Scientific Committee on Health and Environmental Risks

SCHER

Scientific Committee on Emerging and Newly Identified Health Risks

SCENIHR

Scientific Committee on Consumer Safety

SCCS

Toxicity and Assessment of Chemical Mixtures

The SCHER approved this opinion at its 15th plenary of 22 November 2011

The SCENIHR approved this opinion at its 16th plenary of 30 November 2011

The SCCS approved this opinion at its 14th plenary of 14 December 2011

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: 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) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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SCHER

Opinions on risks related to pollutants in the environmental media and other biological and physical factors or changing physical conditions which may have a negative impact on health and the environment, for example in relation to air quality, waters, waste and soils, as well as on life cycle environmental assessment. It shall also address health and safety issues related to the toxicity and ecotoxicity of biocides.

It may also address questions relating to examination of the toxicity and ecotoxicity of chemical, biochemical and biological compounds whose use may have harmful consequences for human health and the environment. In addition, the Committee will address questions relating to the methodological aspect of the assessment of health and environmental risks of chemicals, including mixtures of chemicals, as necessary for providing sound and consistent advice in its own areas of competence as well as in order to contribute to the relevant issues in close cooperation with other European agencies.

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This Committee deals with questions related to emerging or newly identified health and environmental risks and on broad, complex or multidisciplinary issues requiring a comprehensive assessment of risks to consumer safety or public health and related issues not covered by other Community risk assessment bodies. Examples of potential areas of activity include potential risks associated with interaction of risk factors, synergic effects, cumulative effects, antimicrobial resistance, new technologies such as

nanotechnologies, medical devices including those incorporating substances of animal and/or human origin, tissue engineering, blood products, fertility reduction, cancer of endocrine organs, physical hazards such as noise and electromagnetic fields (from mobile phones, transmitters and electronically controlled home environments), and methodologies for assessing new risks. It may also be invited to address risks related to public health determinants and non-transmissible diseases.

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ABSTRACT

The EU Chemicals legislation is based predominantly on assessments carried out on individual substances. Since humans and their environments are exposed to a wide variety of substances, there is increasing concern in the general public about the potential adverse effects of the interactions between those substances when present simultaneously in a mixture. Based on their analysis of the available scientific literature, the non-food Scientific Committees of the European Commission reached the following conclusions:

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

2. Chemicals with common modes of action will act jointly to produce combination effects that are larger than the effects of each mixture component applied singly. These effects can be described by dose/concentration addition.

3. For chemicals with different modes of action (independently acting), no robust evidence is available that exposure to a mixture of such substances is of health or environmental concern if the individual chemicals are present at or below their zero-effect levels.

4. Interactions (including antagonism, potentiation, and synergies) 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 are 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 allow a focus on mixtures of potential concern is necessary. Several criteria for such screening are offered.

6. With regard to the assessment of chemical mixtures, a major knowledge gap at the present time is the lack of exposure information and the rather limited number of chemicals for which there is sufficient information on their mode of action. Currently, there is neither an agreed inventory of mode of actions, nor a defined set of criteria how to characterise or predict a mode of action for data-poor chemicals.

7. If no mode of action information is available, the dose/concentration addition method should be preferred over the independent action approach. Prediction of possible interaction requires expert judgement and hence needs to be considered on a case-by-case basis.

Based upon these conclusions, a decision tree for evaluating the risk of chemical mixtures is proposed.

Keywords: SCHER, SCCS, SCENIHR, scientific opinion, toxicity, risk assessment, mixtures, chemicals, combination effects

Opinion to be cited as:

SCHER, SCCS, SCENIHR, Opinion on the Toxicity and Assessment of Chemical Mixtures, 2012.

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1. BACKGROUND

The EU Chemicals legislation, in common with the situation in other parts of the world, is based predominantly on assessments carried out on individual substances. However, in reality humans are exposed to a wide variety of chemicals throughout their lives as indeed are animals and plants. While current assessment methods incorporate safety factors to take account of a range of uncertainties, the Commission is concerned with ensuring that EU Chemicals' legislation takes proper account of the latest scientific information on mixture toxicity.

In the Council conclusions from 22nd December 2009, the Commission was invited, drawing on existing and future research and paying appropriate attention to the costs and benefits, 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.

2. TERMS OF REFERENCE

In the light of the above considerations, SCHER/SCCS/SCENIHR are asked to advise the Commission on the following issues related to chemical mixtures[1]:

1) Is there scientific evidence that when organisms are exposed to a number of different chemical substances, that these substances may act jointly in a way (addition, antagonism, potentiation, synergies, etc.) that affects the overall level of toxicity?

2) If different chemical substances to which man/environment are exposed can be expected to act jointly in a way which affects their impact/toxicity on/for man and the environment, do the current assessment methods take proper account of these joint actions?

3) Several approaches for the assessment of the mixture effects of chemicals already exist such as dose addition and independent action. What are the advantages and disadvantages of the different approaches and is there any particular model that could be considered as sufficiently robust to be used as a default option?

4) Given that it is unrealistic to assess every possible combination of chemical substances what is the most effective way to target resources on those combinations of chemicals that constitute the highest risk for man and the environment?

5) Where are the major knowledge gaps with regard to the assessment of the toxicity of chemical mixtures?

6) Does current knowledge constitute a sufficiently solid foundation upon which to address the toxicity of chemical mixtures in a more systematic way in the context of EU legislations?

In developing its opinion on the questions set out above the Committee is requested to take account of the latest scientific information and to consult with prominent experts and with relevant agencies such as EFSA, EEA, EMA and ECHA as well as experts and organisations outside the EU.

[1] For the purposes of this request, mixtures of chemicals are considered to be:

- Substances that are mixtures themselves (multi-constituent substances, MCS; materials of unknown or variable composition, complex reaction products or biological materials, UVCB)
- Products that contain more than one chemical e.g. cosmetics, plant protection products;
- Chemicals jointly emitted from production sites, during transport processes and consumption or recycling processes;
- Several chemicals that might occur together in environmental media (water, soil, air), food items, biota and humans as a result of emission from various sources and via multiple pathways.

The Commission services would in particular refer the Committee to the final report of study contract 070307/2007/485103/ETU/D.1 "State of the Art of Mixture Toxicity".

In addition, the EFSA Panel on Plant Protection Products has produced a number of highly relevant opinions on cumulative and synergistic risks from pesticides[2].

3. OPINION[3]

3.1 Problem formulation

Humans and ecosystems are continually exposed to a very complex mixture of chemicals the composition of which is always changing. However, in the great majority of risk assessments only a single chemical is considered and there are no generally applicable guidelines as to when assessment of combinations of chemicals should be carried out.

There are many research publications on various aspects of mixtures. These generally fall into two categories:

i) Investigations of the combined effects of a few pure chemicals that, based on their chemical and/or biological properties, might be expected to show an enhanced effect.
ii) Testing of "real world" complex mixtures in various biological systems, e.g. diesel exhaust fumes, tobacco smoke.

In view of recent publications dealing with risk assessment methodologies for chemical mixtures, i.e. combinations of two or more chemicals that retain their own chemical identity in the mixture, it has become necessary to evaluate whether new EU guidelines should be developed for the assessment of chemical mixtures and the regulatory framework be strengthened. Such an evaluation would need to particularly take account of potential mixture effects at realistic exposure levels in the environment, the possible influence of background exposure in the environment or diet, and of health risks at low-dose exposures to multiple chemicals.

3.2 Scope of the opinion

This opinion does not specifically address drug-drug interactions for human health assessment because they are within the remit of the European Medicines Agency (EMA). Pharmaceuticals have been considered for the environmental assessment and the indirect exposure of the general population. However, this opinion does not address essential metals and nutrients for human health. Furthermore, it does not address metals for environmental combination assessment because these assessments require the use of specific approaches, e.g. essentiality, background concentrations, bioavailability.

[2] Scientific Opinion of the Panel on Plant Protection Products and their residues (PPR Panel) on a request from the EFSA to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to human health with a view to set maximum residue levels (MRLs) for those pesticides in the frame of Regulation (EC) 396/2005. The EFSA Journal 2008; 704:1-85.
EFSA PPR Panel Scientific Opinion on a request from EFSA on risk assessment for a selected group of pesticides from the triazole group to test possible methodologies to assess cumulative effects from exposure through food from these pesticides on human health. The EFSA Journal 2009; 7: 1167.

[3] In the absence of internationally harmonised terminology for the assessment of chemical mixtures, a definition of terms as used in this opinion is provided in the glossary annexed to this document.

3.3 General principles of mixture toxicology

More than 50 years ago, three basic types of action for combinations of chemicals were defined (Bliss 1939, Loewe and Muischnek 1926, Plackett and Hewlett 1948, Plackett and Hewlett 1952):

- Similar action (dose/concentration addition);
- Dissimilar action (independent action), and
- Interactions.

Quantifiable responses: independent action and dose/concentration addition

For mixtures of similarly acting chemicals the effects can be estimated directly from the sum of the doses/concentrations, scaled for relative toxicity (dose/concentration addition).

For mixtures of independently acting chemicals the effects can be estimated directly from the probability of responses to the individual components (response addition) or the sum of biological responses (effects addition).

Both concepts (independent action and dose/concentration addition) are based on the assumption that chemicals in a mixture do not influence each other's toxicity, i.e. they do not interact with each other at the biological target site. Such chemicals can either elicit similar responses by a common or similar mode of action, or they act independently and may have different endpoints and/or different target organs.

Both concepts have been suggested as default approaches in regulatory risk assessment of chemical mixtures. In reality, however, chemical mixtures are rarely composed of either only similarly or of only dissimilarly acting substances. A brief overview of these two concepts is presented below, and a more detailed description can be found in the review by Kortenkamp *et al*. (2009).

Dose/concentration addition (similar action, similar joint action) occurs if chemicals in a mixture act by the same mechanism/mode of action, and differ only in their potencies. Different methods exist for the dose/concentration approach, which mainly differ in the required knowledge about mode of actions and toxicological similarities of the mixture components (for details see Methodology section 3.4 of this document). In principle, doses or concentrations of the single components are added after being multiplied by a scaling factor that accounts for differences in the potency of the individual substances.

The mixture dose/concentration (D_{mix}) is the sum of the adjusted doses/concentrations (aD_i) of the individual components D_i:

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

The effect of a mixture of similarly acting compounds is equivalent to the effects of the sum of the potency-corrected (adjusted) doses/concentrations of each compound.

Dose additivity is assumed over the entire dose range, including doses/concentrations below the individual no observed adverse effect levels/concentrations (NOAEL/Cs) of the mixture components.

It is noted that the dose-additivity approach relies on a correct grouping of "similar" chemicals. Although guidance on grouping of chemicals has been issued (ECHA, OECD, EFSA), there is currently no general agreement on the scientifically best approach and grouping of chemicals is most often done by expert judgement on a case-by-case basis.

As reviewed by Kortenkamp et al. (2009), there is evidence that dose/concentration addition can "…produce reliable estimates of combined effects, if the components share either a strictly identical molecular mechanism of action [...] or belong to the group of so-called baseline toxicants". Faust and Altenburger published two studies on the chronic algal toxicities of binary mixtures (Altenburger et al. 1996, Faust et al. 1994). Altenburger studied 137 binary mixtures of different pesticides and surfactants with the result that concentration addition provided the better overall prediction for the observed toxicity data than the independent action model. A similar result was obtained by Faust who concluded that the toxicity of 66% of the 38 binary pesticide mixtures tested was predictable by concentration addition, although all the test mixtures were composed of a herbicide and an insecticide or fungicide.

Feron and Groten (2002) concluded in their review on mixture toxicity that dose addition is indeed appropriate for risk assessment of a mixture of chemicals with simple similar action. The addition of doses implies that toxicity can be expected if the summed dose is high enough to exceed the threshold of toxicity of the mixture, even when the dose level of each individual chemical is below its own effect threshold. For example, in a 4-week toxicity study, rats were exposed to a combination of four different but similarly acting nephrotoxicants (tetrachoroethylene, trichloroethylene, hexachloro-1:3-butadiene and 1,1,2-trichloro-3,3,3-trifluoropropene). Kidney effects of the mixture were seen at dose levels not showing renal toxicity of the individual compounds. Thus, the study provided support for the assumption of dose additivity for mixtures of similarly acting systemic toxicants under conditions of concurrent, repeated exposure at dose levels below the toxicity thresholds of the individual constituents.

A dose-additive approach was, also, used by Wolansky *et al.* (2009) who showed that sub-threshold doses of individual pyrethroids, when combined in a mixture, produced measurable neurotoxicity in rats.

With regard to carcinogenicity, a few studies are available on dioxins that generally demonstrate the concept of dose additivity when using Toxic Equivalency Factors (TEFs) adjusted dose and tumours as the endpoint (Walker *et al.*, 2005).

Oestrogens may form another group of substances that act by the same mode of action and there are indeed some *in vitro* studies available which demonstrate dose-additivity if the individual compounds act through the same receptor (ERα or ERβ) to produce either inhibitory or stimulatory effects (Charles *et al.* 2002, Payne *et al.* 2001). These findings are also supported by *in vivo* studies (Jobling *et al.* 2009).

