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Assessment of Mixtures - Review of Regulatory Requirements and Guidance

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Abstract

Humans and the environment are continuously exposed to a multitude of substances via different routes of exposure. However, the risk assessment of chemicals for regulatory purposes does not generally take into account the "real life" exposure to multiple substances, but mainly relies on the assessment of individual substances. This report summarises the different methodologies that are used to assess the toxic effects of mixtures. It also provides an overview of current legislation in the European Union (EU) that deals with the safety assessment of chemicals in different matrices and the extent to which the current legislation addresses the toxicological risk of mixtures. Relevant Guidance Documents from the EU and other countries (USA, Canada) and international organisations, such as the World Health Organisation (WHO) and the Organisation for Economic Cooperation and Development (OECD) are also included in this review.

Executive Summary

Humans and the environment are continuously exposed to a multitude of substances via different routes of exposure. However, the risk assessment of chemicals for regulatory purposes does not generally take into account the "real life" exposure to multiple substances, but mainly relies on the assessment of individual substances. This report summarises the different methodologies that are used to assess the toxic effects of mixtures (Chapter 1). It also provides an overview of current legislation in the EU that deals with the safety assessment of chemicals in different matrices and the extent to which the current legislation addresses the toxicological risk of mixtures (Chapter 2). Relevant Guidance Documents from the EU and other countries (USA, Canada) and international organisations, such as the World Health Organisation (WHO) and the Organisation for Economic Cooperation and Development (OECD) are also included in this review (Chapter 3).

Some mixtures that need to be assessed are intentional and thus have a known composition, e.g. personal care products, food additives and pesticides. However, in many cases, mixtures are unintentional and (largely) of unknown composition, e.g. the combination of dozens to hundreds of substances in surface water, drinking water and air. Two approaches are used to assess mixture toxicity: testing of the whole product/mixture, and testing of the individual substances followed by mathematical modelling to predict the combined effect. Testing of the total mixture is frequently applied for environmental samples, as it has the advantage of assessing the toxicity of mixtures of unknown composition. However, the substances driving the overall response frequently remain unidentified. A more common approach is to consider the toxicity of the individual constituents, but this requires more information regarding identity, concentration and toxicity (including mode of action) of the substances, which is frequently lacking. To some extent, missing information regarding concentration and toxicity of known chemicals can be estimated using computational models.

Several mathematical models are commonly applied to estimate the combined toxicological effect of known substances. Based on the mode of action, these models either assume that substances act independently (independent action, IA) or additively (dose or concentration addition, CA). IA models regard a combined effect to be the result of statistically independent random events, and no risk is anticipated as long as exposure concentrations do not exceed zero-effect levels. In contrast, CA models are based on pharmacological concepts like receptor binding and assume that the response of substances are additive if they share the same biological target. Generally, models assuming dose addition are the most frequently applied, because they provide reliable estimates of combined effects and are regarded to be more conservative than independent action models. Other models can also take into account antagonistic and synergistic effects, although the relevance of these interactions at the low concentrations generally encountered is still matter of debate.

Several pieces of EU legislation are in place to regulate single and multiple compounds. These compounds and mixtures can be intentional (i.e. products) and they are generally relatively simple in their composition. Hence, their toxicity can usually be assessed relatively easily based on knowledge of their individual components, or by toxicity tests on the final

product mixture. The following pieces of EU legislation have been identified that apply to intentional mixtures:

- Industrial chemicals and related intentional mixtures are assessed under REACH (Regulation No 1907/2006). Risk assessment focuses on human and environmental (mainly aquatic) risk. Substances and mixtures are divided into different hazard classes based on the Classification, Labelling and Packaging regulation (Regulation No 1272/2008), which has specific provisions for assessing mixtures.
- Plant Protection Products (PPPs) (Regulation No 1107/2009, 283/2013 and 284/2013) and biocides (Regulation No 528/2012) are generally mixtures composed of one or more active substances. Their safety is mainly assessed based on the individual substances and the final products. Attention is given to cumulative and synergistic effects.
- Human and veterinary pharmaceuticals are generally applied as mixtures. The risk assessment of human pharmaceuticals (Directive 2001/83/EC) focuses on humans, including potential interactions with other pharmaceuticals. An environmental risk assessment is also required but no attention is given to cumulative or mixture effects. Veterinary products (Directive 2001/82/EC) are assessed with emphasis on animal, human and environmental risks. The ecotoxicity assessment of veterinary mixtures is more extensive than for human pharmaceuticals, but joint occurrence with other pharmaceuticals or other pollutants is not taken into account.
- Cosmetic products (Regulation 1223/2009) usually consist of several substances, and their risk can be assessed on the basis of individual ingredients, combinations thereof or the whole product. Animal testing is not allowed. The anticipated exposure to the individual ingredients has to be taken into account, including possible interactions. Environmental risks are not addressed but are considered to be assessed via REACH.
- Food and feed stuff can be regarded as complex mixtures. Additives (Regulation No 1333/2008) are assessed for toxicity, but the assessment of mixtures is mainly based on individual compounds. Cumulative toxic effects have to be taken into account, but how the cumulative assessment should be conducted is not specified.
- Human risk assessment via toys (Directive 2009/48/EC) focuses on individual compounds that should not be used in toys, with emphasis on the sensitivity of the target group (i.e. children). Only individual chemicals (beside nitrosamines and nitrosable compounds) are taken into account. For the toxicological properties, reference is made to REACH and the classification and labelling legislation.

In contrast to intentional compounds and mixtures, unintentional mixtures (contaminants, by-products and environmental pollutants) are much more challenging to assess, because they are of varying and often complex composition, and many of the substances present are unidentified and toxicity data are lacking. Legislation generally includes limit values for some of the known individual constituents. The following pieces of

(mostly environmental) legislation have been identified that address the toxicological risk of unintentional mixtures:

- Regulations on contaminants in food (Regulation No 315/93/EEC and follow-up regulations) contain limit values for individual contaminants and generally do not take mixture toxicity into account, with the exception of dioxins and dioxin-like compounds. Food contact materials are addressed separately (Regulation No 1935/2004), considering cumulative effects (undefined) but not mixture toxicity. Pesticide residues in food and feed are also regulated separately (Regulation No 396/2005), which acknowledges the need for cumulative and mixture toxicity assessment. This has recently been addressed by EFSA.
- Water contaminants in general are regulated under the Water Framework Directive (Directive 2006/60/EC), with the related Groundwater Directive (Directive 2006/118/EC) in place to address groundwater specifically. Both focus on limit values for individual chemicals and do not specifically address mixtures or aggregated exposure. However, reference is made to Environmental Quality Standards (EQS) (Directive 2013/39/EU), which do address mixture toxicity. The marine environment is covered separately by the Marine Strategy Framework Directive (2008/56/EC), which emphasises that risk assessment should also consider cumulative and mixture effects but without further specification.
- The Drinking Water Directive (98/83/EC) regulates the maximum levels of contaminants in drinking water. It includes a list of individual compounds but mixture toxicity and cumulative exposure are not specifically addressed.
- Soil quality is addressed by several regulations. A thematic strategy for soil protection is in place (COM(2006)231) and a Directive has been proposed (COM(2006)232). The focus of the proposed directive is on soil function, pollution and risk, but mixture toxicity is not specifically addressed.
- The Directive on ambient air quality (Directive 2008/50/EC) contains target values as limits for individual compounds, but no special attention is given to mixture toxicity.
- Waste production is addressed by the Waste Framework Directive (Directive 2008/98/EC). It does not directly address the toxicological assessment of waste, but rather the management and refers to the legislation on classification and labelling for toxicity assessment. It covers general waste streams but excludes some specific industrial wastes and other waste streams which are covered by other (environmental) legislation. The Waste Framework Directive is linked to the Directive on Integrated Pollution Prevention and Control (IPPC) (Directive 2010/75/EU), which addresses (waste) emissions to the air, including waste incineration. The IPPC does not address mixtures with the exception of dioxins and furans.
- To ensure the safety of workers against chemical agents at work, Directive 98/24/EC specifies maximum levels for individual substances. It also refers explicitly to chemical agents in combination, covering both intentional and coincidental mixtures.

- A separate directive (Directive 2011/92/EU) is in place for the environmental impact assessment of large scale public and private projects (e.g. motorways, airports) that are likely to have significant effects on the environment. This includes estimations of emissions of pollutants, including cumulative effects, but mixture toxicity is not specifically addressed.

In addition to the existing pieces of EU legislation, several guidance documents and reviews are available that provide information on how to apply risk assessment of mixtures. These are mostly based on combined exposure to different substances with a similar mode of action. Special attention is given to exposure via different (dietary) routes and mathematically based physiological models are recommended to determine the potential uptake. The European Food Safety Authority (EFSA) has proposed a tiered approach for cumulative risk assessment, focusing on human exposure to pesticides. However, in principle the concepts developed could be applied to other groups of chemicals (e.g. PAHs and flame retardants). An opinion of the European Commission's non-food scientific committees (SCCS, SCHER and SCENIHR) acknowledged the relevance of joint exposure, but emphasised that regarding human health the assessment of individual substances is sufficient for dissimilar acting substances. However, it was concluded that environmental effects cannot be excluded, even when concentrations are below the individual no effect concentrations. Major knowledge gaps exist however, regarding mode of action, environmental or human concentrations, and toxicity of individual components. Approaches that have been put forward generally apply the CA concept, as is recently applied in an EU regulation on biocidal products.

Several non-EU guidance documents are also available that address mixture toxicity. Especially in the USA, guidance documents have been in place for decades that focus on the toxicity of mixtures, and several governmental bodies including the Environmental Protection Agency (EPA), Food and Drug Administration (FDA) and the Agency for Toxic Substances and Disease Registry (ATSDR) are involved in the assessment of mixtures. Several acts are in place requiring cumulative risk assessment, like the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), the Food Quality Protection Act and the Safe Drinking Water Act. The US EPA has published several guidelines dealing with the risk assessment of chemicals, including mixtures. These generally do not consider interactions, but focus on grouping of chemicals and on the basic concept of addition. In 2008, the National Research Council emphasised the need for mixture toxicity assessment and the grouping of compounds based on their adverse outcome rather than structural similarity. The Canadian Environmental Assessment Review Office has a reference guide on cumulative environmental effects, under the Canadian Environmental Assessment act. It considers the combined effects of human activities on the ecosystem, not only including chemical pollution but also for example global warming and loss of biodiversity and takes aggregated exposure into account.

In addition to legislative initiatives above, the WHO, together with the FAO, has also been working on several projects that address the human and environmental risk assessment of chemicals, including (sometimes specific) mixtures. Others focus solely on

ecotoxicological effects, e.g. addressed in recent publications by ECETOC or OSPAR. All stress the relevance of mixture toxicity assessment and acknowledge the lack of information that is hampering the practical application of these concepts and approaches.

In conclusion, while many pieces of EU legislation are in place to protect humans and the environment against adverse effects of chemicals including mixtures, in many cases it remains unclear how this is to be carried out and only few explicitly consider (real life) exposure to mixtures. In cases where mixtures are considered, the assessment is frequently limited to some well-known components. Several mathematical models and approaches have been developed to assess the toxicity of mixtures, but their routine application is hampered by considerable information gaps.

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1 - Introduction

Recent decades have witnessed an important increase in the synthesis, manufacture and use of chemicals worldwide, in both developed and developing countries. As a consequence, humans and the environment are exposed continuously to large numbers of chemicals simultaneously or sequentially and through multiple exposure routes often in low concentrations; i.e. they are exposed to combinations of chemical in time and in space, from different sources, and most often chronically. The term "chemical mixtures" refers to such a combined exposure to multiple chemicals, and this term is defined as any set of multiple chemicals, regardless of their source, that may or may not be identifiable and that may contribute to joint toxicity in a target population (Mumtaz et al., 2010).

Chemical mixtures can be separated into three types:

-"Intentional mixtures", which are mixtures that are intentionally manufactured as such (i.e regulated and manufactured products such as pesticide formulations or laundry detergent)

-"Generated mixtures" contain additional compounds that are by-products of processes involved (e.g. smelting, drinking water disinfection, fuel combustion); they are usually originating from a single source

-"Coincidental mixtures" are composed of unrelated chemicals from different sources, but having the potential to reach the same "receptor population", e.g. by their presence in or migration into the same medium (e.g. groundwater), or through multiple pathways (ATSDR, 2004).

A distinction is also made between simple and complex mixtures based on their composition: a simple mixture consists of a relatively small number of chemicals (e.g ten or fewer), and its composition is qualitatively and quantitatively known (ex: cocktail of pesticides, a combination of medicines or a group of allyl alcohol esters), whereas a complex mixture is a mixture that comprises tens, hundreds or thousands of chemicals, the composition of which is qualitatively and quantitatively not fully known (ex: a workplace atmosphere, drinking water, welding fumes but also products based on natural ingredients like plant extracts) (Feron and Groten, 2002).

The US Agency for Toxic Substances and Disease Registry (ATSDR) also defines the term "similar mixtures" as mixtures having the same chemicals but in slightly different proportions or having most but not all chemicals in common and in highly similar proportions. Similar mixtures are thus expected to have similar fates, transports, and health effects (ATSDR, 2004).

Thus, long term exposures to low doses of chemical mixtures are now the rule rather than the exception, which implies multiple potential interactions between chemicals, and risk assessment must cope with such challenges. This is especially prominent when it comes to endocrines disruptors (EDs) exposure, as they can be present at very low doses and originate from multiples sources (e.g. drinking water, diet, residential environment). Thus they lead to a final exposure which is much higher than the individual concentrations. Moreover, the mixture may display a complex mode of action compared to its constituents due to different

interactions (i.e. mixture effects), which are usually ignored (Danish Ministry of the Environment, 2009). This is also true for any kind of long term exposure to bioaccumulating substances, which could over time result in a final high level of exposure of the individual, although the environmental concentrations are low.

Nevertheless, human health risk assessment in a regulatory context is still mainly based on *in vivo* toxicity data generated from exposure to a single substance through a single exposure route and medium. Uncertainty factors are then applied to those data to allow for interspecies and inter-individual variation, but they generally do not take into account the possibility of combined actions or interactions between substances. However, from a public health point of view it is essential to take into account the combined exposure, including potential interactions that can influence the overall effect. Another approach consists in testing the mixtures itself (whole mixture testing), especially in the case of the registration of chemical mixtures under sectorial regulations such as the plant protection products and biocidal products regulations. However, for ethical and economical reasons, and in the light of the Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU), the EU policy now aims at reducing the use of laboratory animals and at promoting alternative methods: thus, *in vivo* testing of the whole mixture is not an option any more. Moreover, testing of individual mixtures is also hardly possible due to the almost infinite number of possible different combinations.

It should also be highlighted that an individual substance, depending on its type and/or expected use, can be subject to various EU and national legislation and might need to be specifically authorized for a certain use. The actual risk assessment requirements are defined for a certain use within specific pieces of EU legislation (e.g. as an active ingredient in a plant protections product or pharmaceutical) and do not take into account that the end user could be the same and exposed to higher doses of a specific substance than foreseen in the individual risk assessments for different uses.

In other words, current regulatory requirements do not generally address the exposure to a single substance by multiple pathways and routes of exposure, following its possible different uses (i.e the so-called "aggregated exposure"). Exposure to multiple components sharing a common mechanism of action (i.e the so-called cumulative exposure) might also pose a health problem even if the individual components are present at levels below their respective NOAELs (No Observed Adverse Effects Levels); however the risk assessment regimes under the different existing regulations do often not take into account such risks.

For all these reasons, there is a need to develop a consistent, cross-sector approach to deal with the combined exposure to multiple chemicals.

On 22nd December 2009, the Council expressed some concerns on the "combination effects of chemicals" and invited the Commission, *"... to assess how and whether relevant existing Community legislation adequately addresses risks from exposure to multiple chemicals from different sources and pathways, and on this basis to consider appropriate modifications, guidelines and assessment methods, and report back to the Council by early 2012 at the latest."* On 31 May 2012 the Commission reported to the Council and engaged to launch a new process to ensure that risks associated with chemical mixtures are properly

understood and assessed (EC, 2012a). Under the new approach, the Commission will identify priority mixtures to be assessed, ensure that the different strands of EU legislation deliver consistent risk assessments for such priority mixtures, and tackles some of the data and knowledge gaps to improve understanding of the mixtures to which people and the environment are exposed. The new Commission approach draws heavily on the recent opinion of the three non-food scientific Committees (EC, 2011b) as well as on the "State of the Art Report on Mixture Toxicity" (Kortenkamp et al., 2009) a study contracted in 2007 by DG Environment and completed in 2009, which review the current scientific knowledge and regulatory approaches.

In this context, the objective of this review was to update the work previously done on the analysis of EU regulations and risk assessment regimes and requirements regarding mixtures toxicity. The first part of this review summarises the different theoretical and mathematical approaches used in the risk assessment of mixtures, whereas the second part of the review focuses on the requirements across EU legislative sectors for both human health and the environment. Finally, the third part reviews activities in the area of development of guidance and risk assessment methodologies by Member State (MS) authorities, agencies and international organisations.

2 - Theory and methodology underlying the risk assessment of chemical mixtures

2.1 Source of exposure and environmental fate

Regarding human health, exposure to mixtures can result from the use of specific (intentional) chemical mixtures (i.e. one single source of exposure to the several components of the mixture), in a particular context (i.e. occupational settings) or from diverse environmental sources of one or more chemicals (e.g. food, water, air). This is the case of the exposure of the public, in which exposure may occur via multiple pathways, routes, and media (aggregate exposure). Therefore, in order to do a realistic risk assessment, aggregated and cumulative exposure from all sources to multiple chemicals may need to be considered.

When assessing the risk for occupational exposure, the chemical composition of the resulting mixture to which the worker is exposed, is often known and direct measurements and various models for estimating the exposure are available. This is not the case when assessing general public exposure, in which case the chemical composition of the mixture is often unknown and no, or very limited measured data, are available. Therefore, it is necessary to rely on assumptions and simplification and to use modelling to provide relevant exposure estimates. For a worst case estimate, it is possible to assume maximum exposure to each component of the mixture based on the assessment of daily exposure from all sources, although information to support such an assessment is rarely available (EC, 2011b).

Environmental exposure is even more complex, as it results from intricate patterns depending on emissions, point releases, fate, distribution and persistence of chemicals in the different compartments (i.e. water, sediment, air, soil and biota). The environment is continuously exposed to a wide range of anthropogenic compounds (and their breakdown products), including industrial mixtures (e.g. plant protection products) containing several active substances with possibly different environmental behaviour, but also chemical mixtures emitted by specific human activity (such as complex effluent), and resulting mixtures from multiple emission sources (i.e the combination of all the emissions of human activities in a given territory). In the case of commercial mixtures and mixtures emitted by specific human activities, the composition of the mixture is likely to be known and characterised, at least partly and at a local scale (i.e. emission point). However, the composition may change with time due to differences in environmental fate of each substance. Moreover, regarding plant protection products for example, even if theoretically the risk of a mixture of active substances in one commercial formulation is considered in the RA process, in practice most of the time different formulations are combined before field application and the resulting mixtures are not the same as those assessed in the authorisation process. In the case of mixtures resulting from multiple emission sources, the composition of the mixtures is rarely known, and probably even fluctuates in time and space as it might concern a bigger territory (i.e. a hydrographic basin). Whereas the two first cases could be linked to the implementation of prospective risk assessment schemes using modelling to predict mixture toxicity, the third case could be addressed by retrospective risk assessment

approach, aiming at assessing the impact of an unknown mixture on an ecosystem when compared to a reference state.

Thus, due to e.g. specific and different physico-chemical properties of the components, their potential for biodegradation, abiotic and biotic environmental factors, the climate, the geography and the time of the year, the composition of any kind of mixture in the environment at a given time may be completely different from those of the originally emitted mixture, and significant differences should be expected between the mixture which is released and the mixture to which organisms are exposed.

To simplify the mixture issue, instead of considering every single substance, one approach consists in grouping chemicals in "blocks" of chemicals having similar structure, since they might have similar physico-chemical and environmental degradation properties, similar distribution and similar environmental fate. These blocks should also contain substances with a similar mode of action and a narrow range of toxicity in order to make this approach reliable. Once the blocks are defined, PEC values (Predicted Environmental Concentrations) can be calculated for each block in each environmental compartment (King et al., 1996). This approach, the so-called "hydrocarbon block method", is used to perform risk assessments of complex petroleum substances (ECHA, 2008a).

Moreover, the distribution in different environmental compartments can be predicted by modelling, and is usually based on individual substances (i.e the FOCUS models for pesticides). Mixture compositions may then be obtained through their combination (EC, 2011b), as the assumption is made, at environmentally relevant concentrations, that the distribution of each component of a mixture is not influenced by the physico-chemical properties of the other components, although biodegradation of one component can be influenced by the presence of others. Up to now, no satisfactory model has been developed for modelling mixtures' biodegradation, which is probably due to the complexity of the biochemical mechanisms implicated in this process (substrate competition, metabolism, enzyme induction etc.).

2.2 Whole-mixture analysis and component-based analysis

The hazard of chemical mixtures can be assessed as a whole (whole-mixture analysis, or top-down approach), or based on the individual components of the mixture (components-interaction analysis or bottom-up approach).

Whole mixture effects can be assessed by testing the mixture itself, but can also be based on data generated with a mixture of similar composition (i.e. close in composition regarding components and proportions). If adverse effects are found in relevant toxicity studies, a quantitative assessment can then be carried out directly from these data. This approach allows consideration of any unidentified materials in the mixtures and any interactions among mixture components, but it does not identify the chemicals responsible for interactions, and does not provide any information on the toxicity of individual mixture components. Moreover, this approach is restricted to mixtures that do not significantly change in their composition, and is therefore not recommend using as a general approach (EC, 2011b).

A more detailed approach, which is generally used when the components of the mixture are known, is to assess the combined action of the components. In this analysis, the choice of the experimental design to follow is dependent on the complexity and number of components of the mixture, and the main consideration is whether the mixture components act by the same mode of action (MOA) or whether they are functionally independent (Groten et al., 2001). Its optimal use is therefore dependent on the knowledge of the MOA of the individual components, or on the information regarding their association with groups of chemicals demonstrating similar or identical MOA (assessment groups). Such information may be based on chemical structures and structure-activity relationships (either qualitative or quantitative), molecular modelling, structural alerts or on toxicological responses or effects (EC, 2011b).

Another component-based approach consists in grouping chemicals according to their toxicological effects. In this approach, hazard information is used to identify and group chemicals that have similar endpoints and a common toxic effect, to then assess the cumulative effects of the group of chemical, even if the underlying MOA are not known.

2.3 Mathematical approaches used in the component-based approach

Three basic types of action for combination of chemicals are defined: (i) dose or concentration addition, which implies a similar MOA of the substances in the mixture; (ii) independent action or response addition, which implies a dissimilar MOA of the substances in the mixtures; and (iii) interactions in between substances in the mixture. The term interaction includes all forms of joint action that deviate from either dose or response addition. Hence, the combined effect of two or more substance is either greater (synergistic, potentiating, supra-additive) or less (antagonistic, inhibitive, sub-additive, infra-additive) than that predicted on the basis of dose addition or response addition. The two first concepts are based on the assumption that substances do not influence each other's toxicity by interacting at the biological target site, and they have been suggested as default approaches in regulatory risk assessment of chemical mixtures (EC, 2011b), although chemical mixtures are rarely composed of either only similarly or of only dissimilarly acting substance. Those three approaches for hazard assessment and risk characterisation of multiple chemicals are mostly applied to component-based approaches and further described below.

2.3.1 Dose or concentration addition

Synonyms: simple similar action, similar joint action, relative dose-addition, or concentration addition.

This model assumes that the effects can be estimated directly from the sum of the dose or concentrations of similarly acting substances in the mixture, scaled for their potencies. This model is based on the pharmacological concepts of ligand binding site theory, affinity, potency, and receptor occupancy: receptor occupancy is proportional to the concentration of the ligand and its affinity for the receptor. Thus, the magnitude of the biological response to the chemical mixture can be predicted by summing the doses of the components after adjusting for the differences in potencies (USEPA, 2007a; EFSA, 2008b). In practice, doses or concentrations of the single substances are added after being multiplied by a scaling factor accounting for differences in the potency of the individual substances.

According to the literature, this model provides reliable estimates of combined effects and is appropriate for risk assessment of a mixture of chemicals if the substances share either a strictly identical molecular mechanism of action or belong to the group of baseline toxicants (acting by nonpolar narcosis) (Feron and Groten, 2002; Kortenkamp et al., 2009). However, for other authors, the evaluation of dose-additivity or non additivity is a matter entirely for observation using measured dose-response curves, with no required consideration of mechanism of action (NRC, 2008). Those authors also highlight several observations of particular interest about the definition of dose addition:

- The dose additivity of a particular mixture does not imply the dose additivity of other mixtures of the same components. Mixtures of the same components may be non-dose additive for different component doses, indeed, may be synergistic at some combinations of component doses, and antagonistic at others.
- Conclusions about dose addition, synergism, or antagonism may not be the same for different levels of effect even for similar mixture ratios.

Methods for dose/concentration addition approaches most frequently used in human health are the hazard index (HI), the reference point index (RfPI, or PODI for Point of departure index), or the toxic equivalency factor (TEF). They are presented in Table 1. The toxic unit (TU) model is often used in environmental toxicology (EC, 2011b).

2.3.2 Independent action (response addition)

Synonyms: independent action, effect addition, simple independent (or dissimilar) action and independent joint action.

Response addition occurs when the toxicological effects of the individual substances in a mixture are a consequence of separate mechanism or MOA, and possibly, but not necessarily, when the nature and sites of toxic effects differ between the chemicals in a mixture (USEPA, 2007a; EFSA, 2008b). This model assumes that a combination effect can be calculated from the responses of the individual components using the statistical concept of independent random events; response addition refers to the sum of probabilistic risks or incidence, whereas effects addition means the sum of biological responses. Excepted for non-threshold effect, no health risk is anticipated as long as the various exposure concentrations do not exceed respective zero-effect levels.

The toxicity of a mixture is expressed in terms of probability of an individual to be affected. For the ecological assessment, the reference value is the Predicted No Effect Concentration (PNEC), set at the population or community level, which therefore does not exclude effects on individuals. The population/community response is the aggregation of the individual effects, which depends on the biology of each species and their ecological role. Therefore a mixture of substances with independent action at levels below the ecological thresholds set at the population/community level, but above the threshold for producing effects on individuals, may have effects at the population/community level due to the "aggregated" outcome of the effect on each individual. This is not currently considered in the derivation of the PNECs and environmental quality standards (EQSs) and new scientific

developments are required for a scientifically sound assessment of this "aggregated" outcome (EC, 2011b).

2.3.3 Interaction: synergism and antagonism

Biological interactions might occur at multiple levels, and interference can occur at either the toxicokinetic level (i.e. occur during the processes of uptake, distribution, metabolism and excretion, for example chemicals modifying the absorption or active transport of other) or the toxicodynamic level (i.e. interactions between the biological responses resulting from exposure to the chemical and effects of chemicals on the receptors, cellular target or organ). For example, compounds that influence the amount of biotransformation enzymes can have large effects on the toxicity of other chemicals, as well as competition between substrates for the same pumps or for the biotransformation enzymes themselves. Interactions are translated by an effect differing from additivity based on the dose-response relationship of the individual components, and are categorised as less than additive (antagonistic, inhibitive, masking) or greater than additive (synergistic, potentiating); they may vary according to the relative dose levels, the route(s), timing and duration of exposure, and the biological target. Thus, for a realistic and accurate risk assessment, the interactions' consideration and their integration into the toxicity assessment of a mixture is important.

Antagonism happens when the combined effect of the mixture is less than explained by the total additive effect of the individual components. Masking occurs when components produce opposite or functionally competing effects on the same organ system, and diminish the effects of each other, or one overrides the effect of the other (ATSDR, 2004; USEPA, 2007a; EFSA, 2008b). The result being a decreased toxicity, antagonism is not an issue for risk assessment and the use of additive model could constitute a worst-case.

Synergism occurs when the effects of the mixture is greater than that estimated for additivity on the basis of the toxicities of the components. Potentiation occurs when a component that does not have a toxic effect on an organ system increases the effect of a second chemical on that organ system.

A toxicologically significant synergistic effect can happen in the following cases (EC, 2011b):

- if one or more components significantly enhance the uptake or significantly inhibit the excretion/clearance of other components
- if one or more components exert their action via the formation of toxic metabolites and induce the drug metabolising enzymes involved in the formation of these metabolites
- if two or more components act on different enzymes in an important metabolic pathway
- if two or more components act on different elements of cellular protection mechanisms or cellular repair mechanisms.

Recently, guidelines and guidance have incorporated chemical interaction concepts and have suggested methods to evaluate the possible influence of such interactions on the overall joint toxicity of chemical mixtures (ATSDR, 2004; USEPA, 2004; WHO, 2009; EC, 2011b).

Table 1: **Mathematical approaches for assessing chemical mixture effects (Boobis et al., 2008; EC, 2011b)**

	Hazard Index	Hazard Index Interaction	Reference Point Index
Principle	Sum of the hazard coefficient (HQ : ratio between exposure and respective reference value, RV) for each compound in the mixture	Incorporate available interaction data by converting them into a numerical score (based on expert judgement or WoE evaluation)	Sum of the exposures to each compound expressed as a fraction of its respective reference point (RP) for the common toxic effect *(e.g. the dose that causes a 10% effect, BMD10; or the NOAEL, etc…)*
Formula	$HI = \sum HQ$ $= (Exp_1/RV_1) + (Exp_2/RV_2) + (Exp_3/RV_3)\ldots$	$HI_{int} = HI \,.\, UF^{WoE}$ *- WoE value<0 for antagonistic interactions and >0 for synergism* *- UF: default value=10*	$RPI = Exp_1/RP_1 + Exp_2/RP_2 + Exp_3/RP_3\ldots$
Interpr -etation	- HI < 1: combined risk acceptable - HI > 1: potential health concern to be considered.	- HI < 1: combined risk acceptable - HI > 1: potential health concern to be considered.	If RPI*UF < 1: combined risk acceptable (UF: chosen group uncertainty factor, usually a default value of 100)
Advantage	- Is directly related to a RV, a long-used and well-understood index of acceptable risk - Can be quickly applied - Can accommodate the application of CSAFs early in the process	Takes into account the nature of interaction (synergism/antagonism), the quality of the data, the plausibility of the interaction under exposure condition, the relevance for human health	Sums the exposures to the different components in relation to their relative potencies - A single group UF can be applied as the last step in the process - CSAFs can be applied if needed.
Disadvantage	- RVs obtained by application of an UF that might incorporate policy and scientific judgements: → does not represent a true measure of the relative toxicological potency of the compounds. - Requires HBGV to be available for all members of the assessment group, which is often not the case	-Provide only a numerical score of the potential risk -HI and HI_{int} are strongly affected by a subjective evaluation -The intrinsic uncertainties affecting RVs are combined and amplified in HI_{int} derivation	Interpretation of the results relies on the value of the UF, for which the criteria are undefined
Nota	- An adjusted HI (aHI) can be calculated when the UF used to derive the RV is partly unrelated to the common toxic effect (Boobis et al., 2008). - Preferred approach when extensive mechanistic information is not available -A refinement of the hazard method, the Target-organ toxicity dose (TTD) method, was developed in order to accommodate the assessment of mixtures whose components do not all have the same critical effect, and to take into account the reality that most components of waste-site-related mixtures affect other organs at doses higher than those that cause the critical effects (Mumtaz et al., 1997)	An additional factor (M, ratio between the observed effective dose (ED) and the ED predicted from dose addition approach) can also be introduced, to include a quantitative evaluation of the interaction	Reciprocal of the RPI: combined margin of exposure $MOET = 1/[(1/MOE_1)+(1/MOE_2)+(1/MOE_3)\ldots]$ with MOE=RP /Exp When MOET>100 (or other value considered appropriate by the risk manager) the combined risk from exposure to the compound is considered acceptable.

UF: Uncertainty factor, HI: Hazard Index; HQ: Hazard coefficient, RV: reference Value, RP: Reference Point; CSAFs: Chemical-Specific Adjustments Factors; Dmix: mixture dose; aDi/Di: adjusted dose/dose for the substance i; TS_i: toxicity of the substance i; T_{ID}: toxicity of the IC; HBGV Health Based Guidance Value; WoE Weight of Evidence;

Table 1: Mathematical approaches for assessing chemical mixture effects (Boobis et al., 2008; EC, 2011b) (continued)

	Relative Potency Factor (RPF) or Toxic Equivalency Factor (TEF) or Potency Equivalency Factor (PEF)	Toxic Unit Model (TUs)	Maximum cumulative ratio (MCR)
Principle	Sum of effects expressed as total equivalent exposure by potency-normalizing individual doses. One substance is defined as the index compound (IC) and the potency of all chemical are normalized to the single potency scale of the IC.	Ratio between the concentration of a component in a mixture and its toxicological acute or chronic (e.g. LC_{50} or NOEC) endpoint. The toxic unit of a mixture (TU_m) is the sum of TUs of individual chemical.	Ratio between the toxicity of the mixture and the toxicity of the most toxic chemical in the mixture. Hence, **MCR does not predict risk** but provides a tool for investigating data on cumulative exposures to human and ecological receptors and identify instances where cumulative risk assessments are most needed.
Formula	$Dmix = \sum aD_i = \sum D_i * RPF_i$ With $RPF_i = TS_i/T_{ID}$	$TU_m = \sum TU_s$	MCR= TUm/Highest individual TU 1<MCR<n *n=number of chemical in the mixture*
Interpretation	-Health effect of the mixture assessed by reporting the Dmix on the dose-response curve of the IC. The Dmix can be readily compared to the HBGV	-Used to quantify the toxicity of a mixture on the basis of its composition. An acute lethal TU_m=10 means that a dilution 10% of the mixture would produce 50% of lethality.	MCR ≈ 1: one chemical completely dominates the toxicity; MCR ≈ "n": each chemical is present in "equitoxic" concentrations. MCR indicates the amount of an individual's CR missed by not doing a CRA: MCR=4 → a chemical-by-chemical approach would underestimate the toxicity of a mixture by a factor of 4. MCR is the measure of the fraction of toxicity that comes from the most toxic component: MCR=2 →1 chemical is providing 50% of the mixture's toxicity, MCR=1.1 →1 chemical is providing 90% of the mixture's toxicity
Advan -tage	Method transparent, easy to understand, because it separates potency correction from exposure consideration.	- Refers to a toxicological endpoint - If the shape of the curve is known, TU_m can be used to estimate the expected effect	- Allow to decide either or not a cumulative assessment is necessary for a mixture - Can be integrated into tiered approaches to assess mixtures
Disad- vantage	Determination of the risk posed by the combined exposure places great emphasis on the quality of the toxicology database of the IC	Does not refer to the ecosystem, but only to a specific organism assumed to be representative of a group of organisms ecologically or taxonomically relevant for the ecosystem.	- Data analysed so far are limited, more work is to be done to validate the model - How to predict MCR values for chemicals not yet on the market?
Nota	- Toxic Equivalency factor (TEF) is a special case of this method, initially developed for dioxins and other aryl hydrocarbon receptor (AhR) agonist. - The Potency Equivalency Factor is a more general method that has been used for compounds such as polycyclic aromatic hydrocarbons and certain pesticides.	Frequently used in ecotoxicology. Conceptually, TU_m is comparable to HI when applied to environmental concentrations, but referring to tox. endpoints and not to RV. The RV in ecotox is the PNEC, so ∑PEC/PNEC ratio could be comparable to HI; however PEC/PNEC for components in a mixture may not be homogeneous as PNEC are derived by applying an AF to tox. endpoints obtained for the most sensitive organisms, which may be different for each chemical in the mixture. Nevertheless, this approach is known to be slightly more conservative than the $\sum TU_s$, and could be used as a first-tier when applying CA.	Low MCR values imply additive and independence models will give similar estimates of tox. (when one chemical is the driver of a mixtures tox. both models give similar answers) High MCR values imply that the two model's answers will be different i.e it is more important to establish "assessment groups" where dose additivity occurs. It has been empirically demonstrated that MCR is inversely correlated with the toxic potency of the mixture: very dangerous mixture are driven by very few chemical Can be applied to biomonitoring data with the following equation (exposure based): MCR= Total TEQ/ Maximum TEQ = Σ (Conc. * TEF)/Max (Conc. * TEF) *TEQ: Toxicity equivalents, TEF: Toxicity equivalent factors*

UF: Uncertainty factor, ED: effective dose, RV: reference value, PEC: predicted environmental concentration, PNEC: predicted no effect concentration, MEC: measured no effect concentration, LC_{50}: Lethal Concentration 50, NOEC: No observed effect concentration, AF: Adjustment factor, CA: Concentration addition; CR: Cumulative risk. Tox: Toxicity; ecotox: ecotoxicity; WoE: Weight of Evidence

3 - Regulatory requirements across EU legislative sectors (human health and environment)

A review of EU risk assessment requirements regarding chemical mixtures has been conducted, to assess if and how the different EU directives and regulations take into account risks arising from mixture toxicity.

3.1 Regulatory requirement linked to intentional mixtures

In the case of intentional mixtures, the composition of the mixtures is well known and the assessment is based on the properties of the constituents supplemented, where appropriate, by tests carried out on the entire products. This applies to plant protection products, biocides, pharmaceuticals, food additives and cosmetics for example.

3.1.1 Plant protection products (Regulations 1107/2009, 283/2013, 284/2013)

This regulation defines plant protection products as any products "*consisting of or containing active substances, safeners or synergists, and intended for one of the following uses*" (Article 2):

(a) protecting plants or plant products against harmful organisms (or preventing their action);

(b) influencing the life processes of plants, such as their growth (other than as a nutrient);

(c) preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives;

(d) destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants;

(e) checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants.

