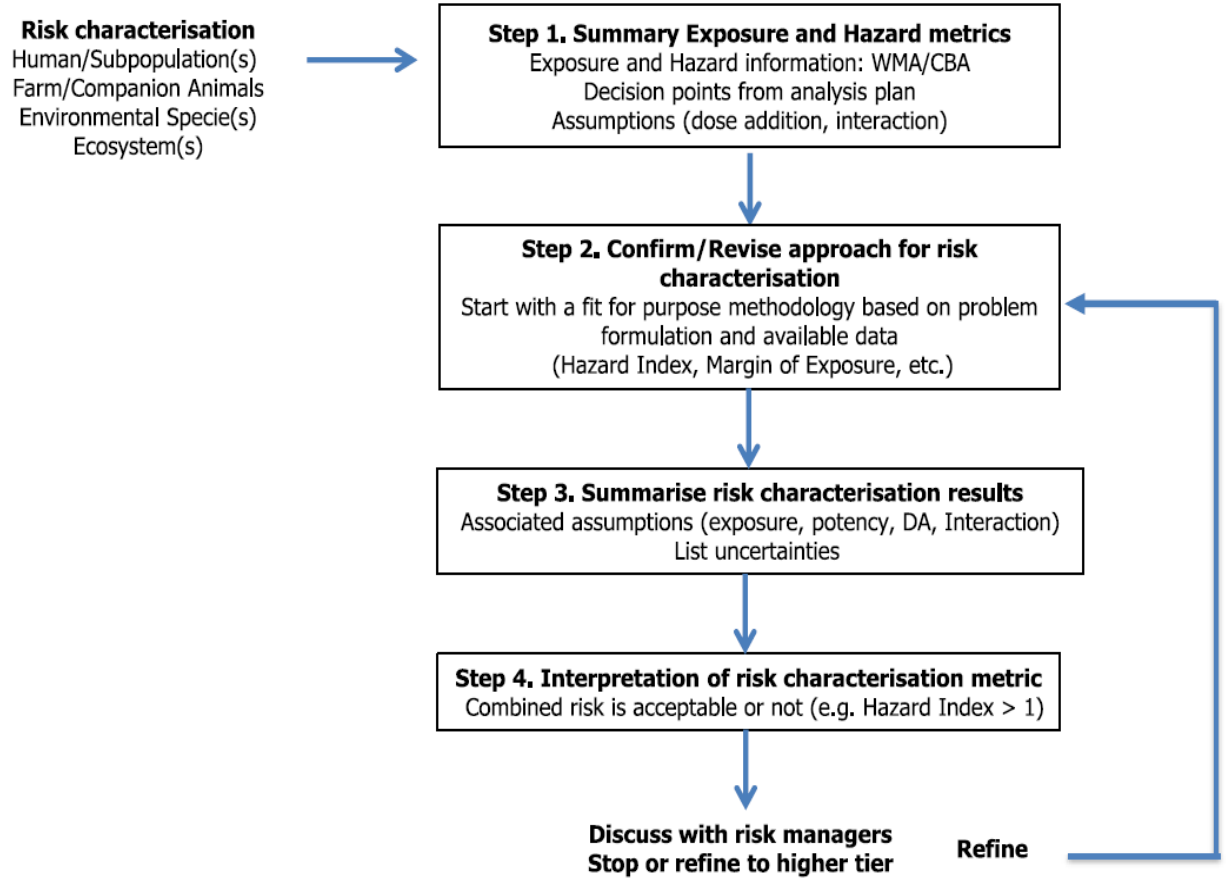


Figure 9: Stepwise approach for risk characterisation of combined exposure to multiple chemicals

Again the **whole mixture** is essentially treated as a **single chemical substance**. If a reference value has been derived, the aim is to identify whether the estimated exposure exceeds that reference value or results in an (in)adequate MoE or Hazard Quotient (HQ).

Methodologies and associated calculations for risk characterisation of combined exposure to multiple chemicals using dose addition

At **lower tiers** it is common to use the **Hazard Index (HI)** approach, especially when all the chemicals in the group are taken together, independently on the information on the MoA or AOP.

The HI is defined as the sum of the hazard quotients (HQ) of the individual components of an assessment group, in which each of the hazard quotients is calculated as the ratio between exposure to a chemical and the respective reference values (i.e. ADI, TDI).