Kortenkamp *et al.* (2009) reviewed the literature for deviations from expected additivity and found that - in human and mammalian toxicology studies - such deviations "were observed quite rarely" (section 4.9 in Kortenkamp *et al.*, 2009).

In this context, Kortenkamp *et al.* (2009) cite a study by Nesnow *et al.* (1998) and summarise the study as follows: "Nesnow *et al.* (Nesnow *et al.* 1998) analysed mixture effects of five polycyclic aromatic hydrocarbons on lung tumours in A/J mice, with mixture ratios representative of ambient air levels of these carcinogens. At low doses, greater than additive effects were seen. At high doses the observed responses fell short of additivity expectations which were derived from independent action in a response surface analysis. However, the observed deviations were rather small". The original paper by Nesnow *et al.* (1998) however states that "A comparison of the additive responses derived from the constrained model (no interaction parameters) indicated close agreement with the additive responses obtained from the sum of the individual PAH dose responses".

As a further example of a deviation in response from expected dose additivity, Kortenkamp *et al.* (2009) report a study by Walker *et al.* (2005) in which three dioxin-

like compounds were administered to rats singly or as a mixture. The conclusion by Kortenkamp *et al.* (2009) was based on an unpublished re-analysis conducted by Kortenkamp (unpublished). Details of this re-analysis are not available, except for the fact that "…the concept of dose-addition was applied directly without utilising WHO TEF values". Note that an analysis of the data produced by the study authors themselves (Walker *et al.*, 2005) and using the WHO TEF values supported the dose-additivity concept.

A few studies indicated antagonisms in the joint effects of estrogenic agents (Charles *et al.* 2007, Rajapakse *et al.* 2004), but these deviations were rather small. Similarly, the study by Hass *et al.* (2007) on the feminizing effects of androgen receptor antagonists on male offspring of dams dosed during gestation indicated a weak synergism with respect to induction of nipple retention. Similar deviations from additivity were not observed with other endpoints of evaluation that were used in the same study.

Tichy *et al.* (2002) observed deviations from concentration additivity for a mixture of benzene and ethanol in a short-term assay with *Tubifex*. However, the observed EC50 only deviated by a factor of 1.5 from the predicted EC50. Since the authors did not calculate the prediction according to independent action for the mixture, it remains unclear whether the combined effect is better described by independent action.

Independent action (response addition, effects addition) occurs if chemicals act independently from each other, usually through different modes of action that do not influence each other. This type of action is also referred to as simple dissimilar action. Response addition refers to the sum of probabilistic risks. Effects addition means the sum of biological responses.

The toxicity of a mixture in terms of the probability of an individual being affected can be expressed as:

$p_M = 1 - (1-p_1)\,(1-p_2)\,(1-p_3)\ldots\,(1-p_n)$

with p_M being the response to the mixture and p_1, p_2, …, p_n being the responses due to exposure to the individual components C_1, C_2, … C_n when present in a specified concentration.

This equation can also be written as:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(C_i))$$

With $E(C_{mix})$ being the combined effect at the mixture concentration (C_{mix}), and $E(C_i)$ being the effect of the individual mixture component (i) applied at the concentration (c_i). Effects are expressed as fractions of a maximum possible effect ($0\% \leq E \leq 100\%$).

According to the above equation, any substance for which $E(C_i)$ is equal to zero does not contribute to the joint effect of the mixture. Consequently, mixtures of independently acting chemicals pose no health concern, as long as the doses/concentrations of each individual component remain below their individual zero-effect levels (concentrations). It is important to note that NO(A)ELs and NO(A)ECs derived from experimental studies do not always represent zero-effect levels. The NOAEL(C)s and NOECs estimated in toxicity and ecotoxicity studies, respectively, are often associated with effect levels in the range of 5 to 20% and hence no "zero-effect levels" (EFSA 2009a, Kortenkamp *et al*. 2009). It cannot be assumed that in all cases $E(C_i)$ is equal to zero for exposures at the NOAEL(C) or NOEC of a particular study. As the NOAEL(C) or NOEC do not necessarily represent a value for which $E(C_i) = 0$, exposures equal to these levels may contribute to mixture effects also for dissimilarly acting substances.

Although Kortenkamp *et al.* (2009) report that significant joint effects of dissimilarly acting toxicants at or below individual NOAEL(C)s were found in four studies, the Scientific Committees conclude that such interpretation is not warranted and is of the opinion that these studies simply confirm that, as expressed above, the NOAEL(C) does not necessarily represent a value for which $E(C_i) = 0$, and therefore exposures at the NOAEL(C) level may contribute to mixture effects also for dissimilarly acting substances.

Details on the four studies cited by Kortenkamp *et al.* (2009) are given below. The studies include a study with fish (Hermens *et al.* 1985), two studies with algae (Faust *et al.* 2003, Walter *et al.* 2002), and one study using human breast cells (Payne *et al.* 2001).

Based on four studies, Kortenkamp *et al.* (2009) conclude: "In demonstrating that dissimilarly acting chemicals too have the propensity to produce significant mixture effects when combined at levels below NOECs, these studies contradict received expert opinion and falsify the hypothesis we set out to examine". The Working Group has evaluated these studies and concludes that they do not allow such interpretation:

Hermens *et al.* (1985) exposed guppies to a mixture of 11 non-reactive, non-ionised organic chemicals, 11 chloroanilines and 11 chlorophenols, all well known aquatic pollutants with presumably different modes of action, at concentrations of 4% of the individual LC50 values. These concentrations were assumed to be below the individual NOECs; however, the NOECs were not determined in this study. The joint acute toxicity of the mixture was almost completely concentration additive. As NOECs were not estimated in this study and toxicity data taken from previous studies, one of which (Hermens *et al.*, 1984) stated that the toxicity of the chemicals was partly calculated, it is possible that, as also concluded by Kortenkamp *et al.* (2009) - some chemicals may have been present at levels above their NOECs and hence may have contributed to mixture effects. The Working Group notes that acute toxicity effects at doses close to the NOECs are not suitable for evaluating low dose effects.

aust et al. (2003) tested a mixture of 16 different biocides, which specifically interact with different target sites in algae, for inhibition of reproduction in algae. When these chemicals were combined at concentrations equivalent to 6.6-66% of their NOECs, a significant, combined effect of 18% was observed. Significant joint effects were also demonstrated when the chemicals were combined in concentrations below individual NOEC values that were statistically estimated to elicit insignificant individual effects of only 1%. Nevertheless, the assumption of independent action yielded accurate predictions, irrespective of the mixture ratio or the effect level, whereas the alternative concept of concentration addition overestimated the joint toxicity.

In a similar approach taken by Walter et al. (2002), the effects on algal reproduction of a mixture of 11 structurally dissimilar aquatic pollutants with mostly unknown modes of action were assessed. Statistical estimates of effect concentrations lower than the corresponding NOECs were derived by regression analysis of concentration response data, down to effect levels of 1%. When combined at individual NOECs a joint effect of 64% was produced. The study results allowed two conclusions: (i) the combined effect on reproduction was higher than that of any individual compound; and (ii) the magnitude of this effect was more precisely predicted by the model of independent action than by concentration addition.

The studies by Faust et al. (2003) and Walter et al. (2002) hence confirm the concept of non-dose additivity by concluding that for multi-component mixtures of substances with strictly different specific mechanisms of action the assumption of

independent action proves to be superior.

Payne et al. (2001) assessed combinations of *o,p'*-DDT, *p,p'*–DDE, beta-HCH and *p,p'*-DDT in the induction of cell proliferation in MCF-7 cells. All four compounds induce cell proliferation in oestrogen-dependent breast cancer cells either as receptor agonists or independent of oestrogen receptor mediated pathways. Although the concentration-response plots showed marked differences in shape and position, the combined effect could be predicted on the basis of the concentration-response relationships of the single compounds. The combination effects were stronger than those of the most potent compound so the combined effects may be called additive or synergistic. In this study, a common endpoint was investigated. Evidence that the compounds trigger cell proliferation via two different mechanisms does not however allow the conclusion that agents with diverse modes of action produce a combination effect.

The evaluation of the four studies by the Working Group showed that the Kortenkamp document over-interprets the outcome of these studies. The Working Group does not consider the studies indicative of a deviation from the commonly accepted criteria of independent action and dose/concentration addition, at low (environmentally relevant, human exposure relevant) doses.

In summary, the re-analysis of the four studies supports the conclusion that at relevant human exposures the compounds acting by independent action are very unlikely to produce a biologically relevant increase in response.

Interactions: synergism and antagonism (mechanistic considerations)

Interaction describes the combined effect of two or more chemicals as stronger (synergistic, potentiating, supra-additive) or weaker (antagonistic, inhibitive, sub-additive, infra-additive) than would be expected on the basis of dose/concentration addition or response addition. Interactions may therefore vary according to the relative dose levels, the route(s), timing and duration of exposure (including the biological persistence of the mixture components), and the biological target(s).

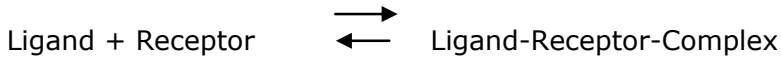
Examples for interactions include:
- Toxicokinetic interactions; these are a common cause of deviations from additivity. Examples are chemicals modifying the absorption of others (e.g., skin penetration enhancing substances in cosmetics) or chemicals competing for active transport mechanisms (uptake, clearance);
- Metabolic interactions: chemicals modifying the metabolism of other mixture components;
- Toxicodynamic interactions: interactions between the biological responses resulting from exposure to the individual chemicals, for example resulting from similar targets (e.g., ligand-receptor interaction)

Kortenkamp *et al.* (2009) focussed on the combination effects of substances interfering with the endocrine system, in particular oestrogen-receptor binding compounds and their potential to synergise in combination. Background information on competitive interactions with the receptor is therefore presented in the following box:

Receptors are components of an organism which bind molecules of diverse chemical structures. These molecules are ligands that activate or inhibit the receptor function and thereby elicit a physiological response. Ligands that activate a response are agonists; those that block the response are antagonists. Receptors are rather large proteins located at specific sites on or within cells, at which chemicals (agonists) react to produce responses.

Classes of receptors are various hormone- and neurotransmitter-receptors. The specific binding of a ligand at its receptor is a prerequisite for its action and triggers a cascade of events.

The receptor-ligand interaction follows the law of mass action and its kinetics are similar to the Michaelis Menten equilibrium except that the products of the Michaelis Menten type of interaction are metabolites whereas interactions of the agonist at the receptor usually do not result in a change of chemical structure of the agonist. Interaction of a ligand with a receptor is described by:



Replacement of a physiological ligand, *i.e.* an oestrogen from the receptor by a competitor, i.e. a xenoestrogen, depends on its relative affinity to the receptor and its concentration. For example, replacement of the physiological ligand from the receptor by a compound of 1000-fold lower affinity requires a 1,000-fold higher concentration than the endogenous compound. Although this oversimplifies competitive interaction of compounds at a receptor, it demonstrates the need for information on the relative binding affinities of the compounds in question and their concentration in the organism.

In 1999 the Scientific Committee on Toxicology, Ecotoxicology and the Environment (CSTEE 1999) compared the potency of xenoestrogen concentrations detected in human blood as a surrogate for concentrations at the receptor with the potency of oestradiol concentrations in blood. Data on the oestrogen activities have been taken from experiments with the following test systems: competitive binding to oestrogen receptors of MCF-7 cells, proliferation of MCF-7 human breast cancer cells (E-SCREEN) or expression of a reporter gene in the yeast oestrogen system (YES). Based on the EC_{50} values (effective dose: the lowest reported concentration inducing 50% of the maximum estrogenic activity *in vitro*) the relative potencies of *o,p'*-DDE, PCBs, nonylphenol, and dieldrin as compared to those of oestradiol are about a million-fold lower than that of estradiol. As presented in the NTP-CERHR Expert Panel Report on Bisphenol A (2007) concentrations in the blood of German, US and Japanese pregnant women average between 0.43 and 4.4 Bisphenol A µg/L with individual concentrations between 0.2 and 18.9 µ/L. The relative potencies of the average values are about 570 to 5,800 fold below that of oestradiol. The highest value of 18.9 µg/L is still about 125 times lower than that of oestradiol. From this it was concluded that an interaction of the compounds at the receptor with physiological consequences is unlikely.

It has to be noted that endocrine disruption is a very general term used for different mechanisms or modes of action, also involving different receptors. The only commonality of these mechanisms is that they may produce adverse effects on reproduction, development, growth or other functions regulated by hormonal activities. It is therefore necessary to differentiate between the mechanisms or modes of action of endocrine disrupters, including their specific receptor interactions before such substances can be grouped into assessment classes.

Conditions under which synergism may be expected

An example of a synergistic action is the combination of a chemical which causes a mutation with one that induces proliferation in the carcinogenic process. This represents the classical initiation–promotion model. Chemicals that interfere with cell cycle regulation, increase the permeability of skin/mucosa or alter the boactivation/detoxication equilibrium might synergise with classical carcinogens.