Regulation (EC) No 1107/2009 lays down rules on the evaluation, authorization, placing on the market and control of plant protection products (PPP) in the EU. It aims at ensuring a "*high level of protection of both human and animal health and the environment at the same time to safeguard the competitiveness of Community agriculture*". This regulation concerns both the substances and the preparations made with those active substances (i.e " *mixtures or solutions composed of two or more substances intended for use as a plant protection product or as an adjuvant"*, Article 3 §3). However, the requirements for individual substances and formulated products are not the same: usually a first RA for active substances is performed at EU level based on a wide range of data regarding human health and environmental effects. At this stage only the active substance is tested, although the formulation might also be tested for some organism groups for ERA. Authorised active substances can then be used in formulations for which authorisations are granted at Member State level. Usually the authorisation of formulations is largely based on data provided for the active substance where possible to avoid unnecessary testing. Formulations are generally only assessed for acute toxicity to humans (by oral and dermal route, by inhalation, skin irritation, eye irritation and skin sensitization), as well as for organisms in the environment that may come into direct

contact with the product. This might be of concern as often formulations are designed to be more toxic than the active ingredient itself, through the use of synergist and surfactant that increase the bioavailability/toxicity of the formulation. These regulatory requirements are described in Regulations EC 283/13 (EC, 2013a) and 284/13 (EC, 2013b) for active substances and formulations respectively. The usual approach for formulation RA is the direct testing of the PPP itself (whole-mixtures approach), but this regulation also promotes the "*development of non-animal test methods in order to produce safety data relevant to humans and to replace animal studies currently in use*" for the risk assessment of preparations.

Regarding human health, Regulation (EU) No 283/2013 on active substance requirements states that sufficient information should be given on the active substance and on the other component(s) of the PPP, as well as on one or more plant protection products containing the active substance, to permit both "*a risk assessment of consumer exposure"* and "*an estimation of the exposure to operators, workers, residents and bystanders"* including, *"where relevant, the cumulative exposure to more than one active substance*" (Introduction to the Annex, Point 1.11). The need to mention the possible presence of pesticide residues arising "*from sources other than current plant protection uses of active substances (for example use of active substances resulting in common metabolites, use as biocide or veterinary drug)*" is also put forward, as the necessity to take into account their aggregate exposure and the cumulative exposure to more than one active substance, where relevant (Annex, Part A, Point 6.9).

Regulation (EC) No1107/2009 is closely linked to the Regulation (EC) 396/2005 on maximum residue levels (MRLs) of pesticides in or on food and feed (see § 2.2.9), as the setting of adequate MRLs might be a condition for granting authorisation for PPP to be used on plants or plant products to be used as feed or food (Art 29 §1i). Moreover, this regulation introduces a clear requirement for the consideration of potential mixture effects of plant protection products and their residues on human health through different sources of exposure, as PPP and their residues*"…shall not have (…) harmful effect on human health, (…), directly or through drinking water (taking into account substances resulting from water treatment), food, feed or air (…), taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available"* (Article 4, §3b and §2a) for the PPP and their residues respectively.

However, it is not clear if this requirement is also valid for the assessment of hazards and risks for the environment, as the only requirement stated by this regulation is that PPP and their residues *"… shall not have any unacceptable effect on the environment…"* (Article 4, §2b, and §3e), and as the amendment recommended by the European Parliament's Committee on the Environment, Public Health and Food Safety to take into account cumulative and synergistic effects and all relevant exposure routes to organisms in the environment has not been accepted during the legislative procedure (Kortenkamp et al., 2009). Nevertheless, Article 29 of this same regulation states that "*interaction between the active substance, safeners, synergists and co-formulants shall be taken into account in the evaluation of plant protection products*", which directly mentions both the interaction between component and

the evaluation of the PPP (i.e. as a mixture) without restricting it to the human health assessment. Moreover, the introduction of Annex of Regulation (EU) No 284/2013 (point 1.3) states that "*Any information on potentially unacceptable effects of the plant protection product on the environment, on plants and plant products shall be included as well as known and expected cumulative and synergistic effects*", which clearly implies the need to take into account the expected cumulative and synergistic effects on the environment.

Following these new regulations and to address the corresponding requirements, guidance documents for environmental risk assessment have been updated. To achieve an adequate consideration of mixture toxicity in the environmental risk assessment of PPP for birds and mammals, for which toxicity data with formulated products or mixtures of active substances are not always available, especially for birds, a 4-step approach for combined effects of simultaneous exposure to several active substances has been proposed (EFSA, 2009a). This approach does not refer to an assessment of formulation toxicity as such, but of the expected effects from exposure to a mixture of active substances (and possibly also toxic co-formulants) in the environment resulting from the use of the formulation (See Chapter 3.1.2). In an EFSA scientific opinion on the science behind the risk assessment of PPPs on bees (EFSA, 2012d), a chapter focuses on the evidence on cumulative and synergistic effects of pesticide mixtures in bees, and recommendations are made for risk assessment purposes. The developed risk assessment scheme also proposes to investigate for each compound whether there are any indications of cumulative effects. More recently, the EFSA guidance on the tiered risk assessment of plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013d) recommends to determine if there is any synergistic effect in order to complete an adequate risk assessment (See Chapter 3.1.2).

3.1.2 Biocides (Regulation 528/2012)

This regulation applies to biocidal products defined as "*any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action"* ((EC, 2012b), Article 3 §1a). The same definition for the words "substances" and "mixtures" is used as in the REACH regulation. This regulation seeks to simplify the procedures concerning the authorisation of biocidal products without reducing the high level of protection for the environment and human and animal health. Biocidal products must have "*no immediate or delayed unacceptable effects"* on "*the health of humans including that of vulnerable groups, or animals, directly or through drinking water, food, feed, air, or through other indirect effects";* and *"no unacceptable effects itself, or as a result of its residues, on the environment"* (Article 19 §1). To achieve these protection goals, extensive toxicity testing and risk assessments are carried out for both the individual active substances and the formulated biocidal products, taken into account "*cumulative and synergistic"* effects (Article 19 §2).

Annex II and III of the regulation set out the data requirements for active substances and formulated biocidal product respectively; besides toxicological / ecotoxicological data and

assessment, Annex III also requires that "*for biocidal products that are intended to be authorised for use with other biocidal products, the risks to human health, animal health and the environment arising from the use of these product combinations shall be assessed* "(Point 8.5.4). Annex VI of this Regulation establishes the common principles for the evaluation of biocidal products, and includes again the explicit requirement to take into account cumulative and synergistic effects (Annex VI point 3) and the necessity to assess the overall toxicity and ecotoxicity of a biocidal product that may contain various substances of concern (Point 53: "*In each of the areas where risk assessments have been carried out, i.e. effects on man, animals, and the environment, the competent authorities shall combine the results for the active substance together with the results for any substance of concern to produce an overall assessment for the biocidal product itself. This should take account of any likely synergistic effects of the active substance(s) and substances of concern in the biocidal product*" and point 54: "*For biocidal products containing more than one active substance any adverse effects shall also be combined to produce an overall effect for the biocidal product itself.*"), a substance of concern being defined as "*any substance, other than the active substance, which as an inherent capacity to cause an adverse effect, immediately or in the more distant future, on humans (…), animals or the environment and is present or is produced in a biocidal product in sufficient concentration to presents risks of such an effect*" (Article 3 f).

Moreover, during the evaluation process, when the evaluating competent authority considers "*that there are concerns for human health, animal health or the environment as a result of the cumulative effects from the use of biocidal products containing the same or different active substances*", it shall "*document its concerns in accordance with the requirements of the relevant parts of Section II.3 of Annex XV to Regulation (EC) No 1907/2006 and include this as part of its conclusions*" (Article 8 §3).

As for plant protection products, hazard and risk assessments of formulated biocidal products are preferably based on toxicity tests with the final product: biocidal products are generally assessed for acute toxicity, skin and eye irritation, and skin sensitization, although other endpoints and other toxicological aspects may be assessed based on the individual substances present in the formulations. This acute toxicity is usually assessed by a whole mixture approach. The complete future guidance structure under the BPR will be implemented in full at a later stage, and some very specific topics such as cumulative and synergistic effects should be addressed[1]. This regulation is also link to other regulation concerning residues in food and feed as Article 19 states that "*where appropriate, maximum residue limits for food and feed have been established with respect to active substances contained in a biocidal product*" in accordance with such regulation[2].

[1] ECHA, Guidance on biocides legislation. Future Guidance Structure Volume V. http://echa.europa.eu/web/guest/guidance-documents/guidance-on-biocides-legislation/future-guidance-structure

[2] Reg. (EEC) No 315/93 laying down Community procedures for contaminants in food, Reg. (EC) No 1935/2004 on materials and articles intended to come into contact with food, Reg. (EC) No 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin, Reg. (EC) No 470/2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin or Directive 2002/32/EC on undesirable substances in animal feed.

3.1.3 Pharmaceuticals: Human medicines (Directive 2001/83/EC) and Veterinary medicines (Directive 2001/82/EC)

Both directives share some basic features. In both cases, the risk assessment follows a *risk-benefit balance* approach, in which the applicant is required to demonstrate that the potential risks are outweighed by the therapeutic efficacy of the product (Dir. 2001/83/EC Recital 7 and Dir. 2001/82/EC Recital 11). If the risk-benefit balance is not considered to be favourable, the marketing authorisation will be refused or an existing authorisation may be suspended, withdrawn or modified. Human and veterinary medicinal products may be single substances, but are more typically combinations of substances. However, the toxicological assessment and the environmental assessment of those two types of medicines slightly differ.

Human medicines

Two types of risks are considered when dealing with medical products and human health: risks to the patients' health and risks to public health. Toxicity studies required for their authorization are either performed for the active substance or the combination of active substances present in the product or for the finished product (Annex I, Part 1, Section 4.2.3). Particular attention is paid to wanted and unwanted interactions of substances combined within a medicinal product, interactions of a medicinal product with other medicinal products administered concomitantly as well as interactions with alcohol, tobacco, and foodstuffs: non-clinical and clinical studies on pharmacokinetic and pharmacodynamic interactions are part of the standard dossier requirements (Annex I, Part 1, section 4.2.1, 4.2.2, 4.2.3, 5.2.3 and 5.2.4). Thus, the toxicity of the whole product is taken into account and potential interactions are deeply assessed.

An environmental risk assessment is also required, since the application must be accompanied by an "*evaluation of the potential environmental risks posed by the medicinal product"*, and, *"on a case-by-case basis, specific arrangements to limit it shall be envisaged"* (Article 8, §3(ca)). This risk assessment overview should evaluate *"possible risks to the environment due to use and/or disposal of the medicinal product"* (Annex I, Part I, section 1.6). A guidance document has been released in 2006 (EMEA, 2006) but it does not specifically address any aspect of mixture toxicity concerning environmental risk assessments.

Veterinary products

Three types of risks are considered when dealing with veterinary medicinal products: 1) risks to the animal, 2) risks to human health, and 3) risks of undesirable effects on the environment. Toxicity and ecotoxicity studies and assessments required for the authorization of veterinary medicinal products are performed for the product, its active substances and relevant metabolites (Kortenkamp et al., 2009). They aim at assessing the potential toxicity of the product and any dangerous or undesirable effects to the target animal, potential harmful effects to humans of residues of the product or substance in foodstuffs, potential risks to directly exposed humans (e.g. during administration), and potential risk to the environment resulting from the use of the product (Annex I, Title I, Part 3, Chapter I). Thus, the toxicity and ecotoxicity of the entire product is taken into account.

Attention is also paid to interactions with other medicinal products or feed additives with respect to effects in the target animals, but this point is less deeply assessed than for medicinal products intended for human use (Kortenkamp et al., 2009).

In contrast, for ecotoxicity assessments, the information requirements are more specific and detailed in comparison to medicinal products for human use (Annex I, Title I, Part 3, Chapter I, section 6). A two phase risk assessment is required: the first phase (which is mandatory) focuses on the potential extent of the exposure by the active substances or their metabolites; in the second phase, further specific investigation of the fate and effects of the products on particular ecosystems is performed, when deemed necessary. However, the environmental toxicity of the mixture, potentially resulting from the joint occurrence of different residues of veterinary products or from the joint occurrence of pharmaceuticals and other pollutants, is not taken into account in the environmental assessment.

3.1.4 Cosmetics (Regulation 1223/2009)

Regulation 1223/2009 defines the term "mixture" as having *"the same meaning as the term "preparation" previously used in the community legislation*" (Recital 21), and as being a "*solution composed of two or more substances*". Thus cosmetic products are typically a mixture of substances. In order to ensure the safety of the cosmetic product for human health, a safety assessment must be performed (Art. 3) which should take into account the intended use of the cosmetic product and the anticipated systemic exposure to individual ingredients in a final formulation (Article 10 a). Moreover, this directive clearly promotes the use of alternative methods by prohibiting the placing on the market of cosmetic products for which "*ingredients, combinations of ingredients or the final formulation have been the subject of animal testing using a method other than an alternative method*" after such alternative method has been validated in the EU (Art. 18.1). According to recital 41 and to limit animal testing, "*the safety of finished cosmetic products can already be ensured on the basis of knowledge of the safety of the ingredients that they contain*", thus safety assessment or testing should be performed either on ingredients, combinations of ingredients, or the finished cosmetic product. An appropriate weight-of-evidence (WOE) approach should also be used for reviewing data from all existing sources (Art 10b), although the regulation does not precise what is an appropriate WOE. This regulation also prohibits or restricts the use of certain chemicals of concern, listed respectively in Annex II and III, and lays down the condition of use of certain colorants and preservatives (Annex IV and V). More generally, the use of Carcinogenic, Mutagenic and Reprotoxic (CMR) products 1A, 1B and 2 (Recital 32) is prohibited, except for CMR 2 substances that have been found safe for use in cosmetic products by the Scientific Committee for Consumer Safety (SCCS) and that are regulated in the previously mentioned Annexes of the Regulation, and for CMR 1A or 1B substances *"in the exceptional case that these substances comply with food safety requirements, and that no suitable alternative substances exist",* and that they have *"been found safe by the SCCS".*

The requirements for the toxicological safety assessment are laid down in Annex I Part 8. This includes cosmetic product safety information, with a toxicological profile of the substances (Part A) in which "*all significant toxicological routes of absorption shall be considered"* and in which "*particular consideration shall be given to any possible impacts on*

the toxicological profile due to interaction of substances". Additionally, the safety assessment of the cosmetic product itself (Part B) must also be assessed, including possible interactions of the substances contained in the cosmetic product.

According to Recital 33, the safety assessment of substances, particularly those classified as CMR 1A or 1B substances previously mentioned, should consider the overall exposure to such substances stemming from all sources, which implies the development of a harmonised approach to the use of such overall exposure estimates. Thus, the SCCS has published and regularly revises the "*Notes of Guidance for Testing of Cosmetic Ingredients and Their Safety Evaluation by the SCCS*", a document designed to provide guidance and to improve harmonized compliance with the EU cosmetics legislation, and is currently developing guidance to a harmonized approach to the development and use of overall exposure estimates in assessing the safe use of CMR substances (SCCS, 2012).

Beside human health, the environmental concerns that substances used in cosmetic products may raise are not addressed in this regulation but should be considered through the application of Regulation (EC) No 1907/2006 (REACH), which enables the assessment of environmental safety in a cross-sectorial manner (Recital 5 of the Cosmetics Regulation).

3.1.5 Classification, labelling and packaging of substances and mixtures (Regulation 1272/2008)

The Classification, Labelling and Packaging (CLP) regulation entered into force on the 20 January 2009 and replaces the former Directive 67/548/EEC (dangerous substances) and also Directive 1999/45/EC (dangerous preparations), in a stepwise manner until finally repealed from 1 June 2015. This Regulation is intended to ensure "*a high level of protection of human health and the environment as well as the free movement of substances, mixtures and articles*" (Art. 1 §1) and aims at determining "*which properties of substances and mixtures should lead to a classification as hazardous, in order for the hazards of substances and mixtures to be properly identified and communicated"*, including physical hazards, hazards to human health and the environment as well as to the ozone layer. This regulation covers all chemicals and mixtures placed on the European market, with the exception of radioactive substances and mixtures, non-isolated intermediates, substances and mixtures for scientific research, waste products, cosmetics, medicinal and veterinary products, and food and feeding stuff (Art.1, §2.5). The terms and definitions used in the CLP Regulation are consistent with those set out in REACH: i.e. a mixture is defined as a deliberate combination of two or more individual substances for producing a marketed final chemical product. Moreover, this regulation has a strong impact within the EU, due to downstream legislation, (such as worker protection or SEVESO), which would deal with substances or mixtures on the basis of their classification under CLP.

This regulation also confirms the EU's intention to contribute to the global harmonisation of criteria for classification and labelling, at UN level, through implementation of the internationally agreed GHS (UN Globally Harmonised System of Classification and Labelling of Chemicals) criteria into EU law.

Each substance and/or mixture is classified into hazard classes, depending on the nature of the hazard, and into a hazard category, indicating the severity of the hazard. The different human health hazard classes and environmental hazard classes are detailed in Tables 2 and 3 respectively, along with their corresponding classification principles.

For the hazard classification of a mixture, all relevant, reliable and scientifically valid available information on the mixture and on its components should be identified in order to determine "*whether the mixture entails a physical, health or environmental hazard*" (Art. 6). According to recital 20, the supplier "*should not be obliged*" to produce any new (experimental) data. Thus, when experimental data on the mixtures are available, classification and labelling should be obtained by using those data (whole-mixture testing). When there is no experimental data available, the classification of the mixture should be based on the information on the mixture components or on the "*bridging principles*", using information on a similar mixture (Recital 23, Annex I). Moreover, if the mixture contains carcinogenic, germ cell mutagenic or reproductive toxic substances or if biodegradation and bioaccumulation properties are evaluated (in the "hazardous to the aquatic environment" class), the classification of the mixture should be mainly based on the information on the mixture components (Recital 22). The supplier should also adjust accordingly the classification of a substance or mixture if any new information becomes available, and a new hazard evaluation is necessary when the composition of a mixture is changed outside specified limits (Art. 15). However, the classification of a mixture should not be affected where the evaluation of the information indicates that the substances in the mixture react very slowly with other substances in the mixture to form different substances at low concentration (Art. 14).

The classification of a mixture is based on the application of generic or specific concentration limits (SCLs) for human health hazards, M-factors (see below) for environmental hazards and cut-off limits. Generic concentration limits are limits assigned to a substance indicating a threshold at or above which the presence of that substance in the mixture leads to the classification of the mixture as hazardous. For human health and environmental hazards, they are given in Parts 3 and 4 of Annex I, respectively[3]. Specific concentration limits must be set by the applicant where adequate and reliable scientific information shows that the hazard of a substance is evident when the substance is present at a level below the generic concentration limits. In exceptional circumstances, specific concentration limits may also be set by the applicant where adequate, reliable and conclusive scientific information indicate that the hazard of a substance classified as hazardous is not evident at a level above the generic concentration (Art. 10 §1). When set in accordance with those rules, specific concentration limits shall take precedence over the generic concentration limits for classification in the relevant sections of Parts 3 and 4 of Annex I (Art. 10 § 3).

For the hazard class "hazardous to the aquatic environment", SCLs are not applicable. Instead, the M-factors (Multiplying factors) concept is used. This concept has been

[3] Annex I: Classification and labelling requirements for hazardous substances and mixtures, Part 3: Health hazards, Part 4: Environmental hazards.

established to give an increased weight to very toxic substances when classifying mixtures. M-factors are only applied to the concentration of a substance classified as hazardous to the aquatic environment (acute category 1, with acute toxicities below 1 mg/l, or chronic category 1, with chronic toxicity below 0.1 mg/l if non-rapidly degradable and 0.01 mg/l if rapidly degradable), and are used to derive by the summation method the classification of a mixture in which the substance is present. They are substance-specific and should be established for acute and long-term hazards separately by manufacturers, importers and downstream users.

Cut-off values are the concentrations above which any substance classified as hazardous (either as a component, an identified impurity or an additive) in a mixture, should be taken into account for the purposes of classification (Art.11). They are ranging from 0.1 to 1% depending on the hazard class (Annex 1 §1.1.2), unless there is a presumption that lower concentrations are still relevant.

The classification of a mixture must also take into account all available information on synergistic and antagonistic interaction among the components of the mixture (Art. 12 §1c). Although not mentioned in the legal text, these terms seem to be defined in relation to the expectation of concentration-additive behaviour of the compounds (Kortenkamp et al., 2009).

For environmental hazards, the classification of a substance or mixture focuses exclusively on the hazard towards aquatic organisms; however GHS experts have requested the OECD to explore the needs for a classification with respect to hazards for the terrestrial environment. It should also be noted that in contrast to the original GHS system, which distinguishes three hazard classes for acute toxicity, the European system only considers one acute toxicity category (Table 3). Moreover, even the classification for chronic toxicity into the chronic categories (1-4) is also based mainly on acute toxicity data (EC_{50} values). This is based on an acute to chronic extrapolation, although this approach is not directly followed for mixtures, as their chemical composition is assumed to undergo significant changes over prolonged exposure times (Kortenkamp et al., 2009).

Table 2: Hazard class and category for human health hazard classification under the CLP regulation

Hazard class	***Category***				***Classification principles***
Acute toxicity	***Cat 1***	***Cat 2***	***Cat 3***	***Cat 4***	- Priority is given to the use of data on the mixture, if available. - If no data, use of bridging principle from similar mixtures if available - If no similar mixture, calculation (CA) if data available for all ingredients for estimating the toxicity, considering ingredients present at a concentration of ≥1% (unless there are indications that the compounds are relevant for classification even at lower concentrations, i.e substance classified as acute toxic, category 1-3, for which a fraction of 0.1% is to be considered for the mixture classification) -If no data data available for all ingredients, use other data available (extrapolation, evidence, QSARs…) to estimate conversion values for classification and convey hazards of the known ingredients
Acute oral tox (mg/kg bw)	ATE ≤ 5	5≤ ATE ≤50	50 ≤ ATE ≤ 300	300 ≤ ATE ≤ 2000	
Acute dermal tox (mg/kg bw)	ATE ≤ 50	50≤ ATE ≤200	200 ≤ ATE ≤ 1000	1000 ≤ ATE ≤ 2000	
Acute inhalation tox: Gases (ppmV) Vapours (mg/l) Dust and mists (mg/l)	ATE ≤ 100 ATE ≤ 0.5 ATE ≤ 0.05	100 ≤ ATE ≤ 500 0.5 ≤ ATE ≤ 2.0 0.05 ≤ ATE ≤ 0.5	500 ≤ ATE ≤ 2500 2.0 ≤ ATE ≤ 10.0 0.5 ≤ ATE ≤ 1.0	2500 ≤ ATE ≤ 20000 10.0 ≤ ATE ≤ 20.0 1.0 ≤ ATE ≤ 5.0	
Skin corrosion and irritation	**Cat 1: Corrosive** *(3 subcategories)*		**Cat 2: Irritant** *(One single category)*		The mixture will be classified using the criteria for substances. -Priority is given to data on the full mixture; moreover the use of existing alternative tests and of a tiered weight of evidence strategy is encouraged in order to avoid unnecessary animal testing (Annex I 3.2.3.1.2). -If no data on the full mixture are available, use of bridging principles are applied, including the use of a summation method for estimating the overall potency of the mixture with cut-off values (Tables 3.2.3 and 3.3.3 of Annex I). The "*theory of additivity*" is mentioned (Annex I, 3.2.3.3.2 and 3.3.3.3.2), but no specific guidelines on their application is given. Ingredients are considered relevant if present at a concentration of ≥1% unless there is a presumption that an ingredient present at a concentration of less than 1% can still be relevant for classifying the mixture (Table 1.1 and Annex I, 3.2.3.3.1 and 3.3.3.1) If strong acids and bases are present, the pH should be used as a classification criterion (Table 3.2.4 Annex 1)
	Corrosive in > 1 of 3 animals: **1A**: If response is noted following up to 3 min exposure and up to 1h observation **1B**: If response is noted between 3 min and 1h of exposure and observation up to 14 d **1C**: If response is noted between 1h and 4h of exposure and observation up to 14 d		- At least 2 of 3 animals have a mean score between 2.3 and 4 for erythema/eschar or for oedema, *or* - Inflammation that persists to the end of the observation periods (14 d) in a least 2 animals, *or* - If there is a pronounced variability of response among animal, with very definite positives effects in a single animal but less than the criteria above.		
Serious damage to the eye and eye irritation	**Cat 1: Irreversible effect on the eye**		**Cat 2: Irritating to eyes**		
	If the application produces: -at least in one animals effect on the cornea, iris or conjunctiva that are not expected to reverse or have not fully reversed within 21 days and/or -at least in 2 of 3 animals a positive response of corneal opacity ≥3 and/or iritis >1.5 (mean scores following grading at 24, 48 and 72h)		If the application produces: -at least in 2 of 3 animals a positive response of corneal opacity ≥1 and/or iritis >1 and/or conjunctival redness ≥2 and/or conjunctival oedema ≥2 (mean scores following grading at 24, 48 and 72h after application, and which fully reversed within 21 d)		
Respiratory or skin sensitisation	**Respiratory sensitizer Cat 1**		**Skin sensitizer Cat 1**		-When reliable experimental data or good quality evidence from human experience are available for the mixture, these can be used to classify the mixture by weight-of-evidence. If not, use bridging principles on similar tested mixtures or individual ingredients -Use of generic concentration limits: should be categorised into this class if at least one of the components is classified into the hazard class "*respiratory or skin sensitisation*" and is present at >0.2% (gas) or >1% (solid/liquid) (Table 3.4.3 Annex I)
	-if there is evidence in humans that the substance can lead to specific respiratory hypersensitivity / sensitisation by skin contact in a substantial number of persons respectively and/or - if there is positive result from an appropriate animal test				

Table 2: Hazard class and category for human health hazard classification under the CLP regulation (continued)

Hazard class	***Category***		***Classification principles***
Genotoxicity			
	Cat 1	**Cat 2**	
Germ cell mutagenicity	Substance to be regarded as if they induce heritable mutations in the germ cells of humans. **1A**: based on positive evidence from human epidemiological studies. **1B**: based on positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or from *in vivo* somatic cell mutagenicity tests in mammals and some evidence that the substance has potential to cause mutations to germ cells; or positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny.	Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans	- Priority is given to the relative content of components in the mixture that are themselves classified (summation method with cut-off criteria, Tables 3.5.2 of Annex I). An ingredient classified germ cell mutagens 1A or 1B and present at more than 0.1% trigger the classification of the mixture as Germ cell mutagen 1A or 1B, an ingredient classified Germ cell mutagens 2 and present at more than 0.1% trigger the classification of the mixture as Germ cell mutagen 2. - Data on the mixture itself are only considered on a case-by-case basis (sections 3.5.3.2.1, of Annex I) when "*demonstrating effects that have not been established from the evaluation based on the individual ingredients*". - The bridging principles according to section 1.1.3 are also considered, if applicable. CA is not applied for any classification purposes.
Carcinogenicity	Known or presumed human carcinogens on the basis of epidemiological and/or animal data. **1A**: known to have carcinogenic potential for humans, classification is largely based on human evidence, or **1B:** presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.	Suspected human carcinogens Classification on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B.	- Priority is given to the relative content of components in the mixture that are themselves classified (summation method with cut-off criteria, Tables 3.6.2 of Annex I). An ingredient classified Carcinogen cat.1A or 1B and present at more than 0.1% trigger the classification of the mixture as Carcinogen 1A or 1B, an ingredient classified Carcinogen 2 and present at more than 0.1% trigger the classification of the mixture Carcinogen 2. - Data on the mixture itself are only considered on a case-by-case basis (sections 3.6.3.2.1 of Annex I) when "*demonstrating effects that have not been established from the evaluation based on the individual ingredients*". - The bridging principles according to section 1.1.3 are also considered, if applicable. CA is not applied for any classification purposes.
Reproductive toxicity	Known or presumed human reproductive toxicant (i.e clear evidence of an adverse effect on sexual function and fertility or on development). In addition, effects on lactation are allocated to a separate hazard category. **1A**: Known human reproductive toxicant; classification is largely based on evidence from humans. **1B**: Presumed human reproductive toxicant; classification is largely based on data from animal studies.	Suspected human reproductive toxicant Classification based on some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1.	- Priority is given to the relative content of components in the mixture that are themselves classified (summation method with cut-off criteria, Tables 3.7.2 of Annex I). An ingredient classified Reproductive Toxicant cat.1A, 1B, 2 or "additional effect on or via lactation" and present at more than 0.3% trigger the classification of the mixture as Reproductive Toxicant cat.1A, 1B, 2 or "additional effect on or via lactation" respectively. - Data on the mixture itself are only considered on a case-by-case basis (sections 3.7.3.2.1, of Annex I) when "*demonstrating effects that have not been established from the evaluation based on the individual ingredients*". - The bridging principles according to section 1.1.3 are also considered, if applicable. CA is not applied for any classification purposes.

Table 2: Hazard class and category for human health hazard classification under the CLP regulation (continued)

Hazard class	*Category*			*Classification principles*
Specific target organ toxicity				
Specific target organ toxicity – single dose	**Cat 1** Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure Classification on the basis of: (a) reliable and good quality evidence from human cases or epidemiological studies; or (b) observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations.	**Cat 2** Substances that can be presumed to have the potential to be harmful to human health following single exposure Classification on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.	**Cat 3** Transient target organ effects This category only includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2. These are effects which adversely alter human function for a short duration after exposure and from which humans may recover in a reasonable period without leaving significant alteration of structure or function.	- Primary classification criterion is the data on the mixture itself, although specific care is advised by the guideline to ensure that "*the dose, duration, observation or analysis does not render the results inconclusive*" (sections 3.8.3.2.1 and 3.9.3.2.1 of Annex I). - The application of bridging principles is put forward in sections 3.8.3.3 and 3.9.3.3 of Annex I. - If no reliable data are available on the mixture itself and the bridging principles cannot be used, the mixture is classified for its specific organ toxicity using the cut-off values given in Tables 3.8.3 and 3.9.4 of Annex I: an ingredient classified as Cat 1 Specific target organ toxicant and present at more than 10% triggers the classification of the mixture as Cat 1 Specific target organ toxicant 1A; if present between 1 to 10%, it triggers the classification Cat 2. An ingredient classified as Cat 2 Specific target organ toxicant and present at more than 10% triggers the classification of the mixture as Cat 2 specific target organ toxicant. - When extrapolating toxicity of a mixture that contains Specific target organ toxicity – single exposure Category 3 ingredient(s), a generic concentration limit of 20 % is appropriate; however, this concentration limit may be higher or lower depending on the Category 3 ingredient(s) and that some effects such as respiratory tract irritation may not occur below a certain concentration while other effects such as narcotic effects may occur below this 20 % value, therefore expert judgement is required (section 3.8.3.4.5). - CA is not applied for any classification purposes. - Care shall be exercised when toxicants affecting more than one organ system are combined that the potentiation or synergistic interactions are considered, because certain substances can cause target organ toxicity at < 1 % concentration when other ingredients in the mixture are known to potentiate its toxic effect (sections 3.8.3.4.4 and 3.9.3.4.4)
Specific target organ toxicity – repeated dose	**Cat 1** Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Classification on the basis of: - Reliable and good quality evidence from human cases or epidemiological studies; or - Observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.	**Cat 2** Substances that can be presumed to have the potential to be harmful to human health following repeated exposure. Classification on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.		
Aspiration hazards	**Cat 1:** Substances known to cause human aspiration toxicity hazards or to be regarded as if they cause human aspiration toxicity hazard Classification based on (a) based on reliable and good quality human evidence, or (b) if it is a hydrocarbon and has a kinematic viscosity of 20,5 mm2/s or less, measured at 40ºC.			The classification of a mixture into the hazard class "*aspiration hazard*" follows the same outline: use of data on the whole mixture of interest, bridging principles, and cut-off values for individual compounds (section 3.10.3. of Annex I).

Table 3: Hazard class and category for environmental hazard classification under the CLP regulation

Hazard class	*Category*				*Classification principles*
Acute aquatic toxicity	***Cat 1*** Classification on the basis of acute aquatic toxicity data only (EC50 or LC50). - A substance or a mixture (on the bases of a whole-testing approach) is classified as acute category I if its EC50 is ≤ 1mg/L (Table 4.1.0) -Summation methods: if the mixture contains ≥ 25% of compounds classified into acute toxicity category I, the mixture is also classified into Cat 1.				-Priority is given to the data on the whole mixture (if available) -If no information available, the classification can be based on bridging principle. -If this is not possible, the "summation method" is applied; highly toxic compounds are given increased weight for the summation by the application of M-factors (Table 4.1.3, Annex I). -If the mixture contains not yet classified compounds for which adequate toxicity data are available, CA is used for the assignment of an acute category to that portion of the mixture (§ 4.1.3.5.2 of Annex I), which is then used in applying the summation method for the whole mixture. If relevant compounds are present in the mixture that are not classified for their acute toxicity and for which no toxicity data are present the classification of the mixture shall be based on the assessable rest of the mixture (§4.1.3.6.1 of Annex I).
Chronic aquatic toxicity	***Cat 1*** If EC50 ≤ 1mg/L **and** the substance is not rapidly biodegradable **and/or** the experimental determined BCF ≥ 500 (or log K_{ow}≥4) **Summation methods:** If ∑(comp. Chronic Cat 1 * M) ≥ 25 % M= multiplying factor, dependant on the LC50 of the component	***Cat 2*** If is 1 mg/L ≤ EC50 ≤ 10mg/L **and** the substance is not rapidly biodegradable **and/or** the experimental determined BCF ≥ 500 (or log K_{ow} ≥ 4), unless the chronic toxicity >1mg/L. **Summation methods:** If ∑[(M*10* comp. Chronic Cat 1) + Chronic Cat 2] ≥ 25 %	***Cat 3*** If is 10 mg/L ≤ EC50 ≤ 100 mg/L **and** the substance is not rapidly biodegradable **and/or** the experimental determined BCF ≥ 500 (or log K_{ow}≥4), unless the chronic toxicity > 1mg/L **Summation methods:** If ∑[(100*M* comp. Chronic Cat 1) + (10*M* Chronic Cat 2] ≥ 25 %	***Cat 4*** 'Safety net' classification for use when the data available do not allow classification under the formal criteria but there are nevertheless some grounds for concern ∑[Chronic Cat 1+ Chronic Cat 2+ Chronic Cat 3+ Chronic Cat 4] ≥ 25 %	Classification of a substance into the chronic categories combines two types of information, i.e. acute aquatic toxicity data and environmental fate data (degradability and bioaccumulation data). Data on the chronic toxicity data (NOEC values) of the whole mixture and/or the classification information on the individual compounds are used for the classification of the mixture into chronic aquatic toxicity category 1-4 (§4.1.3.5.5.4 of Annex I).

ATE: acute toxicity estimate (LD/EC50), ppmV: parts per million per volume, CA: Concentration Addition, Exp: exposure; Obs: Observation, min: minutes, d: days

Thus, in most cases the CLP regulation relies on the concentration addition method to classify mixtures into acute toxicity to human health classes or environmental hazard classes, and does not mention the concept of independent action. This seems to be a defendable approach as there is empirical evidence that the differences between the mixture toxicity according to CA and IA is usually small, and because CA is usually slightly more conservative (Kortenkamp et al., 2009). The summation method (i.e the classification in dependence of the relative amount of already classified compounds) also plays an important role in this regulation. This is an easy-to-use method which allows a rapid classification of any mixture; however, according to Kortenkamp et al (2009) it includes a substantial risk for underestimating the toxicity of the mixture. For example, a mixture would be classified for "*acute toxicity (environment)*" category 1 by the summation method if it contains 25% at least of compounds classified Acute Aquatic Toxicity cat.1. Thus, if it contains only 24% of these compounds, the mixture would not be classified (with assumed multiplying factors of 1). However, if the acute toxicity classification for all the classified compounds is based on an acute EC_{50} of 0.11 mg/L, CA would predict an overall toxicity of the mixture of 0.46 mg/L which implies the classification of the mixture. Indeed, the CA methods would allow no more than 11% of "acutely toxic to the environment" classified compounds with an EC_{50} of 0.11 mg/L (for which the M factor is still 1) in the mixture before classifying it, whereas the summation methods allow 24% of these compounds in the mixtures before reaching the classification threshold, i.e. a 2.5 times higher concentration. It could even be worse if the remaining 76% of the mixture are made of compounds with an EC_{50} slightly superior to 1 mg/L (i.e not classified but showing a low acute aquatic toxicity); in this case CA would calculate a mixture toxicity of 0.34 mg/L.

Thus, this regulation provides guidance on the hazard classification of commercial chemical mixtures for human health and the aquatic environment according to four different classification methods:

1) Use of test data on the whole mixture and classification of the mixture as if it were a single substance

2) Use of the "*bridging principle*s" and classification of a mixture on the basis of a similar mixture that is already classified

3) Use of the summation methods when the amounts of individual classified substances in the mixture of interest are known

4) Use of concentration addition if there are toxicity data available for the mixture components, even though the individual components are not classified

As such, even though the regulation expresses the need to take into account the possible interaction effects between ingredients of a mixture, none of the above-mentioned calculation methods does so, and those interaction effects are only taken into account if the classification is based on test data on whole mixture.

3.1.6 REACH (Regulation 1907/2006)

REACH aims at ensuring the chemical safety assessment (CSA) of all commercial chemicals that are not specifically covered by other sectorial regulations, through their hazard, exposure and risk assessment. It aims to ensure "*a high level of protection of human health and the environment*" and "*the free movement of substances, on their own, in mixtures and in articles*" (EC, 2006b).