HI (Hazard Index)

$$HI = Conc_1/RfD_1 + Conc_2/RfD_2 + \ldots + Conc_n/RfD_n$$

HQ_1

HQn

When HI>1 there is a potential risk and a refinement is necessary (e.g. considering grouping)

When information on target organ or even better MoA or AOP are available or a refinement is necessary it is better using other methodologies

RPF (Relative Potency Factor) used e.g. for OPT pesticides (chlorophos and AChE inhibition as reference chemical -most studied, and generally most potent member of the group- and critical effect)

TEF (Toxic Equivalent Factor) used for dioxin like compounds (e.g. 2,3,7,8,-TCDD and binding to the AhR-receptor as reference chemical and critical effect)

To assess the effects of a mixture of individual substances Si (i=1,2,…n), a substance has to be defined as the index compound (Sind) in order to calculate the component (and exposure route specific) relative potency factors (RPF):
RPF1 = TS1 / TSind, where TS1 is the toxicity of the individual substance (S1) and TSind is the toxicity of the index compound (Sind).

The dose (concentration) is then adjusted: aD1 = D1 x RPF, and the mixture dose (Dmix) is calculated from the sum of the adjusted doses:

$$D_{mix} = \sum_{i=1}^{n} aD_i$$

The health effect of the mixture is then assessed by using the dose-response curve of the index substance.

The “Toxic Equivalent” (TEQ) scheme weighs the toxicity of the less toxic compounds as fractions of the toxicity of the most toxic TCDD. Each compound is attributed a specific “Toxic Equivalency Factor” (TEF). This factor indicates the degree of toxicity compared to 2,3,7,8-TCDD, which is given a reference value of 1, having the other values<1.

Relative potency factors (RPFs) for per- and polyfluoroalkyl substances (PFAS) have previously been derived based on liver effects in rodents for the purpose of performing mixture risk assessment with primary input from biomonitoring studies.

Congenere	TEF	Congenere	TEF

SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 14 June 2018

AMENDED: 18 February 2019

doi: 10.2903/j.efsa.2018.5333

Risk for animal and human health related to the presence of dioxins and dioxin-like PCBs in feed and food

- **The current WHO_{2005}-TEFs should be re-evaluated in order to take into account new in vivo and in vitro data. In particular, more insight into the relative potency of PCB-126 in humans is required.**

1,2,3,6,7,8-HxCDF	0,1	PCB 156	0,00003
1,2,3,7,8,9-HxCDF	0,1	PCB 157	0,00003
2,3,4,6,7,8-HxCDF	0,1	PCB 167	0,00003
1,2,3,4,6,7,8-HpCDF	0,01	PCB 189	0,00003
1,2,3,4,7,8,9-HpCDF	0,01		
OCDF	0,0003		

The 2005 World Health Organization Re-evaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-like Compounds. Toxicological Sciences 93(2), 223-241 (2006)]

An example from the literature regarding pesticides: Blaznik et al, Public Health Nutrition 19(3):1-7 (2015)

Table 1 Relative potency factors (RPF) applied in the cumulative assessment

Pesticide	RPF	Pesticide	RPF
Acephate	1	Fenitrothion	0·028
Azinphos-methyl	1·25	Methiocarb	0·76
Chlorpyrifos	0·752	Methomyl	2·041
Chlorpyrifos-methyl	0·063	Oxamyl	5·556
Dimethoate	4	Phosmet	0·25
Ethion	4·167	Pirimicarb	0·084
Fenamiphos	13·5	Tolclofos-methyl	0·003

The cumulative acute assessment of selected pesticides was calculated with **acephate as index compound**. The residue concentration for a pesticide was multiplied by the RPF value for this substance to obtain an equivalent content of the acephate.

All the pesticides in the table are AChE inhibitors (same MoA).

RPF shown in Table 1 were derived from the literature and considered the benchmark dose at 10 % AChE inhibition (BMD10) in either brain or red blood cells or comparing the NOAEL; RPF of fenamiphos was derived from the lowest observed adverse effect level (LOAEL) for AChE inhibition in red blood cells (the best is to use the same metrics, this approach can have some limitations)

Whether and how to account for potential interactions between components?

Interactions are defined as joint action between multiple chemicals that differ from dose addition categorised as less than additive (antagonism, inhibition, masking) or greater than additive (synergism, potentiation), with synergy being of greater concern for decision-making in the food and feed area than antagonism

- **Toxicokinetic interactions**: these are a common cause of deviations from additivity. Examples are chemicals modifying the absorption of others (e.g., skin penetration enhancing substances) or chemicals competing for active transport mechanisms (uptake, clearance);

- **Metabolic interactions**: chemicals modifying the metabolism of other mixture components;

- **Toxicodynamic interactions**: interactions between the biological responses resulting from exposure to the individual chemicals, for example resulting from similar targets (e.g., ligand-receptor interaction)

A potential for a toxicologically significant synergistic effect should be considered under the following conditions:

- Can one or more components significantly enhance the uptake of other components?
- Can one or more components inhibit significantly the excretion/clearance of other components?
- Do one or more of the components exert their toxic action via the formation of an active metabolite(s) and moght one or more of the components induce the drug metabolising enzymes that may be involved in the formation of these active metabolites?
- Can two or more components act on different enzymes in an important metabolic pathway?
- Can two or more components act on different elements of cellular protection mechanisms or cellular repair mechanisms?