A potential for a toxicologically significant synergistic effect should be considered under the following conditions:
- Can one or more components significantly enhance the uptake of other components?
- Can one or more components inhibit significantly the excretion/clearance of other components?
- Do one or more of the components exert their toxic action via the formation of an active metabolite(s) and moght one or more of the components induce the drug metabolising enzymes that may be involved in the formation of these active metabolites?
- Can two or more components act on different enzymes in an important metabolic pathway?
- Can two or more components act on different elements of cellular protection mechanisms or cellular repair mechanisms?

The Panel on Plant Protection products and their Residues (PPR) of the European Food Safety Authority (EFSA) concluded from a limited review of the available literature that significant toxic interactions between chemicals are "…much less likely to occur at doses below the effect levels for individual component compounds than at higher doses." (EFSA 2008). The SCHER opinion on indoor air quality (SCHER 2007) cites Cassee *et al.* (1998) and states: "Interactive effects giving rise to possible health concern have been reported, starting from concentrations around the LOAEL".

An example for synergism starting around the effect levels of the individual compounds can be found in the publication by Christiansen *et al.* (2009). Christiansen *et al.* (2009) showed that four antiandrogens, combined at their estimated NOAELs for nipple retention, did not provoke appreciably elevated external sex organ malformation rates. The 5-fold higher mixture dose, however, induced malformations in approximately 25% of the males. At the levels present in the 10-fold higher mixture dose, three of the single chemicals alone did not induce discernable effects, but vinclozolin on its own caused malformations in about 5% of the affected males. Yet, when combined at the doses equivalent to 10-fold NOAELs, malformations were found in nearly all male offspring.

Examples for low-dose synergy were reported by Boobis *et al.* (2011). Of the 90 relevant toxicity studies found by an extensive literature search, only six provided "useful quantitative estimates of synergy". For these six studies, the magnitude of synergy at low doses was within a factor of 4 of the levels predicted by additive models.

Two of these six studies were performed by Moser and co-workers using a mixture of five organophosphate pesticides (Moser *et al.* 2005, Moser *et al.* 2006). The administration of this mixture containing the individual components in a ratio reflecting the relative dietary exposure estimates of the general population to adult and pre-weanling rats resulted in a greater than additive response (synergism) at the lower doses of the mixture corresponding to non-effective dose levels of each of the components. The predicted effective doses (ED_{20}, ED_{50}) for different endpoints, among which blood and brain cholinesterase and behaviour pattern, were about half that predicted by additivity. Detoxification factors and kinetic interaction were suggested as the major cause for the observed synergy.

The only example for low-dose synergism, also reported in Kortenkamp et al. (2009), is a study on rats by Crofton et al. (2005) using 18 thyroid-disrupting chemicals (two dioxins, four furans, 12 PCBs) in a ratio that reflected that found in human breast milk and food. The exposure at the highest mixture dose was at or below the no observed effect levels (NOELs) of the individual components for serum thyroxin concentration. Dilutions ranged to 100-fold lower levels. No evidence of synergy or antagonism was found at the lower doses. At the three highest doses the measured effects were under-predicted by the dose additivity models, i.e. there was a 2.5-fold synergy.

The fourth study was a study on rats assessing EC_{50} rotarod performance after acute inhalation of toluene and xylene. Synergy was found at doses >1,000 ppm (Korsak et al. 1988). No synergy was however found by the same group in a subsequent subchronic study with lower exposures (Korsak et al. 1992). Synergy between the organochlorine pesticide mirex and the phorbol ester TPA in producing skin tumours in DMBA-initiated mice was reported in a study by Meyer et al. (1994). The last of the six studies found was an epidemiology study investigating arsenic levels in drinking water in combination with cigarette smoke (Chen et al. 2004).

An example for the relevance of the sequence of exposure and its impact on the toxicity, very likely due to kinetic interaction, is reported by Kacham et al. (2006), Karanth et al. (2001), and Karanth et al. (2004) following exposure of rats to the organophosphorus compounds chlorpyrifos and parathion.

With regard to ecotoxicity, it was shown that mixtures of organophosphates and carbamates produced dose-additive or synergistic AChE inhibition in fish *in vivo* (Laetz *et al.*, 2008*)*, indicating interactions and the importance of mechanistic information when grouping chemicals.

The relevance of the approaches at population and community level

The three types of action described above are applicable to the responses of individual organisms exposed to combinations of chemicals.

However, in ecotoxicology, the objective is not the protection of individuals but the protection of higher hierarchical levels (population, community). Many standard ecotoxicological test procedures are specifically intended to produce information at the level of population dynamics (e.g. algal growth test, *Daphnia* fertility test). Even short-term acute toxicity tests are interpreted, in ecotoxicology, as community tests. A given level of mortality in an acute test in toxicology represents the probability that an individual would be affected. On the contrary, in ecotoxicology, it represents the fraction of the population that would be affected.

On the other hand, one must be aware that the effects of a toxic chemical on population dynamics may be assumed as the result of the effects on individuals. The measured effects in a *Daphnia* reproduction test are the consequence of the reduction of fertility of the tested individuals.

It follows that the general principles of mixture toxicology are applicable in ecotoxicology for predicting effects at population level. However, the concepts of "independent action", "dose/concentration additions" and "synergistic action" should be understood at the population (in addition to the individual and sub-individual) level.

Different considerations must be made for the effects at community level that depend on the complex interactions among different populations and can hardly be predicted only on the basis of single species tests.

At the community level, an additional concept of "synergism" is also possible, considering the combined effects of different chemicals on different taxonomic groups and the

indirect consequences on the structure and functioning of the community. An example could be the effect of the combination of a herbicide and an insecticide, acting independently, if the toxicological point of view is considered, but producing combined effects at the ecological level interacting on the prey-predator relationships.

In this opinion, only the ecotoxicological effects at the population level will be taken into account. The assessment of the complex consequences at the community level cannot be performed using only toxicology-based approaches. It requires ecologically-based approaches accounting for indirect ecological effects and ecological interactions. These problems and the possibility for considering them in risk assessment procedures are matter for another Working Group established within DG SANCO (SCENIHR/SCCS/SCHER Working Group on New Challenges for Risk Assessment).

3.4 Methodology

Risk assessments in the EU deal mainly with individual substances with the exception of "complex substances" falling under the REACH regulation, pesticide and biocidal formulations, and cosmetic products. . At EU level there is currently no generally accepted approach for the methodology to conduct a risk assessment for chemical mixtures and a case-by-case approach is followed depending on the mixture under review. Guidance for conducting cumulative risk assessments has been published by the Environmental Protection Agency of the USA (USEPA 2002), the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT 2002), the Norwegian Scientific Committee for Food Safety (VKM 2008), and the German CVUA (CVUA 2007).

A framework for the risk assessment of combined exposures to multiple chemicals has been proposed by the WHO/IPCS (WHO 2009b, figure 1). General support for this framework and associated terminology was expressed at an OECD-WHO-ILSI-HESI Workshop in 2011 (OECD 2011).

Application of the framework for consideration of risk from exposure to multiple chemicals is an iterative process, involving stepwise consideration of both exposure and hazard in several tiers of increasingly data-informed analyses. The approach involves decision-based analysis which takes into account relevant information at an early stage as a basis to scope additional assessment and recommend any required data generation. Early consideration of potential for exposure (prior to any consideration of hazard potential) is essential in determining critical next steps. At this stage the first estimate of the combined exposure could be compared to the Threshold of Toxicological Concern (TTC). The extent of assessment and nature of recommendations for generation of additional data are dependent upon the extent of the knowledge base, the magnitude of public health concern (*i.e.* taking into account margins between exposure and effect) and the needs of the risk assessment. It is envisaged then, that approaches will range from predictive methodologies and conservative assumptions in early tiers to more realistic estimates of risk and rigorous descriptions of uncertainties in later tiers, based on increasingly data-informed and probabilistic approaches.

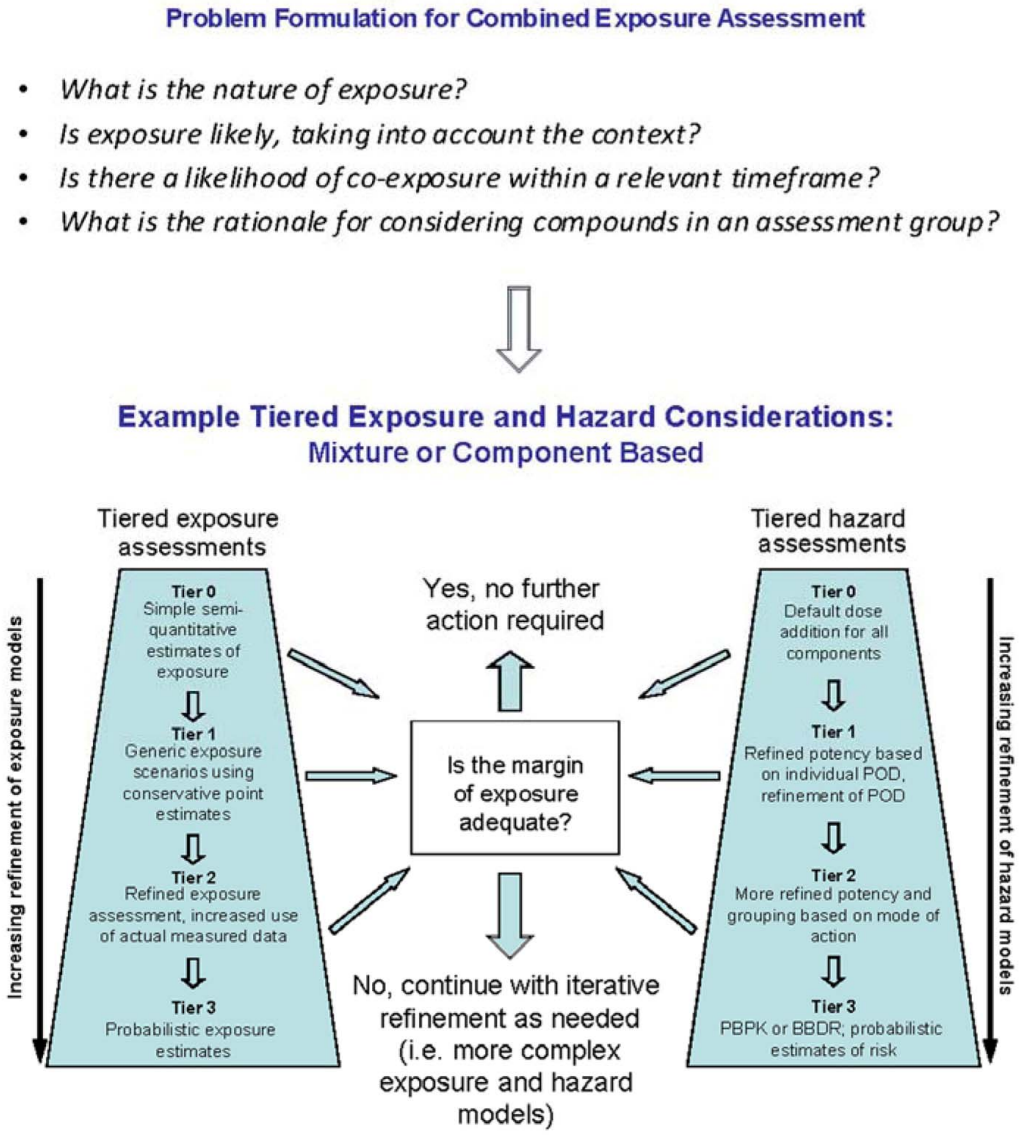


Figure 1. A conceptual representation of the framework.
(Reprinted with permission from Meek *et al.* 2011)

In this framework, the point of departure (POD) is a selected measure of effect. It may be a no observed (adverse) effect level or a lowest observed (adverse) effect level (NO(A)EL or LO(A)EL) or a dose or concentration associated with a specified increase in the incidence of an effect (*e.g,* a benchmark dose [BMD_{10}] or concentration [BMC_{10}] associated with a 10% increase in incidence of an effect). Mode of action (MOA) is a biologically plausible sequence of key events leading to an observed effect, supported by robust experimental observations and mechanistic data[4]. The margin of exposure (MOE) is the ratio of the selected measure of effect to the estimated exposure dose or concentration. In considering the elements of the framework, it is important to understand that the tiers are provided principally as examples. They are not fixed and will vary, depending on available data. There may also be additional iterations of the tiers; for some compounds, for example, earlier iterations of Tier 0 may be sufficient. Mixtures containing components with different modes of action require separate analyses for each. As one moves toward a more rigorous analysis, greater support is obtained for

[4] Where there is reason to believe that compounds would act on the same physiological system, such as the androgen system, it might also be appropriate to consider these as sharing a mode of action (EFSA,2008)

the nature of the combined effect, dose addition, greater or less than dose-additive effects. Also, the preliminary grouping of chemicals into a common group becomes additionally refined in later tiers, often leading to some substances being dropped and or sub-grouped.