The term "chemical mixture" under REACH is used synonymously with "preparation" (ECHA, 2008c), that is, a deliberate combination of two or more individual substances. Thus, they are "intentional mixtures" of non-reacting chemicals for producing final chemical products. REACH registration requirements apply to each of the individual chemicals in the preparation, but not to the preparation itself.

However, Multi-Constituent Substances (or MCS) which are substances resulting from a chemical reaction in which several constituents are present at >10%, and UVCB (substances of Unknown or Variable composition, Complex reaction products or Biological materials) which are mixtures that cannot be completely identified by their chemical composition, are included in the legal "substance" definition. Thus these are generally treated as a single substances under REACH, "*provided that the hazardous properties do not differ significantly and warrant the same classification*", and the testing of hazard and fate properties is made on the mixture itself. Moreover, an individual chemical (or mono-constituent substance) can contain up to 20% arbitrary by-products without the need for specific consideration.

The Chemical Safety Assessment under REACH implies the following steps:

1) Determining the intrinsic hazard toxicity (ECHA, 2008b) of the substance or the mixture, through the hazard classification and the characterisation of the dose/concentration response. This step implies either the estimating of Derived No-effect-Levels (DNEL) for human health or Predicted No Effect Concentration (PNEC) for environmental assessment, as well as to assess the persistence, bioaccumulation and toxicity (PBT) properties of the substance/mixture.

2) Determining the exposure assessment, by building exposure scenarios and estimating exposure levels

3) Characterizing the risk

Regarding exposure, it has to be stressed that under REACH, a registrant is not obliged to take into account an exposure to the same substance from activities from other producers or importers (ECHA, 2008c), although there might be multiple sources of exposure to the same substance in real life (i.e. aggregate exposure). Moreover, there is no specific hazard assessment required for chemical mixtures, preparations, MCS or UVCBs, unless they have PBT/vPvB properties. These mixtures are defined as PBT or vPvB[4] substances if they contain more than 80% of a substance with PBT/vPvB properties (R 11.1.1.2 (ECHA, 2008a), but management measures have to be considered as soon as a substance contains or degrades to

[4] PBT: Persistent, Bioaccumulative, toxicity; vPvB: very persistant, very bioaccumulative; vPvB are characterized by a particular high persistency and a high tendency to bio-accumulate, but not necessarily proven toxicity

PBT or vPvB substances above the threshold of 0.1%. A detailed guidance for their assessment is given in the chapter R11 (R 11.1.4.2 , (ECHA, 2008a), and is summarized in Figure 1.

The first step is a chemical characterisation of the mixture. Since it might be difficult to identify all components down to the 0.1% threshold amount, it is advised to define "representative structures" used in the following steps for describing the properties of whole blocks of the mixtures. Then, the available information for the PBT assessment for each constituent/block of the mixtures is compiled and assessed (Kortenkamp et al., 2009). A case-by-case approach for substances containing many constituents is necessary, and only general guidance can be given. The REACH guidance gives an example based on the Hydrocarbon Block approach in Appendix R11-3 (ECHA, 2008a).

Besides the testing of the substance/mixture itself, the REACH guidance also foresees the use of other methodological approaches for predicting the overall risk based on information on the individual components (EC, 2011b).

Thus, REACH is a typical substance-oriented regulation, providing mainly for individual chemical substances but also for commercial chemical mixtures resulting from chemical reactions or that are natural products such as UVCBs and MCSs, which are included in the legal definition of "substances" under REACH.

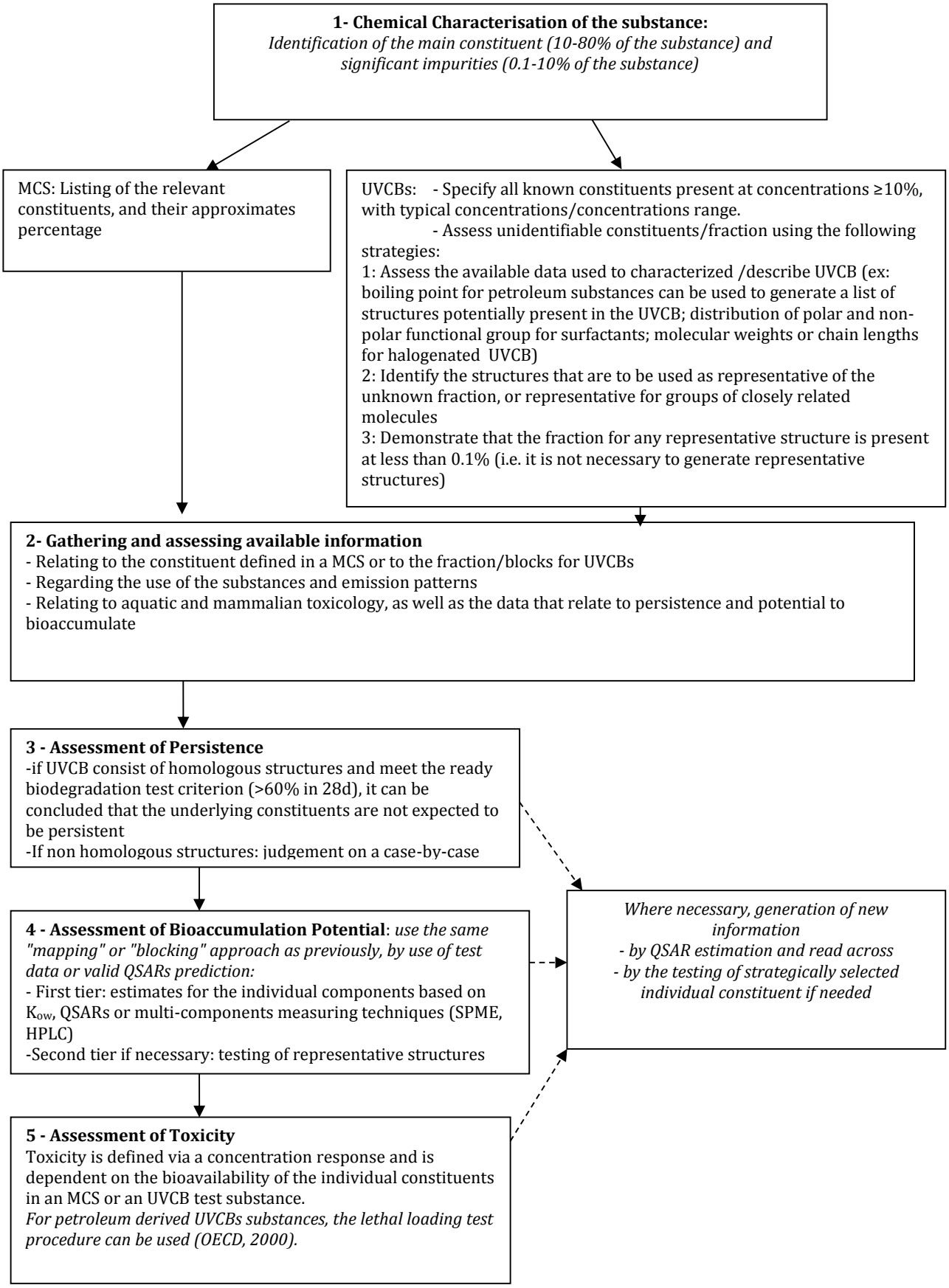


Figure 1: PBT assessment of MCS (Multi-Constituent Substances) and UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) under REACH (according to ECHA, 2008a, § 1.4.2 and appendix R11-3, modified)

Source: European Chemicals Agency

3.1.7 Food law

Regulation (EC) No 178/2002 is the general European food law which lays down general principles and requirements in this field, as well as procedures in matters of food safety. This regulation also established the European Food Safety Authority (EFSA), which became responsible for carrying out corresponding safety evaluations.

Food additives – Regulation (EC) No 1333/2008

The legislation on food additives aims at, amongst others, *"ensuring a high level of protection of human health and a high level of consumer protection, (…) taking into account, where appropriate, the protection of the environment*" (Article 1). Accordingly, Article 6 specifies that food additives must meet three general conditions: consumer safety, technological need, and no misleading of the consumer, as well as, where relevant, *"other legitimate factors, including environmental factors*". Three additional regulations establish complementary rules for *food enzymes* (Regulation (EC) No 1332/2008), *food flavourings* (Regulation (EC) No 1334/2008) and a *common authorisation procedure for food additives, food enzymes and food flavourings* (Regulation (EC) No 1331/2008). Regulation 1331/2008 requires that, in accordance with the framework for risk assessment in matters of food safety established by Regulation (EC) No 178/2002, the authorisation to place substances on the market must be preceded by an independent scientific assessment of the risks that they pose to human health, which must be carried out under the responsibility of the Authority (i.e. EFSA) and must be followed by a risk management decision taken by the Commission (Recital 12).

According to Regulation 1333/2008 a food additive should not, "*on the basis of the scientific evidence available, pose a safety concern to the health of the consumer*" (Article 6, §1a). Established procedures for the safety assessment of food additives are based on Acceptable Daily Intake values (ADI), and permitted use levels are considered to be safe when the intake of additives does not exceed the corresponding ADI values. When necessary, maximum use levels are fixed in Annexes II and III of the regulation.

However, although the previous regulation on food additives provided a basis for mixture toxicity assessments, stating that the evaluation of a food additive *"should also take into account, for example, any cumulative, synergistic or potentiating effect of its use*" (Directive 89/107/EEC, Annex II, §3), the current Regulation 1333/2008 does not mention any of the terms *cumulative, synergistic or potentiating* and does not allude to the need for mixture toxicity assessments. Besides, established procedures for the safety assessment of food additives on the basis of ADI values for single substances do not specifically consider joint actions or interactions between additives and food consumption (Groten et al., 2000). Nevertheless, food safety requirements defined in Regulation (EC) No 178/2002 apply, and state that: "*In determining whether any food is injurious to health, regard shall be had (…) to the probable cumulative toxic effects*" (Article 14, §4). However, this Regulation does not define the term "*cumulative toxic effects*", which could mean, in a narrow sense, a toxic effect resulting from repeated exposure to a single toxicant from the same, similar or different sources via the same or via different routes of exposure; or in a wide sense a toxic effect resulting from simultaneous or sequential exposure to different toxicants and thus be used as

a synonym for *mixture toxicity* (Kortenkamp et al., 2009). In the context of the general food law, the term has often been used in the narrow sense of repeated doses, and without any further indications or specifications the term "*cumulative toxic effects*" cannot be interpreted as a legal requirement for mixture toxicity assessments (Kortenkamp et al., 2009). Moreover, Recital 7 and Article 11 §1b i) states that maximum levels of a food additive should take into account the intake of the food additive from other sources, stating that "*any acceptable daily intake, or equivalent assessment, established for the food additive and the probable daily intake of it from all sources*" should be taken into account when establishing the maximum level of use. This formulation avoids the term *cumulative* and the possible confusion that may arise from it, and is in line with the narrow sense that could be given to this term. Thus, Regulation (EC) 1333/2008 neither excludes the need for mixture toxicity assessments nor does it explicitly define such a need (Kortenkamp et al., 2009).

Feed additives (Regulation (EC) No 1831/2003) and feed additive assessment (Directive 2001/79/EC and Regulation (EC) No 429/2008)

The purpose of Regulation (EC) No 1831/2003 is "*to establish a Community procedure for authorising the placing on the market and use of feed additives and to lay down rules for the supervision and labelling of feed additives and premixtures*" (Article1§ 1). To be placed on the market and used, feed additives must undergo a harmonised scientific safety assessment by EFSA (Recitals 4 and 14) and they must be authorised by the Community (Articles 3 and 9). Moreover, the procedures and rules established by this Regulation are intended to "*... provide the basis for the assurance of a high level of protection of human health, animal health and welfare, environment and users' and consumers' interests in relation to feed additives, whilst ensuring the effective functioning of the internal market"* (Article 1, § 1), which explicitly includes the environment in the protection goals. Accordingly, feed additives should be authorised only if they do "*not have an adverse effect on animal health, human health or the environment*", do "*not harm the consumer by impairing the distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal products*", is "*not presented in a manner which may mislead the user*", and it "*favourably affect the characteristics of feed*" to which it is added and "*the characteristic of animal products*" (Article 5).

If EFSA's opinion is in favour of authorising the feed additive, it shall provide a proposal for the establishment of corresponding Maximum Residues Limits (MRLs) in the relevant foodstuffs (Article 8, §4 e). The methods and procedures to be used for these assessments are not specified in the Regulation, but it is EFSAs' general task to provide "*the best possible scientific opinions*" and to develop "*uniform risk assessment methodologies in the field falling within its mission*" (Regulation (EC) No 178/2002, Article 23 a and b).

The feed additives regulation also covers mixtures of additives sold to the end-user (Recital 9; Article 1; Article 2), whereby "*the marketing and use of those mixtures should comply with the conditions laid down in the authorisation of each single additive*" (Recital 9), However this regulation does not address the topic of mixture toxicity, and does not include provisions for taking into account hazards and risks arising from mixture toxicity (Kortenkamp et al., 2009). As mentioned previously, the general requirement to consider

"*probable cumulative toxic effects"* in food safety assessments (Regulation (EC) No 178/2002) may apply, but cannot be interpreted as a mandatory requirement for mixture toxicity assessments. Moreover, this regulation does not prescribe how scientific risk assessments should be performed.

Commission Regulation (EC) No 429/2008 establishes the detailed rules for "*the preparation and the presentation of applications and the assessment and the authorisation of feed additives"*, and provides the requirements and the studies to be submitted to demonstrate the feed additive efficacy and "*its safety for humans, animals and the environment"* (Recital 2). It lists the physico-chemical, toxicological, and ecotoxicological data and studies that may be required to support safety assessments in relation to target species, workplace exposure, consumers ingesting residues of feed additives or their metabolites, and the environment. This regulation also provides guidance for the derivation of proposals for standard safety criteria from toxicological and ecotoxicological test results, in particular NOAEL, ADI, MRL, and PEC/PNEC values (Annex II).

This regulation explicitly addresses hazards and risks that may arise from mixture toxicity, as it requires that "*Where an additive has multiple components, each one may be separately assessed for consumer safety and then consideration given to the cumulative effect (where it can be shown that there are no interactions between the components). Alternatively, the complete mixture shall be assessed"* (Annex II, section *General Aspects*, subsection *Safety assessment*). However, no further guidance on the practical performance of *cumulative effect* assessments is included in the document.

3.2 Regulatory requirements linked to generated or coincidental mixtures with unknown/varying composition

Discharge to the environment during the production, transport, use or disposal of goods often implies a mixture of chemical substances. Depending of the cases, the composition can either be known, in which cases the assessments can be based on the knowledge of the constituents, or unknown. The release of such mixtures is of concern for legislation such as the Water Framework Directive or waste-related regulation. Other mixtures originating from various sources are unintentional and coincidental. Such a situation could be related either to: a) water/soil/air-related regulation; b) the exposure of workers in the workplace, for which a risk assessment is required for all hazardous chemicals, including in combination; or c) the exposure of humans to multiple chemicals from food and drinking water. The assessment of multiple substances from multiple sources is the main issue raised by the European Commission when dealing with the assessment of chemicals mixture (EC, 2012a).

3.2.1 Environmental impact assessment (Directive 2011/92/EU)

This Directive "*on the assessment of the effects of certain public and private projects on the environment"* states that competent national authorities in EU Member States must carry out an environmental impact assessment before giving consent to "*projects likely to have significant effects on the environment*" (Article 2). The directive covers a broad range of project categories, from agricultural and industrial installations to infrastructure projects, including certain facilities which are or may be a source of emission of chemical pollutants during construction or operation. The Environmental Impact Assessment (EIA) shall "*identify, describe and assess (…) the direct and indirect effects*" on "*human beings, fauna and flora, soil, water, air, climate and the landscape, material assets and the cultural heritage*", as well as "*the interaction between the factors*" (Article 3).

Regarding chemicals, the information required from the *developer* of a project shall, include (i) *an estimate, by type and quantity, of expected residues and emissions (water, air and soil pollution, …*), and (ii) a *description of the likely significant effects (…) on the environment resulting from (…) the emission of pollutants (…) and the elimination of wastes*, (iii) a description of *forecasting methods used to assess the effects* (…) and a description of *measures envisaged to prevent, reduce and where possible offset any significant adverse effects* (…) (Annex IV, § 1, 4, 5 and 6). Based on this information, the EIA of the competent authority shall *identify, describe and assess* the *effects* in *an appropriate manner* (Article 3), but the directive does not prescribe any specific procedures, methods, or criteria and the specification and methodology of the effects assessment is left to the choice of the competent national authorities and in accordance with national legislation. As a support to Member States, the European Commission has published guideline documents for the implementation of the EIA[5], but these guidelines also address procedural aspects on a very general level and do not detail any rules for assessing the effects of pollutants (Kortenkamp et al., 2009).

[5] Available at: http://ec.europa.eu/environment/eia/eia-guidelines/g-screening-full-text.pdf; http://ec.europa.eu/environment/eia/eia-guidelines/g-scoping-full-text.pdf; http://ec.europa.eu/environment/eia/eia-guidelines/g-review-full-text.pdf

Regarding mixture assessment, Annex IV states that the *description of the likely significant effects* required shall *cover the direct effects and any indirect, secondary, cumulative, short, medium and long-term, permanent and temporary, positive and negative effects of the project* (Footnote 1 to §4), and considerations should be given to *the cumulation with other projects* in deciding about the need for an EIA for a specific project (Annex III, § 1), therefore, *likely significant effects* from mixtures of pollutions, if relevant for specific projects, *should* be covered. However, whether and how such mixture effects might be identified, described and assessed is left to the competent national authorities and corresponding national legislation. As mentioned above, a guidance document on *the Assessment of Indirect and Cumulative Impacts as well as Impact Interactions* has been published (EC, 1999), but this guideline only sets out rules and options on a general procedural level of EIA enforcement and is of no practical relevance for mixture toxicity assessments (Kortenkamp et al., 2009). Thus, this leads to the question of whether assessments of mixture toxicity actually play any role in the practice of environmental impact assessments at the Member States level, and if so, how this is done. This has been partly addressed in Part 3 of the Kortenkamp report (2009), dealing with approaches and practical experience in assessing the mixture toxicity of complex environmental samples and wastes samples in the EU.

3.2.2 Integrated pollution prevention and control (Directive 2010/75/EU)

The Integrated pollution prevention and control (IPPC) Directive, 2010/75/EU, which repeals Directive 2008/1/EC from 7th January 2014, aims at preventing or reducing *"emissions in the air, water and land (...) in order to achieve a high level of protection of the environment taken as a whole"* (Article 1). In order to do so, this directive defines the activities with a high potential for pollution that require a permit (Article 4, Annex I), and adopts an *integrated approach*: emissions into air, water or soil, water management, energy efficiency and accident prevention are controlled in a single permit, with the aim to avoid the shifting of pollution between various environmental media, which otherwise might be encouraged by a fragmented approach (Recitals 3).

Application of the "*best available techniques"* (BAT; i.e. using established techniques which are the most effective in achieving a high level of environmental protection as a whole) (Article 3a) and setting of "*Emission limit values"* (ELV) - usually based on the BAT principle without any direct considerations of toxicity - are the key principles for pollution reduction under this directive. Every permit shall include such ELVs *for polluting substances (...) likely to be emitted from the installation concerned in significant quantities* (Article 14, §1a) but toxicological hazard and/or risk assessments do not play a direct role in this approach. Approaches used in mixture toxicology play however an indirect role, as they may be used in the setting of ELVs, such as the Toxic Equivalence Factor (TEF) in the setting of ELVs for dioxins and furans. Another interesting aspect of the IPPC in the context of mixture toxicity comes from the interrelation with the European Pollutant Emission Register (EPER) and the European Pollutant and Transfer Register (E-PRTR), based on the IPPC directive, which document information on pollutant emissions from IPPC establishments. These databases may provide valuable information for modelling approaches to the assessment of

cumulative exposure to multiple pollutants, one of the current bottlenecks in assessing potentially resulting cumulative risks (Kortenkamp et al., 2009).

3.2.3 Waste and waste streams (Directive 2008/98/EC)

A general framework for waste management is given in the Waste Framework Directive (Directive 2008/98/EC), which repealed the older individual directives on waste (2006/12/EC), hazardous waste (91/689/EEC) and waste oils (75/439/EEC). It "*lays down measures to protect the environment and human health by preventing or reducing the adverse impacts of waste"* (Article 1), and it generally states that waste should be managed "*without harming the environment and, in particular (a) without risk to water, air, plants and animals"* (Article 13). Several types of waste are excluded absolutely from the Directive: gaseous effluents emitted into the atmosphere, decommissioned explosives, agricultural or forestry material used in farming, forestry or production of biomass, and radioactive waste (addressed in 2011/70/EURATOM). Others are only excluded to the extent that they are covered by other legislation, like waste waters (addressed in the Water Framework Directive and related directives), animal by-products and carcasses (covered by Directive 1774/2002), waste from specialized industries, e.g. extractive industries like mining (Directive 2006/21/EC).

A list of the different types of waste is defined in Decision 2000/532/EC, which established a classification system for waste including a distinction between hazardous waste and non-hazardous wastes. It is closely linked to the list of the main characteristics which render waste hazardous contained in Annex III to the Waste Framework Directive. The Waste Framework Directive does not address the toxicological assessment of waste, but rather refers via Annex III to the Directive on classification and labelling for classification of the toxicological properties. For disposal operations to seas and oceans, reference is made to international conventions, in particular the Convention on the Prevention of Marine Pollution by dumping Waste and Other Matter (London, 1972 and the Protocol thereto as amended in 2006) (Recital 21). Waste emissions to the air, including waste incineration, are covered by the Directive on Integrated pollution prevention and control (IPPC) (2010/75/EU) (see § 3.2.2), which addresses mixture toxicity for dioxins and furans specifically based on the TEF concept.

3.2.4 Water Framework Directive (2000/60/EC) and Groundwater Directive (2006/118/EC)

The Water Framework Directive aims to establish the basic principles of sustainable water policy in the European Union, and to assess, maintain or improve the chemical status of European waters. In order to do so, this directive aims at progressively reducing the emissions of hazardous substances to water; with the ultimate aim of eliminating priority hazardous substances (Recital 27 and Article 16.1) or reducing concentrations to near natural background concentrations. In this context, this directive both identifies priority hazardous substances presenting a significant risk to or via the aquatic environments on the basis of a scientific assessment of the risk carried out under Regulation No 793/93, the PPP regulation or the biocidal products regulation (Article 16.2 and 16.3), and sets common environmental

quality standards (EQS) and emission limit values for chemicals or certain groups or families of pollutants (Recital 42 and Article 16.7). The identified priority substances are listed in Annex X of this regulation, and Annex V describes a procedure for the setting of chemical quality standards in surface water (Point 1.2.6), established from the application of safety factors on ecotoxicity data. The first list of priority pollutants was described in 2455/2001/EC and the initial EQS, as well as a (revised) set of priority pollutants, are described in Directive 2008/105/EC. Recently, the EQS together with the list of priority pollutants have been amended (Directive 2013/39/EU), adding new compounds to the list of priority pollutants. Although the focus remains on a selected set of compounds, it does add an additional "watch list" of compounds under consideration (Article 8b). This watch list currently includes several pharmaceuticals, a group of compounds regarded as relevant for mixture toxicity but not currently covered by other regulations (nor assessed under REACH), which is why the Commission aims to develop by the end of 2015 *a strategic approach to pollution of water by pharmaceutical substances (Article 8c).* Moreover, a more complete guidance document was published in 2011, presenting the methodology for the establishment of environment quality standards (EQS) for a new list of priority substances and for river-basin-specific pollutants, based not only on the water column but also on sediment or biota matrices (EC, 2011a). This technical guidance document describes a methodology consistent *"with the guidance for effects assessments performed for chemical risk assessment under REACH*", that should account for "*all direct and indirect exposure routes in aquatic systems i.e. exposure in the waterbody via water and sediment or via bioaccumulation, as well as possible exposure via drinking water uptake*".

Although the Water Framework Directive considers families or groups of chemicals, it does not mention chemical mixtures or mixture effects but aims to focus on a limited number of substances, including temporarily in a watch list. Nevertheless, the EQS guidance document recognises that in some circumstances, such as the release of known and largely constant composition mixtures (biocides, pesticides) or other mixtures with a partly unknown, reasonably constant composition, that both change after entry into environment, an EQS for mixtures of substances may be preferable to deriving EQSs for the individual constituent substances (§ 2.7). Section 7 of the guidance document briefly outlines how to estimate EQS for mixtures, using the toxic unit (TU) approach for well-defined mixtures, and the hydrocarbon blocks and the use of non-testing methods such as PETROTOX[6] for the derivation of EQS for petrochemical mixtures of unknown or variable composition (See also Part 3 of this report).

To ensure a proper strategy against groundwater pollution, as specified in Article 17 of the Water Framework Directive, the Groundwater Directive has been developed. In Annex I, this Directive sets a provisional limit of 0.1 µg/l for active substances in pesticides (plant protection products and biocidal products as defined in Directive 91/414/EEC and 98/8/EC respectively) as well as a sum limit of 0.5 µg/l in total. Additional thresholds can be set however that "*take into account human toxicology and ecotoxicology knowledge" (Article 3)*

[6] PETROTOX (CONCAWE) is a tool using QSAR modelling to assess aquatic toxicity hazard of complex petroleum and related substances. https://www.concawe.eu/Content/Default.asp?PageID=778

and reference is made to the priority substances mentioned in the WFD. Annex II sets guidelines for the establishment of threshold values and includes a minimum list of pollutants for which threshold values have to be considered. As Annexes I and II have to be reviewed every six years after implementation, they have been reviewed in 2013, and the following changes to the groundwater directive have been approved and are expected to be published in 2014: a "watch list" will be set up, similar to the watch list for surface water pollutants, to collect more information on pollutants, including emerging contaminants, and identify which need to be addressed in future. Moreover, because of their contribution to eutrophication, nitrite and total phosphorous are being added to Annex II of the directive, which lists the substance member states should consider setting national threshold values for. Finally, the directive's guidelines for reporting the chemical status of groundwater have been rewritten and more guidance provided on determining threshold values[7].

3.2.5 Marine Strategy Framework Directive (Directive 2008/56/EC)

The Directive on a marine environmental policy is in place to ensure the implementation of "*a thematic strategy for the protection and conservation of the marine environment"* (Article 4) which "*addresses all human activities that have an impact in the marine environment"* (Article 5). It builds on other Directives that are already in place, including Directive 92/43/EEC (Conservation of natural habitats and of wild fauna and flora), Directive 79/409/EEC (Conservation of wild birds) and the Water Framework Directive (2000/60/EC) and aims to "*contribute to coherence between different policies and foster the integration of environmental concerns into other policies, such as the Common Fisheries Policy, the Common Agricultural Policy and other relevant Community Policies"* (Article 9). Although it focuses on a wide range of pressures, special attention is given to the impact of pollutants, while not restricting itself to a limited list of pollutants but treating the list as indicative (Articles 8.1.b.i, 9.1 and 10.1) and taking into account potential cumulative and synergistic effects (Article 8.1.b.ii). The indicative list of compounds is reviewed and updated every six years (Article 17.2.b) or sooner *in the light of scientific and technical progress* (Article 24.1). The directive is quite straightforward in that the compounds present may not pose any risk to the marine environment, but it was recognized that the scientific understanding to support this Directive was still lacking. Therefore, it was decided that before implementing the Directive, more research was needed *for assessing good environmental status in a coherent and holistic manner to support the ecosystem-based approach* (Commission Decision 2010/477/EU, Article 3). It was still emphasized however, that it is important to consider cumulative and synergistic effects, not only by compounds listed in the Water Framework Directive (Directive 2000/60/EC) and others, but also compounds that *may entail significant risks to the marine environment from past and present pollution* (Descriptor 8.iii).

3.2.6 Drinking Water Directive (Directive 98/83/EC)

This directive aims at ensuring a good quality of water intended for human consumption, by setting individual parametric values for substances that are of health concern at a level

[7] Draft directive at: http://www.endseurope.com/docs/140217a.doc

strict enough to ensure "*the protection of human health from the adverse effect of any contamination of water*" (Article 1). The parametric values, listed in Annex I of the Directive, should be based *"on the scientific knowledge available*" and on a risk assessment method, and should take into account microbiological risk, chemical risks and public-health considerations (Recital 13 and 14). They should also have been "*selected to ensure that water intended for human consumption can be consumed safely on a life-long basis, and thus represent a high level of health protection*" (Recital 13).

Member States are in charge of ensuring that water intended for human consumption respects the minimum requirements of the Directive (Article 4) and of setting values for those parameters which shall not be less stringent than those set out in Annex I. They shall also, where required by human health protection, set values for additional parameters not included in this Annex. It is also the duty of the Member States to ensure the efficiency of the disinfection treatment applied and that regular monitoring of the quality of drinking water is carried out (Article 7). Where disinfection is carried out, any contamination from disinfection by-products must be kept as low as possible without compromising the disinfection (Article 7).

In case of non-compliance with the required parametric values, the Member State concerned shall ensure that the necessary remedial action is taken as soon as possible to restore the water quality (Article 8), and should assess the extent to which the relevant parametric value has been exceeded as well as the potential risk to human health (Article 8 and Recital 27).

Thus, this regulation requires the monitoring of individual parameters in drinking water but does not address chemical risk assessment or any mixture issue, although disinfection products or by-products might be of concern for human health (Nieuwenhuijsen et al., 2009; Jeong et al., 2012).

3.2.7 Soil Thematic Strategy and Proposal for Soil Framework Directive

Given the importance of soil and the need to prevent further soil degradation, the Sixth Environment Action Programme called for the development of a Thematic Strategy on Soil Protection, which was adopted on September 2006 (COM(2006)231). Soil is subject to a series of degradation processes or threats. These include erosion, decline in organic matter, local and diffuse contamination, sealing, compaction, decline in biodiversity, salinisation, floods and landslides. Along with the Soil Thematic Strategy, a Soil Framework Directive (SFD)[8] was also proposed (COM(2006)232), although this has not yet been adopted. Within the proposal, many different threats to soil are covered. Among these threats is also the risk of soil contamination and related actions for remediation. The SFD "*should contribute to the prevention and reduction of the introduction of dangerous substances into soil to avoid soil contamination and to preserve soil functions*". However, the problem of soil contaminations is mostly already tackled at national level. SFD Article 9 on Prevention of soil contamination

[8] Proposal for a Directive of the European Parliament and of the Council establishing a framework for the protection of soil and amending Directive 2004/35/EC (COM(2006) 232 final)

states *"For the purposes of preserving the soil functions referred to in Article 1(1), Member States shall take appropriate and proportionate measures to limit the intentional or unintentional introduction of dangerous substances on or in the soil, […] in order to avoid accumulation that would hamper soil functions or give rise to significant risks to human health or the environment*." An inventory of contaminated sites should be developed (Article 11 (2) "*the competent authorities shall have identified the location of at least the sites where the potentially soil-polluting activities referred to in Annex II are taking place or have taken place in the past*.") No specific mention on how to deal with mixtures could be found, even if mixtures will have potentially the same relevance for soil as for water. Annex II of the proposal lists some potentially soil polluting activities, with most of them being clearly linked to contamination with multiple chemicals (e.g. ports, military sites, mining installations, landfills and waste water treatment installations). For soil status reports, "*a chemical analysis determining the concentration levels of the dangerous substances in the soil, limited to those substances that are linked to the potentially polluting activity on the site*" has to be included.

3.2.8 Ambient air quality and cleaner air for Europe (Directive 2008/50/EC)

Several of the above mentioned pieces of legislation are also linked to air quality, however, there is a specific directive on ambient air quality and cleaner air for Europe (Directive 2008/50/EC). Article 1 §1 of the Directive states: *"This Directive lays down measures aimed at the following 1. defining and establishing objectives for ambient air quality designed to avoid, prevent or reduce harmful effects on human health and the environment as a whole"*. This Directive focuses mainly on sulphur dioxide, nitrogen dioxide and oxides of nitrogen, particulate matter (PM10 and PM2.5), lead, benzene and carbon monoxide. In Annex I of the Directive, target values are set as limits for the above mentioned pollutants individually, but exposure to multiple chemicals is not specifically addressed. It does mention however the concern regarding polyaromatic hydrocarbons (PAHs), and in Commission Decision 2011/850/EU, PAHs are mentioned specifically. Although the PAHs in this directive are addressed individually, this group of compounds are generally recognized as being relevant for mixture toxicity and have been addressed accordingly by e.g. the US EPA[9]. The pollutants covered by Directive 2008/50/EC should have been reviewed in 2013, taking into account progress in obtaining scientific information regarding the presence and effects of air pollutants.

3.2.9 Protection of the health of workers from chemical agents at work (Directive 98/24/EC)

This Directive lays down minimum requirements for the protection of workers "*from risks to their safety and health arising, or likely to arise, from the effects of chemical agents that are present at the workplace or as a result of any work activity involving chemical agents*" (Article 1). The employer has the responsibility to assess any risk to the safety and health of workers arising from the presence of hazardous chemical agents at the work place (Recital 14 and Article 4), taken into consideration the level, type and duration of exposure,

[9] http://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=194584

and to set out the necessary preventive and protective measures. The given definition of “hazardous chemical agents” includes both individual chemical agents and chemical preparations "*with the meaning of directive 88/379/EEC*"[10] (Article 2b), i.e, what is now called a “chemical mixture” in the REACH regulatory context (intentional mixture). Moreover, Article 4 (§4) requires that "*In the case of activities involving exposure to several hazardous chemical agents, the risk shall be assessed on the basis of the risk presented by all such chemical agents in combination*". Thus, the risk assessment should take in consideration both exposures to intentional mixtures and exposures to coincidental mixtures in the workplace. Moreover, it is also the employer's duty to eliminate or reduce to a minimum the risk associated with hazardous chemical agents (Article 5 §2) by the design and organization of work, the provision of suitable equipment for work with chemical agents, and by reducing to a minimum "*the number of workers exposed or likely to be exposed, (...) the duration and intensity of exposure, (...) the quantity of chemical agents present at the workplace to the minimum required for the type of work concerned*".

According to this directive, practical guidelines for the determination and assessment of risks shall be developed and updated according to technical and scientific progress (Article 4 and 12§2). To address this requirement, and to facilitate compliance in the EU Member States, a (non-binding) guideline document has been published in 2006 on the protection of the health and safety of workers from the risks related to chemical agents at work (EC, 2006a). This document provides practical tools on how to measure and evaluate air concentrations, on the risk assessment methodologies, on the general principles of protection and prevention and on the surveillance of the health of workers exposed to lead. It also contains information on labelling and safety data sheets of chemical products, occupational limit values, biological limit values and information on the hierarchy of prevention measures. A simplified risk assessment methodology is given in Annex 2 and a quantitative evaluation of exposure to chemicals is given in Annex 4. This Annex 4 requires the setting of "Homogeneous exposure group" (HEG) defined as the association of a job and a chemical agent, or several chemical agents that produce the same effect, in a given environment. Thus, among workers who do the same job, there will be as many HEGs as there are chemical agents with independent effects to which they are exposed. Daily exposure (DE) is then calculated for each HEG, as well as short term exposure (SE, defined as the mean concentration in any 15-minute period in the working day). DE and SE can be then compared to the corresponding Limit value ($LV\text{-}_{DE}$ or $LV\text{-}_{SE}$). Thus, this methodology takes into account the simultaneous exposure to different chemicals having the same effects by summing their concentrations.

[10] Council Directive 88/379/EEC of 7 June 1988 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labelling of dangerous preparations, repealed by Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labelling of dangerous preparations.

3.2.10 Food contact materials (Regulation 1935/2004)

The purpose of this Regulation is "*to ensure functioning of the internal market*" for food contact materials "*whilst providing the basis for securing a high level of protection of human health and the interest of consumers*" (Article 1, §1). It complements the general principles and requirements of food law in Regulation (EC) No 178/2002, defines general requirements for food contact materials (Article 3) as well as specific requirements for "active" and "intelligent" food contact materials (Article 4), and sets the frame for specific measures for specific groups of materials or articles (Article 5)[11].

The Regulation is focussed on the protection of human health and environmental risks are not considered. As a general requirement, food contact materials and articles "*shall be manufactured (…) so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: (a) endanger human health; or (b) bring about an unacceptable change in the composition of the food; or (c) bring about a deterioration in the organoleptic characteristics thereof*" (Article 3, §1), but this regulation does not explicitly or implicitly address the topic of mixture toxicity. The general requirement to consider *probable cumulative toxic effects* in food safety assessments as laid down in Regulation (EC) No 178/2002 (see above) may apply, but this cannot be interpreted as a mandatory requirement for mixture toxicity assessments. Moreover, this regulation does not prescribe how corresponding scientific risk assessments should be performed.

3.2.11 Contaminants in food (Regulation 315/93/EEC) and feed (Directive 2002/32/EC)

While the above-mentioned regulations on food and feed additives refer to compounds and mixtures that are intentionally added to the product, various regulations relate to the presence of unwanted compounds and/or mixtures in food (Regulation 315/93/EEC) and feed (Directive 2002/32/EC). The provisions regarding the presence and levels of undesirable compounds have been revised many times, generally for specific types of pollutants (e.g. Regulation 744/2012 and 1275/2013). Regulation 2002/32/EC is one of the few regulations that cover some specific toxic mixtures, in this case the combined effects of dioxins and dioxin-like compounds like furanes and dioxin-like PCBs. The combined effects are based on the toxicological concept of Toxic Equivalency Factors (TEF) (Van den Berg et al., 1998), a mode of action (in this case Ah-receptor binding) based approach to assess the combined effects of a mixture of similarly acting (dioxin-like) compounds. It relies on equivalency factors for individual components that serve as an order of magnitude estimate of the toxicity of individual compounds relative to 2,3,7,8-TCDD. These values have been calculated for several species and are reviewed regularly. Similarly, TEF values are also used to monitor combined dioxin-like effects in industrial emissions (Directives 2008/1/EC and 2010/75/EU).