A generic “Mixture Assessment/Allocation Factor” (MAF) was proposed as a risk management measure by Swedish (KEMI) and Dutch (RIVM) EU Member State authorities and taken up by the European Commission in the “EU Chemicals Strategy for Sustainability” (European Commission 2020).

A generic MAF reduces the acceptable exposure limit (AEL) by a factor of, for example, 2, 5 or 10. The AEL is usually determined as the ratio between exposure of an individual and a health based guidance value (HBGV) such as the acceptable/ tolerable daily intake (ADI/TDI) or the derived no-effect level (DNEL) representing the highest dose not expected to cause adverse effects in humans.

In contrast to a specifc data-driven approach, a generic MAF is equally applied to all substances to which humans are exposed, regardless of their individual potential to contribute to mixture effects. However, before implementing untargeted and universal measures, scientific evidence, mechanistic plausibility and the uncertainties of mixture toxicity should be explored.

Bloch et al, Archives of Toxicology (2023) 97:3005–3017

There is currently neither experimental evidence nor a plausible mechanism supporting the “revolting dwarfs” hypothesis. Consequently, there is also no need for generic protective approaches against health impacts from multi-chemical exposures at very low levels.
Instead, we highlight the need to further develop and refine concepts for targeted mixture toxicity assessment.
The study did not find convincing proof that human health is at risk due to the combined exposure to many substances that are below their individual HBGV at current exposure conditions and hence the need to use a generic MAF

Archives of Toxicology (2023) 97:3005–3017
https://doi.org/10.1007/s00204-023-03565-6

GUEST EDITORIAL

Basic concepts of mixture toxicity and relevance for risk evaluation and regulation

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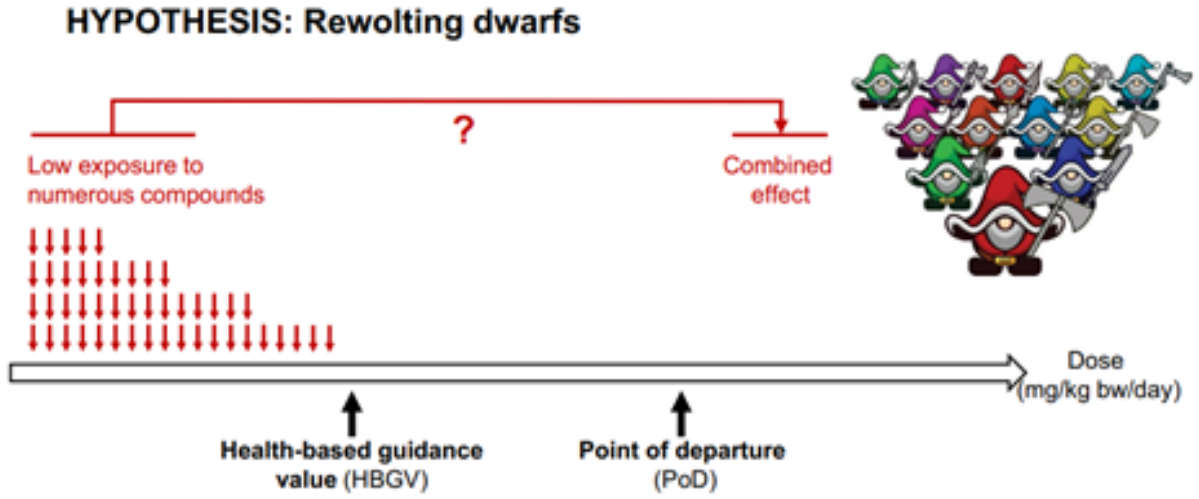
Abstract
Exposure to multiple substances is a challenge for risk evaluation. Currently, there is an ongoing debate if generic “mixture assessment/allocation factors” (MAF) should be introduced to increase public health protection. Here, we explore concepts of mixture toxicity and the potential influence of mixture regulation concepts for human health protection. Based on this analysis, we provide recommendations for research and risk assessment. One of the concepts of mixture toxicity is additivity. Substances may act additively by affecting the same molecular mechanism within a common target cell, for example, dioxin-like substances. In a second concept, an “enhancer substance” may act by increasing the target site concentration and aggravating the adverse effect of a “driver substance”. For both concepts, adequate risk management of individual substances can reliably prevent adverse effects to humans. Furthermore, we discuss the hypothesis that the large number of substances