A general approach for mixtures directly manufactured as such and falling under the "substance" definition under REACH (the legal definition for "substance" includes simple (multi-constituent substances, MCS) and complex reaction products or biological materials (substances of unknown or variable composition, UVCB) is the testing of hazard and fate properties of the "complex substance", i.e. the mixture itself. The REACH guidance however also allows the use of other methodological approaches for predicting the overall risk based on information on the individual components.

Under the current EU pesticides and biocides regulation, for example, both the formulations of plant protection and biocidal products are generally assessed for acute toxicity, skin and eye irritation, and skin sensitisation. Other endpoints and other toxicological aspects may be assessed based on the individual substances present in the formulations. A cumulative risk assessment will be required in the approval process, referring to exposure to multiple residues.

3.4.1 Effect assessment

In principle, the hazard of chemical mixtures can be assessed as a whole, after fractionation or based on the individual components of the mixture.

Whole-mixture approaches

If toxicity data on the mixture itself are available, a quantitative assessment can be carried out directly from these data. An assessment may also be based on data generated with a mixture of reasonably similar composition or a "surrogate mixture", i.e., a mixture close in composition (components and proportions) to the mixture under evaluation.

Whole-mixture approaches have the advantage of accounting for any unidentified materials in the mixture and for any interactions among mixture components (Boobis *et al.* 2011). They have been used for poorly characterised but stable mixtures, for effluent toxicity assessments and for specially designed mixtures. Testing whole mixtures does however not provide specific information on interactions or the toxicity of individual mixture components.

If a mixture cannot be assessed in its entirety, it may be possible to separate fractions (e.g., mixtures of petroleum hydrocarbons into aliphatic fractions of certain chain length ranges and aromatic fractions) and to assess the toxicities of the fractions. This approach has, for instance, been used for diesel exhaust (gaseous fractions and particulate matter fraction).

A major limitation of the whole-mixture approach is that its applicability is restricted to mixtures that do not significantly change in their composition; the Working Group therefore does not recommend using this as a general approach.

Component based approaches

If the components of a mixture are known, a component-based approach is usually performed. Information on the mode of action should be used to assess the type of combined action (independent action, similar action) applicable. The optimal approach for a component-based risk assessment of chemical mixtures is therefore dependent on:

- Knowledge of the modes or mechanisms of action of the individual components, including dose-response information; or
- Information regarding their association with groups of chemicals demonstrating similar or identical modes of action (assessment groups). Such information may be based on chemical structure and structure-activity relationships (SARs), either qualitatively or quantitatively, molecular modelling, structural alerts or on toxicological responses or effects.

In the absence of sufficient information on the mode of action of the mixture components, the independent action concept is often used as a default in human toxicology mixture assessments; whereas it appears that a concentration addition approach is more often used as default in environmental risk assessments. Both concepts include a general assumption that interactions either do not occur at all or are small enough to be insignificant to the risk estimate.

To study the toxicity of individual mixture components, full or fractional factorial designs are used (for review see, *e.g.*, Greim and Snyder 2008).

Grouping of mixture components based on structural similarities

Assessment groups can be formed by grouping mixture components and/or their metabolites based on structural similarities.

If (eco)toxicological data are lacking on the individual components of a mixture and on the mixture as a whole, a (Q)SAR-based approach could be used as a first approach. The chemicals in a mixture can be grouped into assessment groups or "blocks" on the basis of their chemical structure using tools such as the OECD (Q)SAR Application Toolbox (OECD 2009). For each group (this can also be an individual chemical) a representative limit value needs to be derived. This value can be based on the limit value of a representative substance in a group derived by read-across or by the application of QSARs or a TTC-approach.

In the former case, a quantitative read-across, the value of a particular parameter for tested analogue(s) is used to estimate the toxicity for the untested chemical with the assumption that the potency of the effect of interest is shared by both the tested and untested analogue. Quantitative read-across works best for homologous series of chemicals where the metric needed to extrapolate from one substance to another can be linked to a particular property of the category (van Leeuwen et al. 2009).

The use of QSARs for the derivation of human limit values for more complex endpoints such as repeated dose toxicity and reproductive toxicity is not advanced sufficiently (Cronin et al. 2003a, Cronin et al. 2003b). A recent JRC-review revealed considerable differences in the availability of models depending on the endpoint. At one extreme, there is a huge amount of literature and a range of software tools for predicting genotoxicity and carcinogenicity, and at the other extreme, there are few or no models for organ and system-specific toxicities. In many cases, promising models were identified but they are still at the research stage. It was concluded that for routine application in a regulatory setting further efforts will be needed to explore the applicability of such models for specific purposes, and to implement them in a practically useful form (JRC 2010).

QSARs for the derivation of ecotoxicological limit values based on short-term (algae, *Daphnia* and fish) and long-term (*Daphnia* and fish) predictions of ecotoxicity are available (e.g. ECOSAR) and find more general acceptance than those for human health (Cronin et al. 2003a, Cronin et al. 2003b), although once again their validity and the available tools have to be explored further (Hulzebos and Posthumus 2003).

Grouping by toxicological or biological responses / effects

Hazard information is used to identify and group chemicals that have similar endpoints and a common toxic effect, including dose descriptors for critical effects such as benchmark doses, LOAELs or NOAELs.

When considering which chemicals to assess together, the possibility of metabolic interaction should also be addressed.

Relative potency factors (RPFs) and/or a point of departure index can be calculated. However, the selection of index compounds may require an intensive search of the toxicological database and expert judgement. The advantage of this approach is that for many chemicals such information is available. However, there is currently no definition of "a common endpoint", which may refer to identical target organs, identical cell types affected, identical pathology or identical biological/biochemical responses.

Dose/concentration addition approaches

Methods for dose/concentration addition approaches most frequently used are the hazard index (HI), the reference point index (RfPI, also known as point of departure index (PODI), the relative potency factor (RPF), or the toxic equivalency factor (TEF). These methods are briefly discussed below. A more detailed review can be found in Boobis et al. (2008). A further approach is the toxic unit model, often used in environmental toxicology.

A. Hazard index and adjusted hazard index

The hazard index (HI) is the sum of the hazard quotients (HQ), i.e. the ratios between exposure and the reference value (RV) for each component to be evaluated. When the RV of a certain compound is based on an effect that is not the group effect (common toxic effect), or the applied assessment factor includes adjustments not related to the endpoint of concern, then the HQ can be refined by identifying the RV for the group effect and adjusting the hazard quotient, accordingly. In this situation an adjusted HI (aHI) is then calculated. When the HI is less than 1, the combined risk is considered acceptable; values higher than 1 would indicate potential health concern to be considered. The reciprocal of the HQ can also be used; the cumulative risk index is the reciprocal of the sum of the HQs.

The HI has the advantage of relating directly to a RV, which is a long-used and well-understood, transparent index of acceptable risk, and can be (relatively) fast and simply applied when individual RVs are readily available. It can accommodate the application of chemical-specific adjustment factors (CSAFs) earlier in the process. However, RVs are obtained by application of an uncertainty factor (UF) that may incorporate policy (e.g., default extra UF for children or severity of effect) and scientific (e.g., on the quality of the database that might not be directly related to the relevant toxic effect) judgments. As such, it does not necessarily represent a true measure of the relative toxicological potency of the different compounds.

When extensive mechanistic information is not available, the HI is the preferred approach.

When interaction data are available, these can be incorporated in the HI approach, by converting the available information into a numerical score on an expert judgement basis or a weight of evidence (WoE) evaluation, according to appropriate tables elaborated by the US EPA. The score takes into account the nature of the interaction (synergism or antagonism), the quality of available data, the plausibility of the interaction at actual exposure conditions and the relevance for human health. The interaction-based HI can be calculated as follows:

$HI_{int} = HI \cdot UF^{WoE}$

The WoE value is negative for antagonistic interactions and positive for synergisms; and UF is an uncertainty factor whose default value is 10. An additional factor (M) (obtained as the ratio between the observed effective dose (ED) and the ED predicted from dose addition approach) can be also introduced, to include a quantitative evaluation of the interaction.

The limitations of this approach are: i) HI_{int} provides only a numerical score of the potential risk related to a chemical mixture exposure; ii) HI and HI_{int} are strongly affected by a subjective evaluation; and iii) the intrinsic uncertainties affecting RVs are combined and amplified in HI_{int} derivation.

B. Reference Point index

The reference point index (RfPI) differs from the HI because it represents the sum of the exposures to each chemical component expressed as a fraction of their respective RfPs (also known as point of departure) for the relevant effect (e.g. the dose that causes a 10% effect, BMD_{10}; or the NOAEL). When the RfPI multiplied by the chosen group uncertainty factor (UF) is lower than 1, the combined risk is considered acceptable.

The reciprocal of the RfPI is the combined margin of exposure (MOET), where the individual margin of exposure (MOE) is the ratio of the RfP to the level of exposure in humans (measured or estimated). MOET is calculated as the reciprocal of the sum of the reciprocals of the individual MOEs. If the MOET is greater than 100 or another alternative value specified for the MOE by the risk manager, the combined risk is considered acceptable.

The RfPI has the advantage that it sums the exposures to the different components in relation to their relative potencies, expressed as the RfP. A single group UF can be applied as the last step in the process, or alternatively, chemical specific adjustment factors (CSAFs) can be applied earlier in the process, if needed.

C. Relative potency factor methods/toxic equivalency factor/potency equivalency factor

To assess the effects of a mixture of individual substances Si (i=1,2,…n), a substance has to be defined as the index compound (S_{ind}) in order to calculate the component (and exposure route specific) relative potency factors (RPF):

$RPF_1 = TS_1 / TS_{ind}$, where TS_1 is the toxicity of the individual substance (S_1) and TS_{ind} is the toxicity of the index compound (S_{ind}).

The dose (concentration) is then adjusted: $aD_1 = D_1 \times RPF$, and the mixture dose (D_{mix}) is calculated from the sum of the adjusted doses:

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

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

The RPF method is transparent, and easy to understand, particularly because it separates potency correction from exposure considerations. Thus, it provides a better basis for standardising toxic dose metrics for the various chemicals. However, it should be noted that determination of the risk posed by the combined exposure places great emphasis on the quality of the toxicology database of the index compound. The index compound

would normally be chosen among the compounds with a toxicological database that provides the lowest uncertainty.

A special case of the RPF method (USEPA 2002) is the toxic equivalency factor (TEF) method, which was initially developed for dioxins and other aryl hydrocarbon receptor (AhR) agonists (Haws et al. 2006, van den Berg et al. 2006). The potency equivalency factor (PEF) is, like the RPF, a more general method that has been used for compounds such as polycyclic aromatic hydrocarbons and certain pesticides.

D. The toxic unit model

The model of toxic units (TUs) is frequently used in ecotoxicology. It represents the ratio between the concentration of a component in a mixture and its toxicological acute (e.g. LC_{50}) or chronic (e.g. long-term NOEC) endpoint. The toxic unit of a mixture (TUm) is the sum of TUs of individual chemicals. The TU model does not refer to the ecosystem, but only to a specific organism representative of a group of organisms ecologically or taxonomically relevant for the ecosystem (e.g. algae, *Daphnia*, and fish, for the freshwater ecosystem).

The TU concept can be used to quantify the toxicity of a mixture (assuming the dose/concentration addition principle) on the basis of its composition, i.e. an acute lethal TUm = 10 means than a dilution of 10% of the mixture would produce 50% of lethality. If the slope of the concentration/effect curve is known, the TUm can be used to estimate the expected effect.

In addition, when the TU model is applied to environmental concentrations (predicted PECs, or measured, MECs) it is conceptually comparable to HQ (and TUm to HI) with the difference that it refers to a toxicological endpoint and not to a reference value (RV) derived by extrapolation (e.g. application factors) from the endpoints. The RV in ecotoxicology is the PNEC, so the sum of PEC/PNEC ratios could be assumed as comparable to HI. It must be considered that a PNEC is derived by applying an AF to toxicological endpoints obtained for the most sensitive organism that may be different for different chemicals. Therefore PEC/PNEC for a component of a complex mixture may be non-homogeneous. However, this approach is known to be slightly more conservative than the summation of TUs (which would be the scientifically more correct approach). Hence, for practical reasons, the sum of PEC/PNECs could be used as a first-tier when applying concentration addition.

<u>Independent action approaches</u>

Independent action (response addition, effects addition) occurs where the modes 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. With the exception of non-threshold effects, no health risk is anticipated as long as the various exposure concentrations do not exceed respective zero- effect levels.

If the effect measure is an incidence (or likelihood) and the mixture components act independently but have similar health effects (for example, different types of cancer), the combined effect can be calculated through response addition.

For the ecological assessment, the reference value (i.e. the PNEC) is set at the population/community level, and does not exclude effects on individuals, even if the information used for deriving it is usually obtained through toxicological tests at individual/population level. The population/community response is the outcome of the aggregated response of each individual. The "aggregation" of the individual effects largely depends on the biology of each species and their ecological role within the community, which may vary among ecosystems. 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 effects 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.