[11] It is complemented by a list of specific supplementary acts, defining specific rules for specific food contact materials such as regenerated cellulose film, ceramics, plastic materials, vinyl chloride, plasticisers in States relating to ceramic articles intended to come into contact with foodstuffs gaskets in lids, epoxy derivatives, and nitrosamines from rubber and migration testing. Respectively Directive 2007/42/EC, Directive 84/500/EEC, Directive 2002/72/EC, Directives 78/142/EEC, 80/766/EEC, and 81/432/EEC, Regulation (EC) No 372/2007, Regulation (EC) No 1895/2005, Directive 93/11/EEC, Directives 82/711/EEC, and 85/572/EEC

3.2.12 Maximum residue levels of pesticides (Regulation 396/2005)

This regulation aims at ensuring that pesticide residues are not present in food and feed products "*at levels presenting an unacceptable risk to humans and, where relevant, to animals*" (Recital 5). It focuses only on human risk assessment and environmental risk assessment is out of scope. The Regulation establishes the maximum quantities of pesticide residues permitted (maximum residue levels, or MRLs) in products of animal or vegetable origin intended for human or animal consumption. These MRLs include MRLs that are specific to particular foodstuffs intended for human or animal consumption and, for products and/or pesticides for which no specific MRLs are set, a default value of 0.01 mg/kg applies (Article 18, paragraph 1(b)). Risk assessment is the responsibility of EFSA (Recital 6, Articles 10 and 24), which provides Reasoned Opinions on each intended new MRL, amendment or removal.

Established procedures for setting MRLs on the basis of ADI values and food consumption patterns are focused on single substance assessments, although the Regulation explicitly addresses the need for carrying out "*further work to develop a methodology to take into account cumulative and synergistic effects"* and states that MRLs should be set in "*view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health*" (Recital 6).

As a consequence, support measures related to harmonized pesticide MRLs shall include "*studies and other measures necessary for the preparation and development of legislation and of technical guidelines on pesticide residues, aimed, in particular, at developing and using methods of assessing aggregate, cumulative and synergistic effects*" (Article 36, § 1 (c)), and Commission decisions concerning the MRLs shall take account of "*the possible presence of pesticide residues arising from sources other than current plant protection uses of active substances, and their known cumulative and synergistic effects, when the methods to assess such effects are available*" (Article 14, § 2 (b)).

To address these requirements, the EFSA *Panel on Plant Protection Products and their Residues* (PPR) published a report of a colloquium held on 2006 on the *Cumulative Risk Assessment of Pesticides to Human Health: the Way forward* (EFSA, 2007), as well as an opinion paper on methods for cumulative risk assessment of pesticides (EFSA, 2008b) (see chapter 3). The latter report reviews existing methodologies for mixture toxicity assessment and combines these in a tiered approach. It also suggests applying this for cumulative assessment groups (CAG) of pesticides with a common mode of action. As a next step, EFSA applied the proposed strategy to the group of triazole fungicides (EFSA, 2009c). Although EFSA concluded that the proposed approach was appropriate, its report also highlights the following needs before being able to apply this approach on a routine basis: (*i*) an international consensus on which groups of pesticides could be looked at together through a cumulative risk assessment approach; (*ii)* further work on the application of new cumulative risk assessment methodologies in order to address uncertainties; and (*iii*) development of guidance for appropriate exposure assessments. To answer these needs, EFSA has recently published a guidance document on the use of probabilistic methodology for modelling dietary exposure to pesticide residues (EFSA, 2012c) and a scientific opinion on the identification of

pesticides to be included in cumulative assessment groups on the basis of their toxicological profile (EFSA, 2013c) (See Chapter 3).

3.2.13 Toys (Directive 2009/48/EC)

The safety of toys is addressed by Directive 2009/48/EC, which covers many aspects of safety of toys including chemical safety. It is in place to *ensure a high level of protection of children against risks caused by chemical substances in toys.* It is also stressed that the provisions from REACH should be *adapted to the particular needs of children, who are a vulnerable group of consumers (Recital 21).* Regarding the toxicological properties of the chemicals, the Directive refers to REACH (Regulation 1907/2006) and the Regulation on Classification & Labelling (1272/2008) (§ 3.1.6 and 3.1.5 respectively). Particularly substances that are classified as carcinogenic, mutagenic or toxic for reproduction (CMR compounds), allergenic substances and certain metals are of concern. A list is provided of allergenic fragrances that are not allowed or should be clearly labelled to be present when concentrations exceed 100 mg/kg. *S*pecific limit values are given for certain substances in directive 88/378/EEC, which should be updated as scientific knowledge increases. It is encouraged to replace dangerous substances by less dangerous equivalents where technically and economically possible. Several compounds that are particularly toxic should not be used intentionally (arsenic, cadmium, chromium VI, lead, mercury and organic tin) and *limit values should be set at levels half of those considered safe (Recital 22).* Specific limit values can be adopted for *toys intended for use by children under 36 months, and in other toys intended to be put in the mouth (Recital 24 and Article 46)* (though none have been defined) and there is a legal obligation to assess *the likelihood of the presence in the toy of prohibited or restricted substances (Recital 35).* In some situations, CMR compounds can be used if the individual concentrations are below what is specified in Appendix B2, or when the substances are inaccessible to children when used as intended or in a foreseeable way and use is not prohibited for consumer articles under the REACH regulation or is compliant with the regulation on food contact materials. Migration limits are specified for several elements, although these do not apply for toys or components which clearly exclude any hazard due to their mass, size, volume etc.

Mixtures are mentioned specifically in Directive 2009/48/EC, and are defined as a solution composed of two or more substances (definition adopted from Directive 67/548/EEC). For the classification of substances and mixtures, a clear distinction is made between substances and mixtures. For substances, reference is made to the classification under Regulation 1272/2008, while for mixtures Directive 1999/45/EC and Directive 67/548/ECC applies until 31 May 2015, from which point Regulation 1272/2008 will apply. Nitrosamines and nitrosable substances are treated as a group and are not allowed if the total migration of the substances is equal to or higher than 0.05 mg/kg for nitrosamines and 1 mg/kg for nitrosable substances.

Directive 2009/48/EC solely addresses human health concerns, environmental concerns are addressed by horizontal environmental legislation and the legislation on waste.

3.3 Conclusions

Table 4 and Table 5 summarise the different EU requirements across sectors regarding chemical monitoring/risk assessment and mixture risk assessment, respectively for intentional mixture and generated or coincidental mixture. Chemical mixtures are predominantly assessed and regulated in relation to intentional mixtures under EU legislation. Some complex mixtures discharged/emitted to the environment from a single source are also subject to controls, but there are very few requirements for, and examples of, control and risk assessment being carried out in relation to several substances originating from different sources and through different pathways. Moreover, whereas a product can be subject to several sectorial regulations, each sectorial regulation requires a risk assessment for its own type of use only, without taking into account the possible additional exposure to other type of use under other sectorial regulation. As such, current EU legislation does not provide a mechanism for a systematic, comprehensive and integrated assessment of cumulative effects of different chemicals taking into account different routes of exposure and different product types (EC, 2012a).

Therefore, although there is a very extensive corpus of legislation aiming at ensuring that humans, animals and the environment are exposed to individual chemicals within safe limits, there is a need to examine whether or not an exposure to a mixture of chemicals substances, originating from different sources and through different pathways, in which each of the substances is present at a concentration below its threshold of concern, could have negative effects on human health or the environment.

Table 4: Summary table. Chemical and mixture assessment requirement in the European regulation. Intentional mixtures.

		Chemical monitoring and analysis	**Encouraging alternative methods to animal testing**	**Chemical risk assessment**		**Mixture assessment**		**Prospective RA**	**Retrospective RA**
				Human health	Environmental health	Human health	Environmental health		
Plant protection products	Reg 1107/2009	No (Environment)/ Yes (Residues)	Yes	Yes	Yes	Yes – WM approach (for formulation)		Yes	No
PPP- AI Requirement	Reg 283/2013								
PPP- Formulation Requirement	Reg 284/2013								
Biocides	Reg 528/2012	No (Environment)/ Yes (Residues)	Yes	Yes	Yes	Yes – WM approach, plus risk assessment if the biocidal product is intended to be used in combination with other biocidal product.		Yes	No
Human medicines	Dir 2001/83/EC	No	Not specified	Yes	Yes	Yes	No	Yes	No
Veterinary medicines	Dir 2001/82/EC	No (Environment)/ Yes (Residues in foodstuff)	Not specified	Yes	Yes	Yes	No	Yes	No
Cosmetics	Reg 1223/2009	No	Yes	Yes	Through REACH	Yes	No	Yes	No
CLP	Reg 1272/2008	No	Yes	Yes	Yes	Yes	Yes	Yes	No
REACH	Reg 1907/2006	No	Yes	Yes	Yes	Yes	Yes	Yes	No
Food additive	Reg 1333/2008	Yes (Intake)	No	Yes	No	No	No	Yes	No
Feed additive and feed additive assessment	Reg1331/2008; Reg 429/2008	Yes (Residue)	No	Yes	Yes	Additive with multiple components: consideration is given for the cumulative effects or WM assessment. No consideration for mixture assessment from different sources		Yes	No

Table 5: Summary table. Chemical and mixture assessment requirement in the European regulation. Generated or coincidental mixtures.

		Chemical monitoring and analysis	Encouraging alternative methods to animal testing	Chemical risk assessment		Mixture risk assessment		Prospective RA	Retrospective RA
				Human health	Environmental health	Human health	Environmental health		
Environmental Impact Assessment (EIA)	Dir 2011/92/EC	No	No testing	Yes	Yes	Should be addressed		Yes	No
Integrated pollution prevention and control (IPPC)	Dir 2010/75/EC	Yes	No testing	No	No	No	No	No	No
Waste and waste streams	Dir 2008/98/EC	No, but refers to other legislation, e.g REACH, IPPC	No	Refers to CLP	Refers to CLP	No		No	No
Water Framework Directive (WFD)	Dir 98/83/EC	Yes	No testing	Yes	Yes	Mentioned in Guidance, but not addressed in detail		No	Yes
Groundwater Directive	Dir 2006/118/EC	Yes, with reference to WFD	No	No RA, but contained additional threshold value for pesticides and nitrates	No	No	No	No	No
EQS Directive	Dir 2013/39/EU	Yes	Yes	No	No RA, but QS are derived from ERA studies	No	Yes	No	No
Marine Strategy	Dir 2008/56/EC	Yes, with reference to WFD	No testing	No	Yes	No	No	No	No
Drinking water	Dir 98/83/EC	Yes	No testing	No RA, but parametric values should be based on a RA method	No	No	No	No	No
Soil thematic strategy – Soil framework directive	COM(2006)231/COM(2006) 232 final,2006/0086 (COD))	Yes	No	Yes	Yes	No	No	No	Yes
Ambient Air quality and cleaner air for Europe	Dir 2008/50/EC	Yes	No testing	Yes	Partly	No	No	No	Yes
Food Contact material	Reg 1935/2004	Yes	Not specified	Yes	No	No	No	Yes	No

Table 5: Summary table. Chemical and mixture assessment requirement in the European regulation. Generated or coincidental mixtures (continued)

		Chemical monitoring and analysis	Encouraging alternative methods to animal testing	Chemical risk assessment		Mixture risk assessment		Prospective RA	Retrospective RA
				Human health	Environmental health	Human health	Environmental health		
Food contaminant	Reg 315/93/EEC	Yes (Food)	Not specified	Yes	No	No	No	Yes	No
Feed contaminant	Dir 2002/32/EC	Yes (Feed)	Not specified	Yes	Yes	No	No	Yes	No
Maximum Residue Levels (MRLs)	Reg 396/2005	Yes	Not specified	Yes	No	Yes	No	Yes	No
Protection of the health of workers	Dir 98/24/EC	Yes	Not specified	Yes	No	Yes	No	Yes	No
Toys	Dir 2009/48/EC	Yes	No testing	No RA but threshold value and refers to CLP	No	Refers to CLP	No	No	No

4 - Existing guidance documents on mixture toxicity assessment

The first part of this chapter reviews the recommended approaches to handling mixtures and combined exposures in risk assessment in a regulatory context in EU Member States, based on guidance documents and guidelines. The second part describes approaches to mixture toxicity assessment used in other parts of the world or by international agencies or organizations (such as the World Health Organization, the OECD, or the European Centre for Ecotoxicology and Toxicology of Chemicals).

4.1 Mixture toxicity in European Guidance Documents

4.1.1 Toxicological guidance and mixture toxicity assessment

REACH

A document was published in 2010 as a result of a joint CEFIC/VCI project to develop tools and guidance on exposure assessment for industry (CEFIC, 2010). It deals with mixture exposure assessment and communication in the supply chains, and describes the tasks and obligations of the different actors who handle mixtures (i.e. manufacturer and downstream user such as formulators or end user). Nearly all REACH obligations are related to substances as such or as part of a mixture – but not to mixtures themselves. Although the chemical safety assessment of a substance should cover its whole life cycle and consider the different exposure routes, operational conditions and risk management measures, most of the time the registrant does not know the recipes of the mixtures in which his substance will be used by downstream formulators. In general, the registrant assumes that the use of a substance in a mixture can be seen primarily as a dilution of the substance by other substances, considered to be inert. If substances with the same hazards and/or effects profile are formulated together, the possible synergistic or antagonistic effects have to be considered; if the manufacturer of the substance does not know about this, it is the task of the formulator to take his specific knowledge on the mixture into account. However, this document highlights that REACH sets no formal obligation to perform a cumulative risk assessment of mixtures with exposure estimation and quantitative risk characterisation, and that there is no formal obligation for any actor in the supply chain to develop an exposure scenario for a mixture. In addition, authorities have several possibilities but no obligations to take into account possible effects of aggregated and cumulative exposure to the multitude of substances under their review. Finally evaluation of specific properties of mixtures relies heavily on the expert knowledge of the formulator, which should take into account existing knowledge regarding the function and possible interaction between substances when assessing a mixture. If mixtures have specific properties with relevance for the exposure situation it is likely that they require an advanced evaluation, and information should be given by the formulator to his supplier in order to ensure that these properties are covered by the registration.

More recently, a report describing and discussing approaches for the environmental risk assessment (aquatic compartment) of mixtures under REACH was published (Bunke et al.,

2014). It presents the results of a research and development project of the German Federal Environmental Agency (project short title: "4M: Mixtures under REACH"), which took into account findings regarding the assessment of mixtures under other pieces of legislation. It focuses on technical mixtures and discharge mixtures and considers both cumulative and aggregated exposures. The report also briefly discusses interfaces to other regulations (e.g. Water Framework Directive) and consideration of substances not regulated under REACH (e.g. biocides).

In this project, a tiered component based approach for the risk assessment of technical mixtures is proposed, allowing the environmental assessment of technical mixtures in the aquatic compartment with increasing degrees of precision. Figure 2 shows the structure of the proposed tiered approach for the risk assessment of technical mixtures, which consists of three components: a tiered exposure assessment (left column), a tiered hazard assessment (right column) and a tiered risk assessment (central part). As usual, the resulting risk is calculated as the quotient of the estimates from the exposure assessment and the hazard assessment.

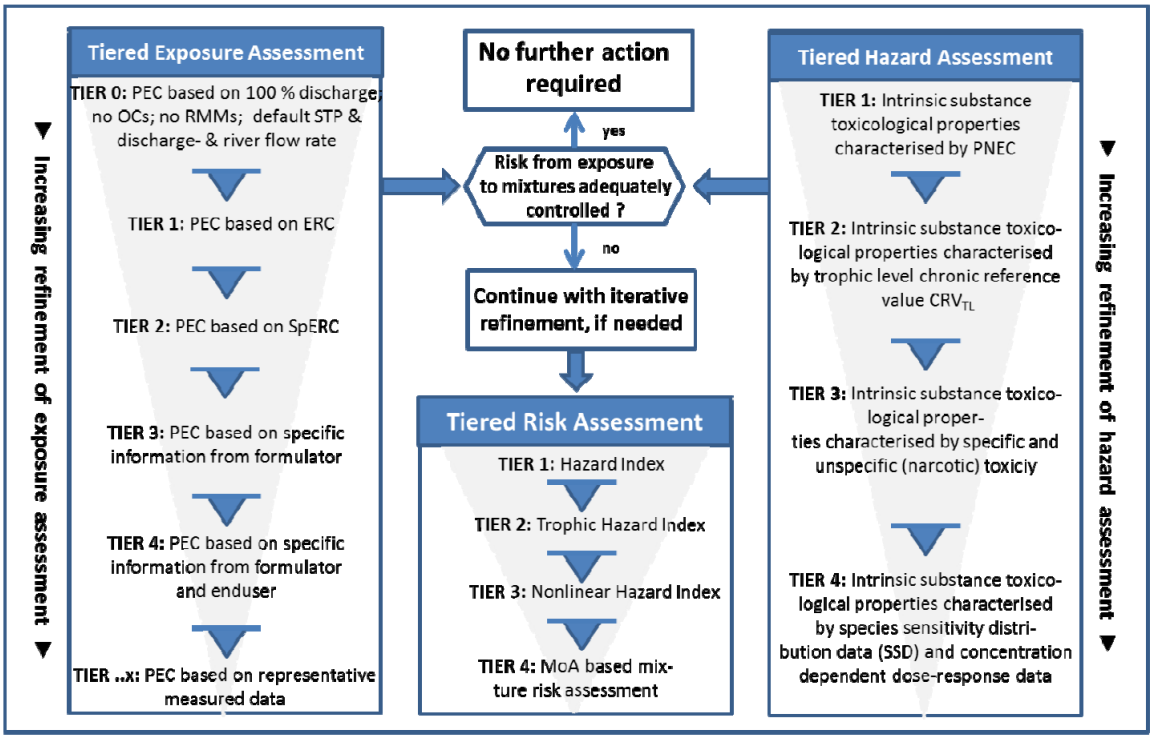


Figure 2: Schematic presentation of a tiered approach for assessment of technical mixtures under REACH (Bunke et al., 2014). *Source: German Federal Environmental Agency (UBA).*

Note that numbering of tiers is flexible and not necessarily identical to tiers as used in similar schemes. This figure only demonstrates the principle factors and hierarchies schematically. For a final guidance, numbering and assigned definitions may have to be adapted.

At tier 1, concentration addition is applied in its simplest form. For each substance in the mixture, risk characterisation ratios are calculated using PECs and PNECS, and the ratios of the substances are summed up (the result of which is commonly called the "Hazard Index",

HI, for the technical mixture). If HI<1, no risk for environmental effects from the exposure to the mixture is expected. This approach is relatively simple but it may overestimate the risk as predicted environmental concentrations often overestimate the real exposure situation. In addition, the PNEC as a single value must be protective for all three trophic levels - algae, daphnia and fish. Species-specific toxicity data (e.g. chronic toxicity values for fish) are not evaluated specifically at this tier.

At tier 2, the assessment is refined by replacing PNECs by the "chronic reference values specific for trophic level" (CRV_{TL}), based on species-specific ecotoxicity data. For each trophic level, such a reference value is calculated for each substance, and Risk Characterization Ratio for the substances are calculated using these reference values and the respective exposure levels. The substance "RCRs" for each trophic level (RCR_{TL}) are added up to calculate a hazard index for the mixture for each trophic level (HI_{TL}). Finally, the highest of these hazard indexes is chosen to characterise the risk posed by the mixture. This index, called the "trophic level hazard index" (THI), is comparable to Hazard Index HI at tier 1, but does not add up effect data from different species to estimate mixture effects, and will usually be lower than the risk quotient calculated at tier 1. This step avoids overestimation of mixture ecotoxicity at least to some degree due to the separate assessment of each trophic level.

At tier 3 and tier 4, more sophisticated elements are introduced on the hazard side, such as differentiation between specific and unspecific (narcotic) toxicity and knowledge on the mode of action. For tiers 1 – 3 of the hazard assessment, concentration addition is assumed as the basic principle for emerging mixture effects. In the fourth tier, independent action (IA) and more detailed information may be used for mixture risk quantification. It is not feasible to complete a hazard assessment at tier 3 and 4 with the set of data usually generated (and published) under REACH, and they require expert knowledge. This tiered approach has been tested on real technical mixtures from a tannery.

As possible supplementary elements, mixture assessment factors (MAFs) and whole mixture testing can be considered. MAFs are sometimes proposed if a tiered approach is not feasible, and used as an additional assessment factor to calculate a PNEC for all those single substances known to be present within the mixture. Whole mixture approaches/whole effluent approaches are proposed to assess effects of mixtures with (partly) unknown substances, or for mixtures of known composition to assess whether and which kind of mixture effects occur. Because of the almost infinite number of resulting potential mixtures, this approach would not be feasible as a routine regulatory procedure to assess mixture toxicity, but may be helpful to: a) validate CA, IA or synergism/antagonism assumptions for specific mixtures with known substances; and b) compare such assessments based on known substances with the additional influence by unknown further substances within a mixture; and c) assess constant emission scenarios, where substances are present in stable compositions in environmental media.

This document also identifies and acknowledges current limitations for risk assessment of technical mixtures under REACH, i.e. the generic and very crude substance exposure levels (PECs) generated by REACH risk assessment tools, the disparity in the availability of

suitable data across the supply chain limiting the possibilities of different actors to assess mixture risks, and the missing link between the responsibilities of the single REACH actors and the real environmental risk of the actual local coincidental mixture in the receiving water volume.

As priority setting is essential for deciding in which cases an additional risk assessment of technical mixtures should be performed, this document also describes two different concepts for priority setting: a) nomination of "Mixture Assessment Triggering Substances" (or MATS, defines as a substance which causes concern, because it already occurs in relevant concentrations in the environment in relation to its ecotoxicity) and b) the identification of "priority mixtures" having specific properties (from composition or critical uses/exposures) which increase the likelihood of mixture effects. Thus, the concept of MATS aims to identify substances which occur already in relevant concentrations in the environment (the proposal foresees that in most cases such substances will be nominated and selected by a regulatory decision from authorities), whereas the starting point for the identification of "priority mixtures" is the composition of the mixtures and their uses. The authors also propose that priority substances from the Water Framework Directive could be used as long as no official MATS are nominated.

This report also describes several options to assess technical mixtures under REACH, which refer not only to registrants, but also to formulators and end-users; e.g. a formulator could use a tiered approach as an indicator for safe use of a specific technical mixture, whereas an end-user could assess the aggregated exposure if he uses the same substance in different technical mixtures. Also options to act for the national competent authorities are presented – related to substance evaluation, restriction and authorisation. A first and preliminary, qualitative assessment of the feasibility of the options was conducted, and their implementability without changes of the legal text was assessed.

Finally, the following five activities were recommended by the authors:

- Assessment of aggregated exposures by end-users
- Communication and use of existing knowledge on mixture effects in the supply chain
- Development of prioritisation criteria for mixtures
- Application and further development of the tiered approach by formulators
- Mixture assessment as an element of the tasks of authorities

The two first activities could be implemented in the short term, as they require fairly low efforts and have benefits regarding an improved risk management (aggregated exposures) and knowledge dissemination and awareness raising. All other options identified as possibly feasible should be subject to further assessment, testing and discussion before implementation.

External experts have been invited to discuss major findings of the project at a workshop, and the results and conclusions of those discussions are presented in the final report. Possible next steps for validating and refining the proposed mixture risk assessment strategy and for implementation are described, and the need for more coordination with other

pieces of legislation in order to develop a common strategy not only for REACH, but including also other regulations.

Cumulative risk assessment and MRLs

Regulation (EC) No. 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin emphasises the importance of developing a methodology to take into account cumulative and possible synergistic effects of pesticides to human health.

In this context EFSA organised a Colloquium in 2007 to allow for an open scientific debate on methods available for conducting a CRA (i.e. referring to the assessment of the risk from exposure to more than one pesticide) for pesticides with a common MOA, and to explore the scientific basis for grouping pesticides, not sharing a common MOA, in a hazard assessment (EFSA, 2007). Participants discussed the choice of data and methodology for combined exposure assessment, and possibilities for joining efforts internationally to further develop harmonised approaches.

They agreed that a number of methodological options exist with respect to food consumption, residue and effect data; and that it was important to start the CRA process in a step-wise approach in order to develop a pragmatic strategy within the EU. The first priority would be substances sharing a common MOA for which evidence indicates that they act with dose addition and for which data were already available in the US. They also recommended that sources of exposure other than the dietary route should be included in the longer term, and that EFSA should not restrict CRA issue to pesticides because CRA is a general issue which goes beyond exposure to pesticides alone.

Based on the output of this colloquium, the PPR Panel published an opinion on specific actions needed for the near future (EFSA, 2008b), in which they evaluated existing methodologies for assessing risks of exposure to two or more pesticides in combination, particularly in the context of setting MRLs (Reg. (EC) No. 396/2005).

Although the Panel's opinion is that all sources (e.g., PPP, veterinary drugs, human medicines), pathways (e.g., food, drinking water, residential, occupational) and routes of exposure should be considered, they highlighted that appropriate data on levels of exposure to pesticides from pathways and sources other than PPP residues in food are not generally available. Therefore the PPR Panel restricted its consideration of CRA to exposures from PPP residues in food. Having considered the potential relevance of the different forms of combined toxicity (i.e. dose addition, independent action or interaction) to RA for pesticide residues in food, and having noted that there is no empirical evidence for interactions occurring at the expected levels of exposure, they also restricted this opinion to the possible impact of dose addition when consumers are exposed to combinations of pesticide residues that share (or might share) the same MOA.

The PPR Panel identified criteria for selecting groups of compounds for consideration in a CRA, such as frequency of detection in monitoring programmes, high use, evidence of "high" intake from biomonitoring data, or high exposures relative to reference values. In addition they noted that the assessment of specific pesticide combinations might be carried out if there were strong evidence that certain compounds may interact below their respective

NOAELs, and that the presence of relevant non-food sources of exposure might require an assessment of the margin of exposure for the food part.

The PPR Panel also proposed criteria for the grouping of pesticides into a cumulative assessment group (CAG), such as chemical structure or mechanism of pesticidal action, common toxic effect, or ultimately toxic MOA. Amongst the various methods described for CRA, the Panel concluded that the most useful were, in increasing levels of complexity and refinement, the hazard index, the reference point index, the relative potency factor method and physiologically-based toxicokinetic (PBTK) modelling.

Acute and chronic exposure scenarios for RA were considered, both in the context of MRL-setting and in relation to actual exposures based on monitoring data; and they discussed which data source should be used in each exposure scenario. Proposals were made on how to deal with residues below the Limit of Detection (LOD), Limit of Quantification (LOQ), or Limit of Reporting (LOR). An overview of the food consumption data available at the European level and how to use them in cumulative exposure assessment was also provided. They presented general issues on uncertainties as well as those specific to CRA, related to residue data, consumption and toxicity evaluation, and reported a qualitative estimate of their relevance.

The PPR Panel also performed a critical overview of CRA already done, and noted that some compounds initially included in a CAG on the basis of toxicological considerations were further excluded from the CRA on the basis of exposure considerations.

Finally, they proposed a tiered approach for both hazard assessment and exposure assessment in order to make the most efficient use of available resources. This tiered approach is presented in Figure 3.

In this Opinion, they also highlighted the necessity to establish a continuing dialogue between toxicologists, exposure assessors, and risk managers, in order to identify the relevant issues, to best use available resources, and to consider collection of data more representative of actual exposure to pesticides forming CAG. Moreover, the raw survey data from monitoring programmes of pesticide residues and from national food consumption databases should be accessible for RA purposes and they suggested to perform a harmonized consumption survey at the European level along the lines of the four European GEMS/Food cluster diets. Moreover, they recommended adopting a case-by-case approach to assess combined effects in case of biological plausibility for an interaction between pesticides at low, non-effective doses.

As a next step, the PPR Panel worked on a concrete example and the proposed methodology was applied to a selected group of triazole fungicides. They applied the tiered approach in different exposure scenarios of relevance for risk management, for both acute and chronic cumulative effects: these were actual acute exposure, actual chronic exposure, acute exposure relevant for MRL-setting and chronic exposure relevant for MRL-setting. Risk characterisation was then performed for each of the four scenarios by calculating HI, adjusted HI (with several tiers of refinement on the exposure side), and applying the RPF method to either NOAELs or BMDs as the reference point. The results were reported in a

separate opinion (EFSA, 2009c), in which they described progressive steps of refinement in CRA:

(i) establishment of a CAG through the analysis of the specific toxicological effects common to triazole pesticides and their underlying biochemical mechanisms

(ii) refinement of the hazard characterisation, making use in successive tiers of regulatory reference values, reference values based on the common specific toxicological effects and benchmark dose modelling,

(iii) refinement of the cumulative exposure assessment making use of deterministic and probabilistic methodologies in successive tiers.

It was recognised that, while risk assessment for single compounds is typically based on the most sensitive adverse effect, this will not necessarily hold true for risk assessment of multiple compounds which ideally should be based on an endpoint selected on the basis of a common MOA.

Based on this exercise, the PPR Panel proposed a simplification of the overall tiered approach. The CAG should be as refined as the data would allow at an early stage, and exposure assessments should ideally be restricted to one deterministic and one probabilistic tier.

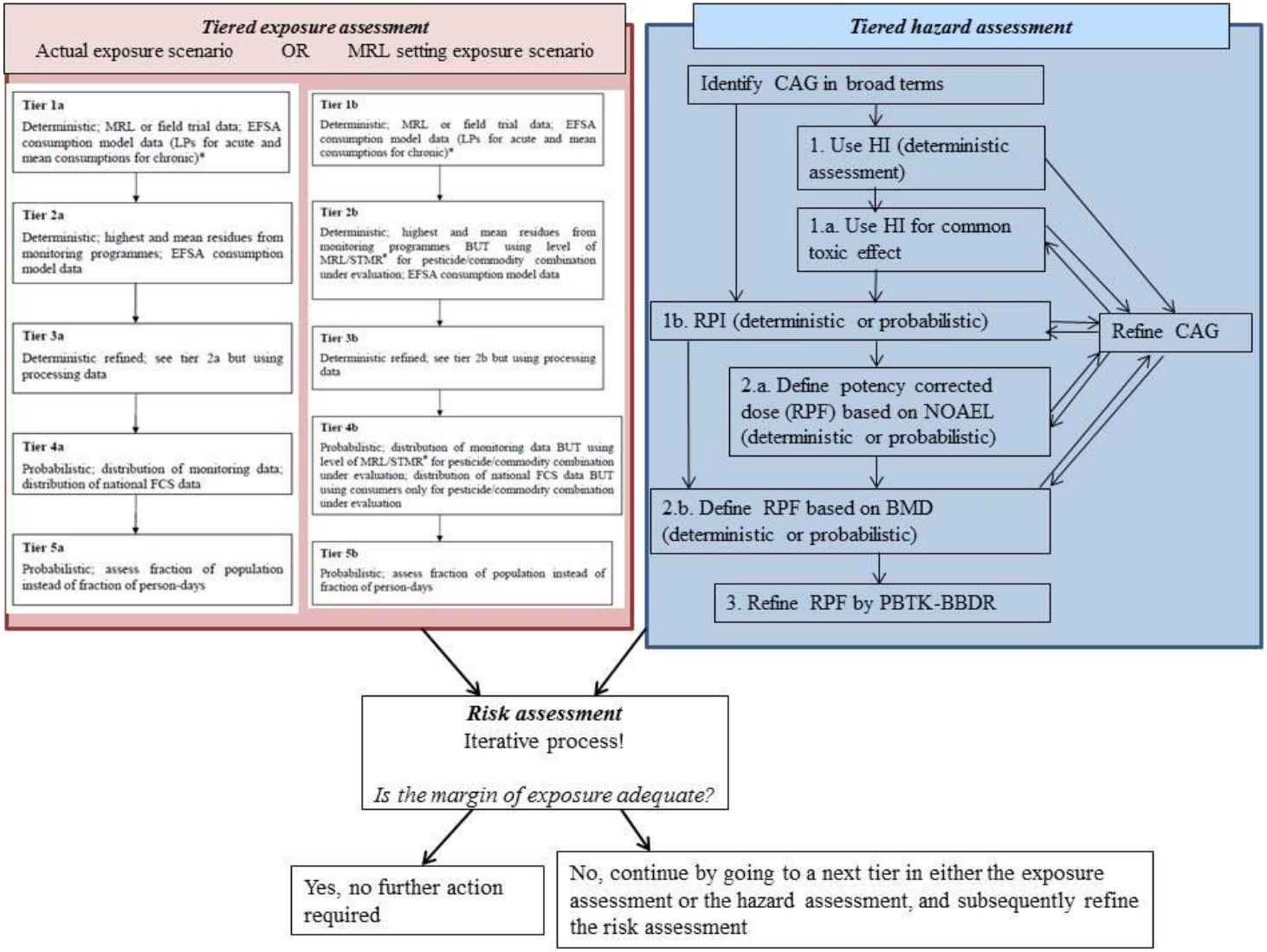


Figure 3: Proposed cumulative risk assessment process, using a tiered approach both on exposure and hazard assessment (EFSA, 2008b, modified)

Source: European Food and Safety Authority

Overall, it was concluded that this tiered approach was an appropriate way to address cumulative dietary risk assessment, but that the following issues must be addressed before it could be applied on a routine basis: the establishment of CAGs at the European level, the definition and agreement on desired levels of protection, and the improvement of the robustness of methodologies of cumulative exposure assessment and development of guidance on their appropriate use.

To start addressing these issues, EFSA asked the PPR Panel to provide guidance on probabilistic dietary exposure assessment, and to develop a methodology to identify pesticides to be included in CAGs on the basis of their toxicological profile.

In this context, the PPR Paned published in 2012 guidance on probabilistic dietary exposure assessment of single and multiple active substances. This guidance aims at complementing and supplementing the standard deterministic methodologies currently used in the EU (EFSA, 2012c). These deterministic methods, although having the advantage of being simple and fast to use, only allow estimation of the exposure of one single virtual consumer, whereas probabilistic methods allow estimation of the distribution of intakes[12] among multiple individuals in a specified population, taking into consideration the variability in food consumption (both between and within individuals) and in occurrence of residues in food commodities. Therefore, probabilistic methodologies are necessary for higher tier assessment where deterministic approaches are insufficient to reach a risk management decision.

In the same document, the PPR Panel provided specific guidance for performing probabilistic modelling of both acute and chronic exposures assessment of single active substances in the contexts of authorisation, MRL setting, enforcement actions, and periodic reviews of monitoring data on actual exposures. The recommended approach makes a distinction between basic and refined probabilistic assessment, and provides specific guidance for basic probabilistic assessments but not for refined assessments, for which specialised expertise is required to select appropriate methods.

A separate section deals with additional approaches required for modelling exposure to multiple substances (cumulative assessment), and addresses two aspects of cumulative assessment that have been identified as impacting the methodology for probabilistic modelling: the methodology for cumulation of toxicity, necessary because cumulative risk is assessed by combining the exposures of different compounds expressed as functions of their toxicities; and the need to quantify co-occurrence of residues in food.

The methodology for cumulation of toxicity refers to the methods described in previous guidance i.e. in order of increasing complexity, the Hazard Index (HI), the adjusted Hazard Index (aHI), the Reference Points Index (RfPI), the Relative Potency Factors (RPF), and the physiologically based toxicokinetic and toxicodynamic modelling (PBTK and PBTD) approaches (EFSA, 2008b, 2009c). The assumptions and uncertainties of each of these methods are highlighted. PBTK and PBTD either separately or in combination can be

[12] Refers to the amount of chemical taken up by the dietary route, i.e., dietary exposure.

considered as options for refined assessment, and can be used to explore possible types of combined action other than dose addition.

Regarding co-exposure of residue assessment, it is necessary to take account of any correlations that may exist between the concentrations of different members of the CAG in the same food sample; therefore, it is necessary to have data where the different CAG members are measured in the same samples. In case of complete residue datasets, in which the same substances are measured in every sample, the PPR Panel recommends to apply RPFs to combine all the substances into a single measure of cumulative potency for each sample, and to generate the cumulative acute assessment in the same way as for a single substance assessment. When the substances analysed differ between samples so that the matrix of samples by substances is incomplete and contains a mixture of positives, non-detects and missing values, the Panel recommends a procedure for basic probabilistic assessment of acute exposure, which captures the correlations present in the available data and replaces missing values in a way that should over-estimate the degree of positive correlation. This avoids the need to estimate the level of correlation, but this also over-estimates exposure and risk.

The guidance also includes a checklist of key issues to be considered when writing or peer-reviewing reports on probabilistic exposure assessments, a list of desirable characteristics of software for probabilistic exposure modelling, and case studies illustrating some of the recommended approaches. Finally, the PPR Panel also provide some recommendations on further work that will be required to make the methods available to end-users in a more practical form, including software and more specific user instructions.

In 2013, the PPR Panel published another opinion dealing with the identification of pesticides to be included in cumulative assessment groups (CAGs) on the basis of their toxicological profile (EFSA, 2013c). As there are often few or no data available on MOA and as many compounds affect the same target and/or cell population, they suggest an approach for grouping of pesticides based on phenomenological effects, which can be applied even when the underlying biochemical events are not understood. The approach also takes cumulative effects into account in the decision on applications concerning MRLs of pesticides in or on food and feed. However, as interactions (synergisms or antagonisms) are not expected to occur at the low exposure levels of residues that are observed, the PPR Panel considers that mainly dose additive effects of substances are normally relevant to CAGs that may be used in the context of MRL setting. Moreover, when insufficient or no information is available, it is assumed that chemicals with the same effects may have a similar MOA, even though they exhibit a wide range of chemical structural features. This is based on empirical evidence that chemically unrelated substances may have a common effect in target organs/systems, which can be well approximated by dose addition (Kortenkamp et al., 2009). The methodology that has been developed for grouping addresses both acute and chronic dietary effects, and comprises four main steps, shown in Figure 4.