Cyanotoxins and mixture toxicity: a case study

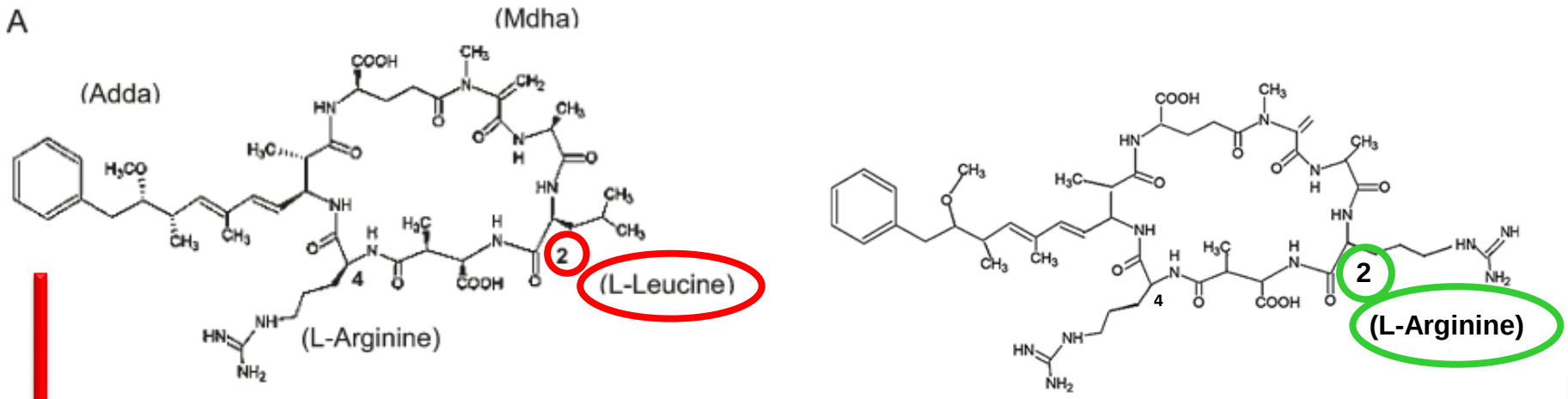
A single MC-producing cyanobacteria specie can contain and/or release in water a pattern of different variants (more than 200 known so far). Various species can be concurrently present in water producing diffent pattern of variants.

In raw waters MCs are therefore present as a mixture of MC variants and the efficiency in removal to produce drinking water is not necessarily the same for all the variants.
MC toxicity is variant specific

By adopting a conservative approach, **acute toxicity may be referred to MC-LR equivalents** (considering all the variants having exactly the same toxicity of MC-LR).

Since this congener is the most acutely toxic, the toxicity of the mixture is likely to be overestimated by this approach.

However, in case this **tier 0** approach gives rise to exposures not exceeding the HBGV, there is no need to proceed.