Higher-tier assessments

Physiologically-based modelling may be useful for a higher-tier assessment. PBTK modelling can provide an estimate of the concentration of the compound at the target site for a toxicological effect. Such models may also help in extrapolating experimental data from high-to-low-dose real life situations, and from route-to-route. Haddad and co-workers (2000) used a PBTK modelling method for the extrapolation of two-component-mixture data to a BTEX mixture (benzene/toluene/ethylbenzene/m-xylene).

A physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PBPD) model was, for instance, developed for the organophosphorus insecticides chlorpyrifos and diazinon. It was anticipated that at low environmentally relevant doses the pharmacokinetics would be linear, and the cholinesterase inhibition dose-additive (Timchalk and Poet 2008). Evaluation and use of PBPK/PD models, if available and appropriate, has been suggested by the ATSDR in their guidance document for the assessment of joint toxic action of chemical mixtures (ATSDR 2004).

Physiologically-based approaches provide a highly refined methodology. However, they are resource intensive and demand special expertise, and are therefore unlikely to be routinely used in the near future.

Epidemiological studies in cumulative risk assessment

A framework for inclusion of epidemiological evidence into cumulative risk assessment has been proposed by Levy (2008), who states that "Although the lack of sufficient human evidence often necessitates reliance on toxicological evidence, it is noteworthy that epidemiology received little mention in any of the cumulative risk assessment guidance or applications to date. The Framework for Cumulative Risk Assessment (1) provides no guidance for the use of epidemiology and only describes it as an "area of complexity". On this basis Levy (2008) proposes several criteria for inclusion of epidemiological evidence into cumulative risk assessment: "An epidemiological study maximally informative for cumulative risk assessment would have the following attributes:

(1) It provides quantitative dose-response relationships within the exposure range of interest for all key stressors, with consideration of interactions or other joint effects. The stressors considered are related to risk management concerns and hypothesized to contribute to a specified disease process and health outcome.

(2) It explicitly and quantitatively addresses all relevant dimensions of vulnerability, potentially including differential exposure, susceptibility/sensitivity, preparedness to withstand the insult of the stressor, and ability to recover from the effects of the stressor.

(3) It is based on a population similar in vulnerability and exposure characteristics to population of interest, or at least includes all relevant subpopulations across these dimensions with adequate stratified analyses".

These criteria will almost never be fulfilled. However, they provide a basis for the development of a framework allowing the best use of epidemiological data, as all key stressors will never be known, nor all factors that may make individuals or populations more sensitive to one of the exposures under consideration.

Exposure measurements in epidemiological studies have greatly improved over the past decade, and many studies are looking into the effects of combined exposures. However, it is a challenge to get cumulative exposure estimates, but methods have been developed to do this e.g. in air pollution epidemiology.

Much of the available epidemiological evidence is derived from the occupational setting, where the exposure is much higher than the exposure for the general population, which has to be taken into account when generalizing to the general public.

For example, air pollution exists as a complex mixture that varies spatially and temporally, which complicates an analysis of an interactive effect using epidemiological studies. Furthermore, in many cases there is a co-variation of the different pollutants depending on the source of emission, and the pollutant level will also depend on climatic conditions. In addition, as these pollutants exert their adverse health effects by different mechanisms, these effects may not be expressed simultaneously.

An epidemiological method to study interactions should be based upon sufficiently comparable populations; measurement of potential co-exposures as a test of synergy requires measurement of the effects of each component and the combined compounds under identical conditions (Mauderly and Samet 2009). Synergism is then assessed by statistical methods using multivariable models.

There are several epidemiological studies reporting a synergism of interaction of chemical factors, e.g. alcohol and smoking on certain cancer endpoints, smoking and asbestos in development of lung cancer. However, it has to be noted that the exposure in these studies was in the area of effect levels for the individual substances.

Vainio and Boffetta (1994) reviewed several epidemiological studies on the interaction of asbestos and tobacco smoke and found that the magnitude of interaction was not uniform, but that the interaction was clearest in studies of workers exposed to high levels of asbestos. A recent analysis using a Bayesian approach to assess evidence of an interaction between asbestos exposure and tobacco indicated that the relationship is closer to multiplicative than additive (Wraith and Mengersen 2007).

In the absence of adequate epidemiological data, the effects of chemical mixtures on human health are based on experimental studies. However, their qualitative and quantitative transposition from the laboratory animal data to human populations can be addressed using science-based safety factors.

Specific aspects relating to ecological effect assessments

Even if the general concepts of mixture toxicity (CA, IA, synergism, etc) may be assumed to be the same for man and for the environment, one must be aware that there are substantial conceptual differences between toxicology and ecotoxicology, which may also affect the possible application of the CA and IA approaches.

The most important difference is the objective of the protection. The goal of human toxicology is the protection of individuals. On the contrary, the goal of ecotoxicology is protecting structure and functions of biological communities and ecosystems. The death of individuals is accepted in the frame of natural selection processes. A huge number of individuals must die before attaining the reproductive age (particularly in "r strategic" populations) in order to modify a steady state of the population dimension and of the community structure. The death of part of the population reduces competition and increases the probability of survival for the remaining individuals.

It follows that relevant endpoints may be different in human toxicology and in ecotoxicology. Ecotoxicological endpoints are relatively broad and are related to ecologically-relevant parameters such as massive mortality, reduction of fertility and any other effect affecting reproductive capability. Some effects which are extremely important for individuals, but produce a moderate effect on population dynamics (e.g. carcinogenicity) are of negligible relevance in ecotoxicology. Therefore, precise endpoints

that in human toxicology are often referred to a specific target organ are meaningless in ecotoxicology.

Moreover, in ecotoxicology, knowledge of the toxicological mode of action on all the different types of organisms that may be present in an ecosystem is largely incomplete. Even for chemicals developed with the objective of a specific activity (e.g. pesticides) the toxicological mode of action is well known for target organisms but not for the non-target ones. Pesticides exert their effect on a particular physiological or metabolic function that, usually, is not common to all living organisms present in a biological community (photosynthesis inhibitors, AChe inhibitors, etc.). Therefore, for non-target organisms, taxonomically far from the target ones, the effect of the chemical is likely to be of the narcotic-type (baseline toxicity). Examples are given in figure 2, where the relationship between algal toxicity and octanol-water partition coefficient (Kow) is reported for some compounds belonging to different chemical groups with specific or non-specific toxic effects on algae. It appears from the figure that chemicals with specific toxic effect on animals (organophosphate and chlorinated insecticides) behave on algae as "baseline", narcotic-type, toxicants, while the toxicity of triazines, specific photosynthesis inhibitors, is orders of magnitude higher. Ankley *et al*. (2010) provides a useful IPCS-like mode of action framework for grouping substances in ecological risk assessments.

Therefore, the concept of "common mode of action" may have a different meaning in ecotoxicology in comparison with human toxicology, and should be referred to broader end-points, such as reproduction impairment, population growth, mortality, etc.

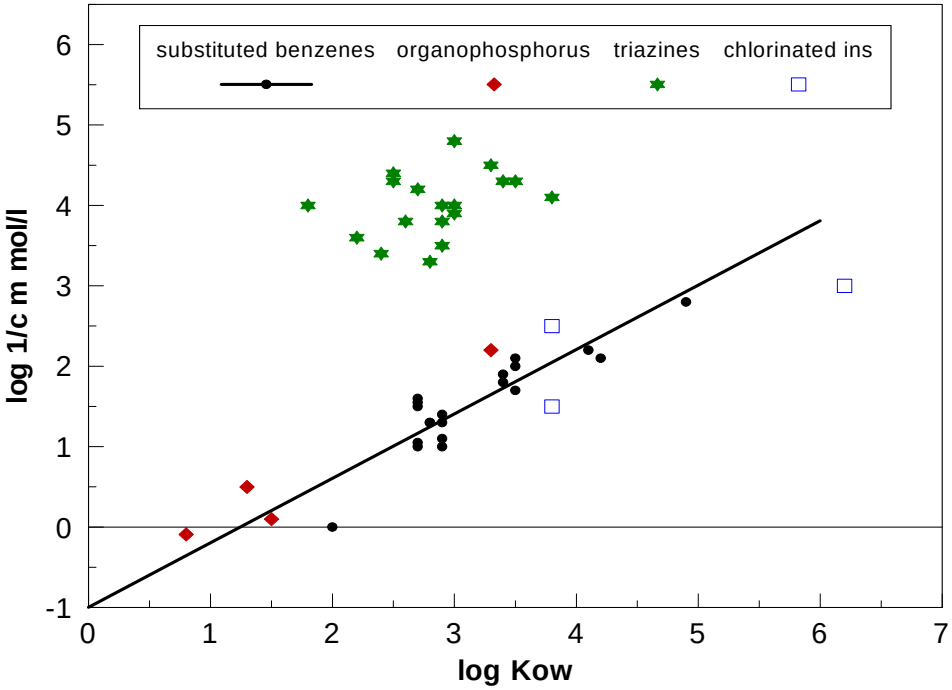


Figure 2 – Relationship between toxicity on algae (Log 1/EC50) and Log Kow for chemicals with a specific (triazines) and a non-specific (all others) toxicological mode of action on algae (Vighi 2006).

The assessment of effects of mixtures is particularly relevant and complex at low or even very low dose/concentration exposures. Each single organism, including humans, is exposed via the environment to a huge number of different substances, generally at low or very low levels. Therefore, any assessment should start with the identification of the relevant components to be assessed. For substances with specific mechanisms of actions, the sensitivity among tested species can differ by several orders of magnitude. As a

consequence, the relevant components to be selected for the mixture assessment may differ for each species as well as with time. The general concepts of mixture toxicity (CA and IA) at levels close to the no effect level (NEL) are applicable to individuals/species, but difficult to implement when moving to population/community effects.

3.4.2 Exposure assessment

Human Health

Exposure to mixtures can be from specific chemical products, or from particular sources, e.g. food, water, air. Also, aggregated and cumulative exposure from all sources to multiple chemicals may need to be considered. Cumulative exposure assessment will, for example, be required under EU pesticide regulation,

Exposure assessments with regard to chemical mixtures are generally complicated, and must often rely on simplifications and assumptions. Recently, a framework for the assessment of combined exposures to multiple chemicals has been suggested by the WHO/IPCS (WHO 2009a, WHO 2009b), "involving stepwise consideration of both exposure and hazard in several tiers of increasingly data-informed analyses" (Gomes and Meek 2009; see also introduction to Methodology section 3.4).

An exposure assessment may be relatively simple in occupational settings for which direct measurements and various models for estimating the exposure exist (see, for example, REACH guidance documents). However, this is much more complex for the general public, in which exposure may occur via multiple pathways, routes, and media (aggregate exposure).

Furthermore, the chemical composition of a mixture is often, at least in part, unknown and the levels of particular components in a mixed exposure may vary with time and environmental conditions. Assessments of the exposure to a mixture generally use relevant available data, such as emissions data, measurement of the components (or a lead component) in environmental media, and biomarker information. Fate and transport of the mixture components in the environment, routes of exposure and pharmacokinetics of components once in the body may all be considered in the exposure assessment.

In cases where measured data (which are the preferred exposure data) are not available or if such data are too limited, it is necessary to rely on assumptions and to use modelling to provide relevant exposure estimates.

For a "worst case" estimate it may be necessary to assume maximum exposure to each component of the mixture based on the assessment of daily exposure from all sources. In practice, however, information to support such an assessment is rarely available.

Even more complicated is an exposure estimate with regard to the target tissues or organs of toxic substances ("internal dose"). This parameter is influenced by alterations of toxicokinetic processes as a possible consequence of mixture component interactions. As environmental concentrations are typically highly variable over time, the tissue concentrations they produce will also vary over time.

Human biomonitoring data and biomonitoring equivalents may be used to estimate internal or absorbed doses from all exposure routes.

Environment

Environmental exposure is the result of complex patterns depending upon widespread emissions and point releases on one hand, and the fate and the distribution of chemicals in the different compartments on the other. Water, sediment, air, soil and biota (food)

are the main environmental compartments; the latter only for chemicals with bioaccumulation and biomagnification potential. Exposure is usually expressed as concentration instead of dose, particularly in the aquatic environment. The environment is almost never exposed to simple (i.e. well known) mixtures, but their compositions change with time and must be estimated through transport and persistence patterns.

a. Chemicals used as technical mixtures
Some typical examples are commercial industrial mixtures and formulations of plant protection products, biocidal, cosmetics or pharmaceutical products that often contain several active ingredients with different chemical structures and environmental behaviour. Other examples are industrial technical mixtures of congeneric chemicals such as PCBs and PBDEs. It should be noted that multi-constituent substances and substances of variable or unknown composition are treated as substances under regulatory procedures but in reality are mixtures.

b. Chemicals emitted by a human activity
A mixture may be the result of the emission from a specific human activity, such as complex wastes from an industrial typology or production process, combination of pesticides applied on an agricultural crop, etc.
These kinds of emissions may be considered as mixtures of given composition, likely to be characterised, at least on a local basis, in qualitative and quantitative terms, although the composition may significantly change with time.

c. Chemicals likely to be present in the environment as the result of multiple emissions
An environmental system may be exposed to complex mixtures of chemicals resulting from the combination of all the emissions of human activities in a given territory (e.g. a mixture present in a river as a result of the emissions in the hydrographic basin). These kinds of mixtures may be assessed at different scale levels: local, regional, global.