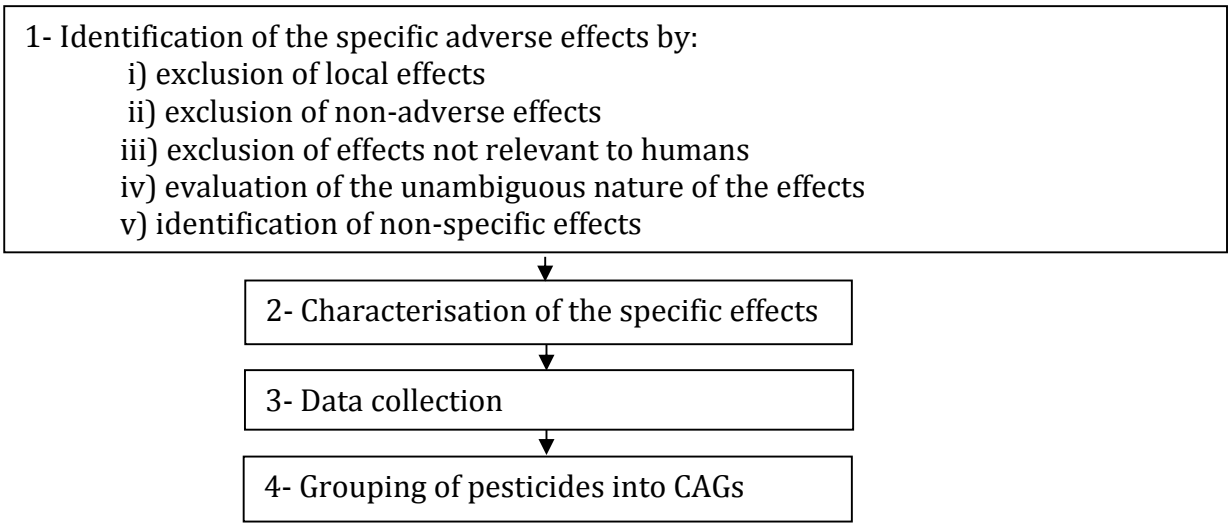


Figure 4: Stepwise methodology for the grouping of pesticides in CAGs based on their toxicological profile

This approach implies the analysis and the interpretation of complex and voluminous data sets, and therefore requires scientific expert judgement. In particular, expert judgement is required to identify and characterise substances that can trigger different outcomes of the same toxicity pathway or that may cause toxic effects at multiples sites by a single mode of action. Moreover, the PPR Panel recognized that grouping based on toxic effects rather than on MOA leads to more uncertainties in predicting possible combination effects; nevertheless, when limiting CAGs to known common MOA (i.e. excluding pesticides for which information on MOA is not available), the degree of uncertainty in CRA would also increase. Thus, a higher level of protection can be afforded by using an effect-based approach, although this introduces some uncertainties around combination effects, until information on precise MOA becomes available. Moreover, further refinement of grouping might be achieved when data on the precise toxicological MOA are available.

The methodology has been applied to establish CAGs for pesticides having adverse effects on the thyroid and nervous system: for the thyroid system, active substances were allocated to CAGs for effects either on C-cells/the calcitonin system or on follicular cells/the T3/T4 system; and active substances exhibiting neurotoxic properties were allocated to CAGs for both acute and chronic effects on motor, sensory and autonomic divisions of the nervous system and neurochemical endpoints. The allocation of pesticide active substances into CAGs was based on a standardised and thorough review of the dataset of oral toxicity studies reported in draft assessment reports (DARs).

The PPR Panel noted that the resulting groups encompass many pesticides and that individual pesticides could appear in several groups; however, although some CAGs contain a large number of pesticides, little indication of cumulative risk may be inferred from the size of CAGs *per se*; and even within large CAGs, cumulative effects are likely to be driven mainly by a few active substances within the group.

According to EFSA, this methodology should be considered specific for pesticides, and cannot be generalized to other types of chemicals in food, however CAGs derived from this methodology could in principle be used to support CRA resulting from non-dietary exposure (i.e operator, worker, bystander and resident exposure). The Panel recommended the implementation of this methodology for all major organ/systems.

ECHA Guidance on biocidal products

In support of Regulation No 528/2012 concerning the making available on the market and use of biocidal products, ECHA published a guidance document for human health risk assessment (ECHA, 2013). This ECHA document provides guidance on risk assessment from combined exposure to multiple biocidal substances within a single biocidal product on the basis of the tiering principles of refinement as described within the WHO/IPCS Framework on Combined Exposures (cf. §4.2.3)[13].

This approach, as shown in Figure 5, includes 3 tiers:

- Tier 1 is the assessment of each substance within the mixture, to verify risk acceptability substance by substance, following all exposure scenarios which are relevant to the product use. The decision-making criterion for acceptability of risk is that the estimated level of exposure to each substance must be lower than its Acceptable Exposure Level (AEL), or another European validated value (e.g. DNEL for the purpose of REACH implementation). The hazard quotient (HQ), defined by the ratio of internal exposure and AEL, can also be used: if HQ<1: the risk from the individual components is considered acceptable and the effects of the biocidal product/mixture must be assessed (Tier 2); whereas if HQ>1: the risk from the individual components is not considered acceptable and before proceeding to Tier 2 refinement of hazard and/or exposure assessment needs to be performed first so that the HQ is lower than 1.

It is noteworthy that this methodology can be applied only in order to assess systemic risks, and is not relevant for local effects of mixtures, in which cases CLP rules would apply.

- Tier 2 involves assessing the combined exposure to the substances of the mixture/biocidal product by concentration (dose) addition by default, if no synergistic effects have been reported, and relies on worst-case scenarios. Hazard Quotients (HQ) for each substance will be used to calculate the Hazard Index (HI) for the mixture/biocidal product. The risk related to use of the mixture will be considered acceptable if $HI \leq 1$, whereas it will be considered unacceptable if $HI > 1$ which would trigger risk refinement, considering risk management measure (RMM) or performing Tier 3. When RMM are considered, taking into account that the conditions related to the different uses should remain realistic, Hazard index is re-calculated using the new estimate of internal exposure of each substance.

If synergistic effects have been identified or are suspected, the risk related to use of the mixture will be considered acceptable if the value of HI is less or equal to a reference HI (HI_{ref}), derived on a case by case basis on the available data. If data is too limited a worst case

[13] Guidance for Human Health Risk Assessment, Volume III Part B, Section 4.4.1

pragmatic factor of 10^{14} could be used. Consequently, the value of this reference HI_{ref} would be below to 1. As a result, the decision-making criterion is in this case:

-If $HI \leq HI_{ref}$ the risk related to use of the mixture will be considered acceptable;

-If $HI > HI_{ref}$ the risk related to use of the mixture will be considered unacceptable

- Tier 3 is more complex than Tier 2 but is considered to be more realistic. Therefore, if a risk is considered acceptable in Tier 2, Tier 3 will not be necessary. Tier 3 is divided in 3 steps of refinement, Tiers 3 A, B and C.

Tier 3A deals with combined exposure assessment by grouping the substances with common target organ/MOA (with the non-refined AEL of each substance): for each group of substances with similar target organ/MOAs, HQ_{to} are summarized for each substance and an "approximate" HI_{to} is calculated. The decision-making criterion is the same as previously, i.e. all adjusted HI_{to} values must be ≤ 1 to consider the risk as acceptable. If one or more $HI_{to} > 1$, risk is considered unacceptable and Tier 3B could be envisaged.

Tier 3B assesses combined exposure assessment with specific AEL by target organ/MOA: in each group for which risk is not acceptable, specific AELs for each identified target organ/MOA and each substance ($AEL_{a.s.-to}$) are determined. Based on the exposure estimates calculated in Tier 1, $HQ_{a.s.-to}$ by target organ will be then calculated for each substance and for each common target organ/MOA, and an "adjusted" HI for each common target organ/mode of action (HI_{to}) will be calculated. As above, all adjusted HI_{to} values must be less than 1 in order for the risk to be considered acceptable (or less than the reference HI defined in the second tier if synergistic effects were identified). If one or more adjusted $HI_{to} > 1$, risk is considered not acceptable.

In this case, it might be possible to refine the risk assessment, by considering either hazard assessment if data are available and allow to perform refinement (for example skin absorption data for the mixture if default values were used in Tier 1, or exposure assessment data under actual conditions of use). The principles of higher tier refinement as described for active substances in this guidance document should also be investigated for applicability in refinement of risk assessment also for mixture assessment.

Tier 3C uses the knowledge of the mechanism of action to refine the HI_{to}, although it will be very rare in practice to have this information in the dossier.

If there is no target organ or MOA in common, the concentration (dose) addition is not confirmed and the effects are considered dissimilar. Consequently, independent action is the rule and the risks are, in this case, covered by Tier 1 of this approach (assessment made substance by substance).

[14] This value of 10 is conservative based on the publication of Boobis *et al.,* 2010 showing that the magnitude of synergy at low doses did not exceed the levels predicted by additive models by more than a factor of 4.

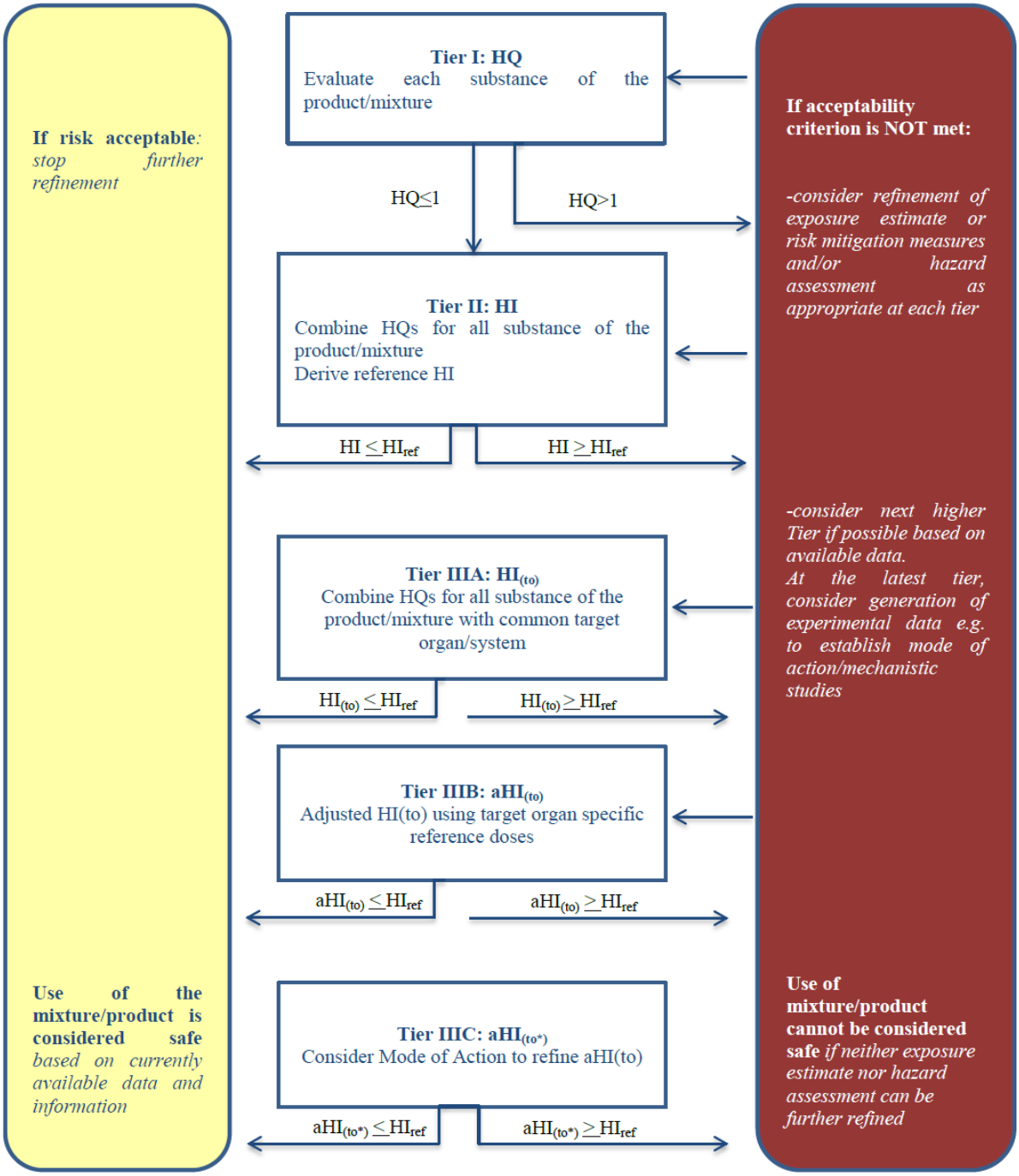


Figure 5: Simplified overview of the assessment method (ECHA, 2013)

Source: European Chemicals Agency

According to this document, similar methodology can be considered to assess combined exposure to multiple substances by different sources of release and/or uses, provided that modifications take into account the various exposure scenarios and cumulative effects.

Moreover, in case of multiple biocidal product types containing the same active substance, aggregated exposure could be assessed by combining the exposure estimates from uses/releases from the different product types. However this is a complicated assessment since the consumer may be exposed to a vast range of treated articles for which the use frequency as well as the ratio of first-time use versus repeated use (and thus the leaching rate) needs to be considered.

Finally, further guidance still needs to be developed regarding procedural aspects on when combined exposure to multiple chemicals (cumulative assessment) needs to be performed for active substances under the BPR.

Risk assessment for exposure to multiple contaminants by the EFSA Panel on Contaminants in the Food Chain (CONTAM)

EFSA's CONTAM Panel deals with contaminants in the food chain (other than pesticide residues). Depending on the contaminant(s) assessed, it has applied both the whole mixture approach and component-based approaches mostly using dose addition as the main assumption for the combined effects of multiple contaminants.

For polycyclic aromatic hydrocarbons, the CONTAM Panel concluded that the TEF approach could not be applied because of the inadequacy of the toxicological database and evidence for different MOA between PAH congeners (EFSA, 2008a). Hence, the Panel initially used an index chemical approach with BaP as a marker of both the carcinogenicity and occurrence of PAHs in a MOE approach. The approach was then refined to use the sum of four specific PAHs as markers of both carcinogenicity and occurrence since BaP was sometimes not present when other important PAHs were.

Regarding mineral oil saturated hydrocarbons (MOSH) detected in food, since information on the occurrence of individual MOSH was not available, and since toxicological data were available only for a few MOSH complex mixtures, the whole mixture approach was applied (EFSA, 2012b). Although a common critical effect was identified (formation of hepatic microgranulomas) for most of the MOSH mixture tested, the MOA could not be clearly established. The NOAEL for the most potent MOSH mixture was compared to dietary exposure in the general population for the RA. This conservative approach was justified by the substantial level of uncertainty regarding the chemical composition of the MOSH mixture tested and the overall range of MOSH to which humans are exposed.

A whole mixture approach was also applied to the flame retardants hexabromocyclododecanes (HBCDDs), predominantly consisting of three stereoisomers (EFSA, 2011a). A BMDL10 for neurodevelopmental toxicity was derived using toxicological data from a single administration of the technical mixture in mice which was then adjusted to body burden to take into account toxicokinetic differences between mice and humans. The body burden was then converted to an estimated chronic human dietary intake using half-life and human gastrointestinal absorption. MOEs were then derived using this estimate and human exposure.

A TEF approach was applied by the CONTAM Panel to the evaluation of several groups of marine biotoxins, based on the rationale that the toxins have a common neurotoxic MOA mediated either by the disruption of the voltage-gated sodium channels or by cytotoxicity through perturbation of the actin cytoskeleton depending on the group of toxins (EFSA, 2009b).

For pyrrolizidine alkaloids (PAs), evidence that PAs with the common key chemical feature of double C-C bond in position 1-2 of the pyrrolizidine ring undergo a similar bioactivation leading to a common active metabolite responsible for the DNA-adduct formation led the Panel to consider the 1,2-unsaturated PAs group using the dose addition approach (EFSA, 2011b). Since specific data on carcinogenicity were available for two substances only, no TEF approach could be applied and a MOE calculation was carried out by dividing the BMDL10 for the most potent PA for which data were available, by the combined exposure to all the detected 1,2-unsaturated PAs as a conservative approach. Similarly, dose addition was also used in the CONTAM Panel opinion on ergot alkaloids (EAs) by deriving a group Acute Reference Dose (ARfD) and a group TDI considering all the relevant EAs to be equally potent as the most active EA tested (ergotamine) (EFSA, 2012a).

Opinion of the three non-food Scientific Committees of the European Commission

The Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) are three independent non-food Scientific Committees made up of external experts. They aim at providing the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment, and at drawing the Commission's attention to the new or emerging problems which may pose an actual or potential threat. Those committees were asked to advise the Commission on the issue related to chemical mixtures by addressing several questions. After having reviewed and analysed the available scientific literature on the general principles and methodologies for mixture toxicology, they reached the following conclusions (EC, 2011b):

1. Under certain conditions, chemicals will act jointly in a way that the overall level of toxicity is affected.

2. In a mixture, chemicals with common MOA will act jointly to produce combination effects that are larger than the effects of each chemical applied singly. These effects can be described by dose/concentration addition.

3. For human health effects, if the intended level of protection is achieved for each individual substance, the level of concern for mixtures of dissimilarly acting substances should be assumed to be negligible. However, for ecological effects, the exposure to mixtures of dissimilarly acting substances at low, but potentially relevant concentrations should be considered as a possible concern, even if all substances are below the individual PNECs. Consequently there is a need to improve the current knowledge and methodologies, and to

develop holistic approaches for the ecological risk assessment of chemicals under realistic conditions.

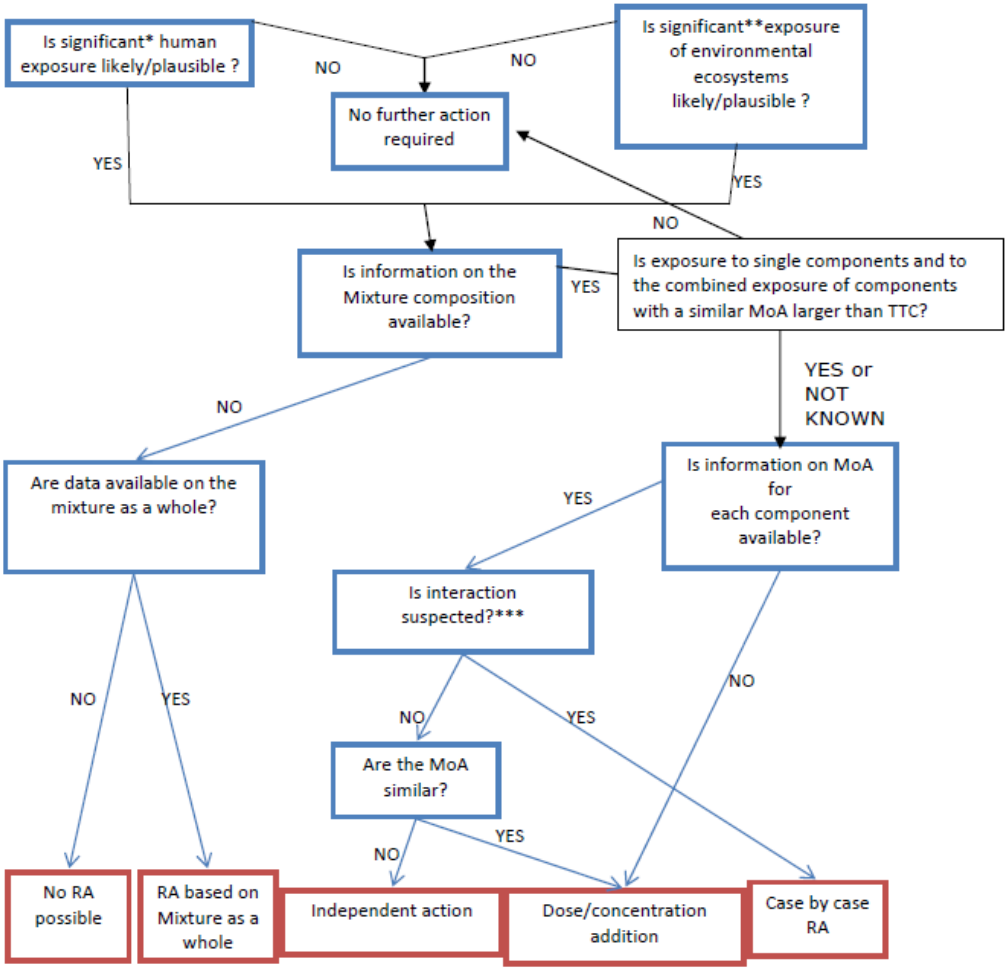
4. Interactions usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or toxicologically insignificant.

5. In view of the almost infinite number of possible combinations of chemicals to which humans and environmental species are exposed, some form of initial filter to focus on mixtures of potential concern is necessary. Several criteria for such screening were offered, such as exposures at significant levels (e.g. close to the HBGVs, DNELs, or PNECs for several components); multi-constituent substances or commercial mixtures with component/substance of concern; potential serious adverse effects of one or more chemicals at the likely exposure levels; frequent or large scale exposure of the human population or the environment; persistence of the chemical in the body/the environment; known information of potential interaction at realistic levels of exposure; predictive information that chemicals act similarly (QSAR and structural alert); or presence of one or more components assumed to have no threshold for effect.

6. A major knowledge gap at the present time is the lack of exposure information and the limited number of chemicals for which there is sufficient information on their MOA, as well as the absence of an agreed inventory of MOA, and of a defined set of criteria how to characterise or predict a MOA for data-poor chemicals.

7. If no MOA information is available, the dose/concentration addition method should be preferred over the independent action approach. The likelihood of synergistic interaction at actually relevant exposure levels has to be assessed on a case-by-case basis from MOA information on the individual chemicals.

They also proposed a decision tree for evaluating the risk of chemical mixtures, presented in Figure 6 .



*"Significant" exposure is determined by the frequency, the duration, and the magnitude of exposure.
**For the environment, an exposure driven assessment without at least a preliminary risk characterisation, as well as the TTC model, is hardly acceptable. Therefore, it must be considered as significant any exposure produced by emissions capable to modify the natural background conditions.
***Evidence for interaction can be found at various steps of the decision tree (e.g. comparing product information with compound-based assessment).

Figure 6: Decision tree for the risk assessment of mixtures (EC, 2011b)
Source: European Commission

International frameworks dealing with human risk assessment of combined exposure to multiple chemicals (EFSA, 2013b)

Although not a guidance document, this is a scientific report reviewing the terminology, methodologies and frameworks developed by national and international agencies for the human risk assessment of combined exposure to multiple chemicals and providing recommendations for future activities at EFSA in this area. It refers to "risk assessment of combined exposure to multiple chemicals" in order to support such harmonisation in terminology as proposed by the International Programme on Chemical Safety (IPCS) of the World Health Organisation (WHO). However, the use of this terminology is not restrictive; for example, the term "cumulative risk assessment" is used in the pesticide field for the settings of Maximum Residue Levels (MRLs) under Regulation (EC) No 396/2005.

It reviews the different steps of a risk assessment of combined exposure to multiple chemicals (i.e. problem formulation defining the relevant exposure, hazard and population to be considered; exposure assessment, hazard assessment and risk characterisation) and the different existing methodologies (i.e. whole mixture approach or component-based approaches for hazard assessment, depending on the toxicological data available). Additivity is the most common assumption through either dose addition with a similar MOA or response addition with a dissimilar MOA. Methods using the dose addition assumption include the Hazard Index approach with variants such as the target-organ toxicity dose, the reference point index/point of departure index, the relative potency factor and the toxicity equivalency factor approaches. For chemicals with a dissimilar MOA, the probability of observing a toxic response for each chemical component in the mixture is first estimated and components are then summed to estimate total risk from combined exposure to the multiple chemicals. Using the assumption of interactions (toxicokinetic and toxicodynamic), combined toxicity for the multiple chemicals is categorised as either less than additive (antagonism, inhibition, masking) or greater than additive (synergism, potentiation). Methods for deriving risk estimates for interactions include interaction-based Hazard Index and Hazard Index modified for binary interactions. For higher tier risk assessment (tier 3), full probabilistic models can be developed for exposure assessment and physiologically-based toxicokinetic (PB-TK) models and physiologically-based toxicokinetic-toxicodynamic (PB-TK-TD) models for hazard assessment. A typical example of the use of a PB-TK includes the derivation of interaction-based Hazard Index using tissue doses accounting for multiple toxicokinetic interactions between the multiple chemicals. The choice of the tier depends on data availability, the purpose of the risk assessment and the resources available. Uncertainty analysis included in this methodology aims at identifying the sources and magnitude of uncertainty in a tiered manner (qualitative, semi-quantitative or probabilistic) associated with exposure assessment, hazard assessment and risk characterisation, and help to identify data gaps, strengths and limitations of the assessment and to make recommendations on future research.

This report shows that most national and international frameworks described apply stepwise decision trees and tiered approaches for the risk assessment of combined exposure

to multiple chemicals based on the original framework developed by the US EPA. Key differences include the separation of two different frameworks for cancer and non-cancer effects (ASTDR) versus the use of single tiered approach (three Non-Food Committees, WHO) and the use of dose addition as the default approach unless evidence demonstrates otherwise (WHO, three Non-Food Committees, EFSA).

The report recommends the development of harmonised terminology and methodologies for human risk assessment of combined exposure to multiple chemicals, as well as a consistent approach to the assessment of priority mixtures across the different EU regulations and directives. The development of methodologies for risk assessment of exposure to multiple chemicals combined with other stressors (e.g. biological hazards, physical agents) is also proposed as a longer term objective.

This report concludes with several recommendations regarding each step of the risk assessment process: for problem formulation, priority chemicals should be identified in a way that takes account of differences in legal frameworks (i.e. regulated substances versus contaminants).

For exposure assessment, it is recommended to collect occurrence data for multiple priority chemicals in individual food samples, to develop case studies/training sets to compare deterministic versus probabilistic methods, and methodologies for aggregate exposure assessment for priority chemicals.

For hazard assessment, further exploration of the scientific basis for both the whole mixture approach and the setting of assessment groups for component-based approaches should be carried out, and better information regarding MOA/MEA (Mechanism of Action) of multiple substances should be generated, from toxicokinetic and toxicity studies as well as from the use of predictive and alternative methodologies. To improve the basis for setting CAGs/AGs, toxicokinetic and toxicodynamic data for multiple chemicals of priority in humans and major test species should be collected. Finally, this report also recommends the development of a guidance document for uncertainty analysis regarding hazard assessment and risk characterisation for multiple chemicals.

4.1.2 Ecotoxicological guidance and environmental mixture risk assessment

EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA, 2009a)

The basic concept underlying the pesticide RA for birds and mammals is that the exposure of animals occurs mainly via pesticide residues in their food. Appendix B of the EFSA Guidance for RA to birds and mammals (EFSA, 2009a) addresses how to deal with exposure to mixtures of active substances (and possibly also co-formulants) in the environment.

Combinations of active substances (a.s.) and also formulations are rarely experimentally tested in birds, whereas formulation toxicity data are more often available for mammals, since they are also used for classification and labelling. A four-step approach on how to deal

with mixtures of a.s. and formulations is described in the guidance and briefly summarised here.

In a first step, the acute toxicity for mixtures (surrogate LD_{50}) is calculated based on the concentration addition/toxic unit approach. If data are available, the toxicities of co-formulants are also considered in the calculation. The toxicity per fraction is calculated to determine the contribution of each a.s. to the overall mixture toxicity. If one a.s. alone is responsible for a major part of the mixture toxicity it might be sufficient to perform a single substance RA for this a.s. and to avoid further steps 2-4. In the case of higher tier refinements in which consideration of different environmental fate parameters may result in a different residue composition than in the initial mixture, the equation for calculating the mixture toxicity has to be adjusted by using Multiple Application Factors (MAF). If synergisms are expected, targeted studies should be performed and the calculation approach not followed.

In the second step, measured formulation toxicity data are considered if dose-response test data (step 2a) or limit test data (step 2b) are available. If measured data show higher toxicity than the calculated toxicity, the measured data should be used in tier 1 RA to account for any additional effect caused by components not considered in the calculation (e.g. co-formulants) or synergistic effects that might also occur in the environment. The use of calculated toxicities in such cases should be considered only at higher tier refinements and based on careful justification that factors leading to the higher measured toxicity of the formulation would not be relevant under field conditions. If the calculated mixture toxicity is higher than the measured one, the calculated should be used instead.

In the third step sublethal and reproductive effects are also considered. It is generally recommended not to use calculated mixture toxicities for endpoints from long-term and reproductive studies. Reliable results would only be expected if data are available on exactly the same endpoints and ideally based on EC_x values with the same x avoiding variations that could be introduced by using NOAELs. Thus, calculation of mixture toxicities for sublethal and reproductive endpoints is recommended only on case-by-case basis. Then all a.s. in one group could be expressed after weighing against the most toxic representative of the group and the RA would be performed for the whole group by applying the corresponding NOEC for the most toxic compound.

Step 4 describes the determination of the exposure estimates for the mixture risk assessment. In a first step, for single applications, the formulation component concentrations are simply summed up to calculate environmental residues. If several applications have to be considered, the default MAF of Tier 1 should be applied to the mixture. For refinements based on specific properties of individual a.s., the residue composition might be adjusted and does not need to remain as in the original mixture. If the RA is based on measured formulation toxicity data, the exposure refinement is not applicable.

EFSA PPR Panel Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013d)

A guidance document on the risk assessment of plant protection products (PPPs) was developed by the PPR Panel of EFSA and published in July 2013. It specifies how the risk to aquatic organisms in edge-of-field surface waters should be assessed for active substances and plant protection products, i.e. formulations.

Usually, approval of active substances at EU level is performed for single active substances (a.s.), but there is growing concern in several Member States that PPP exposure in the environment will be characterised by simultaneous and sequential exposure to multiple pesticides due to usual crop protection programs on one or also neighbouring fields in agricultural areas. Since the number of PPPs applied on one field over a growing season is highly variable and the potential number of PPP combinations is expected to be huge, it was not considered possible to perform ERA for all possible combinations. To reduce however the potential risk from multiple PPP exposure in the single substance RA, two options can be chosen, i.e. (1) the ecological threshold option (ETO), accepting negligible population effects only, and (2) the ecological recovery option (ERO), accepting some population-level effects if ecological recovery takes place within an acceptable time period. The selection of ETO for the RA of individual PPPs is more likely to avoid stress caused by the multiple uses of different PPPs. Although a RA that considers recovery of sensitive populations may be a reasonable option for surface waters adjacent to crops with a limited PPP input, it is more uncertain if ERO can be achieved when assessing risks for individual PPPs for their use in crop protection programmes characterised by intensive PPP use (simultaneous or repeated use of different PPPs). Thus, strict rules are set out on when the ERO should be avoided in order to be protective.

For the approval of PPP formulations containing more than one a.s., a decision scheme and guidance is included on how to consider mixture effects (Chapter 10.3 of EFSA 2013b). The intention is to improve the mixture RA without increasing testing requirements, so that the use of calculated mixture toxicities should be considered whenever possible and justified.

The toxicity calculation is based on the principle of concentration addition. When measured data are available for the mixture toxicity, these are compared to the calculated mixture toxicity in order to identify whether interactions (antagonism or synergism) occur. It is also important to consider whether the composition of the experimentally studied mixture deviates in composition from the mixture composition that is considered in exposure assessment (predicted environmental concentration for the mixture, PEC_{mix}). In such cases, the mixture toxicity should be calculated for both compositions and compared to the measured mixture toxicity. Depending on the deviation between the calculated and measured mixture toxicity, it is recommended to use one or the other in the RA.

In the calculated mixture toxicity usually only the a.s. are included and the contribution of co-formulants should therefore be carefully considered in these comparisons. If toxicity data for individual co-formulants are available, these can be easily incorporated in the calculations.

Regarding the exposure assessment, as a conservative first step, it is usually assumed that all mixture components appear in the environment with the maximum concentrations at the same time, which are then summed up to determine the PEC_{mix}. If further exposure refinement is needed, predicted exposure profiles can be used and PEC_{mix} can be calculated for a time series of e.g. days, so that also the ratio $exposure_{mix}/toxicity_{mix}$ (ETR_{mix}) is calculated for each day. If refined toxicity data are used from higher tiers that would result in the use of different assessment factors, the RQ_{mix} approach needs to be applied, whereas otherwise the ETR_{mix} approach will be followed. ETR_{mix} or RQ_{mix} are calculated to assess whether the risk is acceptable. In both cases, the assessment is done separately for each organism group (e.g. fish, algae, plants, invertebrates).

EFSA Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees) (EFSA, 2013a)

A guidance document for the risk assessment of plant protection products on bees was published by EFSA in July 2013. It was developed based on the respective Scientific Opinion of the PPR Panel[15]. Chapter 10 of the guidance addresses mixture toxicity and the toxicity of formulated products with more than one active substance. The concept of concentration addition is proposed for calculating mixture toxicity using a Sum of Toxic Units (TU) approach. If measured toxicity values for formulations with more than one active substance are available, these should be compared to the calculated mixture toxicity to verify the applicability of dose additivity. Differences might be explained e.g. by co-formulants that were not considered in the calculation, toxicokinetic interaction or synergism/potentiation of effect. If the measured toxicity of the formulation is lower than the calculated one, the calculated one should be used in the RA in a first tier. On the exposure side, in a first tier the simultaneous co-occurrence of all components at the maximum concentration is assumed as a conservative approach. Mixture exposure estimates can be refined taking into consideration the real concentration profiles predicted for the individual components, which might have peak concentrations at different times.

Environmental mixture risk assessment in the context of the Biocidal Product Regulation

In 2012, the EU council and Parliament adopted a regulation[16] that implements an EU-wide, harmonized system for the authorization for biocidal products. Such products are most of the time multi-component products (i.e. "intentional mixture") and the assessment of possible combination effects between the different components is a critically important step during the regulatory environmental risk assessment of these products. To assess the mixture effects of biocidal products from their ingredients, a tiered approach has been proposed by Bachaus et al., (2013) which accommodates different data situations, optimizes resource usage, limits animal testing as far as possible and should ensure adequate protection of the environment.

[15] EFSA Panel on Plant Protection Products and their Residues (PPR); Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA Journal 2012; 10(5) 2668. [275 pp.] doi:10.2903/j.efsa.2012.2668.
[16] Regulation (EU) No 528/2012. Biocidal Product Regulation (BPR)

This approach is mainly based on concentration addition as a component based approach, as the use of non-testing methods with respect to ecotoxicity data is already stressed in the Biocidal Products Regulation. It also facilitates the re-use of existing data for individual ingredients, which is expected to increase as the BPR promotes data sharing between applicants. CA is either approximated by summing up PEC/PNEC ratios (Figure 7) or as sums of toxic units (Figure 8). It is suggested to take into account potential interactions (i.e synergistic effects) by initially penalizing the CA-based assessment with an additional assessment factor, termed "IF" ("Interaction Factor"), in particular if there is no ecotoxicological data for the product in question. A default value of 2 is provisionally proposed as sufficiently protective, unless available evidence exists showing that the IF might have to be set to a value greater than 2, or decreased to 1 (Figure 7); however the authors recognized that the use and the initial size of this IF might warrant later review and perhaps adjustment.

The minimum requested set of data for a component-based assessment consists of complete information on the product composition, and the PEC/PNEC ratio for the compound of highest concern (typically the active ingredient). As only semi-quantitative data are needed for this purpose, QSAR estimates, hazard classification data according to the CLP regulation or censored data (e.g. from limit tests) and simple exposure estimates should be sufficient. Then, the final risk of the product is estimated by calculating the Risk Quotient of the product. If RQ_{Prod} <1, no further testing or data evaluation would be required; if RQ_{Prod} >1, the PEC, PNEC and/or IF might be refined by providing additional data; the whole mixture might be subject to direct biotesting, or a more detailed component-based assessment might be carried out using quantitative risk estimates for every relevant compound instead of using only the highest concern one. If after having applied these various options, there is still reason for concern, direct product testing or the application of CA in the form of the sum of toxic units (STU) might be performed (Figure 8). The sum of toxic units is calculated for each ecotoxicological endpoint, which is species-specific, and implies that data for all relevant compounds are available for all endpoints. The maximum STU indicates which endpoint for which species is expected to be most sensitive for the biocidal product in question and is therefore used for the final assessment.

Independent action was considered as not suitable for incorporation into a tiered approach without explicit confirmatory studies as it might lead to an underestimation of the actual environmental risk. Whole product testing is considered where appropriate but according to the authors it should be regarded as a "gold standard" for the assessment of acute toxicities or if synergistic interactions are suspected.

An ECHA guidance document based on this proposal was published in 2014 (ECHA, 2014). This guidance is regarded as “transitional” because it was initiated under the “old” Biocidal Products Directive and finalised before the relevant new Biocidal Products Regulation guidance document was fully developed. It is therefore made available as a Transitional Guidance document until the relevant new document is ready for publication.