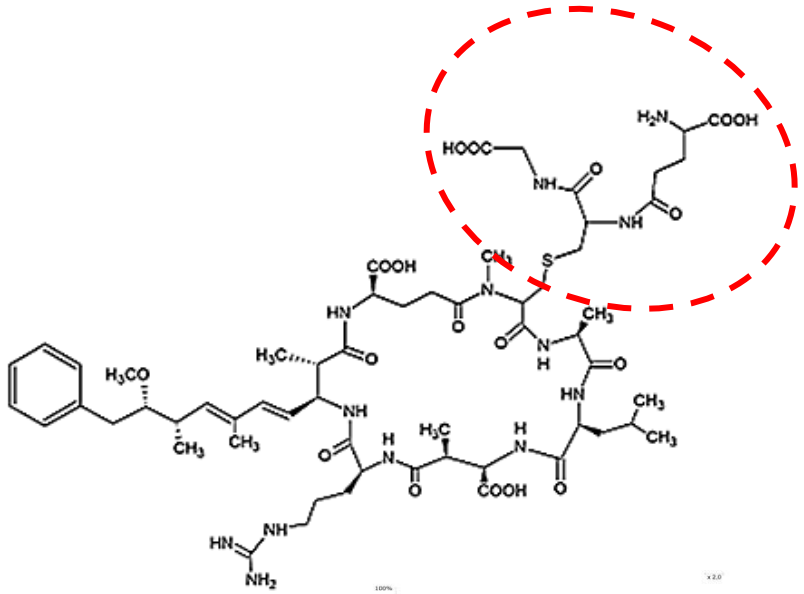


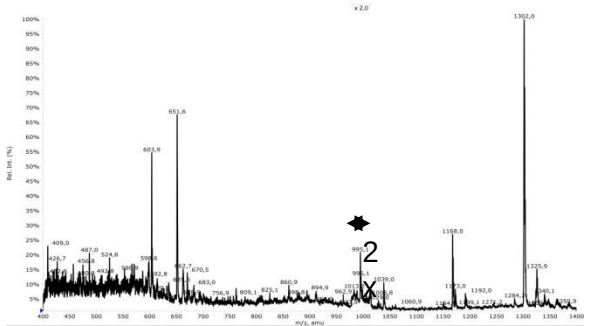
MCs acute hepatotoxic potential is congener-dependent

Toxin	i.p. LD_{50} (µg/kg)	M.W.	Structure
MC-LR	**50**	**994**	**cyclo -(D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)**
[D-Asp^3]MC-LR	**50**	**970**	cyclo -(D-Ala-L-Leu-D-**Asp-**L-Arg-Adda-D-Glu-Mdha-**)**
MC-LA	**50**	**909**	cyclo -(D-Ala-L-Leu-D-MeAsp-**L-Ala-**Adda-D-Glu-Mdha-**)**
MC-YA	**60-70**	**959**	cyclo- (D-Ala-**L-Tyr-D-**MeAsp-**L-Ala-**Adda-D-Glu-Mdha-**)**
MC-YR	**150-200**	**1044**	cyclo -(D-Ala-**L-Tyr-**D-MeAsp-L-Arg-Adda-D-Glu-Mdha-**)**
[6(Z)-Adda^5]MC-RR	**>1200**	**1037**	cyclo- (D-Ala-**L-Arg-**D-MeAsp-L-Arg-**6(Z)Adda-**D-Glu-Mdha**)**
[Dha^7]MC-RR	**180**	**980**	cyclo -(D-Ala-**L-Arg-**D-MeAsp-L-Arg-Adda-D-Glu-**Dha-)**
MC-RR	**500**	**1037**	cyclo -(D-Ala-**L-Arg-**D-MeAsp-L-Arg-Adda-D-Glu-Mdha-**)**

MC are **conjugated with reduced glutathione (GSH)** in the liver of some aquatic organisms, rodents and humans.

The reaction involves the α,β-unsaturated carbonyl of the N-methyl-dihydroalanine moiety (opposed to Adda). The nucleophilic reaction occur spontaneously at alkaline pH and enzymatically, catalysed by **Glutathione-S-transferases**. **The conjugates have negligible phosphatases inhibitory activity** and are excreted in the urine.