Even for substances emitted/released simultaneously, the environmental fate (distribution and persistence) may be different for any individual component of the mixture. Therefore, the composition of the mixture in the environment may be completely different from those of the originally emitted mixture and highly variable in space (particularly in different environmental compartments) and in time.

The exposure and subsequent risk assessments are much more complex as small differences in the behaviour of each component may significantly affect the overall risk. Due to environmental fate differences, significant differences may be expected between the mixture in the industrial environment, the mixture which is released and the mixture to which organisms are exposed. Except for closely related components, the exposure assessment may require information on the individual components, although there are some approaches for using fractionation methods (e.g. Gutiérrez *et al.* 2008).

Difficulties in carrying out an environmental risk assessment for mixtures arise because the individual components have specific and different physico-chemical properties, (eco)toxicological properties, and potentials for being degraded in the environment. Each component will be subjected to different distribution and fate processes on release to the environment. Each component will behave independently and reach its own concentration in each environmental compartment. Therefore, a PEC for the whole mixture could be determined if the individual composition is known. However, since several components of a mixture of similar structure will have similar physico-chemical properties and environmental-degradation potentials, they will have similar distributions and fates within a given environment. It is therefore possible to group or "block" such groups, so that components having similar properties may be considered together (it should be noted that a "block" may consist of a single component or a large number of components with similar fate and distribution properties). Once the blocks for a substance have been established,

PEC values can be calculated for each block for each environmental compartment (King et al. 1996).

Blocks will primarily be defined on the basis of those physico-chemical and degradation properties that are key in determining the distribution and fate of their components. Care should be taken to ensure that blocks are not so wide as to include components without broadly similar fates and distributions after release. Similarly, blocks should, whenever possible, contain substances with a similar mode of action and a narrow range of toxicity. Both the fate and toxicity criteria for block definition need to be satisfied simultaneously. Identification of blocks when applying this approach will frequently be dependent on the use of QSARs for the estimation of physico-chemical properties (e.g. log *Kow*, water solubility, melting point and vapour pressure) and degradation rates (e.g. photodegradation and hydrolysis rates) when measured values are not available. There are reasonably well accepted methods for the generation of these data using readily available databases or QSARs. There are no widely accepted QSARs for biodegradation but it is considered adequate, at least for screening, if experimentally determined rate constants for the blocks of interest are not available, to use QSAR estimates for block identification according the principles laid down in Chapter R.6 of the REACH Guidance (ECHA, 2008).

Distribution in different environmental compartments can be predicted by modelling. The predictions are generally made for individual substances and mixture composition may be obtained through their combination (Finizio et al. 2005, Verro et al. 2009). It is expected that, at concentrations present in the environment, distribution of each component of a mixture is not influenced by the physico-chemical properties of the other components.

The biodegradation of each component of a mixture can be strongly influenced by the presence of other components. Biodegradation is one of the major processes leading to the disappearance of chemicals from aquatic and terrestrial environments. Biodegradability of chemicals is generally assessed on single compounds. Since interactions could be expected to be related to biodegradation rather than to chemical/physical patterns, focus is given to biodegradation processes. However, there are situations that need to be addressed differently. For example, predicting degradation cannot be based on single compound kinetics, as shown by the literature. Desai *et al.* (2008) have demonstrated that this assumption would likely overestimate the rate of disappearance of PAHs. This is the case when the chemicals are competing as substrates for the same microorganisms. However, in other cases, co-metabolism allows degrading complex mixtures. For example, Heath *et al.* (2006) showed that a commercial polychlorinated alkane mixture was appreciably dehalogenated in shake flasks only when 1,10-dichlorodecane was present as a co-substrate.

Enzyme induction also plays a major role in the biodegradation of mixtures of chemicals. Haigler et al. (1992) designed experiments to determine the conditions required for induction of the individual pathways and to establish whether multiple substrates could be biodegraded simultaneously. Their results indicated that induction of appropriate biodegradative pathways in *Pseudomonas* sp. strain JS150 permits the biodegradation of complex mixtures of aromatic compounds.

A number of efforts to develop mathematical models for mixed substrate kinetics have been made. A full review of the literature should be made to prepare a state-of-the-art on mixtures biodegradation modelling. However, it has been shown that simple models published accounting only for one of the mechanisms cited above lead to poor prediction of mixtures biodegradation [Dimitriou-Christidis and Autenrieth (2007), Knightes and Peters (2006), Reardon et al. (2002)].

Mixture composition can be assessed by applying different approaches as a function of the objective of the assessment, in particular for regulatory purposes. For hazardous chemical control (e.g. REACH) the mixture exposure can be estimated as the result of a

given process that would produce a specific emission into a generic environment assumed as being representative of the European, more or less, realistic conditions and using default values. On the contrary, for the EU Water Framework Directive (WFD), the quality assessment of mixtures is the result of site specific measurements and the conditions that would produce effects on the real environment that must be taken into account case-by-case, for each individual water body.

Some examples of methods and tools for assessing ecosystem exposure to mixtures and its variability in space and time are reported in the literature (see for example Verro *et al.* 2009). It is worth noting that all the available experimental evidence indicates that in the mixtures realistically occurring in the environment, the number of individual chemicals covering the largest part of the toxic potency of the mixture is very low. Usually, not more than three to four chemicals cover more than 90% of the total TUs of a mixture (Finizio *et al*. 2005, Junghans *et al.* 2006, Verro *et al*. 2009). This finding is also supported by Price and Han (2011) who introduced maximum cumulative ratio (MCR), defined as the ratio between the toxicity of the mixture and the toxicity of the most toxic chemical in the mixture (using the TU model, MCR=TUm/Largest individual TU; 1<MCR<n, where n is the number of chemicals in the mixture). It has been empirically demonstrated that MCR is inversely correlated with the toxic potency of the mixture: very dangerous mixtures are driven by very few chemicals (Price and Han, 2011). This is very important for practical reasons, because for mixtures driven by few individual chemicals, the difference between the predictions based on concentration addition or on independent action is small or, sometimes, negligible (Altenburger *et al.* 2004).

However, it must be pointed out that the comparison between CA and IA approaches at low levels of chemicals cannot be based on the assumption that low concentrations (that may be assumed as NOEC) correspond to zero-effect level. If a concentration-response curve is developed using suitable statistical approaches (Weibull or comparable), the response at low levels is asymptotic and a value can be calculated however low the concentration is. In this case, an effect different from zero may be calculated using the IA approach and may be compared with the CA approach, even at very low concentrations.

3.5 Uncertainty

The need for uncertainty analysis in the risk assessment of chemicals is now well recognised. However, whilst some guidance exists (e.g. EFSA 2006, IPCS 2006, NRC 2009) the design and conduct of such analysis are still under discussion. In assessing the toxicity of chemical mixtures, in addition to an assessment of the uncertainty and variability associated with the individual chemicals, these need to be assessed for the mixture as well. In such an assessment, there will be sources of uncertainty, in particular related to the assumptions necessary in assessing the combined risk. These include, but are not limited to, the following:

Uncertainties in the exposure assessment of mixtures include

- The level of accuracy with which exposure to mixtures has been characterised.
- The extent and profile of co-exposure to different chemicals. Different chemicals have different persistence in the environment and in the body, and therefore duration of exposure will vary; it may be episodic for one chemical and continuous for another.
- The determination of the identity of the chemicals involved.

Examples of uncertainty in the toxicity assessment of mixtures include;

- The adequacy of the toxicological database.
- Lack of knowledge regarding human relevance.

- The lack of an agreed definition of criteria for "similar modes of action" and of grouping criteria for chemicals into assessment groups. Chemicals may need to be considered in the same assessment group even if the effect does not drive the individual risk assessment.
- Assumptions on the consequences of the combined effect of co-exposure, i.e. dose addition, independent action/response addition, synergy, antagonism.
  - For dose/concentration addition, assumptions regarding similarity in the shape of the dose response curves in some curves.
  - Nature and identification of points of departure for use in combined risk assessments.
  - Assumptions about departures (or absence of departure) from additivity at human relevant exposures to chemicals in an assessment group.

For the ecological assessment of mixtures, additional uncertainties refer to the complexity of ecosystems:

- The structure of biological communities in the exposed ecosystems may be extremely different, with different sensitivity and vulnerability towards toxic chemicals;
- The mode of action of chemicals is usually not the same for the different types of organisms (bacteria, plants, invertebrates, vertebrates) and the knowledge is usually poor;
- The same components of a mixture may have a similar mode of action on a taxonomic group of organisms and different modes of action on another group;
- Toxicological data, when available, are usually limited to a few endpoints on a few indicator organisms;
- The complex effects at the level of population/community, including indirect effects on ecosystem functioning, are largely unknown.

In addition to uncertainty, sources of variability in the risk assessment should be identified, together with an estimate of their potential magnitude, where possible.

3.6 Discussion

There is increasing concern in the general public about the potential toxic effects of chemical mixtures (in the media often referred to as "cocktail-effects"). Current legislation at EU level requires, only in a few instances, the assessment of cumulative risks from the exposure to multiple chemicals (e.g. for pesticides when suitable methodology is available). The use of a dose addition method is recommended in the ECHA guidance document for classification and labelling of mixtures and by the WHO (WHO 2009a, WHO 2009b).

In view of recent publications on the assessment of the ecological and human health risks of mixtures, the question has been raised whether the current approaches offer an acceptable level of protection or need to be changed.

Any change to be implemented in legislation would be expected to result in a higher level of protection of humans and the environment than at present.

When it comes to the question of whether the toxic effects of a mixture can be predicted, it has to be realised that this is only possible under the condition that the individual mixture components are fully identified and that their mode of action, as well as dose

response curves, are known or appropriate assumptions can be made. This is a situation which, in human toxicology as well as in ecotoxicology, is rarely given.

The likelihood of synergistic interaction (the type of interaction that is of most toxicological concern) at actually relevant exposure levels has to be assessed on a case-by-case basis from mode of action information on the individual chemicals.

Except for mixtures composed of substances with a similar mode of action, current evidence does not show significant mixture toxicity at exposures at or below zero-effect levels of the individual components. It is important to note that NO(A)ELs and NO(A)ECs derived from experimental studies do not always represent zero-effect levels. The NOAEL(C)s and NOECs estimated in toxicity and ecotoxicity studies, respectively, are often associated with effect levels in the range of 5 to 20% and hence no "zero-effect levels". It cannot be assumed that in all cases $E(C_i)$ is equal to zero for exposures at the NOAEL(C) or NOEC of a particular study. As the NOAEL(C) or NOEC do not necessarily represent a value for which $E(C_i) = 0$, exposures equal to these levels may also contribute to mixture effects for dissimilarly acting substances.

The question, therefore, is not if exposures to mixtures of substances at the NOAEL or NOAEC for each component represent a potential risk, but if exposures to mixtures well below these levels, and in particular at the level assumed to be safe for each component (TDI, DNEL, PNEC or equivalent) may produce adverse effects. The answer to this question is different for human health and ecological assessments.

The human health assessment is based on the protection of individuals. In deriving health based guidance values (HBGVs), such as tolerable daily intakes (TDI), DNELs or equivalent values, a number of conservative assumptions are made, so that it is unlikely that exposure at the level of the HBGV will result in significant risk (Herrman and Younes 1999). This is implicit in risk analysis policies of organisations such as the European Commission, where HBGVs, such as TDIs and ADIs, derived from the application of a 100-fold uncertainty factor to the NOAEL, are used in risk characterisation. The HBGVs are hence expected to represent a value at which no effects are produced; thus for threshold substances, the assumption is that this value is equal to or lower than the no-effect level; thus an $E(C_i)=0$ should be assumed for exposures at the HBGV level. Consequently, the effects of co-exposure to several substances all below the HBGV value should be assumed to be negligible if all substances have dissimilar modes of action.

There is obvious uncertainty in setting HBGVs. If for a particular threshold substance the TDI, DNEL or equivalent provokes effects (and therefore may contribute to mixture effects), the protection principle is not achieved even for exposures to that substance alone (independently of the co-exposure to other chemicals); thus, the conclusion should be that the HBGV should be recalculated for offering a proper level of protection. For mixtures, however, the probability of needing the full default uncertainty factors continues to decrease as the number of chemicals in the mixture increases (Carlson-Lynch *et al.* 1999).

The situation is different for non-threshold substances, but in this case the level of protection is based on the acceptability of the risks, not on the lack of effects; effects may be expected regardless whether the exposure is to one or several dissimilarly acting substances.

In summary, 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.

The situation is different in the case of ecological assessment where population and community level endpoints are used. Therefore, a PNEC, EQS or equivalent value does

not necessarily represent an $E(C_i)=0$ at the individual effect, and therefore combined exposures to mixtures at the PNEC/EQS level for each component are likely linked to $E(C_{Mix})$ higher than the individual level of effects for each substance. Consequently, population/community effects cannot be excluded. In a simplified example, assuming that population effects are expected when the reproduction rate decreases by 25%, for instance, any PNEC value with $E_{reproduction\_rate} < 25\%$ is appropriate for providing protection for the exposure to a single substance. However, any $E_{reproduction\_rate} > 0$ will contribute to the mixture effects and therefore the $E(C_{Mix})$ may be higher than 25%, resulting in population effects, even if all PNECs for individual substances are sufficient for protecting the population.