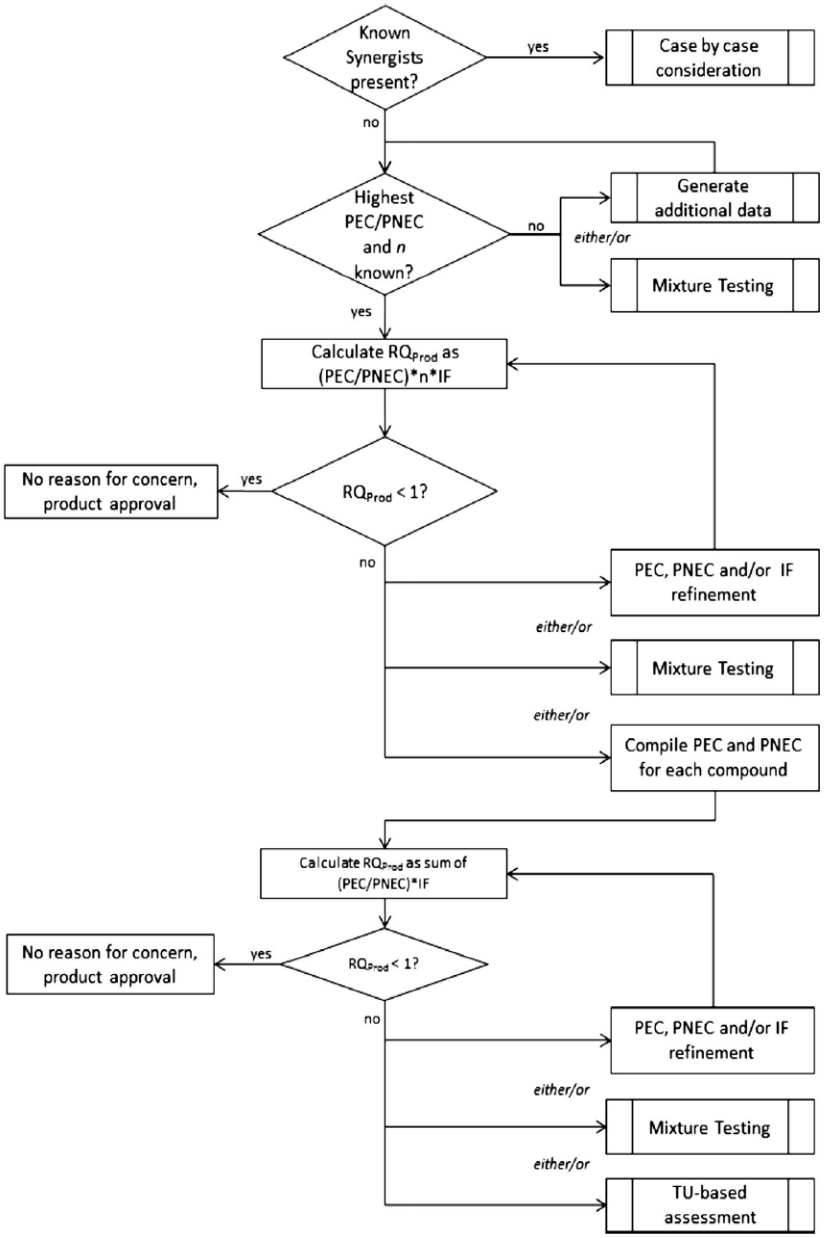


Figure 7: Approach for environmental risk assessment of biocidal products based on PEC/PNEC summations (Backhaus et al., 2013). Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0)

PEC: Predicted Environmental Concentration, PNEC: Predicted No Effect Concentration; RQ_{Prod}: Risk Quotient for the product, TU: Toxic Unit, IF: Interaction Factor, n= number of compounds in the mixture.

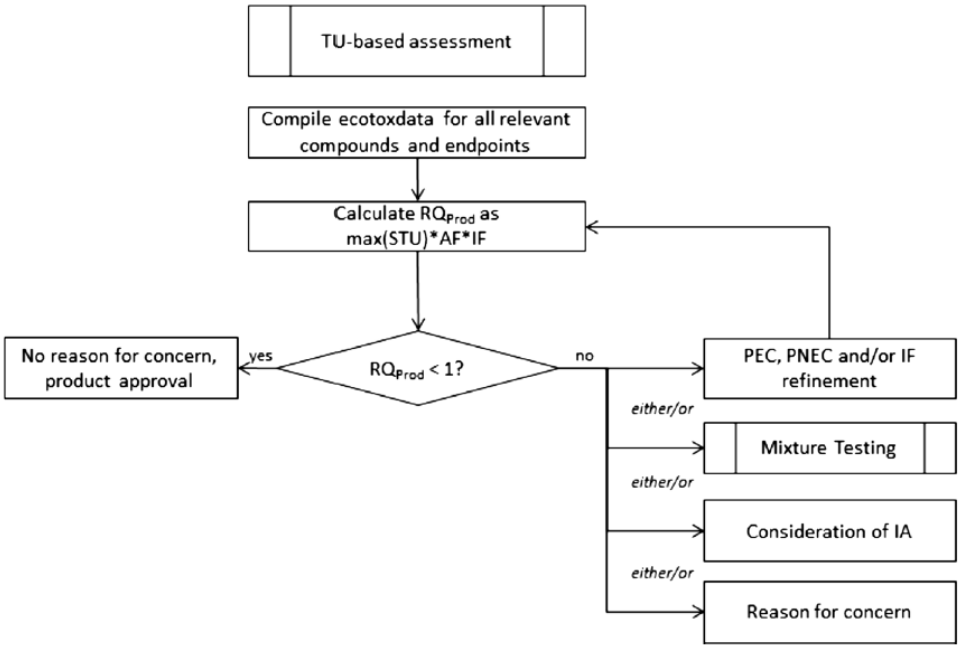


Figure 8: Toxic Unit based approach for environmental risk assessment of biocidal products (Backhaus et al., 2013) Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0)

PEC: Predicted Environmental Concentration, PNEC: Predicted No Effect Concentration; RQ_{Prod}: Risk Quotient for the product, TU: Toxic Unit, IF: Interaction Factor, IA: Independent Action, n= number of compounds in the mixture, AF: Assessment Factor.

This guidance addresses the ERA of mixture toxicity of biocidal products as well as synergistic effects as required by the BPR by applying a tiered scheme, and focuses on component-based approaches and whole-mixture testing, which may be the only viable option in certain cases (i.e when it is suspected that a component in the mixture acts as a synergist, and may cause an interactive type of joint action for which CA -or IA for that matter- is an invalid assumption, or when even higher tier effect modelling predicts unacceptable risk). However, testing should always be the last option, and if this approach is chosen, careful consideration should be taken to determine the most relevant mixture to be tested. As a matter of fact, testing of the product might be useful when the environment is directly exposed to the formulated product, but in most cases, the environment is exposed to a mixture that is different from the original composition of the product[17].

As a first step of this ERA, the concerned environmental compartments and the type of mixture to which it would be exposed (i.e the product itself or a modified mixture) should be identified, as well as the relevant substances of the mixtures (i.e. biologically active chemicals that are present at sufficiently high concentrations and are contributing to the overall toxicity of the respective mixture). In case of indication for synergistic interactions, a case-by-case risk assessment should be performed; if there is no indication for synergistic effects, it is recommended to proceed with the tiered approach.

[17] In this guidance document the terms “mixture” and “relevant mixture ” are used for the product itself and the ecologically relevant mixture, respectively

The tiered approach developed in this guidance is a 4-stages approach which accommodates different data situations, optimises resource usage and limits biotesting as far as possible. It mainly builds on using component-based approaches (CBAs) based on the concept of CA for mixture toxicity prediction, which is either approximated by summing up PEC/PNEC ratios or implemented as sums of Toxic Units (STU). As previously, the concept of IA was assessed as not being suitable for incorporation into a tiered approach without explicit confirmatory studies, as it might otherwise lead to an underestimation of the actual environmental risk. In addition, IA would lead to higher data demands compared to CA. However, if the applicant can prove that IA adequately describes the toxicity of a given product by submitting appropriate data, e.g. information about the MoAs and the concentration-response relationships of the mixture components, these data should be taken into account for mixture toxicity assessment and assessed according to expert judgment.

Each of the upper tiers involves a less conservative and more accurate assessment than the previous tiers but requires also more resources, including additional exposure and toxicity data. The tiers must not be performed step by step for a respective product, e.g. in case the data for Tier 3 are available in the beginning, the assessment can be started with Tier 3.

Tier 1 implies the summation of the PEC/PNEC ratios if there are available for all relevant ingredients, to calculate the risk quotient of the mixture ($RQ_{Product}$)[18]. If $RQ_{Product} <1$, the risk is considered as acceptable; if it is >1, a refinement of the PEC- and/or PNEC-values by providing additional information on the exposure and/or hazard characterisation of the compounds can be performed, or the RA can continue with tier 2, 3 or 4, or effective Risk Mitigation Measures (RMM) should be defined. Ultimately, the only remaining option is the non-authorisation of the product.

The refinement in Tier 2 and Tier 3 consists of looking separately at the combined risk from all relevant substances towards each separate trophic level, by calculating the Sum of Toxic Units (STU) for each trophic level. Two approaches are presented: First, a modified Toxic Unit Summation (TUS) which can take into account varying data sets for the relevant substances (Tier 2) and secondly, the standard TUS for cases where homogeneous data sets are available for all relevant substances (Tier 3). This method is a more realistic approach than Tier 1, as it combines effects for each trophic level (e.g. separate risk-ratios are calculated for all relevant substances for algae, daphnids and fish). The only difference between the two Tiers is that Tier 2 gives the opportunity to use different AFs for each relevant substance, whereas a common AF factor is being used in Tier 3, selected depending on the amount of available data according to the rules set up in the TGD.

If in Tiers 2 or 3 the criterion for an acceptable risk for the environment is still not met, i.e. $RQ_{Product} > 1$, a refinement of the PEC- and/or ECx-values by providing additional information on the exposure and/or hazard characterisation of the compounds, or the definition of effective Risk Mitigation Measures (RMM) might be an option. If this is not

[18] It should be pointed out that this summation is fundamentally different from the concept of CA, of which one assumption is that all individual toxicity data refer to same biological endpoint and organism; yet the PNECs from the various compounds might be based on data from completely different endpoints and species. Consequently, the use of PEC/PNEC sums derived from a set of different species or endpoints are only recommended for first-tier CA assessment in the opinion on mixture toxicity assessment as put forward by the EU scientific committees.

possible, ultimately the RA can proceed to Tier 4 with the direct testing of the mixture of concern (either the biocidal product, if there is a direct release of the product into environment or the ecologically relevant mixture in case the composition of the product changes radically before release to environment). If the direct biotesting of the mixture of concern, i.e. the product and/or the ecologically relevant mixture is not possible and other options such as a further refinement of the single substance data or the definition of effective RMMs are not applicable, the only remaining option is the non-authorisation of the product.

EU and Endocrine Disrupting Chemicals (EDCs)

In 2011, a "State of the art assessment of endocrine disrupters" was published, commissioned by the European Commission (Kortenkamp et al., 2011). This report is not a guidance document, but aims at summarising advances in the state of the science since the WHO IPCS report of 2002 and mapping out the currents requirements regarding endocrine disruptors in different pieces of EU chemicals regulation (i.e. PPP regulation, REACH and the Biocide Regulation).

It highlights the fact that there is good evidence that several EDCs can work together to produce combined effects. Especially when there is simultaneous exposure to chemicals affecting the same endpoint, EDCs can produce additive effects, even when combined at low doses that individually would not produce observable effects. According to a WHO report, this has the effect of "*making accurate risk assessment difficult or impossible*" (WHO-UNEP, 2012). From a regulatory point of view, it is therefore of great importance to have information about the spectrum of EDCs that are present in relevant exposure scenarios. This information is currently fragmentary, and this lack of information makes it likely that the full extent of risks associated with EDCs might be underestimated.

In 2013, EFSA SC also published a Scientific Opinion on the hazard assessment of EDCs (EFSA, 2013e). Regarding interactions and modes of action, the SC recognises that information may be obtained at the receptor level from *in vitro* studies. However, because of differences in the toxicokinetic properties of the substances, this is not enough to predict the nature of the combined effects at the organism level, even if the EDCs have a similar MOA. Moreover, the current tests are being developed to address single substances, and it is necessary to first develop the tests for single substances with adequate dose response data for reference substances, before addressing combined exposure to multiple substances. Thus, there is also a gap concerning guidance on the assessment of effects triggered by combined exposure to EDCs, and a need to develop mixture methodology in this context.

4.2 Assessment of mixture toxicity in other geographical areas

4.2.1 United States of America

The US Environmental Protection Agency (US EPA) is the main US authority engaged in industrial chemicals and mixtures management, risk assessment and regulation. The Food and Drug Administration (FDA) and the Agency for Toxic Substances and Disease Registry (ATSDR) also play a role, with the FDA establishing tolerance levels for hazardous substances in food and consumer items, and the ATSDR evaluating data on releases of hazardous substances and creating and maintaining registers of exposed people (Kortenkamp et al., 2009). This paragraph reviews the main guidance documents dealing with mixture toxicity issued from those different US authorities, and the context in which they have been developed.

Environmental Protection Agency (EPA)

Cumulative risk assessment (CRA) for contaminated sites

The Comprehensive Environmental Response Compensation and Liability Act (CERCLA) which came into force as early as 1980 specifically requires mixture risk assessment during the evaluation of risks stemming from hazardous waste sites and chemical accidents. These risk assessments are termed "cumulative risk assessment" (CRA) as they consider the combination of risks associated with exposure to multiple chemicals and non-chemical stressors by all routes and pathways (an exposure pathway being here how a chemical moves from the waste site to a human subject), and from all sources, including multiple time frames and multiple health outcomes. In contrast, the term “aggregate risks” is used to describe risks that stem from exposure to one substance by multiple pathways and routes (WHO, 2009); it should be thus reserved for single chemicals to avoid any confusion.

In this context, US EPA has published five guidelines dealing with the risk assessment of chemicals, including one dealing with chemical mixtures, as well as the "Risk Assessment Guidance for Superfund (RAGS)" in 1989 (USEPA, 1987, 1989) in which extensive and detailed guidance on mixture assessment is given. A risk assessment for a mixture is composed of three steps: exposure assessment, toxicity assessment and risk characterisation.

Exposure assessments are made on “reasonably maximally exposed” people, and decision making is based on the worst-case (highest exposure likely to occur). The initial list of chemicals to be evaluated in a typical site risk assessment is given by the EPA’s Contract Laboratory Program (CLP) Target Compound and Target Analyte List (TCP/TALs)[19]. The outcome of exposure assessments for hazardous waste sites are estimates of exposure or dose for each chemical for defined subpopulation disaggregated by time periods and exposure pathways.

[19] http://www.epa.gov/superfund/programs/clp/target.htm. It includes 52 volatile chemicals, 30 pesticides and Aroclors, 24 metals, cyanide, and 67 semivolatile chemicals.

The toxicity assessment is based on the toxicity information for each considered chemical, found in the IRIS database (reference values such as reference dose - RfD[20]- or reference concentration - RfC[21]- are used).

The risk characterisation is then calculated from these inputs, differently for carcinogens and non-carcinogens. Figure 9 gives the basic principles of CRA for Superfund sites, as practiced by US EPA. For carcinogens, it is assumed that there is no dose threshold, and that the dose-response function is essentially linear. Because risk estimates are probabilities, cancer risks associated with different substances can be added together irrespective of whether the substances cause cancer by (1) similar mechanisms, or (2) completely independent mechanisms. Under such conditions the use of simple effect summation for the estimation of a cancer risk estimate produces results similar to independent action, because the predicted cancer probabilities are very much smaller than 0.001 (Kortenkamp et al., 2009).

For non-carcinogenic chemicals, a hazard quotient (HQ) is calculated for each pathway and each averaging period. An overall summary hazard index (HI - Application of dose addition) is then calculated as the sum of HQs for each pathway and each chemical. If $HI \leq 1$, it is assumed that there is unlikely to be a risk of deleterious effects and the analysis is complete. If $HI > 1$, further analysis may be performed to determine whether application of dose additivity to all the chemicals simultaneously is justifiable.

These documents do not really go further than a basic application of additivity concepts. In particular, there is little in the 1989 guidance about how to deal with toxicological interactions (Kortenkamp et al., 2009). In 2000, the US EPA published a Supplementary Guidance for Health Risk Assessments for Mixtures, in which some of these gaps were filled. Table 6 gives an overview of evolution of the US EPA's guidance documents on mixtures whereas brief summaries of the guidance documents are given in the Annex I.

[20] Reference dose (RfD): An estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL, or benchmark dose, with uncertainty factors generally applied to reflect limitations of the data used. http://www.epa.gov/risk/glossary.htm

[21] Reference concentration (RfC): An estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL, or benchmark concentration, with uncertainty factors generally applied to reflect limitations of the data used. http://www.epa.gov/risk/glossary.htm

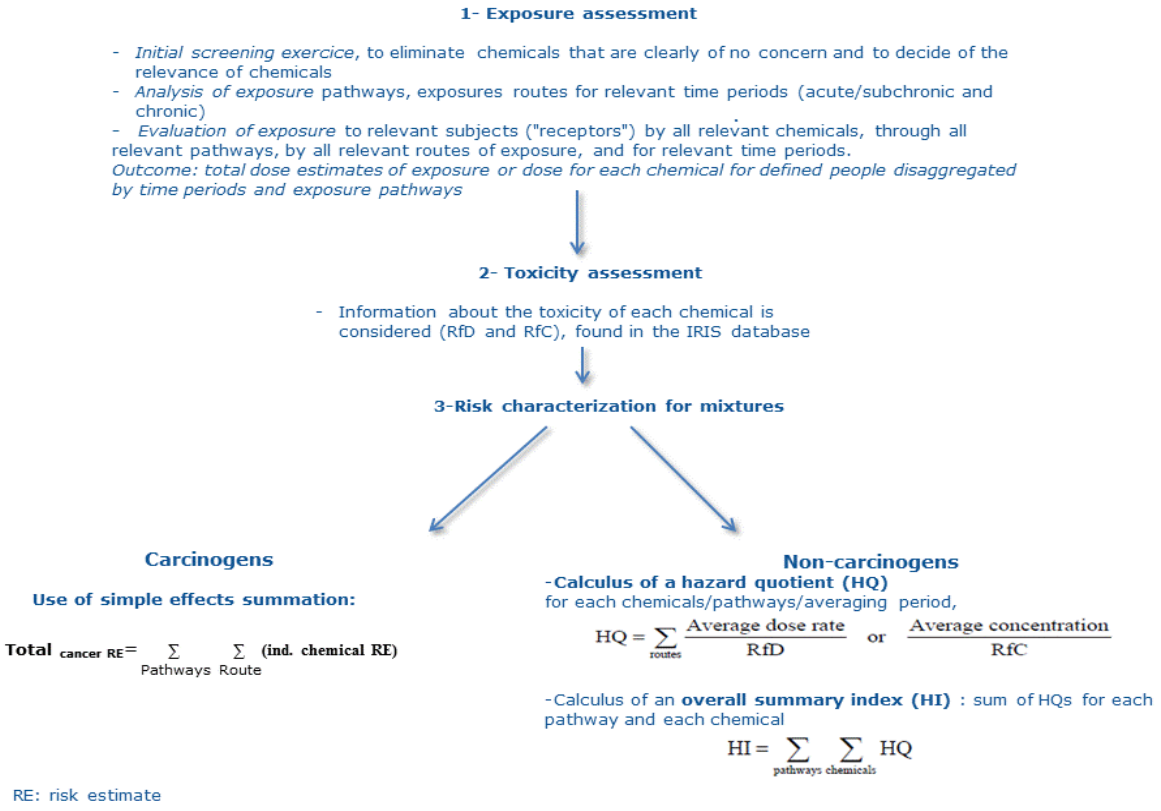


Figure 9: Cumulative risk assessment for waste sites in US EPA Risk Assessment Guidance for Superfund (RAGS)

Source: United States Environmental Protection Agency

(http://www.epa.gov/oswer/riskassessment/ragsa)

Table 6: **US EPA publications on mixture assessment and evolution of the EPA guidance documents**

Guidance	Principles and Strengths	Deficiency
US EPA 1986-Guidelines for the health assessment of Chemical Mixtures	-Use basic application of additivity concepts (dose addition or independent action) -Approach: use data on the mixture of concern, when available; or information about similar mixture; or evaluation pairwise interactions between mixtures constituents, or assume dose additivity for chemicals with the same MOA, or response additivity for chemicals with the same health endpoint but different MOA. -Consider multiple chemicals, exposure routes and effects.	Consideration of chemicals interaction only through the use of interaction coefficient if toxicological data are available.
US EPA 1989-Risk Assessment -Guidance for superfund. Vol 1. Human Health Evaluation Manual. Part A.	- Use basic application of additivity concepts (dose addition or independent action) - Consider multiple chemicals, exposure routes and effects. -Implemented component-based approaches for the assessment of the effects of multiple chemicals - Make a distinction between carcinogens and non-carcinogens: *carcinogenic substances: component risks are added, following the principles of independent action. *non-cancer endpoint: the doses of mixture components are scaled and added (application of the dose addition concept, termed "the hazard index") - Develop the quantitative evaluation of exposures via multiple pathways by using the hazard quotient concept	- Toxicological interaction: a default approach was defined, stipulating application of dose addition or independent action, where appropriate - To produce an hazard index (HI), CRA summed hazard quotients of individual chemicals that had similar adverse health outcomes, but not necessarily similar modes of action - Lack of detail regarding procedures for conducting multi-pathway analyses.
Supplementary Guidance for conducting health risk assessment of chemical mixtures. US EPA, 2000	-Default approach of HI or relative potency for "toxicologically similar" components, independent action for "toxicologically independent" components -Development of a process for the quantitative evaluation of toxic interactions (Interaction Hazard Index)	- Lack of workable procedures for multi-chemical, multi-pathway exposure assessments, as well as for multiple effects produced by mixtures -Interactions are addressed only in terms of altered or joint toxicity.
USEPA, 2002 Guidance on Cumulative Risk Assessment of pesticide chemicals that have a common mechanism of toxicity	-The CRA begins with the identification of a group of chemicals, a CMG (Common Mechanism Group). It is assume that identification of a CMG implies dose addition for its member chemicals, so dose addition is the only possibility considered. - Does not focus on single pathways of exposure (e.g., from pesticide residues in food, water, or residential/non-occupational uses) for individual chemicals, but on the potential for individuals to be exposed to multiple pesticides by all pathways concurrently (i.e. cumulative risk assessment).	-Considers only chemicals that produce an effect by a common MOA, and omits other chemicals that might also induce the same effect of interest, although by different mechanisms. -Does not consider toxic interactions, and mixture effects are by default assumed to be additive.

Table 6: US EPA publications on mixture assessment and evolution of the US EPA guidance documents (Continued)

Guidance	Principles and Strengths	Deficiency
USEPA, 2003. Framework for Cumulative Risk Assessment, Risk Assessment Forum, EPA/630/P-02/001F	-Offers a basic structure and provides starting principles for EPA's cumulative risk assessments, and the basic principles around which to organize a more definitive set of cumulative risk assessment guidance. -Define Cumulative risk as the combined risk from aggregate exposures to multiples agents or stressors -Define Aggregate exposure as the combined exposure of an individual (or define population) to a specific agent or stressor via relevant routes, pathways, and sources).	Give no explicit default approach, only refers to previous documents (particularly EPA 2000)
USEPA, 2006- Considerations for developing alternative health risk assessment approaches for addressing multiples chemicals, exposures and effects	-The approaches extend those ideas to include kinetic modelling to integrate exposures occurring through multiple routes, interactions affecting fate and transport and interactions affecting multi-route joint toxicity -Emphasize the links between the exposed population and the multiple factors being addressed - CRA is defined explicitly as considering the health risks that stem from multiple chemicals, via multiple routes and exposure pathways, within multiple time frames. Multiple health effects are taken into account.	-Currently much information is lacking regarding compound toxicity and kinetics to use in kinetic modelling -Relies on known concentrations for many chemicals which is frequently unknown -Give no explicit default approach, only refers to previous documents (particularly EPA 2000)
USEPA 2007 – Concepts, methods and data source for cumulative health risk assessment of multiples chemicals, exposures and effects: a resource document. EPA/600/R-06/013F, National Center for Environmental Assessment, Cincinnatti, OH.	-Resource document for identifying specific element and approaches for implementing cumulative risk assessments (presentation of concepts, methods and data sources). -Define cumulative risk assessment as the analysis, characterisation and possible quantification of the combined risks to health or the environment from multiple agents or stressors.	Give no explicit default approach, only refers to previous documents (particularly EPA 2000)

CRA and pesticides

The passage of the Food Quality Protection Act (FQPA) in 1996, requiring the estimation of health risks from combinations of pesticides with a common mode of action, also played a role in the evolution of CRA by triggering the development of procedures for pesticide mixture assessment. In order to fulfil this requirement, the US EPA first developed guidelines to determine which pesticides should qualify for inclusion in common mechanism groups (USEPA, 1999), and then published a guidance document concerning the application of the hazard index principle to pesticides, with an aggregate risk formula equivalent to the total hazard quotient in the Superfund guidance (USEPA, 2002a). The 10 steps of the risk assessment for pesticide mixtures are described in Figure 10. The US EPA's pesticide CRA follows the broad principles developed for Superfund sites, although with some modifications: the selection process of chemicals which should be considered was modified and the procedure was extended to deal with simultaneous exposures from food, drinking water and residential (non-occupational) use of pesticides for the general population (Kortenkamp et al., 2009). This risk assessment procedure was used to extensively assess the risk linked to mixtures of organophosphates, carbamates, triazines and chloroacetanilides (USEPA, 2002b, 2006c, b, 2007b).

Figure 10: Cumulative risk assessment of pesticide chemicals that have a common mechanism of toxicity (USEPA, 2002a)

Source: United States Environmental Protection Agency

The first step reflects the need to identify a group of chemicals that are considered to induce a common toxic effect by a common mechanism, a so-called common mechanism group (CMG), and to evaluate the registered and proposed uses for each chemical in the CMG, to further identify potential exposure pathways and exposure routes.

Then, the various endpoints associated with this common mechanism of toxicity are also identified. An important aspect of this assessment step is to determine if the common effect is expressed across all exposure routes for each chemical in the CMG. To avoid useless testing and quantitative dose-response analysis, pesticides that contribute to exposures by minor pathways are excluded from the CMG, forming a subset, termed cumulative assessment group (CAG). For each CAG member, dose response analyses are performed to determine its toxic potency for the common effect. The concept of dose addition is normally used to estimate the combined risks in the CAG, although deviation from this basic principle is permitted if appropriate. Then, one chemical from the CAG is selected to serve as an index chemical, and the relative potencies of the CAG members to this index chemical are defined for the standardization of their common toxicity in terms of relative potency factors (RPF). The index chemical should be well evaluated for its toxicity, because such data are then used to characterise risks as margins of exposure. RPF are used to convert exposures of all chemicals in the CAG into exposure equivalents of the index chemical, rather like the procedure used with TEFs for dioxin-like chemicals.

Then, a detailed exposure assessment is made by developing detailed exposure scenarios for all CAG members, including determination of potential human exposures by all relevant pathways, durations and routes where simultaneous exposure may occur, as well as sequential exposures. The output of this analysis is an aggregation of exposures via all routes and pathways, for each chemical, which is then expressed in terms of an equivalent exposure of the index chemical, by using RPFs.

Finally, in the risk characterisation phase, the exposure assessment yields a dose measure for the mixture that is expressed as equivalent exposure to the index chemical. The results and conclusions of the cumulative risk analysis must be described and the risk contributions from each pathway and route should be evaluated both individually and in combination, in order to identify risk contributors. The risk characterisation step also includes descriptions of variability and major areas of uncertainty and the need for uncertainty and safety factors is determined.

Other applications of CRA by US EPA

The 1996 amendments to the Safe Drinking Water Act also required consideration of chemical mixtures. In this context US EPA was charged with developing new approaches for the assessment of complex mixtures, with particular focusing on disinfection by-products. Again, default assumptions about joint additive effects were adopted, and considerations of synergistic or antagonistic effects remained minimal (Kortenkamp et al., 2009).

Finally, cumulative risk assessment has also been applied to estimate the health effects of air pollutants, in the context of the National-Scale Air Toxics Assessment (NATA)[22], US EPA's comprehensive evaluation of air toxics in the U.S. In this work, the US EPA considered 177 air pollutants, and used dispersion models to estimate their concentrations in ambient air. These were used as input values for the estimation of both cancer and non-cancer risks.

NATA estimated simultaneous exposures to the selected chemicals at the census tract, country or state level at a point in time. The cumulative methods applied were dose addition and independent action. For carcinogens, lifetime cancer risk estimates for inhalation exposures were added (independent action). For non-carcinogens, the common health effect of concern was respiratory irritation and single-chemical hazard quotients for respiratory irritants were added to yield a "respiratory hazard index" (dose addition) (USEPA, 2007).

However, the CRA summed hazard quotients of individual chemicals that had similar adverse health outcomes, but not necessarily similar modes of action. Like the previous applications of CRA, synergistic or antagonistic effects were not considered, nor were non-chemical stressors taken into account (Kortenkamp et al., 2009).

Framework for CRA

In 2003, the US EPA published a Framework for Cumulative Risk Assessment (USEPA, 2003), which provides starting principles for EPA's cumulative risk assessment, for the future development of a comprehensive and detailed guidance on methods for evaluating cumulative risk. This report emphasizes chemical risks to human health including the effects from a variety of stressors, including non-chemical stressors. This was further developed in a the 2006 publication on the "Considerations for developing alternative health risk assessment approaches for addressing multiple chemicals, exposures and effects" (USEPA, 2006a). This document is not a guidance document, but presents concepts that could assist the development of detailed guidance in the future, and provides explicit approaches for addressing some of the complicating "multiples" in cumulative risk assessment. These approaches include new methods and the extension of existing methods to address health risk from multiple chemicals and multiple exposure pathways and times.

Agency for Toxic Substances and Disease Registry (ATSDR)

The ATSDR is the principal federal public health agency involved with hazardous waste issues. The agency is responsible for preventing or reducing the harmful effects of exposure to hazardous substances on human health, and for implementing the health-related parts of the Superfund law and of other laws that protect the public from hazardous wastes and environmental spills of hazardous substances. ATSDR also advises the EPA, as well as other federal and state agencies, community members, and other interested parties, on the health impacts of Superfund sites.

[22] http://www.epa.gov/nata/

Under the CERCLA, the ATSDR assesses whether adequate information on health effects is available for the priority hazardous substances; if not, the ATSDR initiates, in cooperation with the National Toxicology Program, a program of research to determine these health effects. The Act also requires that ATSDR develops methods to determine the health effects of substances in combination with other substances with which they are commonly found, where feasible. Similarly, the FQPA requires that factors be considered in establishing, modifying or revoking tolerances for pesticide chemical residues in food, and that these should include available information on the cumulative effects of substances with a common mode of action (Kortenkamp et al., 2009).

To address these requests, ATSDR's Division of Toxicology has developed a research program for chemical mixtures, which includes a trend analysis to identify mixtures most often found in environmental media, *in vivo* and *in vitro* toxicological testing of mixtures, quantitative modelling of joint action, and methodological development. The agency has also devised a guidance manual that outlines the latest methods for mixture assessment (ATSDR, 2004).

ATSDR Guidance document for the assessment of joint toxic action of chemical mixtures

This guidance manual aims both at assisting the agency in determining whether exposure to chemical mixtures at hazardous waste sites may impact public health and at serving as a basis for the development of interaction profiles (see below).

The approach developed in the mixtures guidance manual is consistent with the US EPA guidance articulated since 1986. This approach is a semi-quantitative screening process. A step-by-step procedure is outlined in a flow chart, before being discussed and illustrated by examples, for the assessment of both non-carcinogenic and carcinogenic effects. These flow charts are presented in Figure 11 and Figure 12.

The strategies for non-cancer and cancer effects are similar. Exposure data and toxicological information on the mixture of concern (or a similar mixture) are the preferred basis for an assessment. If no data are available, no whole mixture studies and no Minimal Risk Levels (MRLs) or comparable health guideline values for the mixture or guidance regarding a health assessment approach, a components-based approach is undertaken.

Component-based mixture assessments make use of the hazard index and are fully compatible with US EPA approaches. The components-based approach focuses on mixture components that are present at toxicologically significant exposure levels. Linked physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) models for two or more components may be used to predict the potential for interactions, or possibly for noncancer or cancer health effects from the mixture. The hazard index method is used to screen for non-cancer health hazards from potential additivity of the components. Cancer risks for the components are summed to screen for health hazards from potential additivity of carcinogenic effects. A weight-of-evidence method is used to evaluate the potential impact of interactions on non-cancer and cancer health effects.

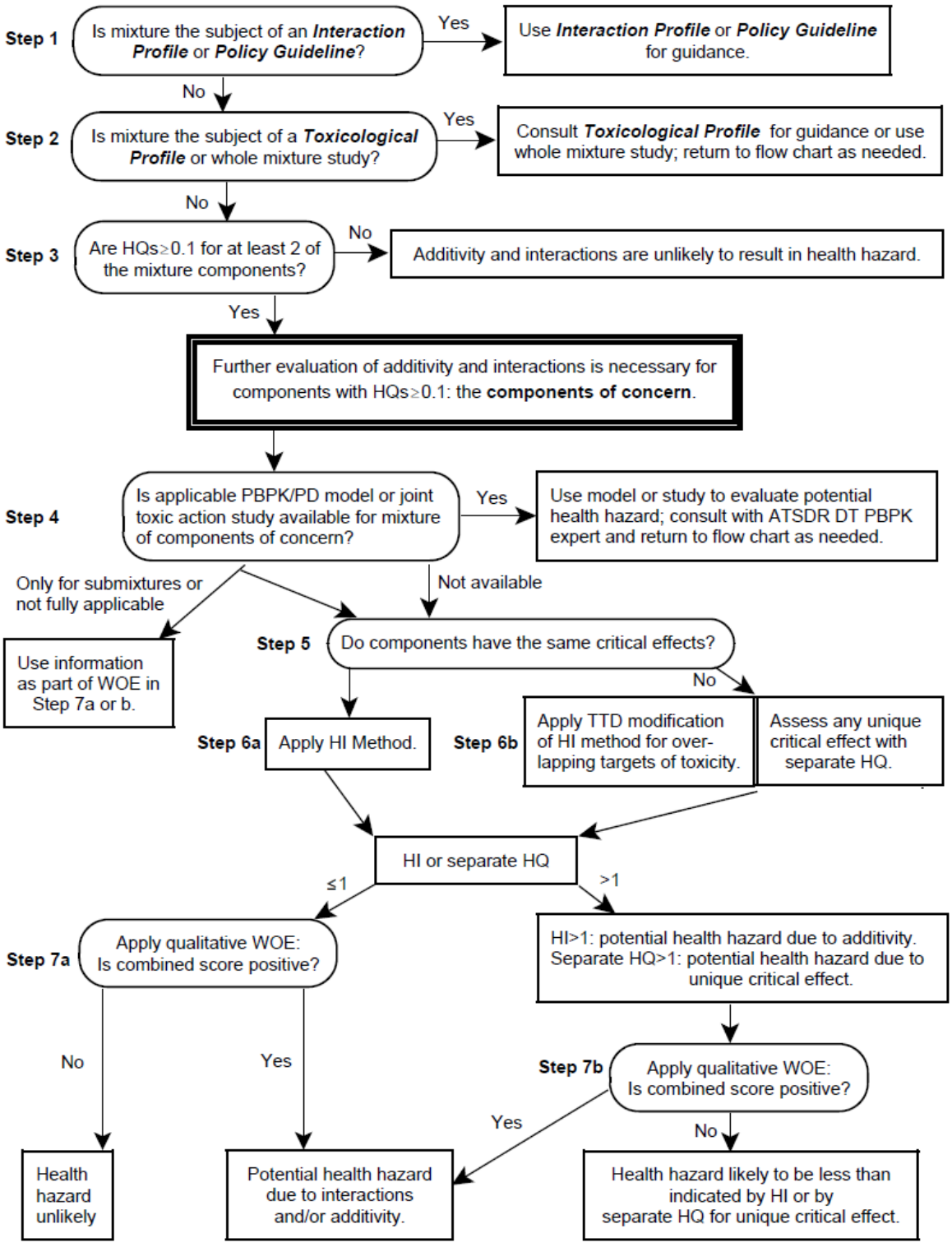


Figure 11: Strategy for exposure-based assessment of joint toxic action of chemical mixtures: non-carcinogenic effects (ATSDR, 2004)

Source: Agency for Toxic Substances and Disease Registry (http://www.atsdr.cdc.gov)

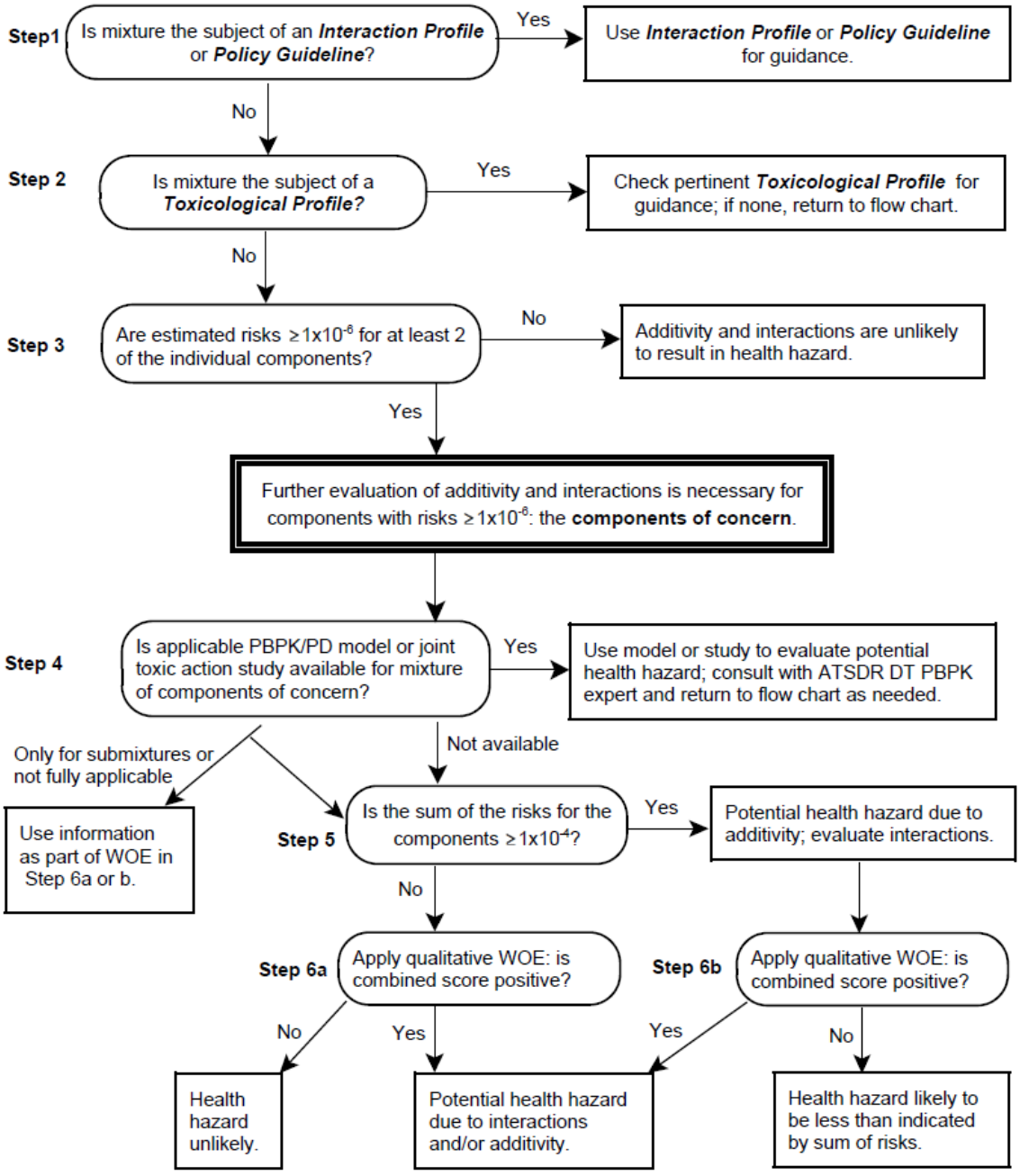


Figure 12: Strategy for exposure-based assessment of joint toxic action of chemical mixtures: carcinogenic effects (ATSDR, 2004)

Source: Agency for Toxic Substances and Disease Registry (http://www.atsdr.cdc.gov)

In deriving hazard quotients, the US EPA recommends the use of RfD also for effects that occur at higher doses, not only the critical effects, which may lead to overestimations of risks. To deal with this potential complication, the target organ toxicity dose (TTD) modification of the hazard index was developed for chemicals that affect an endpoint at a dose higher than for the critical effect (Kortenkamp et al., 2009).