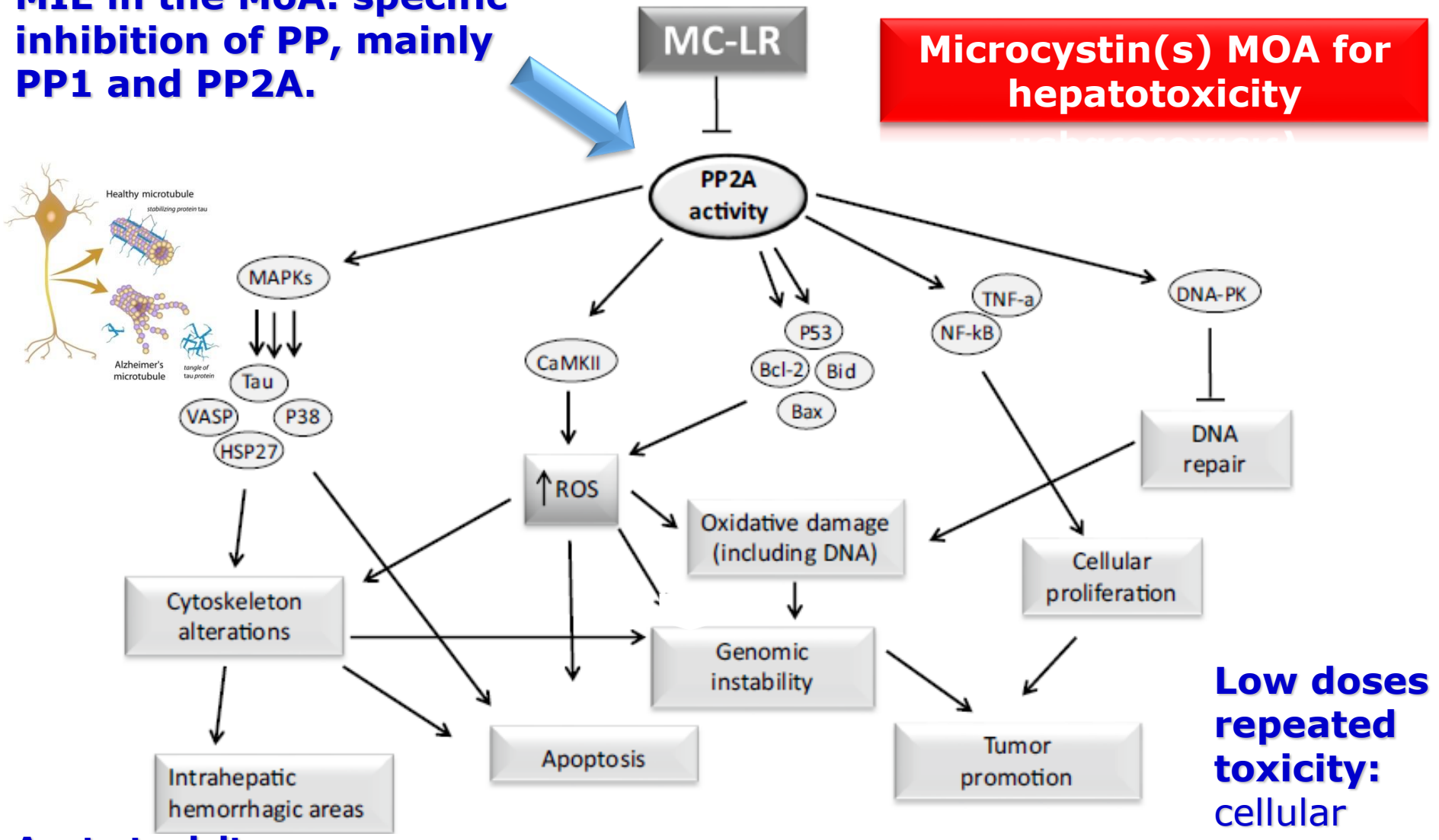




Buratti et al, 2011, 2013

- ✓ The enzymatic reaction adds to the spontaneous one, enabling efficient detoxication.
- ✓ **Differences between species** (Buratti and Testai, 2015)
- ✓ **Differences among variants** In vitro detoxication reaction (spontaneous plus enzymatic) seems to be **favoured by the hydrophilicity of the variant** (the most lipophilic LF is poorly conjugated) (Santori et al, 2020)
- ✓ **Differences among variants in the absorption** due to different affinity for transporters (Turco et al, 2022)

MIE in the MoA: specific inhibition of PP, mainly PP1 and PP2A.



Acute toxicity:
Cascade of events ⇒ damage of sinusoidal capillaries ⇒ intrahepatic hemorrhagic areas ⇒ centrolobular toxicity.

Buratti et al, Arch. Tox. (2017)

Low doses repeated toxicity:
cellular proliferation and hepatic hypertrophy ⇒ tumor promotion

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In raw waters MCs are therefore present as a mixture of MC variants and the efficiency in removal to produce drinking water is not necessarily the same for all the variants.
MC toxicity is variant specific

By adopting a conservative approach, **acute toxicity may be referred to MC-LR equivalents** (considering all the variants having exactly the same toxicity of MC-LR). For MC-LR an acute NOAEL has been derived

Since this congener is the most acutely toxic, the toxicity of the mixture is likely to be overestimated by this approach.

However, in case this **tier 0** approach gives rise to exposures not exceeding the HBGV, there is no need to proceed.

Acute risk: MC-LR

- Acute ip NOEL 25 µg/kg bw (lowest value)
- Correction factor (CF) = x 10 (conservative approach) since there is a 30-100 fold difference between the oral and i.p. routes of exposure
- UF = 100 for inter- e intra-species variability
- **Acute no-effect dose = 2.5 µg/kg bw (150 µg for an adult of 60 kg)**

- Steepness of the dose/response curve: the value very close to the potential LOAEL for hepatic effects in humans after oral administration (= 5 µg/kg bw, obtained by dividing the LOAEL in mice for a UF=100, accounting for inter- and intra-species extrapolation).
- Caution is needed when the exposure to MC-LR is close to the acute no-effect dose.
- The data are however only indicative, and taken as a pragmatic approach: the WHO itself considers the data base not robust enough to derive a HBGV.