In addition, the capacity of the current default assessment factors for providing an adequate level of protection is much more uncertain in the case of ecological assessment; several examples indicate that for substances with specific mechanisms of action some taxonomic groups may be affected at the level estimated through the use of default assessment factors. An additional problem particularly relevant for the environment is the fact that concentrations above the PNEC, EQS or equivalent are really frequent, particularly downstream point emission sources.

In summary, 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 for improving the current knowledge and methodologies, and developing holistic approaches for the ecological risk assessment of chemicals under realistic conditions.

It should be noted that the REACH Regulation is generating the largest database on chemicals in history, and that this information could be used to reduce some of the current uncertainties described above. The information covers not only the hazard identification and the hazard assessment (including dose-response relationships) of each chemical, but also a description of the intended uses and in many cases a Chemical Safety Report with detailed exposure assessments per use. Although the information for each substance is not essentially different from that included in the comprehensive risk assessments conducted under the previous legislation, the main difference is the size of the database: over 4,000 substances already published and a final estimation exceeding 30,000 substances. In addition, much more detailed and categorised information on the uses, use patterns, use conditions and risk management measures applied by the different companies along the supply chain is presented. Specific information on consumers' uses and on incorporation of substances in articles is provided. The information is primarily generated by industry and may require a peer-review assessment in some cases, but could nevertheless be highly valuable for moving forward in a scientifically based assessment of the combined effects of chemical mixtures.

3.7 Conclusions and recommendations

In the light of growing public concern with regard to potential toxic effects of mixtures and recent publications on the state of art methodology in mixture toxicology a scientifically based opinion was requested on whether current approaches provide a sufficient protection level for the environment and human health or whether a regulatory change has to be implemented. For the development of an opinion by the three Scientific Committees (SCHER, SCENIHR and SCCS), six questions were provided by the EU Commission Services ("Terms of Reference", see above), which are addressed in the following:

> **Question 1** – Is there scientific evidence that when organisms are exposed to a number of different chemical substances, these substances may act jointly in a way (addition, antagonism, potentiation, synergies, etc.) that affects the overall level of toxicity?

Yes, under certain conditions, chemicals will act jointly in a way that the overall level of toxicity is influenced.

Chemicals with common modes of action may act jointly to produce combination effects that are larger than the effects of each mixture component applied singly. These effects can be described by dose/concentration addition.

For chemicals with different modes of action (independently acting), no robust evidence is available that exposure to a mixture of such substances is of health concern if the individual chemicals are present at or below their zero-effect levels. It is important to note that NO(A)ELs and NO(A)ECs derived from experimental studies do not always represent zero-effect levels._ The NOAEL(C)s and NOECs estimated in toxicity and ecotoxicity studies, respectively, are often associated with effect levels in the range of 5 to 20% and hence no "zero-effect levels". It cannot be assumed that in all cases $E(C_i)$ is equal to zero for exposures at the NOAEL(C) or NOEC of a particular study. As the NOAEL(C) or NOEC do not necessarily represent a value for which $E(C_i) = 0$, exposures equal to these levels may also contribute to mixture effects for dissimilarly acting substances.

For ecological effects, the exposure to mixtures of dissimilarly acting substances at low but potentially relevant concentrations should be considered, even if all substances are below the individual PNECs. A PNEC is a conservative value, obtained through the application of an assessment factor (AF) to ecotoxicological endpoints (EC50 or NOECs). However, it must be assumed as a level protective for the highest hierarchical levels of the biological organisation (population, community) and does not exclude effects on individuals.

In the examples in which independent action provided a more accurate prediction, dose (concentration) addition slightly overestimated the actual mixture toxicity, which suggests that the use of the dose/concentration concept for risk assessment of chemicals of unknown toxic mechanisms is sufficiently protective.

Interactions (including antagonism, potentiation, and synergies) 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. According to Boobis et al. (2011), "low dose" is defined as at or near or below doses that do not cause statistically significant effects in experimental studies, such as NOALs, NOECs or benchmark dose levels.

> **Question 2** – If different chemical substances to which man/environment are exposed can be expected to act jointly in a way which affects their impact/toxicity on/for man and the environment, do the current assessment methods take proper account of these joint actions?

No, indeed at present risk assessment on the combined effects of chemicals in a mixture is not commonly carried out, nor required by most EU regulations. Direct toxicity testing is performed with mixtures, for some purposes only. For example, direct toxicity testing is performed with certain formulations or waste water effluents and is generally limited to acute effects, whereas joint actions between chemicals on a long-term basis are equally important and far more difficult to estimate.

As outlined in the answer to question 1, different chemical substances may act jointly in a way which affects their toxicity for man and the environment. Current assessment methods for mixtures can take account of joint actions, such as dose/concentration addition or response/effect addition, but are generally only applied under specific circumstances. With these methods, effects of chemical mixtures composed of either dissimilarly or similarly acting substances can be reasonably well predicted. Interactions, however, are generally more difficult to assess and require expert judgement on a case-

by-case basis. Specific conditions under which synergistic actions might be expected, i.e. the most relevant interactions with regard to the toxicological risk, are outlined in the opinion.

The methodology for the (eco)toxicological assessment of chemical mixtures appears to be generally suitable. However, it is often not applied in practice. Assessments of aggregated and combined exposures across different industrial and use sectors, in particular, are rarely performed.

> **Question 3** - Several approaches for the assessment of the mixture effects of chemicals already exist such as dose addition and independent action. What are the advantages and disadvantages of the different approaches and is there any particular model that could be considered as sufficiently robust to be used as a default option?

In view of the huge variety of human exposures to chemical mixtures, the default assumption in human risk assessment had been that the chemicals generally acted by dissimilar modes of action. In cases, however, where information is available to indicate a similar mode of action, a dose/concentration addition approach is appropriate. A dose/concentration addition approach, if applied to chemical mixture components with unknown modes of action, may result in an over-prediction of toxicity; using the independent action approach may however underestimate toxicity. Therefore, in the case of unknown modes of action, the dose/concentration addition approach is also preferable to ensure an adequate level of protection.

Different methods exist for the dose/concentration addition approach (see Methodology section 3.4 for details). When using the RfP or RV, one should be aware that NOAELs/LOAELs are based on single experimental datapoints and the values depend on the dose-spacing used in the experiment. In contrast, BMDs are based on all experimental points and therefore provide more reliable information on the dose response.

In ecotoxicology, any approach should be referred to specific endpoints and to defined taxonomic groups of organisms. The reference values (PNECs) are derived using different sensitive organisms for any type of chemical. Therefore, a combination of PEC/PNEC ratios is less scientifically correct than the sum of TUs. However, it has been proved slightly more conservative and, in some cases, more easily applicable. Therefore, for pragmatic reasons, it may be used as a first-tier conservative approach.

A significant limitation of component-based approaches is that they are only applicable to mixtures of which the major components are known.

> **Question 4** – Given that it is unrealistic to assess every possible combination of chemical substances, what is the most effective way to target resources on those combinations of chemicals that constitute the highest risk for man and the environment?

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 allow a focus on mixtures of potential concern is necessary. The following criteria are proposed for consideration:

- Human and/or environmental exposure at significant levels (e.g., close to the HBGVs, DNELs or PNECs for several components).
- Chemicals that are produced and/or marketed as multi-constituent substances or commercial mixtures with several components and/or active ingredients and/or substances of concern (i.e. as defined by EU legislation, e.g. REACH, CLP, pesticides and biocidal products legislation, food law, *etc.*).

- Potential serious adverse effects of one or more chemicals at the likely exposure levels.
- Likelihood of frequent or large scale exposure of the human population or the environment.
- Persistence of chemicals in the body and/or in the environment. High persistence/bioaccumulation would be a property of importance.
- Known information of potential interaction at levels of human and environmental exposure.
- Predictive information that chemicals act similarly such as (quantitative) structure activity relationships and structural alerts.
- Particular attention should be paid to mixtures for which one or more components are assumed to have no threshold for its effects such as genotoxic carcinogens; a MOE or a lifetime cancer risk approach could be applied.

Exposure to one or more components approaching the threshold levels for adverse effects would mean that the mixture should be given priority for assessment. A TTC-like approach can be used to eliminate combinations that are of no concern [for details on the applicability of a TTC approach for the assessment of chemical mixtures see Boobis *et al.* (2011) and Price *et al*. (2009)].

For the environment, attention should be paid to mixtures of chemicals, individual components of which approach the PNEC. The TTC model may be not appropriate for biological communities, where a threshold of concern may be several orders of magnitude different for different taxonomic groups of organisms (from bacteria to vertebrates). In some cases, for very sensitive taxonomic groups, even very low concentrations which are difficult to assess or measure may be not negligible.

In view of the difficulty and time needed to retrieve or generate an appropriate dataset for hazard characterisation and exposure estimates, a tiered approach, such as proposed by the WHO/IPCS (2009b) or EFSA (2008), may be considered (for details on the tiered approach, see Methodology section 3.4). The identification of the data gaps after the application of the tiered approach should determine the extent of testing of chemical mixtures and study design.

> **Question 5** – Where are the major knowledge gaps with regard to the assessment of the toxicity of chemical mixtures?

With regard to the assessment of chemical mixtures (as defined in the mandate), a major knowledge gap at the present time is the lack of knowledge on where, how often and to what extent humans and the environment are exposed to certain chemical mixtures and how exposure may change over time. There is a need to better understand human and environmental exposures, both through the use of monitoring and modeling (Tornero-Velez *et al.* 2011).

For many chemicals, there is no good information on mode of action. Currently there is neither an agreed inventory of modes of action, nor a defined set of criteria on how to characterise or predict a mode of action for data-poor chemicals or how to group chemicals into assessment groups.

Interactions of chemicals in mixtures are difficult to foresee, particularly for long-term effects. Research is needed to define criteria that predict potentiation or synergy.

In ecotoxicology, the problem is even more complex. A knowledge of all possible modes of action that may occur in the different types of organisms of a complex biological community is difficult (if not impossible) to be attained. On the other hand, it must be considered that ecologically relevant endpoints are generally broader and not so specific

(e.g. toxicity on specific organs, etc.) as in human toxicology. A full review of the literature should be made to prepare a state-of-the-art on mixtures biodegradation modelling.

> **Question 6** – Does current knowledge constitute a sufficiently solid foundation upon which to address the toxicity of chemical mixtures in a more systematic way in the context of EU legislation?

In many cases, knowledge is insufficient for a robust scientific analysis. If toxicologically significant interactions can be excluded, the components of a mixture are identified and known mode of action information is available, either a dose addition or independent action model should be applied. This set of information, in human toxicology, is however rarely available and, in most cases, very cost- and labour-intensive to generate. Often, it may not be possible to obtain the required data because of limitations in existing study designs and analytical methods, for example.

In ecotoxicology, the mode of action should be known for all the relevant taxonomic groups of aquatic and terrestrial ecosystems. Thus, the availability of information is even more to limited; in addition, modes of actions considered dissimilar at the individual level may affect the same population relevant endpoint, and therefore, the dose/concentration addition model may be more appropriate for predicting effects at the population level.

However, in most cases, when applying a dose/concentration addition approach, it is necessary to rely on assumptions such as mode of action, shape and slope of dose response curves of the individual components. These assumptions may be generated by grouping of chemicals into categories and assessment groups. However, no generally agreed criteria for the grouping of substances exist, adding to the uncertainties associated with this approach. Choosing the independent action approach may however underestimate combined effects of similarly acting chemicals. Hence, if no mode of action information is available, the dose/concentration addition method should be preferred over the independent action approach.