Moreover, the hazard index concept assumes dose additivity, and does not take into account potential toxic interactions. In order to fill this gap, another modification of the hazard index approach is also developed in this document, by using additional uncertainty factors to accommodate the possibility of deviations from expected additivity. It evaluates binary mixtures and introduces a classification that indicates the expected direction of interaction (synergistic or antagonistic) by using an alphanumerical scoring system. The scores are then combined with the hazard index.

Other documents from the ATSDR

In addition, a series of documents called interaction profiles[23] were developed for certain mixtures of concern found in environmental media, in food, or in site-specific exposure settings. The purpose of these documents is to evaluate data on the toxicity of the 'whole' priority mixture (if available) and on the joint toxic action of the chemicals in the mixture in order to recommend approaches for the exposure-based assessment of the potential hazard to public health.

Food and Drug Administration (FDA)

The FDA is the US agency responsible for protecting public health by assuring the safety, efficacy and security of human and veterinary drugs, biological products, medical devices, the nation's food supply, cosmetics, and products that emit radiation.

The FDA has issued a guidance document for industry on drug-drug interaction studies (FDA, 2006). The focus of this guidance is to advise on studies aimed at establishing whether one drug influences the absorption, distribution, metabolism, excretion, or the effects of another drug, and the promoted strategy is based on *in-vitro* study, *in-vivo* study and PBPK modelling. The idea is not to determine additive combination effects between drugs, and consequently no component-based approaches are suggested. Guidance relevant to pesticide residues or additives in food could not be located (Kortenkamp et al., 2009).

National Research Council (NRC)

The National Research Council (NRC) is the operating arm of the United States National Academies, which produces reports that shape policies, inform public opinion, and advance the pursuit of science, engineering, and medicine. In 1989, the Safe Drinking Water Committee of the National Research Council (NRC) discussed possible modifications to the approaches for estimating the toxicity of mixtures in drinking water, suggesting to group

[23] Available at: http://www.atsdr.cdc.gov/interactionprofiles/index.asp

mixture components according to toxicity endpoints, such as specific organ toxicity or carcinogenicity, with the aim of assessing their combined hazards and risks (NRC, 1989).

In 1994, while reviewing the methods used for the determination of carcinogenic risks associated with exposure to hazardous air pollutants, the NRC pointed out that people at risk are exposed to a mixture of chemicals, each of which is possibly associated with an increased probability of one or more health effects (NRC, 1994), and that emitted substances might be carried to and deposited on other media, such as water and soil, and cause people to be exposed via routes other than inhalation (e.g. dermal absorption or ingestion). In such cases, data are often available on only one of the adverse effects (e.g. cancer) associated with each chemical: the issue is how best to characterise and estimate the potential aggregate risk posed by exposure to a mixture of toxic chemicals.

The method used by the US EPA of adding the risks related to each chemical in a mixture for developing a risk estimate was considered appropriate when the only risk characterisation needed is a point estimate for use in screening. When a more comprehensive uncertainty characterisation is desired, the NRC recommended that the US EPA uses appropriate statistical (e.g., Monte Carlo) procedures to aggregate cancer risks from exposure to multiple compounds. They further recommended that in the analysis of animal bioassay data on the occurrence of multiple tumour types, the cancer potencies should be estimated for each relevant tumour type that is related to exposure, and the individual potencies should be summed for those tumours.

More recently, the NRC was asked by the US EPA to look into the necessity of conducting a cumulative risk assessment for phthalates, because phthalates make up a chemical class that produce similar effects and have similar chemical structures, and, if necessary, to suggest an approach for such an assessment. In their report (NRC, 2008) the committee strongly advised that risk assessment should group chemicals that cause common adverse outcomes; thus it should consider not only certain phthalates, but also other chemicals that could potentially cause the same health effects, instead of focusing exclusively on chemicals that are structurally or mechanistically similar, which is US EPA's current practice. Accordingly, phthalates and other agents that affect male reproductive development in animals, including antiandrogens, should be considered in a cumulative risk assessment. A focus solely on phthalates to the exclusion of other antiandrogens would be artificial and could seriously underestimate cumulative risk. This is different from current US EPA current practice, which often considers only chemicals that are structurally related, on the assumption that they exert their effects by similar mechanisms leading to a final health outcome. The NRC committee pointed out that this practice ignores how exposures to different chemicals may result in the same health effects.

To the question of whether dose addition, independent action, or some other method should be used for estimating risk associated with phthalates and other antiandrogens, the committee concluded that the answer should be based on empirical data that directly test any proposed method. Results of the conducted mixture studies in laboratory animals with phthalates and/or other antiandrogens indicate that the mixture effects in each case are predicted well with dose addition methods, although a variety of mechanisms are involved.

Moreover, when the model predictions differed significantly, no case could be found in which independent action predicted mixture effects better than dose addition. Thus, the evidence supports the use of dose addition as an approximation in estimating cumulative risk posed by phthalates and other antiandrogens.

There are several approaches for conducting cumulative risk assessment with the dose-addition approach. This report outlines a few options, each having some advantages and disadvantages; it was left to the EPA to evaluate each option and determine which is most appropriate.

The committee also recommended that the conceptual approach taken for phthalates - to consider chemicals that cause similar health effects - should also be applied when completing any cumulative risk assessment. For instance, the US EPA could evaluate the risk of combined exposures to lead, methylmercury, and polychlorinated biphenyls because all contribute to cognitive deficits consistent with IQ reduction in children, albeit by very different mechanisms.

Other US administrations

Other administrations dealing with the assessment of mixture toxicity general use additivity approaches similar to the hazard index. This is the case of The American Conference of Governmental Industrial Hygienists **(**ACGIH), of The Occupational Safety and Health Administration (OSHA) and of The National Institute of Occupational Safety and Health (NIOSH) for the evaluation of occupationally relevant combined exposures. The National Academy of Sciences also recommends a hazard index approach, where the sum of the ratios of the measured concentrations to the acceptable concentrations for the individual components has to be kept at levels equal to, or lower than, unity, to investigate multiple chemical exposures in freshwater aquatic systems (Kortenkamp et al., 2009).

Another example of the use of concentration addition in an environmental context is the development of a Pesticide Toxicity Index (PTI) by the US Geological Survey, in charge of providing scientific information to help facilitate effective management of natural resources. This index combines measures of pesticide exposures of aquatic biota with acute toxicity data derived from laboratory assays to produce a single index for a sample or a site (USGS, 2006). The development of the PTI was limited to pesticide compounds routinely measured and to toxicity data readily available from existing databases. The PTI for a particular sample is the sum of toxicity quotients (measured concentration divided by the median toxicity concentration from bioassays) for each detected pesticide. Thus, the PTI is in effect an indicator of combined effects from pesticides that are to be expected under the assumption of concentration addition, and this approach is very similar to the sum of Hazard Quotients used by US EPA.

The PTI can be calculated for specific groups of pesticides and for specific taxonomic groups, and although it does not determine whether water in a sample is toxic, its values can be used to rank or compare the toxicity of samples or sites on a relative basis for use in further analysis or additional assessments. The index is also useful for assessing the relative

contribution of specific pesticides to an overall assumed effect, but as the median toxicity values are based on short-term laboratory assays with high effect levels (50%), it may limit the usefulness of PTI for estimations of long-term effects. Moreover, the PTI makes allowance only for additive effects and does not take possible synergisms or antagonisms into account. Nevertheless, despite these limitations, the PTI's make best use of available data and are a valuable tool for comparative assessments of water quality.

Conclusion

The US EPA approach to cumulative risk assessment has evolved over the years. However, approaches that can deal with more than additive combination effects are still lacking. Moreover, taking account of multiple chemicals via multiple routes is not always possible, which is both due to a lack of knowledge in key areas, and to an absence of appropriate data. For the estimation of risks from chemical mixtures, the EPA, ATSDR and other relevant bodies employ a variety of approaches, ranging from whole mixture approaches to component-based approaches, depending on the risk assessment context. Dose addition and independent action are both applied, and guidance advises when to use either concept. For specific groups of chemicals, including dioxins, organophosphates and polycyclic aromatic hydrocarbons, toxic equivalency factors are employed.

4.2.2 Canada

In 1994, the Canadian Federal Environmental Assessment Review Office published a reference guide describing an approach for addressing cumulative environmental effects under the *Canadian Environmental Assessment Act* (FEARO, 1994). Specifically, this reference guide reviews the concept of cumulative environmental effects, discusses the relevant requirements of the Act and proposes a framework for addressing cumulative environment effects under the Act.

This reference guide was supplemented in 1999 with the The *Practitioners Guide,* providing further information on cumulative effects (CEAA, 1999). Cumulative effects are defined as "*the effect on the environment which results from effects of a project when combined with those of other past, existing and imminent projects and activities"*, which "*may occur over a certain period of time and distance"*. It considers the combined effects of human activities on ecosystems, including chemical pollution, global warming and loss of biodiversity. Thus, this assessment is not restricted to chemical effects. Moreover, it does not necessarily cover chemical mixture assessment issues. In 2007, the Canadian Environmental Assessment Agency released an updated policy statement (CEAA, 2007).

In 2003, Health Canada issued a science policy notice on aggregate exposure and risk assessments (PMRA, 2003). Aggregate exposure and risk assessments involve the analysis of exposure to a single chemical by multiple pathways and routes of exposure. The pathways of exposure considered include the potential for pesticide residues in food and drinking water, as well as residues from pesticide use in residential, non-occupational environments. All potential, relevant routes of exposure (oral, dermal, inhalation) and pathways (through food,

drinking water and residential use) are analysed within an aggregate exposure assessment; however this document does not consider concurrent exposure to several chemicals.

4.2.3 Guidance from major international bodies

World Health Organisation (WHO) and the International Programme on Chemical Safety (IPCS)

Through the International Programme on Chemical Safety (IPCS), the WHO works to establish the scientific basis for the sound management of chemicals, and to strengthen national capabilities and capacities for chemical safety.

During the last 20 years, the IPCS has established and regularly re-evaluated toxic equivalency factors (TEFs) for dioxins and related compounds, for humans, mammals, birds and fish. These TEFs have been used for the risk management in UN Member States and adopted formally by a number of countries and supranational bodies. TEF values are re-evaluated on a regular basis (preferably at five-year intervals); but the last re-evaluation of human and mammalian TEFs was carried out in 2005. As a result, a number of TEF values have been changed, notably for PCBs, octachlorinated congeners and pentachlorinated furans[24], and the project has also served to update the database summarizing all studies published on the relative potency of dioxins, furans, and dioxin-like PCBs.

Together with the FAO, the WHO also worked on the project to update the principles and methods for the assessment of chemicals in food, through the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). In response to their need for general guidance for risk assessments, the IPCS sponsored the preparation of two Environmental Health Criteria (EHC) monographs, EHC 70 (Principles for the safety assessment of food additives and contaminants in food) and EHC 104 (Principles for the toxicological assessment of pesticide residues in food). FAO and WHO have also initiated a joint Project to update and consolidate principles and methods for the risk assessment of chemicals in food. A website has been set up to provide reports and other information on the project as they become available[25], but the information available on this website does not indicate that combination effects of pesticides and/or food additives will be taken into account (Kortenkamp et al., 2009). Reports have also been published in 2008, 2010 and 2012 to assess the risk from melamine, bisphenol A and histamine exposure through food intake.

In 2001, the IPCS also developed a coherent framework for integrated risk assessment. IPCS define the term integrated risk assessment as a science-based approach that combines the processes of risk estimation for humans, biota, and natural resources into one assessment. The report details this general framework and contains four case studies intended to illustrate the benefits of integrated risk assessment: persistent organic pollutants; UV radiation effects on amphibians, coral, humans and oceanic primary productivity; tributyltin and triphenyltin

[24] http://www.who.int/foodsafety/chem/tef_update/en/index.html
[25] http://www.who.int/foodsafety/chem/en/

compounds; and organophosphorous pesticides in the environment[26]. Considerations of the effects of sequential and simultaneous exposure to several chemicals are an integral part of the framework, but the specifics of mixture hazard characterisation are not described (Kortenkamp et al., 2009).

In March 2007, the IPCS convened an international workshop on current issues in aggregate/cumulative risk assessment (i.e. the combined risk from exposure to one or more agents via all relevant routes and pathways), in order to discuss and review available methods for assessing combined risks from exposures to multiple chemicals, to develop working definitions for the different types of exposures, effects and risks of chemicals, and to initiate the development of a framework for assessment of risks to multiple chemicals.

The workshop agreed on the following working definitions for key terms and concepts:

• Exposure to the same chemical by multiple pathways and routes should be described as "*Single Chemical, All Routes*" (sometimes also referred to as "aggregate exposure").

• Exposure to "*Multiple Chemicals by a Single Route*" should be distinguished from

"*Multiple Chemicals by Multiple Routes*", and both these possibilities are the topic of the framework development.

• Chemicals that act by the same mode of action and/or at the same target cell or tissue display "*Dose Additive*" combination effects.

• Where chemicals act by diverse modes of action or at different target cells or tissues, the combined effects are "*Effects Additive*" or "*Response Additive*".

• *Synergy* and *antagonism* are defined as departures from dose additivity, not response additivity.

• "*Mode of Action*" is a biologically plausible sequence of key events that lead to an observed effect.

• "*Mechanism of Action*", in contrast, involves a sufficient understanding of the molecular basis for an effect so that causation can be established.

The workshop also proposed a preliminary framework for consideration of risk from exposure to multiple chemicals, which is intended as an iterative process involving step-wise consideration of exposures and hazards in several tiers, depending on the data available to support the analysis (WHO, 2009).

The analysis begins with a consideration of the potential for cumulative exposure, before any assessments of hazards take place. In its first tier, the workshop report recommends adopting dose addition, if there is no evidence for synergisms or antagonisms. Chemicals to be subjected to this procedure should be grouped according to their chemical structure, similarity of target tissue and/or similarity in the manifestation of toxicity. Should the combined risks turn out to be not acceptable, the assessment should be refined further by additional consideration of temporal aspects of the common toxic effect, the presence of a common metabolite, analysis of key biological targets and consideration of information about environmentally relevant mixture ratios and exposure levels (Kortenkamp et al., 2009).

[26] http://www.who.int/ipcs/publications/new_issues/ira/en/

The IPCS Framework and several case studies (on carbamates, commercial hexane, food additives, application of TTC in the assessment of chemical mixtures found in surface water, and pharmaceuticals in surface waters) were further developed during 2010 and subsequently published (Meek et al., 2011; OECD, 2011b), and needs for further work on combined exposures were identified (OECD, 2011b).

The WHO also published in 2012, as part of collaboration with the United Nation Environment Programme (UNEP), a "State of the Science of Endocrine Disrupting Chemicals (WHO-UNEP, 2012). This document is not strictly a guidance document but it is an update of the scientific knowledge, including main conclusions and key concerns, on endocrine disruptors. The authors express the view that EDCs are a concern to public and wildlife health and that EDCs represent a challenge, as their effects depend on both the level and timing of exposure, being especially critical when exposure occurs during development. Moreover, this document highlights the reality of simultaneous exposure to many EDCs; thus, it is argued that the measurement of the linkage between exposure to mixtures of EDCs and disease or dysfunction is more physiologically relevant than a focus on linking one EDC to one disease, which it is claimed may severely underestimate the disease risk from such mixtures. EDCs might act at low levels of exposure, and might exhibit dose-response curves that are non-linear and potentially non-monotonic (WHO-UNEP, 2012). According to EFSA's Scientific Committee (EFSA, 2013e) (EFSA, 2013) the debate is evolving in the scientific community as to the existence and/or relevance of low-dose effects and NMDRs in (eco)toxicology in relation to endocrine disruption or other endpoints/modes of action, but still lacks consensus. More work needs to be conducted to agree the definitions of the respective terms, and in practical terms to consider whether or how it could impact upon risk assessment (i.e. assessment of dose response relationships for adverse effects) and testing strategies.

In this context, the WHO report concludes that new approaches are needed to examine the effects of mixtures of EDCs on disease susceptibility and etiology. Moreover, since only a narrow spectrum of chemicals and a few classes of EDCs have been assessed, a more comprehensive assessment of human and wildlife exposures to diverse mixtures of EDCs are needed.

Organisation for Economic Co-operation and Development (OECD)

Over the last 40 years, the OECD Chemicals Programme has been helping its member governments to develop and implement high-quality chemicals management policies and instruments. OECD countries now have science-based, rigorous and comprehensive systems for assessing and managing the risk of chemicals.

A key mission of the OECD is to work for mutual acceptance of data on the hazardous effects of chemicals. In this context, many of the activities focus on the development of guidelines for chemicals testing. However, the OCDE also established a Cooperative Chemicals Assessment Programme (CoCAP), based on the previous High Production Volume (HPV) Chemicals Programme, to better respond to the needs of member countries. The programme specifically addresses a number challenges, such as assessing more

chemicals in a shorter period of time, addressing all chemicals on the market, or avoiding duplication of on-going work in other countries. In this context, the programme put in place a forum to exchange experience among member countries to avoid duplication of effort and identify issues for collaborative work, amongst which the effects from exposure to multiple chemicals[27].

OECD has produced guidance on limiting the number of toxicological tests to be carried out by grouping chemicals into closely related categories (OECD, 2007), a category being "a group of chemicals whose physico-chemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity". The similarities may be based on common functional group(s), common constituents or chemical classes, similar carbon range numbers (which is frequent with complex substances known as "substances of Unknown or Variable composition, Complex reaction products or Biological material" or UVCB substances); an incremental and constant change across the category; or the likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals (e.g. the "metabolic pathway approach" of examining related chemicals such as acid/ester/salt). An updated version of this guidance should be published this year.

In this so-called category approach, data for chemicals and toxicological endpoints that have been tested are used to estimate the corresponding properties of untested chemicals, to avoid supplementary testing. In principle, this approach can also be used to define groups of chemicals to be subjected to mixtures risk assessment. An example is the TEQ approach, used for polychlorinated dioxins and furans (PCDD/F), which was originally designed to estimate the toxicity of untested congeners but which has matured into a framework for assessing mixtures of PCDD/F. In 2011, OECD published a new Series on Testing and Assessment on forming chemical categories based on mechanistic information and on the Adverse Outcome Pathway (AOP) (OECD, 2011a). This document reviews the knowledge on mechanism or MOA in the context of key events or processes that lead to specific adverse outcomes that are used in RA; proposes how to organise scientific data on mechanism or MOA as key events; and examines a series of case studies using adverse outcome pathways. This document also recognized that AOPs provide a means of supporting assessment of combined exposure to multiple chemicals within and across AOPs.

European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC)

Established in 1978, ECETOC is a European industry association that develops and promotes science in human and environmental risk assessment of chemicals.

In its report "Aquatic Toxicity of Mixtures" (ECETOC, 2001), ECETOC emphasized the need for practical methods to deal with the possibility that mixtures of chemicals present in aquatic systems express additive effects, even when individual substances are present at

[27] http://www.oecd.org/chemicalsafety/risk-assessment/oecdcooperativechemicalsassessmentprogramme.htm

concentrations not expected to lead to chronic toxicity on their own. Five approaches were listed and are presented in Table 7 along with their advantages and disadvantages.

More recently, ECETOC published a new guidance document on the same issue (ECETOC, 2011a). Acknowledging the fact that the use of modelling to predict the toxicity of mixtures allows implementation of prospective risk assessment schemes, applicable when environmental mixtures can be reliably predicted (i.e. in product assessment, where the product is a mixture, or where joint emissions can be adequately predicted and quantified), it was recognized that, once released into the environment, relative concentrations of the constituents and the associated risk will change. In this case predicting the chemicals to which an environment is exposed is difficult and often impossible. In this context, this document presents a framework which retrospectively allows the evaluation of the potential impact of chemicals or chemical mixtures in the environment. The suggested approach is shown in Figure 13.

The starting point for assessing the potential ecological impact of a chemical or chemical mixture is having an understanding of the reference condition, and to identify an impact when the "ecological status" of a site is compared to the ecological reference. Then, well established methods such as Whole Effluent Testing (WET) and Direct Toxicity Assessment (DTA) can be successfully employed to confirm or not, that chemicals are responsible. This can then be followed by Toxicity Identification Evaluation (TIE), Effect Directed Analysis (EDA) or Bioassay Directed Fractionation (BDF) to identify the toxic components within effluents or environmental samples allowing effective management.

If an environmental impact has no obvious cause, then causal analysis approaches should be employed to investigate and determine which stressors are the most probable cause of the impact (i.e. habitat, conventional pollutants, natural perturbation such as invasive species, or anthropogenic stressors such as toxic chemicals).

Table 7: Approaches to assess aquatic toxicity of mixtures

Toxic unit summation using actual environmental concentrations	***PEC/PNEC summation***	***Use of a correction factor to modify individual chemical assessments***	***Environmental monitoring***	***Biological field monitoring***
Principles: -Individual substances are identified and their concentrations are measured. -For each chemical, QSAR-based toxicity values (NOECs, LC_{50}…) are derived to establish TU. -TU are summed up, if $\sum TU > 1$, further evaluations are considered. *Advantage:* -Only substances actually present in the environment are included -Used of measured value. *Weakness:* -Approach only viable if the number of chemicals is low, as the identification and quantification of chemicals is time consuming, analytical methods may be lacking, and special problems may arise when the limit of detection is larger than the biologically effective concentrations of chemicals (as is the case with some hormonally active chemicals).	*Principles:* Use of predicted values (PEC; PNEC) which are relatively easily available for a large number of chemicals. *Advantages:* -Straightforward approach, uses of available data - Does not require (initially) environmental measurements. *Weakness:* Predicted values may not always be reliable and may over-estimate risks where individual PEC/PNEC ratios are overly conservative.	*Principles*: This approach aims to adapt and modify existing RA procedures for individual chemicals by applying a "mixtures correction factor" to each individual substance. It is based on conventional RA for deriving PEC/PNEC ratios, but determines ratios of PEC to PNEC times X, where X is the number of chemicals also present in a mixture. *Advantage*: Ease of use, especially on a case-by-case basis *Weakness*: X, the number of chemicals occurring together with the one to be assessed, is largely unknown, and may fluctuate. Assumption that substances are present at concentrations proportional to their PEC/PNEC ratios: more prevalent substances would be weighted in the same way as all others, leading to a skewed analysis of the situation.	*Principles:* Chemical and/or biological monitoring techniques, e.g. biomimetic approaches using membrane devices, can provide valuable surrogate measures of bioavailable substances. *Advantages:* Relatively easy to use *Weakness:* Poorly validated methods	*Principles:* Well established approach to assess whether effects have actually occurred in ecosystems. *Advantages:* Provides an integrated biological picture; chemical measurements are unnecessary if effects are not observed. *Weakness:* In case of noticeable effects, causes are difficult to establish, monitoring techniques are not protective because they can establish effects only after they have occurred.

TU: Toxic Unit, ratio of concentration to toxicity value; PEC: predicted environmental concentrations; PNEC: predicted no-effect concentration

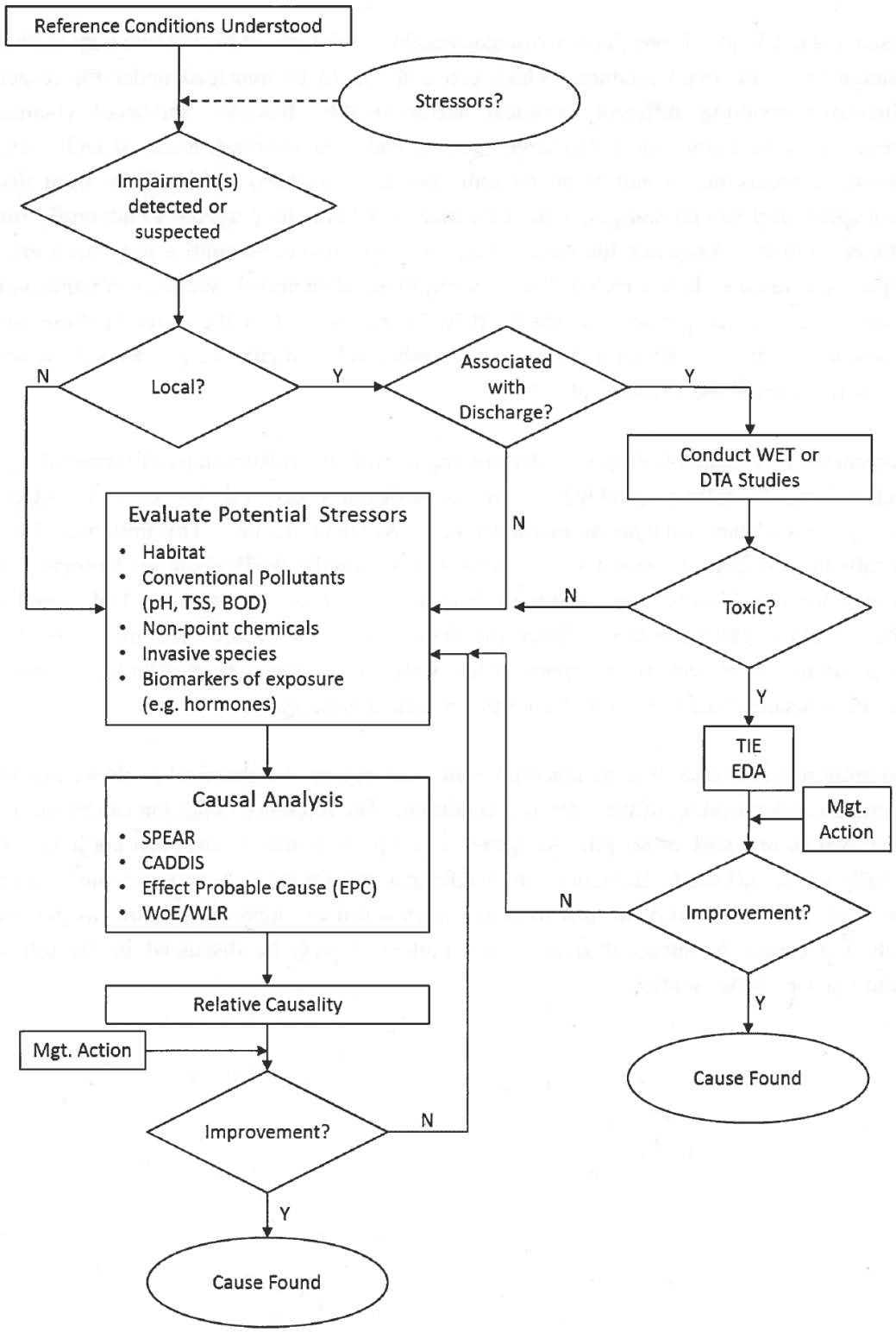


Figure 13: Suggested approach to assessment of ecologic risk of mixtures of chemicals in the aquatic environment (ECETOC, 2011a)

TSS: Total Suspended Solids, BOD: Biological Oxygen Demand; SPEAR: Species at risk, CADDIS: Causal Analysis/Diagnosis Decision Information System; WET: Whole Effluent Testing, DTA: Direct Toxicity Assessment, TIE: Toxicity Identification Evaluation, EDA: Effects Directed Analysis; WoE: Weight of Evidence; WLR: Weighed Logistic Regression

Source: European Centre for Ecotoxicology and Toxicology of Chemicals (http://www.ecetoc.org)

ECETOC also held a *Workshop on combined exposure to chemicals* in 2011(ECETOC, 2011b). The resulting report focuses on the state of the science and on technical aspects of co-exposure, and discusses reliable and pragmatic approaches to risk assessment of combined exposure to chemicals.

It was recognized that according to available evidence, the toxicity of mixtures is often dominated by a few of their components, which should be identified at an early stage. In this context, the Maximum Cumulative Ratio (MCR) approach was suggested as a useful tool for both human health and environmental risk assessments. It was also suggested to develop a tiered approach beginning with limited data and a high degree of conservatism, moving if necessary to a requirement of more realistic data and a reduction in conservatism and increased expert input. TTC or an equivalent approach would be a potentially important element of the lower tier assessment.

Further research needs were also identified, such as better understanding of mode of action, improved methodologies of exposure assessment including assimilation of better databases and data processing methods. The TTC approach and non-testing methods would also needs further development for use in this context; and the development of transparent, scientifically-valid criteria for prioritising combinations that need to be assessed, and considering interactions where exposure to biological or physical stressors is currently near the threshold of effects, was suggested as a next step.

European Chemical Industry Council (CEFIC)

CEFIC also proposed a two-step approach (Price et al., 2012) for the evaluation of human and ecological effects from exposures to multiples chemicals from a single or multiple sources. The first step consists of a screening tool which aims at identifying those cases where effects from combinations of chemicals might be of potential concern and need further risk management measures. If yes, the second step consists of the use of a decision tree proposing a "Tiered risk assessment" and building on existing Europe Scientific Committee's decision tree and the WHO framework for mixtures, and incorporating the recently developed MCR tool (cf. Table 1). This decision tree, shown in

Figure **14**, allows four different groups of combined exposures to be identified, each of which requires different strategies for managing the effects:

Group I: combined exposures of concern because one or more individual chemicals are a concern; thus there is a need to address the chemical specific concerns for the exposures and efforts to refine the assessment need to focus on the chemicals of concern.

Group II: combined exposures of low concern for both individual chemicals and for their combined effects; this group can be set aside as a low concern when evaluating combined exposures.

Group III: combined exposures of low concern for individual chemicals but of concern for the combined effects. This is the critical group for further assessments since the concern for these exposures cannot be identified using a chemical-by-chemical approach. This group is further divided into two subgroups:

- Group IIIA, where one chemical provides the majority of toxicity of the combined exposure; this chemical should be the focus of either refining the risk assessment or reducing exposure.

- Group IIIB, where no chemical dominates the toxicity of the exposure. Group IIIB exposures need to be the focus for refining the assessment by developing data on the modes of action (MoAs) for the chemicals that drive the toxicity of exposures.

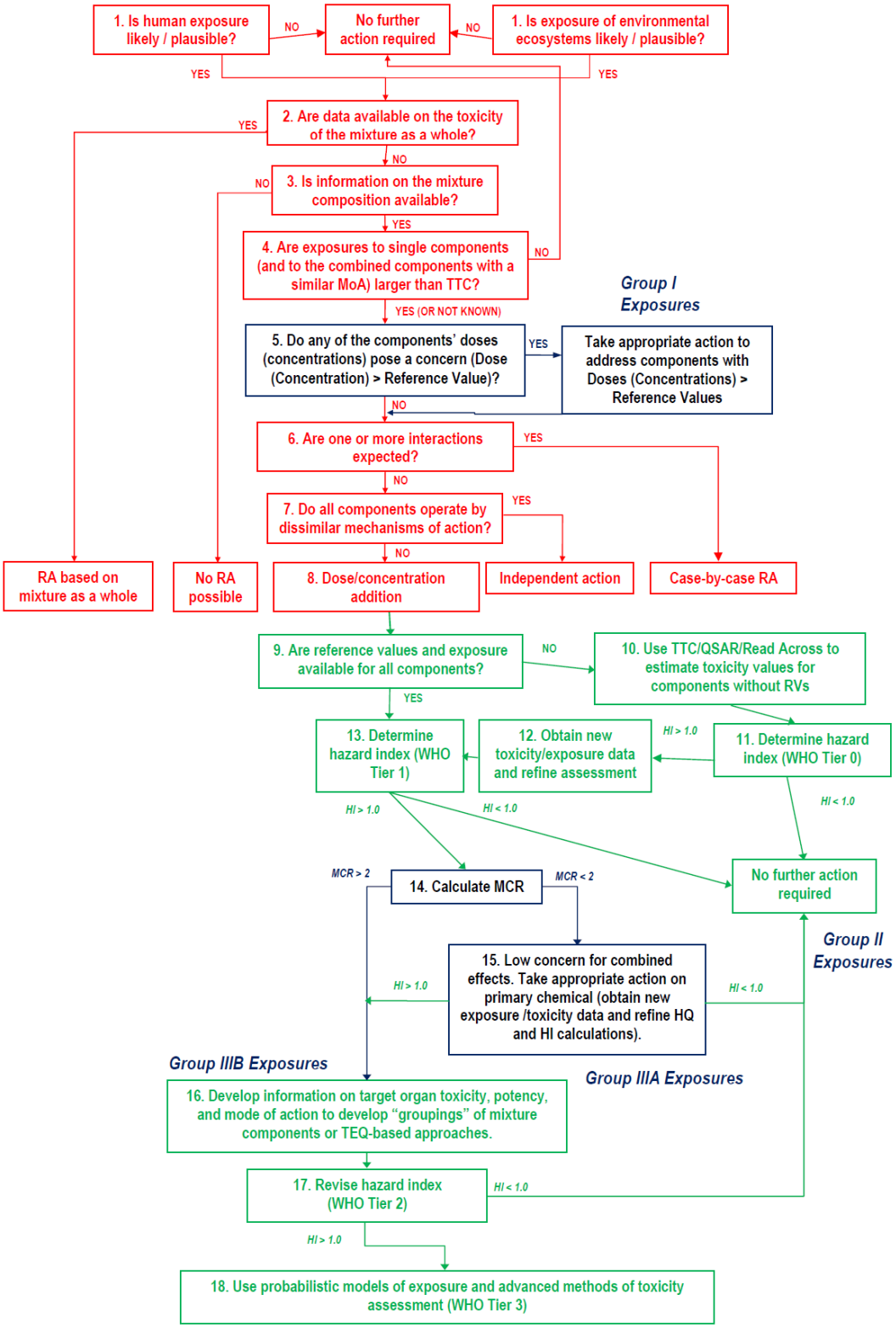


Figure 14: CEFIC decision tree

Red: European Scientific Committees' decision tree; green: WHO framework for mixtures.

Source: The European Chemical Industry Council (http://www.cefic.org/)

Convention for the protection of the marine environment of the North-East Atlantic (OSPAR)

OSPAR is the mechanism by which fifteen governments of the western coasts and catchments of Europe, together with the European Union, cooperate to protect the marine environment of the North-East Atlantic. To answer the requirements set out in §3 of OSPAR 2012/5 Recommendation for a Risk-based Approach (RBA) to the Management of Produced Water Discharges from Offshore Installations (OSPAR, 2012b), OSPAR published guidelines providing general guidance when undertaking ERA for all produced water discharges offshore (OSPAR, 2012a).

The RBA follows principles of environmental risk assessment already in use in the EU (ECHA -TGD) and US (US-EPA Guidance on risk assessment). The RBA approach has as far as possible been aligned with ECHA guidelines on risk assessment for single chemicals, and the assessment of mixtures was developed according to the best scientific practice.