Toxin	Concentration in the mixture (µg/L)
MC-LR	30
MC-RR	100
MC-YR	15
NOD	20
MC-AR	60
Total	225 µg/L

For an adult drinking 2 L in a day the intake is 450 µg

For a 20kg child drinking 1 L in a day the intake is 225 µg (with a no effect threshold of 50 µg)

Some acute risk could be expected

Since the MoA is the same it is possible to use the **"Toxicity equivalent factor"**

Method used for polychlorinated dibenzo[p]dioxins (PCDD), among which the reference compound is the most toxic congener, 2,3,7,8-TCDD, to which a default TEF value of 1 is attributed. The specific TEF for the other congeners is established by comparison of their toxicity potency with TCDD as 1.
The toxicity of the mixture is then obtained by summing up the product of specific TEF with the concentration of the related congeners.

The reference cyanotoxin is MC-LR, with TEF = 1; the TEF of a specific variant (X) is derived as the ratio between the LD_{50} values, according to the equation:

$$\mathbf{TEF_X = LD_{50}\ MC\text{-}LR / LD_{50}X}$$

The total acute toxicity of the mixture is estimated by the sum of all the individual toxicity equivalents obtained as the product between the specific TEF and the toxin concentration.

Application of TEF method to a mixture of MCs and NOD*

Toxin	Concentration in the mixture (μg/L)	i.p. LD_{50} (μg/kg)	TEF	Toxicity Equivalent
MC-LR	30	50	1.0	30
MC-RR	100	500	0.1	10
MC-YR	15	150	0.33	5
NOD	20	50	1.0	20
MC-AR	60	250	0.2	12
Total	225			77

TEF = Toxicity Equivalent Factor. i.p. = intraperitoneal.
*As described by Wolf and Frank (2002).

By applying the TEF approach to the mixture the Toxicity Equivalents for acute toxicity are greatly reduced

An acute risk could be reasonably excluded

Still limitations are present since the data are from i.p. administration and kinetic differences have been observed between i.p. and oral route of exposure
For the moment it is not possible to apply this approach to repeated toxicity, since only data on MC-LR are available, solid enough to compare repeated toxicity potential among variants

TDI of MC-LR

Lowest subchronic NOEL(mouse)=40 µg(kgx d)$^{-1}$

UF= 10 interspecific variability
(≠ TK: ≠ OATP and GST animal/human;
≠TD: ≠ sensitivity of targets)

10 intraspecific variability

10 lack of chronic tox data

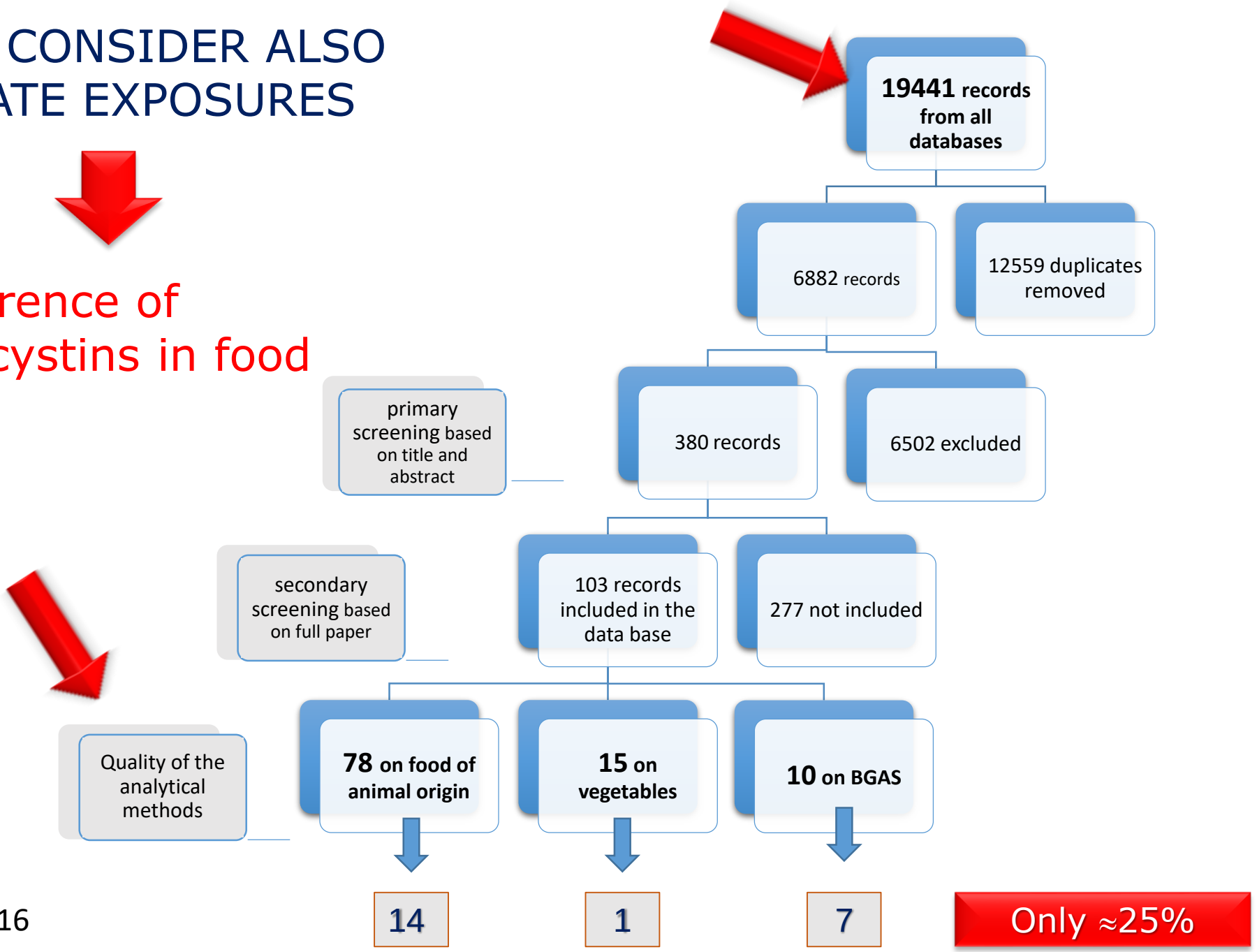
TDI= NOEL/1000=0.04 µg(kg bw x g)$^{-1}$ → 2.4 µg/d per person (60 kg bw)