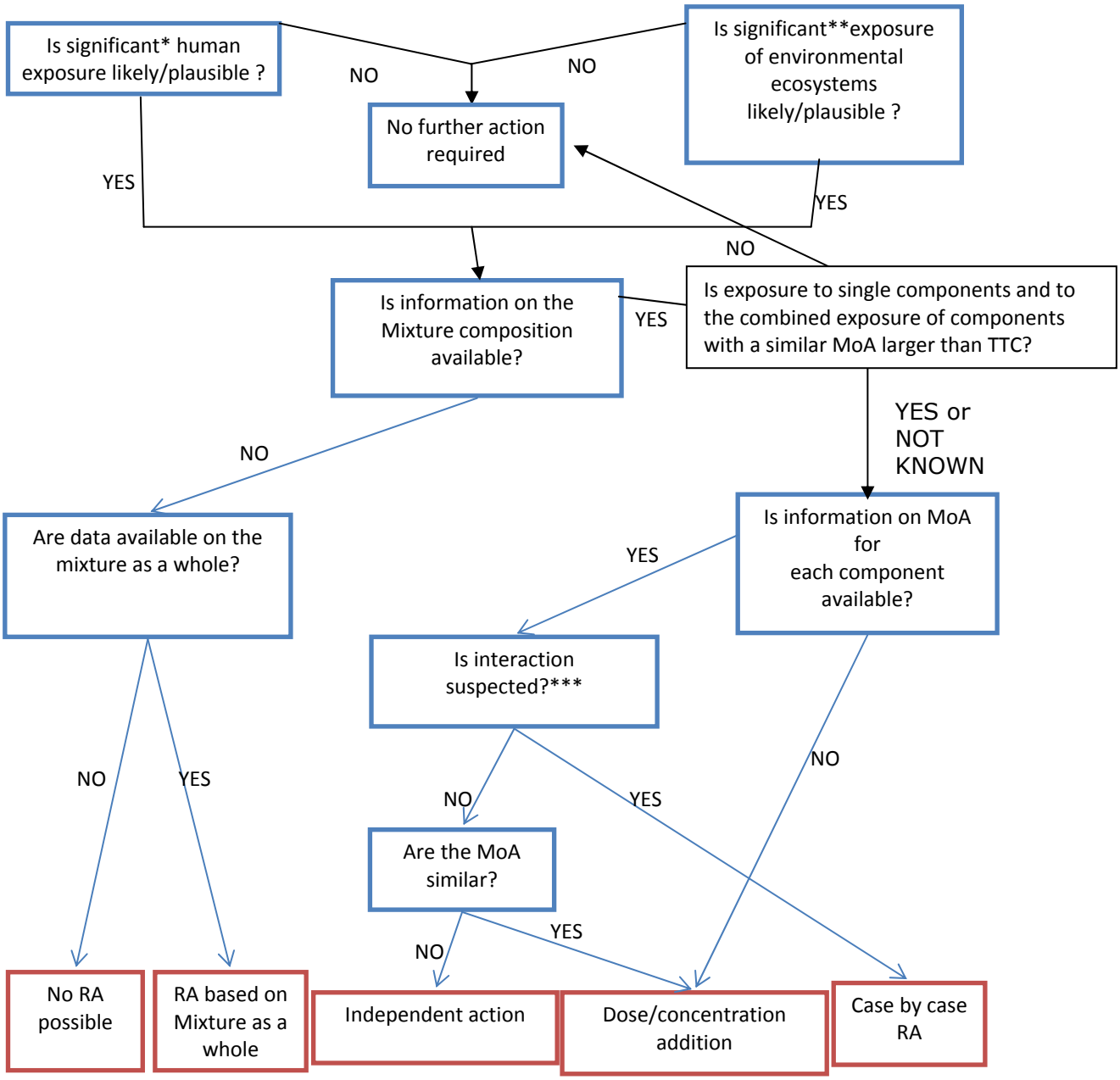
Prediction of possible interaction requires expert judgement and hence needs to be considered on a case-by-case basis.

In future, pathway-based toxicity evaluations (e.g. inflammation - oxidative stress - genotoxicity) based on *in silico* and *in vitro* methodology may become more feasible, enabling these methods to identify common effects. However, the report of a recent meeting of the US National Academic's Standing Committee on Use of Emerging Science for Environmental Health Decision concluded that "many challenges remain to be addressed before the findings from high-throughput screens and *in silico* models may be considered sufficiently robust and informative" (Rusyn and Daston 2010). The Working Group agrees with this conclusion.

In ecotoxicology, a relevant issue may be related to combined effects capable of affecting reproduction, population dynamics and the health of an ecosystem. For some chemicals these effects may become evident even some time after exposure ceased.

Having reviewed the available evidence, the Committees recommend that a mixture-dependent approach is used for the assessment of chemical mixtures as outlined in the following diagram:

Decision Tree for the Risk Assessment of Mixtures[5]



*"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).

[5] Please refer to the accompanying text when consulting this decision tree.

In order to prioritize chemical mixtures for possible assessment it is first necessary to consider whether there is significant human or environmental exposure to the mixture or its components. Unless there are indications for a significant interaction, a dose/concentration addition model could be used if the components of the mixture exert their biological effects via an identical or similar mode/mechanism of action. If the mixture components act dissimilarly, the independent action model would be applied. It further appears justifiable that a dose/concentration addition approach should be used as default approach in cases where neither mode of action nor dose-response information is available to ensure adequate conservatism in the assessment.

4. LIST OF ABBREVIATIONS

AChE	Acetyl Cholinesterase
ADI	Acceptable Daily Intake
AF	Application Factor, Adjustment Factor, Assessment Factor
ATSDR	US Agency for Toxic Substances and Disease Registry
BMC	Benchmark Concentration
BMD	Benchmark Dose
BMDL	Benchmark Dose Lower Confidence Limit
CA	Concentration Addition
COT	UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CSAF	Chemical Specific Adjustment Factor
CSTEE	Scientific Committee for Toxicity, Ecotoxicity and the Environment
CVUA	Chemisches und Veterinäruntersuchungsamt
DA	Dose Addition
DNEL	Derived No Effect Level
EC	Effective Concentration
ECDC	European Centre for Disease prevention and Control
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EQS	Environmental Quality Standard
EU	European Union
HI	Hazard Index
aHI	adjusted hazard index
HBGV	Health Based Guidance Value
HQ	Hazard Quotient
IA	Independent Action
IPCS	International Programme on Chemical Safety
JRC	Joint Research Centre
LD_{50}	Median lethal dose
LO(A)EL/C	Lowest Observed (Adverse)Effect Level/Concentration
MCR	Maximum Cumulative Ratio
MCS	Multi-Constituent Substances
MEC	Measured Environmental Concentration
MOA	Mode of Action
MOE	Margin of Exposure
MOET	Combined Margin of Exposure
MRL	Maximum Residue Level
NEL	No Effect Level
NO(A)EL/C	No Observed (Adverse) Effect Level/Concentration
OECD	Organisation for Economic Co-operation and Development
PAH	Polyaromatic Hydrocarbons
PBPK	Physiologically-Based Pharmacokinetics

PBPD	Physiologically-Based Pharmacodynamics
PBTK	Physiologically-Based Toxicokinetics
PBTD	Physiologically-Based Toxicodynamics
PCBs	Polychlorinated Biphenyls
PEC	Predicted Environmental Concentration
PEF	Potency Equivalency Factor
PNEC	Predicted No Effect Concentration
POD	Point of Departure
PODI	Point of Departure Index
PPR	Palnt Protection Products
QSAR	Quantitative Structure–Activity Relationship
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
RfP	Reference Point
RPF	Relative Potency Factor
RV	Reference Value
SAR	Structure–Activity Relationship
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
TDI	Tolerable Daily Intake
TEF	Toxic Equivalency Factor
TTC	Threshold of Toxicological Concern
TU	Toxic Unit
TUm	Toxic Unit for a mixture
UF	Uncertainty Factor
US EPA	United States Environmental Protection Agency
UVCB	Substances of Unknown or Variable Composition, Complex reaction products or Biological Materials
VKM	Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for Mattrygghet)
WFD	Water Framework Directive
WHO	World Health Organization
YES	Yeast Oestrogen System

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ANNEX

Glossary

In the absence of internationally harmonised terminology for the assessment of mixtures and combinations of chemicals, a definition of terms as used in this opinion is provided in the following section drawn on the work of the EFSA, the US EPA, the WHO, and Kortenkamp *et al.* (2009):

- Aggregated exposure

Aggregated exposure includes all routes, pathways, and sources of exposure to a given chemical.

- Combined exposure

Combined exposure includes all routes, pathways, and sources of exposure to multiple chemicals.

- Concentration addition, see Dose-addition

- Cumulative exposure

Cumulative exposure, in EU terminology, means repeated exposure to one and the same chemical from the same, similar or different sources via the same or different routes of exposure. In a wider sense, it includes combined exposure to multiple chemicals.

- Dose/concentration-addition (similar action, similar joint action, relative dose addition)

Dose/concentration-addition occurs when chemicals in a mixture act in the same way, by the same mechanism/mode of action, and differ only in their potencies. Dose/Concentration-addition implies that the effects of exposure to a mixture of such compounds are equivalent to the effects of the sum of the potency-corrected doses of each component compound.
In ecotoxicology the most frequent exposure pattern is through the concentration of the chemical in the environmental compartment (water, air, soil), not through food. Therefore, concentration is preferred over dose.

- Effects Addition

The sum of biological responses following exposure to a mixture of substances with dissimilar modes of action, see also Independent action.

- Hazard Index (HI)

The HI is a dimensionless figure, corresponding to the sum of the ratios between the exposure level and the reference value of each component. When the RV of a certain compound is based on an effect that is not the group effect (common toxic effect), or the applied assessment factor includes adjustments not related to the endpoint of concern then the HQ can be refined by identifying the RV for the group effect and adjusting the Hazard Quotient (see below), accordingly. In this situation an adjusted HI (aHI) is then calculated.

- Hazard Quotient

The hazard quotient is the ratio of the potential exposure to the substance and the reference value. If the Hazard Quotient is calculated to be less than 1, then no adverse health effects are expected as a result of exposure.

- Independent action (dissimilar action, independent joint action)

Independent action (response addition, effects addition) occurs where the modes 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. Effects addition refers to the sum of biological responses; response addition means the sum of probabilistic risks (for details see section 3.3)

- Interaction

Interaction describes the combined effect of two or more chemicals as stronger (synergistic, potentiating, supra-additive) or weaker (antagonistic, inhibitive, sub-additive, infra-additive) than would be expected on the basis of Dose/Concentration-addition or response-addition.

- Low-dose

This is the dose equivalent to an environmentally-relevant (human-exposure relevant) dose. It does not mean a dose which is close to a NOEL(C) determined in an experimental study (see also no effect level (concentration).

- Margin of exposure

The Margin of Exposure (MOE) is the ratio of the reference point RfP to the level of exposure in humans (measured or estimated).

- Margin of exposure total (MOET)

MOET is calculated as the reciprocal of the sum of the reciprocals of the individual MOEs.

- Mechanism of action

This is a molecular sequence of events that produce a specific biological outcome.

- Mixture

A chemical mixture consists of two or more substances which have been combined such that each substance retains its own chemical identity.

Mixtures of chemicals covered in this opinion include (see "Terms of Reference"):
- Substances that are mixtures themselves (multi-constituent substances, MCS; materials of unknown or variable composition, complex reaction products or biological materials, UVCB);
- Products that contain more than one chemical e.g. cosmetics, plant protection products;
- Chemicals jointly emitted from production sites, during transport processes and consumption or recycling processes;

- Several chemicals that might occur together in environmental media (water, soil, air), food items, biota and humans as a result of emission from various sources and via multiple pathways.

- Mixture effect (combination effect, joint effect)

This is the response of a biological system to a chemical mixture.

- Mode of action (MOA)

MOA is a plausible hypothesis about measurable key events by which a chemical exerts its biological effects. MOA is not intended to build a comprehensive model of a chemical's actions. MOA can include mechanisms of action, but is considered to be broader.

A common MOA is defined in accordance with the EFSA definition (EFSA 2009b) as "involving the same key events leading to an adverse health effect following interaction of the compound with its biological target[s]".

- No observed (adverse) effect level (concentration) (NO(A)EL(C))

A NO(A)EL or NO(A)EC is derived from an experimental toxicity or ecotoxicity study. A NO(A)EL or NO(A)EC does not always represent a zero-effect level (concentration) as NO(A)ELs and NO(A)ECs derived in toxicity and ecotoxicity studies may be associated with effect levels in the range of 5 to 20%. Exposures around the NOAEL(C)s should therefore not be considered as "low-dose".

- Point of departure (POD)

Often no observed adverse effect levels (NOAELs) or no observed adverse effect concentrations (NOAECs) are used as POD. Increasingly, the lower confidence limit of doses or concentrations associated with a specified increase in the incidence of an effect, so-called benchmark doses (BMD) are used as POD. For example, a benchmark dose such as the BMD10 is the dose of the test chemical that leads to a 10% increase in effect.

- Point of departure index (PODI)

The PODI is the sum of exposures divided by the point of departure for each of the individual components.

- Response addition

See "Independent action"

- Response potency factor, see Toxic equivalency factor

- Similarity

Toxicological similarity is the basis for grouping chemicals together in classes or categories. It represents a general knowledge about the action of a chemical or a mixture and can be expressed in broad terms such as at the target organ level in the body (e.g., enzyme changes in the liver). In general, the same or similar modes of action and comparable dose-response curves are assumed for similar components in a mixture. The term group of similar mixtures refers to chemically related classes of mixtures that act by a similar mode of action, have closely related chemical

structures, and occur together routinely in environmental samples, usually because they are generated by the same commercial process.

- Toxic Equivalency Factor (TEF)

The TEF is similar to the Relative Potency Factor (RPF), and describes the potency of organochlorine compounds such as dioxins and mixtures of PCB congeners.

- Toxic Unit (TU)

The TU is a dimensionless figure, calculated as the ratio between the exposure level (e.g. a PEC) and a given acute or chronic endpoint (e.g. EC50 or NOEC). The toxic units for a mixture (TUm) are calculated as the sum of individual TUs.

- Zero-effect Level / Concentration

The true or absolute no-adverse effect level, i.e. level/concentration that is not associated with an adverse effect on health or environment.