The first step in the RBA process is data collation, in which information on the discharge is collected. The risk is determined using combined information from Hazard Assessment (Step 2) and Exposure Assessment (Step 3).

In the hazard assessment step, the inherent capacity of the discharge to cause adverse effects is evaluated, either on the basis of properties of the effluent (with a PNEC based on whole effluent testing and the use of an assessment factor) or of the individual substances. This last case requires derivation of PNECs from single species laboratory toxicity tests (preferably NOEC), and the grouping of substances based on a combination of the chemical structure, toxic mode of action and toxicity of the substance. The toxicity of the group will then be represented by the PNEC of representative chemicals in the group.

In step 3, an exposure assessment is carried out to derive the PECs for the receiving environment around an offshore installation. The assessment can also be based on the effluent as a whole or on the combination of individual substances. The output will be then respectively the concentration of produced water effluent (PEC) in the receiving environment, expressed as a percentage of the original effluent or the concentration of substances discharged with the produced water in the receiving environment (PEC). The PEC can be determined by modelling the concentrations in the receiving environment, by use of a 1-, 2- or 3-dimensional dilution/dispersion model. It should be demonstrated that dilution is not overestimated by the model by use of (peer reviewed) field validation study(s). If available, a model that takes account of different fate processes should be used, in order to provide a more accurate PEC.

In step 4, a risk characterisation is carried out by comparing the predicted environmental concentration of the substance and/or the effluent (PEC) and the hazard (PNEC) at a given distance as a minimum. The risk can be further characterised by identification of the contribution of the individual substances (both naturally occurring and man-added substances) or groups of substances (e.g. through TIE/EDA) to the overall risk. If risk

estimates are calculated on a substance based approach, the PEC/PNEC ratios for the individual identified substances should be combined to calculate the overall risk estimate for the produced water. As ECHA does not provide guidance for mixtures, this guideline recommends a combined approach based on species sensitivity distributions (Appendix 7). If needed, the risk characterisation can be refined by reducing uncertainties (as uncertainties lead to high assessment factors in the derivation of the PNEC), by collecting additional data and/or undertaking additional toxicity testing to obtain more reliable PNECs, or by obtaining more advanced dilution/fate modelling for instance.

Then, in step 5 (risk management) and 6 (monitoring), contracting parties should review management options, evaluate measures and develop and implement site-specific actions to reduce those risks which are not adequately controlled. Monitoring is used in order to verify the effectiveness of any risk management measures. It may also be used to detect changes in the discharge and in the receiving environment.

Conclusions

Although overarching analytical frameworks offer general guidance on ways to evaluate cumulative risk, we often lack adequate scientific knowledge and understanding about exposures, health or environmental effects, and the link between exposure and effects to implement them fully. The reality is that quantitative analyses are impractical in the context of many real-world problems because data on interactions among environmental stressors are scarce, information on place- and population-specific exposures is lacking, and verified mechanistic models relating exposure to effect are unavailable.

The situation is complex and different with regard to prospective or retrospective risk assessment. With regard to prospective risk assessment the definition of environmentally relevant mixtures (i.e. mixture composition: number, identity and concentrations of individual substances) is generally considered easier than for retrospective risk assessment, but still not without problems.

Dose addition (or concentration addition) has found widespread acceptance as an assessment concept for chemical mixtures if there is no evidence for synergistic or antagonistic behaviour and is extensively used by US authorities, regulatory and International bodies.

Less clarity exists in deciding on criteria for choosing the chemicals that are to be subjected to CRA by using dose (concentration) addition. Suggestions include the grouping of substances according to their chemical structure, similarity in toxicological mechanism or mode of action, target tissue and/or similarity in the manifestation of toxicity. However, narrowing similarity criteria might lead to the exclusion of chemicals that also contribute to joint effects, whereas inclusion of too many chemicals might render procedures of cumulative risk assessment unwieldy.

To conclude, the development of harmonised terminology and methodologies for human and environmental risk assessment of combined exposure to multiple chemicals is needed, as well as a consistent approach to the assessment of priority mixtures across the different EU regulations and directives. The development of methodologies for risk assessment of exposure to multiple chemicals combined with other stressors (e.g. biological hazards, physical agents) might also be proposed as a longer term objective.

List of abbreviations

a.s.	Active substance
ADI	Acceptable Daily Intake
AF	Assessment/Adjustment Factor
AOP	Adverse Outcome Pathway
ATE	Acute Toxicity Estimate
ATSDR	Agency for Toxic Substances and Disease Registry
BAT	Best Available Techniques
BDF	Bioassay Directed Fractionation
CAG	Cumulative Assessment Group
CEAA	Canadian Environmental Assessment Act
CEFIC	European Chemical Industry Council
CERCLA	Comprehensive Environmental Response Compensation and Liability Act
CLP	Classification, Labelling and Packaging
CMG	Common Mechanism Group
CMR	Carcinogenic, Mutagenic and Reprotoxic
CRA	Cumulative Risk assessment
CSA	Chemical Safety Assessment
DA	Dose addition
DE	Daily exposure
DNEL	Derived No-effect-Levels
DTA	Direct Toxicity Assessment
EC	European Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European CHemicals Agency
ED	Effective Dose
EDA	Effect Directed Analysis
EDC	Endocrine Disrupting Compound
EFSA	European Food Safety Authority
EIA	Environmental Impact Assessment
ELV	Emission Limit Value
EMA	European Medicines Agency
EQS	Environmental Quality Standard
EU	European Union
FQPA	Food Quality Protection Act
ERA	Environmental Risk Assessment
FAO	Food and Agriculture Organization of the United Nation
FDA	Food and Drugs Administration
FEARO	Canadian Federal Environmental Assessment Review Office
FOCUS	FOrum for Co-ordination of pesticide fate models and their Use

FQPA	Food Quality Protection Act
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
GM	Genetically Modified
GMO	Genetically Modified Organism
HEG	Homogeneous Exposure Group
HI	Hazard Index
HQ	Hazard Coefficient
IA	Independent Action
IPCS	International Programme on Chemical Safety
IPPC	Integrated Pollution Prevention and Control
LC50	Lethal Concentration 50
LOAEL	Low Observed effect Concentration
MEA	Mechanism of Action
MCR	Maximum Cumulative Ratio
MCS	Multi-Constituent Substances
MRL	Maximum Residue Level
MOA	Mode Of Action
MOE	Margin Of Exposure
MS	Member States
NATA	National-Scale Air Toxics Assessment
NOAEL	No Observed Adverse Effect Level
NRC	National Research Council
OECD	Organisation for Economic Co-operation and Development
OSPAR	Convention for the protection of the marine environment of the North-East Atlantic
PBPK/PD	Physiologically-Based PharmacoKinetic/PharmacoDynamic
PBT	Persistence, Bioaccumulation and Toxicity
PCB	PolyChlorinated Biphenyl
PCDD/F	Polychlorinated dioxin and furan
PEC	Predicted Environmental Concentration
PEF	Potency Equivalency Factor
PNEC	Predicted No Effects Concentrations
PPP	Plant Protection Products
PTI	Pesticide Toxicity Index
RA	Risk Assessment
RBA	Risk-Based Approach
RAGS	Risk Assessment Guidance for Superfund
RfC	Reference Concentration
RfD	Reference Dose
RfPI	Reference Point Index
RPF	Relative Potency Factor

RV	Reference value
SCCS	Scientific Committee for Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SE	Short-term Exposure
SFD	Soil Framework Directive
TEF	Toxic Equivalency factor
TIE	Toxicity Identification Evaluation
TTC	Threshold of Toxicological Concern
TTDs	Target organ Toxicity Dose
TU	Toxic Unit
UF	Uncertainty Factor
US EPA	United States Environmental Protection Agency
UVCB	Substances of Unknown or Variable composition, Complex reaction products or Biological materials
vPvB	very Persistent, very Bioaccumulative
WET	Whole Effluent Testing
WFD	Water Framework Directive
WHO	World Health Organization
WOE	Weight-Of-Evidence

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Glossary

Acute toxicity: Adverse effects of finite duration occurring within a short time (up to 14 d) after administration of a single dose (or exposure to a given concentration) of a test substance or after multiple doses (exposures), usually within 24 h of a starting point (which may be exposure to the toxicant, or loss of reserve capacity, or developmental change, etc.).

Additive effect: Effect observed after exposure to two or more chemical agents which act jointly but do not interact. The total effect is the simple sum of the effects of separate exposure to the agents under the same conditions.

Adverse effect: Change in the morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences

Aggregate risk: Risk associated with all pathways and routes of exposure to a single chemical.

ARfD (Acute reference dose): Estimate of the amount of substance in food and/or drinking water, expressed on a body weight basis, that can be ingested over a short period of time, usually during one day, without appreciable risk to the consumer on the basis of the data produced by appropriate studies and taking into account sensitive groups within the population (e.g. children and the unborn).

Chronic effect: Consequence that develops slowly and/or has a long lasting course: may be applied to an effect that develops rapidly and is long-lasting.

Coincidental mixtures: mixtures that are composed of unrelated chemicals from different sources, but having the potential to reach the same "receptor population", e.g. by their presence in or migration into the same medium (e.g. groundwater), or through multiple pathways.

Combined exposure: exposure of the same person to the same substance in the same setting via different routes of entry into the body or from different products containing the same substance. It might be considered to be synonymous with "aggregate exposure".

Combined toxicity: Response of a biological system to several chemicals, either after simultaneous or sequential exposure. It can take three possible forms: dose-addition, response-addition or interaction.

Common mechanism group: Group of chemicals determined to cause a common toxic effect by a common mechanism of toxicity

Common mechanism of toxicity: Pertains to two or more substances that cause a common toxic effect to experimental animals or to human health by the same, or essentially the same, sequence of major biochemical events. Hence, the underlying basis of the toxicity is the same, or essentially the same, for each chemical.

Common toxic effect: Two or more substances that are known to cause the same toxic effect in or at the same anatomical or physiological site or location (e.g. same organ or tissue). Thus, a toxic effect observed in studies involving animals exposed to a pesticide is considered common with a toxic effect caused by another chemical if there is concordance with both site and nature of the effect.

Cumulative assessment group (CAG): Group of active substances that could plausibly act by a common mode of action, not all of which will necessarily do so. The first and most conservative level of grouping is based on the organ or organ system level being the target of the pesticide toxicity (CAG 1). Further refinement to form a second level of grouping (CAG 2) is based on the identification and characterisation of specific effects in the organ or organ system.

Cumulative risk: Several definitions of this term are given depending on the context. In Europe, it is intended to be the risk deriving from the exposure to compounds that share the same mode of action (dose addition) or that have similar effects but do not act at the same molecular target (response addition) and is contrasted to synergistic risk (EFSA 2008b). Regarding pesticides, it has been defined as the risk resulting from exposure to more than one active substance via the diet (EFSA 2013c). The US EPA defines cumulative risk as the combined risk from aggregate exposure to multiple agents or stressors which may include chemical, as well as biological or physical agents (USEPA 2003).

Dissimilar mode of action: Occurs where the mode of action and possibly, but not necessarily, the nature and sites of toxic effects differ between the chemicals in a mixture, and one chemical does not influence the toxicity of another. The effects of exposure to such a mixture are the combination of the effects of each component compound (also referred to as response-addition).

Dose addition: see similar mode of action.

Generated mixtures: mixtures that contain additional compounds that are by-products of processes involved (e.g. smelting, drinking water disinfection, fuel combustion); they are usually originating from a single source.

Hazard: Inherent property of an agent (e.g. pesticide) or situation having the potential to cause adverse effects when an organism, system, or (sub-) population is exposed to that agent or situation.

Hazard assessment: Process that includes hazard identification and characterisation and focuses on the hazard in contrast to risk assessment where exposure assessment is a distinct additional step.

Hazard characterisation: Qualitative and, wherever possible, quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects. This is the second stage in the process of hazard assessment.

Hazard identification: Identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system, or (sub) population. This is the first stage in the process of hazard assessment.

Hazard index (HI): Sum of Hazard Quotients, i.e. ratios between exposure and the reference value for the common toxic effect of each component in a mixture or a CAG.

Index Compound (IC): The chemical used as the point of reference for standardizing the common toxicity of the chemical members of the CAG. The index compound should have a clearly defined dose-response, be well defined for the common mechanism of toxicity, and have a toxicological/biological profile for the common toxicity that is representative of the CAG.

Intentional mixtures: mixtures that are intentionally manufactured as such (i.e. regulated and manufactured products such as pesticide formulations or laundry detergent).

Interaction: Umbrella term for synergies (mixture effects greater than expected) and antagonisms (mixture effects smaller than expected). Interactions can be judged in relation to additivity expectations derived from dose addition or independent action

Mechanism of action: Detailed explanation of the individual biochemical and physiological events leading to a toxic effect

Mechanism of toxicity: Mechanism of toxicity is defined as the steps leading to a toxic effect following interaction of a pesticide with biological targets. All steps leading to an effect do not need to be specifically understood. Rather, it is the identification of the crucial events following chemical interaction that are required to describe a mechanism of toxicity. In the context of this document, mechanism of toxicity refers to the mechanism by which a pesticide is toxic to humans or experimental animals and plants, and not the mechanism by which it is toxic to target or intended species (i.e. its mechanism of pesticidal action). With some pesticides, however, the mechanism responsible for causing toxicity to humans or experimental animals and plants is similar to the mechanism of pesticidal action.

Mode of action (MoA): Biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. It refers to the 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.

Pathway of exposure: The physical course a chemical takes from the source to the organism exposed (e.g., through food/feed, drinking water, emissions etc.).

Response addition: see dissimilar mode of action

Risk assessment: Process intended to calculate or estimate the risk to a given target organism, system, or (sub-) population, including the identification of attendant uncertainties, following exposure to a pesticide or agent of concern as well as the characteristics of the specific target system. It is the first component in a risk analysis process.

Route of exposure: Means by which a chemical enters an organism after contact (e.g., ingestion, inhalation, or dermal absorption).

Similar mixtures: mixtures having the same chemicals but in slightly different proportions or having most but not all chemicals in common and in highly similar proportions. Similar mixtures are thus expected to have similar fates, transports, and health effects.

Similar mode of action: Describes the mode of action when all chemicals in the mixture act by the same mechanism/mode of action, and differ only in their potencies. The effects of exposure to a mixture of these compounds are assumed to be the sum of the potency-corrected effects of each component (also referred to as dose-addition).

Substances: Chemical elements and compounds, as they occur naturally or by manufacture, including any impurity inevitably resulting from the manufacturing process.

Synergism: Pharmacological or toxicological interaction in which the combined biological effect of two or more substances is greater than expected on the basis of the simple summation of the toxicity of each of the individual substances.

Toxic effect: Effect known (or reasonably expected) to occur in experimental animals/plants and presumably in humans that results from exposure to a chemical substance and that will or can reasonably be expected to endanger or adversely affect the human or environmental health.

Toxic equivalency factor (TEF): Ratio of the toxicity of a chemical to that of another structurally related chemical (or index compound) chosen as a reference.

Annex

Guidance Documents
US EPA
Guidelines for the health assessment of Chemical Mixtures. US EPA, 1986
This document aims to generate a consistent approach for evaluating data on the chronic and subchronic effects of chemical mixtures and to set forth principles and procedures to guide EPA scientists in the conduct of risk assessments, acknowledging that most instances of environmental contamination involve concurrent or sequential exposures to a mixture of compounds that may induce similar or dissimilar effects over exposure periods ranging from short-term to lifetime. In these Guidelines, mixtures are defined as any combination of two or more chemical substances regardless of source or of spatial or temporal proximity; a complex mixtures might be made of related compounds produced as commercial products and eventually released to the environment, or consisting of scores of compounds that are generated simultaneously as byproducts from a single source or process. I might also consists of compounds, often unrelated chemically or commercially, which are placed in the same area for disposal or storage, eventually come into contact with each other, and are released as a mixture to the environment. This report emphasizes broad underlying principles of the various science disciplines (toxicology, pharmacology, statistics…) necessary for assessing health risk from chemical mixture exposure, and that risk assessments will be conducted on a case-by-case basis, giving full consideration to all relevant scientific information. Agency scientists will identify the strengths and weaknesses of each assessment by describing uncertainties, assumptions, and limitations, as well as the scientific basis and rationale for each assessment. The Agency may also be required to take action because of the number of individuals at potential risk or because of the known toxicologic effects of these compounds that have been identified in the mixture The prediction of how specific mixtures of toxicants will interact must be based on an understanding of the mechanisms of such interactions. No single approach to risk assessments for multiple chemical exposures is recommended in these Guidelines, but guidance is given for the use of several approaches depending on the type of mixture, the known toxic effects of the components, and the nature and quality of the available data. Given the complexity of this issue and the relative paucity of empirical data from which sound generalizations can be constructed, emphasis must be placed on flexibility, judgment, and a clear articulation of the assumptions and limitations in any risk assessment that is developed. The basic assumption in the recommended approach is that it is preferable to base the risk assessment on data from the mixtures of concern or similar mixture. If data are available on the mixture of concern, the preferred approach for predicting the effects of subchronic or chronic exposure will be to use subchronic or chronic health effects data and adopt procedures similar to those used for single compounds, either systemic toxicants or carcinogens, although keeping in mind that dose-response models used for single compounds are often based on biological mechanisms of the toxicity of single compounds, and may not be as well justified when applied to the mixture as a whole. Factors such as the persistence of the mixture in the environment, the variability of the mixture composition over time and the possible different rates of degradation in the environment should also be taken into account. If data are available on several mixtures of the same components that have different component ratios which encompass the temporal or spatial differences in composition of the mixture of concern, an attempt should be made to determine if significant differences exist

among the chemical mixtures, and ranges of risk can be estimated based on the toxicologic data of the various mixtures. If no significant differences are noted, then a single risk assessment may be adequate.

If data on the mixture are not available, the risk assessment might be based on data available on similar mixtures: (i.e., a mixture having the same components but in slightly different ratios, or having several common components but lacking one or more components, or having one or more additional components), a decision must be made whether the mixture on which health effects data are available is or is not "sufficiently similar" to the mixture of concern to permit a risk assessment. In determining reasonable similarity, consideration should be given to any information on the components that differ between the mixture on which health effects data are available and the mixture of concern. Particularly on any toxicologic or pharmacokinetic data on the components or the mixtures which would be useful in assessing the significance of any chemical difference between the similar mixture and the mixtures of concern.

In the case of a mixture containing carcinogens and toxicants, an approach based on the mixture data alone may not be sufficiently protective, as in a chronic study on a two-components mixture of one carcinogen and one toxicant, the presence of the toxicant could mask the activity of the carcinogen (i.e at doses sufficient to induce a carcinogenic effect, the toxicant could induce mortality so that no carcinogenic effect could be observed). Consequently, the mixture approach should be modified to allow the risk assessor to evaluate the potential for masking, of one effect by another, on a case-by-case basis.

If the only data available are on mixture components, the risk assessment may be based on the toxic or carcinogenic properties of the components in the mixture. When little or no quantitative information is available on the potential interaction among the components, additive models are recommended for systemic toxicants. Nonetheless, dose additive models are not the most biologically plausible approach if the compounds do not have the same mode of toxicologic action. Consequently, depending on the nature of the risk assessment and the available information on modes of action and patterns of joint action, the Federal Register most reasonable additive model should be used.

For systemic toxicants, the risk assessment methodology used for single compounds most often results in the derivation of an exposure level which is not anticipated to cause significant adverse effects. Depending on the route of exposure, media of concern, and the risk assessments type, these exposure levels may be expressed in a variety of ways such as acceptable daily intakes (ADIs) or reference doses (RfDs), levels associated with various margins of safety (MOS), or acceptable concentrations in various media. For such estimates, the "hazard index" (HI) of a mixture based on the assumption of dose addition may be defined as:

$$HI = E_1/AL_1 + E_2/AL_2 + \ldots + E_i/AL_i \quad (2\text{-}1)$$

where: Ei = exposure level to the i^{th} toxicant* and AL_i = maximum acceptable level for the i^{th} toxicant.

Since the assumption of dose addition is most properly applied to compounds that induce the same effect by similar modes of action, a separate hazard index should be generated for each end point of concern. Dose addition for dissimilar effects does not have strong scientific support, and, if done, should be justified on a case-by-case basis in terms of biological plausibility. The assumption of dose addition is justified when the mechanisms of action of the compounds under consideration are known to be the same. In any event, if a hazard index

is generated the quality of the experimental evidence supporting the assumption of dose addition must be clearly articulated.

The hazard index is only a numerical indication of the nearness to acceptable limits of exposure or the degree to which acceptable exposure levels are exceeded. As this index approaches unity, concern for the potential hazard of the mixture increases, if it exceeds unity, the concern is the same as if an individual chemical exposure exceeded its acceptable level by the same proportion.

If dose-response curves are estimated for systemic toxicants, however, dose-additive or response additive assumptions can be used, with preference given to the most biologically plausible assumption.

- For carcinogens, whenever linearity of the individual dose-response curves has been assumed (usually restricted to low doses), the increase in risk P, caused by exposure d, is related to carcinogenic potency B, as:

$P = d\,B$

For multiple compounds, this equation may be generalized to:

$P = \sum d_i B_i$

This equation assumes independence of action by the several carcinogens and is equivalent tothe assumption of dose addition as well as to response addition with completely negative correlation of tolerance, as long as $P < 1$. An index for n carcinogens can be developed by dividing exposure levels (E) by doses (DR) associated with a set level of risk:

$HI = E_1/DR_1 + E_2/DR_2 + \ldots + E_n/DRn$

The less linear the dose-response curve is, the less appropriate are those equations, perhaps even at low doses, and because of the uncertainties in estimating dose-response relationships for single compounds, and the additional uncertainties in combining the individual estimate to assess response from exposure to mixtures, response rates and hazard indices may have merit in comparing risks but should not be regarded as measures of absolute risk.

None of the above equations incorporates any form of interaction; if data are available that suggest that two or more components in the mixture may interact, such information must be assessed in terms of both its relevance to subchronic or chronic hazard and its suitability for quantitatively altering the risk assessment. If chronic or subchronic toxicity or carcinogenicity studies have been conducted that permit a quantitative estimation of interaction for two chemicals, then it may be necessary to use an interaction ratio (observed response divided by predicted response) to treat the two compounds as a single toxicant with greater or lesser potency than would be predicted from additivity. Other components of the mixture, on which no such interaction data are available, could then be separately treated in an additive manner. However, the use of an interaction ratio is useful only in assessing the magnitude of the toxicant interaction for the specific proportions of the mixture which was used to generate the interaction ratio.

The likelihood that other compounds in the mixture may interfere with the interaction of the two toxicants must also be taken into account. If this is likely, then a quantitative alteration of the risk assessment may not be justified, as in such cases, the risk assessment may only indicate the likely nature of interactions, either synergistic or antagonistic, and not quantify their magnitudes.

For each risk assessment, the uncertainties regarding the composition of the mixtures, health effects, or exposures, should be clearly discussed and the overall quality of the risk assessment should be characterised

Assumptions and limitations are discussed in part 3 of the guidelines, regarding information interaction data, additivity models, and exposure. For example, the use of interaction data

from acute toxicity studies to assess the potential interactions on chronic exposure is highly questionable unless the mechanisms of the interaction on acute exposure were known to apply to low-dose chronic exposure. The use of information from two-component mixtures to assess the interactions in a mixture containing more than two compounds also is questionable from a mechanistic perspective, as the addition of a third compound which either chemically alters or affects the absorption of one of the first two compounds could substantially alter the degree of the toxicologic interaction. There is also some concerns with the use of interaction data on experimental mammals to assess interactions in humans. If systematic differences in toxic sensitivity to single chemicals exist among species, then it seems reasonable that the magnitude of toxicant interactions among species also may vary in a systematic manner. Thus, even if excellent chronic data are available on the magnitude of toxicant interactions in experimental mammal, there is uncertainty that the magnitude of the interaction will be the same in humans.

Finally, the guidelines are formulated in part to bridge gaps in risk assessment methodology and data. By identifying these gaps and the importance of the missing information to the risk assessment process, EPA wishes to encourage research and analysis that will lead to new risk assessment methods and data.

Technical Support document on risk assessment of chemical mixtures (1988) United states environmental Protection Agency. Office of research and development. Washington, DC 20460. EPA/600/8-90/064

This document was recommended by the U.S. EPA's Science Advisory Board as a means of providing the broad technical background for the principles and procedures described in the "Guidelines for Health Risk Assessment of Chemical Mixtures".

Chapter 2 discusses the nature of the available information on three general categories of mixtures: complex mixtures, mixtures composed of a single class of chemicals and simple mixtures. This section intends to illustrate the difference between the types of information that are available on the various categories of mixtures. Emphasis is placed on the description of the tests used to assess the toxicity of the mixtures as well as the available methods and feasibility of these methods for quantitatively measuring interactions of the components in the mixtures. To conclude, this chapter discus on additional topics: interactions of carcinogens with other compounds, some results from the Agency's data base on mixtures and quantitative measures of interactions.

An overview of mechanisms of interaction is presented in chapter 3, which discuss the ways in which compounds may interact: direct chemical-chemical reactions that result in the formation of a different chemical species as well as the biological bases of toxicants interactions such as effects on absorption, distributions, metabolism, excretion and receptor site affinity.

Chapter 4 reviews the mathematical models and statistical procedures used to assess toxic interactions, including dose addition, response addition, generalized linear models, and response surface models. This chapter concludes with a critical review of statistical methods used in research article that are covered in the Agency's mixtures data base.

Chapter 5 reviews and reevaluates the guidelines on mixtures based on the agency's experience in applying these guidelines as well as considerations of new information that has been obtained and new approaches that have been proposed since the guidelines where developed. It separately discusses complex mixtures, similar mixtures and simple mixtures.

For complex mixtures, emphasis remains on in vivo bioassays, the applicability of which can be extended by the concept of sufficient similarity. Recognizing the highly variable nature of some complex mixtures as well as the difficulty and expense of obtaining good in vivo bioassays, the relative potency method, the "toxic equivalency factor" method and analogous methods based on in vitro assays, are more strongly endorsed than in the original guidelines. A limitation of dose addition is also discusses, primarily related to limitations of risk assessment of single compounds.

The report concludes (Chapter 6) with a brief outline of research needed to improve or validate the risk assessment procedures for mixtures. Because the reassessment of the guidelines relies heavily on the use of in vitro tests, emphasis is placed on the validation of such tests using whole animal assays.

The two most significant conclusions in the document are that the available literature is extremely poor for use in quantifying the extent of synergism expected from environmental exposures, and that validation of in vitro and short-term in vivo studies seems to be the most promising approach for assessment of complex mixtures.

Risk Assessment Guidance for Superfund. Vol 1. Human Health Evaluation Manual. Part A. US EPA, 1989

The Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA, or "Superfund), establishes a national program for responding to releases of hazardous substances into the environment. The National Oil and Hazardous Substances Pollution Contingency Plan (NCP) is the regulation that implements CERCLA. NCP establishes, among other things, the overall approach for determining appropriate remedial actions at Superfund sites. To help meet this Superfund mandate, EPA has developed a human health evaluation process as part of its remedial response program. The goal of this process is to provide a basic framework for developing health risk information at Superfund sites necessary to assist decision-making at remedial sites, and also gives specific guidance on appropriate methods and data to use.

Chapter 1 summarizes the human health evaluation process conducted during the remedial investigation and feasibility study (RI/FS). This methodology has been established for characterizing the nature and extent of risks posed by uncontrolled hazardous waste sites and for developing and evaluating remedial options. The three main parts of this process - baseline risk assessment, refinement of preliminary remediation goals, and remedial alternatives risk evaluation - are described in details in part A, B and C of the guidance document respectively.

The human health assessment process is involves in the baseline risk assessment and implies the following step: the project scoping, the site characterisation, and the feasibility study.

The purpose of the **project scoping** is to define the appropriate type and extent of investigation and analysis that should be undertaken for a given site. The main objectives of scoping are to identify the types of decisions that need to be made, to determine the types (including quantity and quality) of data needed, and to design efficient studies to collect these data. Potential site-specific modelling activities should be discussed to ensure that modelling results will supplement the sampling data and effectively support risk assessment activities. Project scoping implies the establishment of a conceptual model of the site to assist in evaluating the possible impacts of releases from the site on human health and the environment, considering in a qualitative manner the sources of contamination, potential pathways of exposure, and potential receptors. This is also the starting point for the risk assessment, as exposure pathways are identified for further investigation and quantification.

During **site characterisation,** the sampling and analysis plan developed during project scoping is implemented and field data are collected and analysed to determine the nature and extent of threats to human health and the environment posed by the site, and to develop a baseline risk assessment. Part of the human health evaluation, this baseline risk assessment is an analysis of the potential adverse health effects (current or future) caused by hazardous substance releases from a site in the absence of any actions to control or mitigate these releases, which results are used to help determine whether additional response action is necessary at the site; modify preliminary remediation goals help support selection of the "no-action" remedial alternative, where appropriate; and document the magnitude of risk at a site, and the primary causes of that risk.

After an initial planning stage, there are four steps in the baseline risk assessment process: data collection and analysis; exposure assessment; toxicity assessment; and risk characterisation.

- **Data collection and evaluation** involves gathering and analysing the data relevant to the human health and identifying the substances present at the site that are the focus of the risk assessment process.
- **An exposure assessment** is conducted to estimate the magnitude of actual and/or potential human exposures, the frequency and duration of these exposures, and the pathways by which humans are potentially exposed. In the exposure assessment, reasonable maximum estimates of exposure are developed for both current and future land-use assumptions. Current exposure estimates are used to determine whether a threat exists based on existing exposure conditions at the site. Future exposure estimates are used to provide decision-makers with an understanding of potential future exposures and threats and include a qualitative estimate of the likelihood of such exposures occurring. Conducting an exposure assessment involves analyzing contaminant releases; identifying exposed populations; identifying all potential pathways of exposure; estimating exposure point concentrations for specific pathways, based both on environmental monitoring data and predictive chemical modelling results; and estimating contaminant intakes for specific pathways. The results of this assessment are pathway-specific intakes for current and future exposures to individual substances.
- **The toxicity assessment** considers: (1) the types of adverse health effects associated with chemical exposures; (2) the relationship between magnitude of exposure and adverse effects; and (3) related uncertainties such as the weight of evidence of a particular chemical's carcinogenicity in humans, and typically relied heavily on existing toxicity information. This toxicity assessment is generally accomplished in two steps: hazard identification and dose-response assessment. Hazard identification aims at determining whether exposure to an agent can cause an increase in the incidence of an adverse health effect; it involves characterizing the nature and strength of the evidence of causation. Dose-response evaluation aims at quantitatively evaluating the toxicity information and characterizing the relationship between the dose of the contaminant administered or received and the incidence of adverse health effects in the exposed population. From this quantitative dose response relationship, toxicity values are derived that can be used to estimate the incidence of adverse

effects occurring in humans at different exposure levels.

- **The risk characterisation** summarizes and combines outputs of the exposure and toxicity assessments to characterise baseline risk. During risk characterisation, chemical-specific toxicity information is compared against both measured contaminant exposure levels and those levels predicted through fate and transport modelling to determine whether current or future levels at or near the site are of potential concern.

In situations where the results of the baseline risk assessment indicate that the site poses little or no threat to human health or the environment and that no further (or limited) action will be necessary, the FS should be scaled-down as appropriate.

The purpose of the **feasibility study** is to provide the decision-maker with an assessment of remedial alternatives, including their relative strengths and weaknesses, and the trade-offs in selecting one alternative over another. The FS process involves developing a reasonable range of alternatives and analyzing these alternatives in detail using nine evaluation criteria. Because the RI and FS are conducted concurrently, this development and analysis of alternatives is an interactive process in which potential alternatives and remediation goals are continually refined as additional information from the RI becomes available.

The next two chapters present additional background material for the human health evaluation process. Chapter 2 discusses statutes, regulations, guidance, and studies relevant to the Superfund human health evaluation, and chapter 3 discusses issues related to planning for the human health evaluation. The remainder of the manual is organized by the three parts of the human health evaluation process: the baseline risk assessment is covered in Part A of the manual (Chapters 4 through 10); refinement of preliminary remediation goals is covered in Part B of the manual; and the risk evaluation of remedial alternatives is covered in Part C of the manual.

Chapters 4 through 8 provide detailed technical guidance for conducting the steps of a baseline risk assessment, and Chapter 9 provides documentation and review guidelines. Chapter 10 contains additional guidance specific to baseline risk assessment for sites contaminated with radionuclides.

Supplementary Guidance for conducting health risk assessment of chemical mixtures. US EPA, 2000

This document has been published as a supplement to the EPA Guidelines for the Health Risk Assessment of Chemical Mixtures of 1986, in which the emphasis is on dose-response and risk characterisation. The principles and concepts put forth in the Guidelines remain in effect, but this document intends to provide more details on these principles and their applications.

The primary purpose of this document is to generate a consistent Agency approach for assessing health risks from exposures to multiple chemicals, denoted by the general term "mixtures." The resulting mixtures risk assessments are intended to assist decision makers by characterizing health risks for the particular exposure conditions of interest. Because exposure scenarios and the available supporting data are highly diverse, this document has been developed as a procedural guide that emphasizes broad underlying principles of the various science disciplines (environmental chemistry, toxicology, pharmacology, statistics) necessary for providing information on the relationship between multichemical exposure and potential health effects. This document addresses only risks to human health from multichemical exposures (ecological effects are beyond its scope), and focuses on procedures for dose-response assessment and risk characterisation

After an overview of the background and scope, the document puts forth the risk assessment paradigm for mixtures, considering problem formulation, hazard identification, dose-response assessment, exposure, and risk characterisation. It suggests that a chemical mixture risk assessment should begin with an assessment of available data quality, which would then leads to the selection of a risk assessment method. The major concerns are whether the available data are on components or whole mixtures, whether the data are composed of either similar components or similar mixtures that can be thought of as acting by similar toxicologic processes, and whether the data may be grouped by emissions source, chemical structure, or biologic activity.

It describes detailed procedures for chemical mixture assessment using data on the mixture of concern, data on a toxicologically similar mixture, and data on the mixture component chemicals. The state of the science varies dramatically for these three approaches. The whole-mixture procedures are most advanced for assessing carcinogenic risk, mainly because of the long use of in vitro mutagenicity tests to indicate carcinogenic potency. In vitro test procedures for noncancer endpoints are still in the pioneering stage. In contrast, the component-based procedures, particularly those that incorporate information on toxicologic interactions, are most advanced for noncarcinogenic toxicity.

New guidance is provided that gives more specific details on the nature of the desired information and the procedures to use in analysing the data. Among these are methods for using whole-mixture data on a toxicologically similar mixture, such as the comparative potency methods; methods for incorporating information on toxicological interactions to modify a Hazard Index (HI) into an interaction-based hazard index; and generalized procedures for mixtures involving classes of similar chemicals.

The comparative potency method is based on the use of a set of mixtures of highly similar composition to estimate a scaling factor that relates toxic potency between two different assays of the same toxic endpoint. The mixture of concern can then be tested in one of the assays, and the resulting potency is then adjusted by the scaling factor to estimate the human cancer potency. The comparative potency method involves extrapolation across mixtures and across assays. It is restricted to a set of different assays that monitor the same, single type of health effect, and to different mixtures that are considered toxicologically similar. The basic assumption is that the curves of dose response for the assays are the same shape and that the relationship between any two mixtures will be the same, whichever assay is used. The guidance give an example of this methodology applied to the estimation of human cancer unit risk from exposure to polycyclic organic matter (POM).

The interaction-based hazard index is based on the key assumptions that interactions in a mixture can be adequately represented as departures from dose addition, and that influence of all the toxicologic interactions in the mixture can be adequately approximated by some function of the pairwise interactions. Toxicologic interactions have been mostly studied with binary mixtures. The assumption is made that higher order interactions are relatively minor compared to binary interactions; one way to include interactions in a mixture assessment is then to modify the noninteractive assessment by knowledge of these binary interactions. Thus, this interaction-based HI evaluates binary mixtures and introduces a classification that indicates the expected direction of interaction (synergistic or antagonistic) by using an alphanumerical scoring system. The scores are then combined with the hazard index. Thus, each term is modified according to the influence (interaction) of the other components, and then these modified terms are summed. The full formula for the interaction-based HIINT is the following:

$$HI_{INT} = \sum_{i=1}^{n}(HQ_i * \sum_{j \neq i}^{n} f_{ij} \quad M_{ij}^{B_{ij}\theta_{ij}})$$

where:
HI_{INT} = HI modified by binary interactions data,
HQi = hazard quotient for chemical i (unitless, e.g., daily intake/RfD),
fij = toxic hazard of the j^{th} chemical relative to the total hazard from all chemicals potentially interacting with chemical i (thus j cannot equal i),
Mij = interaction magnitude, the influence of chemical j on the toxicity of chemical i,
Bij = score for the strength of evidence that chemical j will influence the toxicity of chemical i, and
θij = degree to which chemicals i and j are present in equitoxic amounts.

In this report, there are also expanded discussions of the concerns when using only whole-mixture data as well as when using only data on the individual chemical components; moreover no single approach is recommended, but guidance is given for the use of several approaches depending on the nature and quality of the data.

The appendices contain definitions, a discussion on toxicologic interactions and pharmacokinetic models. Method-specific user fact sheets for quantitative risk assessment, which are intended to provide a concise overview of each currently available method, are given. These fact sheets provide the following information relative to the risk assessment approach:

- Type of Assessment
- Data Requirements
- Section(s)
- References
- Strategy of Method
- Ease of Use
- Assumptions
- Limitations
- Uncertainties

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