The TEF approach for subchronic or chronic toxicity cannot be applied as data for variants other than MC-LR are not available. WHO consider the use of MC-LR eq

Limits : extrapolation from one congener to the others, from potency data on i.p. acute toxicity on some of them to potency in chronic toxicity .
Possibility of different target organs based on transporter expression, different detoxication kinetics and different phys-chem properties leading to different repeated toxicity.

NEED TO CONSIDER ALSO AGGREGATE EXPOSURES

Occurrence of Microcystins in food



Testai et al, 2016

A score of reliability for the used analytical methods :
1 = reliable without restriction (validated method),
2 = reliable (fully characterized method),
3 = reliable with restriction (only recovery included),
4 = not reliable (no recovery included) EXCLUDED

The availability of reliable analytical method is one of the major issues considering the topic of cyanotoxins contaminations of complex matrix, as food items are.

1. Free vs bound to protein content
2. Strong matrix effects reported depending on the matrix and on the toxins
3. Different clean-up steps used, % recovery rarely reported
4. Elisa gives only semiquantitative results, with cross reactivity among variants

MC concentrations and relative intakes in crustacean and mussels.

	Crustacean (4 data)		Mussels (4 data)	
	MC conc in µg/kg	MC intake (**µg**) considering a consumption of 250 g (or 400g) of crustacean	**MC conc in µg/kg**	**MC intake (µg) considering a consumption of 250 g (or 400g) of mussels**
Mean	689	**172 (275)**	**19828**	**5000 (8000)**
Median	217	**54 (87)**	**2271**	**600 (900)**
Max value	2250	**563 (900)**	**74700**	**19000 (30000)**
P95	1962	**491 (785)**	**64155**	**16000 (26000)**

MC intakes for fresh-water crustacean species are close to the acute risk threshold 150 µg/day for an adult if the mean consumption is used for estimating the daily intake.

MC intakes by eating mussels (*Mytilus edulis* and *Mytilus gallo-provincialis*, commonly consumed by the general population) are up to 100 fold higher than the acute risk threshold. The possibility that mussels are grown in aquaculture plant in estuarine and coastal areas, in which cyanobacteria can be present, makes this data a possible concern

Need for more 'good quality' data and for a refinement

Open questions……

once established the need to consider specific mixtures in drinking water (see the gatekeeper step in the problem formulation)

Do we have information on the co-occurrence of

1. mixtures of toxins
2. mixtures of other contaminants (e.g. persistent chemicals such as **dioxin like compounds**? **Pesticides**? Metals?)
3. mixtures of toxins and other contaminants

If yes, and starting from the co-occurrence

- is the data base for the toxicological properties of toxins able to support a combined risk assessment?
- Can we group different toxins based on their MoA or target organ?
- Can we identify the data gap and try to prioritise them (also on the basis of what we already know)?
- Which are the other chemicals we need to prioritise? Starting from the often well-known toxicological profile, are present co-exposure and estimated one in changing scenarios the drivers for it?

THANKS!