State of the Art Report on Mixture Toxicity

Final Report

Executive Summary

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Contractor	**The School of Pharmacy** **University of London (ULSOP)**
Sub-Contractors	**Göteborg University (UGOT)** **Faust & Backhaus** **Environmental Consulting GbR (FBEC)**
Responsible Scientists	**Prof. Dr. Andreas Kortenkamp (ULSOP)** **Assoc.-Prof. Dr. Thomas Backhaus (UGOT)** **Dr. Michael Faust (FBEC)**

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Executive Summary

This report details the findings of a project on mixture toxicology and ecotoxicology commissioned by the European Commission, DG Environment. It describes the scientific state of the art in the field, and gives an account of the regulatory state of the art for dealing with combined exposures in the European Union, in major competing economies, including the USA and Japan and in international bodies.

1. Terms of reference, scope, definitions

The specifications of the invitation to tender defined four tasks, and these form the *terms of reference* of this report:

Task 1: Analyzing scientific literature on mixture toxicity
Based on literature searches, with additional information derived from an analysis of EU projects, conference publications and opinions of relevant EU Scientific Committees a critical review was to be prepared with the aim of summarizing the current scientific state of the art of mixture toxicology and ecotoxicology.

Task 2: Analyzing EU risk assessment regimes relevant to mixture toxicity assessments
An analysis of EU risk assessment regimes was to be conducted, with the specific aim of assessing whether EU risk assessment regimes in 21 different EU directives and regulations take into account risks arising from mixture toxicity and if, in which way. The analysis was to result in an overview of relevant provisions, an identification of regulatory gaps, and recommendations for future improvements.

Task 3: Analyzing practical experiences in assessing mixture toxicity, approaches and methodologies used for this purpose in the EU
Practical approaches for assessing the toxicity of environmental samples and/or waste samples currently used in relevant EU member states with respect to assessing the effects of chemical mixtures were to be analyzed. The analysis had to include both whole mixture approaches (i.e. direct toxicity testing of the mixture) and component-based approaches (i.e. estimating the total toxicity from information on identified components only).

Task 4: Analyzing approaches to assess mixture toxicity in major competing economies of EU and international bodies
A systematic overview of approaches to the hazard and risk assessment of chemical mixtures used by competent authorities in the USA, Japan and in international bodies such as the World Health Organisation (WHO), the International Programme on Chemical Safety (IPCS), the International Agency for Research on Cancer (IARC), the Organisation for Economic Cooperation and Development (OECD), and others, was to be conducted.

The *scope* of this report is restricted to the toxicity of mixtures of chemicals after simultaneous exposure. The term *mixture toxicity* is understood as unwanted adverse effects of mixtures of chemicals. In this report, *combination effect*, *combined effect* or *joint action* is used synonymously with *mixture toxicity*. Interactions of chemical factors with physical and/or biological stressors in the environment are beyond the scope of this study. The same applies to wanted beneficial effects of mixtures, such as therapeutic effects of drug combinations.

For the purposes of this report 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 or 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 human tissues, as a result of emission from various sources, via multiple pathways.

2. The scientific state of the art of mixture toxicology

During the last ten years, mixture toxicology has undergone a remarkable and productive development. Whilst earlier experimental studies have focused mainly on combinations of only two chemicals, a significant number of well-designed and decisive studies have been carried out that involve multi-component mixtures. Ecotoxicology has played an important role in advancing mixture toxicology, with human and mammalian toxicology slowly catching up. The planning, conduct and assessment of multi-component mixtures is possible, with clear results. This has extended from *in vitro* assays to *in vivo* studies and even to analyses of mixture effects on biological communities. Multi-component mixtures were composed of both, unspecifically and specifically, acting chemicals, with similar, and to a lesser extent, dissimilar modes of action. The compounds in the mixtures belong to several chemical classes. Among the most frequently studied groups are pesticides, heavy metals, endocrine disrupters, PAHs and general industrial chemicals. A detailed appraisal of the relevant literature is presented in **Part 1** of this report.

The current scientific state of the art of mixture toxicology can be summarized conveniently by addressing issues and questions that arise frequently during the practice of chemical risk assessment.

Is an assessment of the effects of chemical mixtures necessary from a scientific viewpoint?

Humans and all other organisms are typically exposed to multi-component chemical mixtures, present in the surrounding environmental media (water, air, soil), in food or in consumer products. However, with a few exceptions, chemical risk assessment considers the effects of single substances in isolation, an approach that is only justified if the exposure to mixtures does not bear the risk of an increased toxicity. This would be the case, for example, if only one chemical of the mixture is toxic while the others are biologically inert, or if empirical evidence showed that the joint action of chemicals is typically not larger than the effect of the most toxic compound.

However, there is strong evidence that chemicals with common specific modes of action work together to produce combination effects that are larger than the effects of each mixture component applied singly. Fewer studies have been conducted with mixtures composed of chemicals with diverse modes of action, with results clearly pointing in the same direction: the effects of such mixtures are also higher than those of the individual components (see below for a discussion of the special case of low-dose mixtures). The literature shows that this applies to a host of different endpoints of relevance to mammalian toxicology and ecotoxicology, and holds true for a diverse set of chemicals that all are subject to EU regulations (Part 1, 4.1 – 4.10, 5.2).

There is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment might be too simplistic. It is in danger of underestimating the risk of chemicals to human health and to the environment.

Is there not sufficient protection against mixture effects if we make sure that each chemical is present individually at exposures unlikely to pose risks?

Chemical risk assessment provides threshold doses or concentrations of regulatory concern such as acceptable daily intakes (ADI) or predicted no effect concentrations (PNECs) for individual chemicals which are based on so-called points of departure (No Observed Adverse Effect Levels, NOAELs, No Observed Effect Concentrations, NOECs, or benchmark doses). Exposures below these levels are usually considered safe.

The experimental evidence on mixture effects provokes the question as to whether there is sufficient protection also against combined exposures, if each component is present below their individual threshold doses (concentrations). That conjecture has been tested experimentally by combining chemicals at levels commonly used to derive estimates of safe exposures. Early pioneering studies have been conducted with bacteria, daphnids and fish, and were followed up by additional experiments with populations and communities of unicellular organisms. More recently, studies with endpoints relevant to endocrine disruption have been documented for receptor-binding, and receptor-activation assays, tests with mammalian cell lines and higher organisms (fish and rat). Taken together, these studies have produced strong evidence that mixture effects may arise when several chemicals are combined at doses or concentrations around, or below, points of departure (Part 1, 6.4 – 6.7).

The majority of these studies have analyzed the effect of combinations composed of chemicals that interact with the same sub-system of an organism. In such cases, the concept of *dose* or *concentration addition* is applicable. The principles of *dose (concentration) additivity* mean that mixture effects are to be expected even when each chemical is present below zero-effect levels, because it is assumed that all toxicants in the mixture behave as if they were a dilution of one another. Hence, any concentration of any compound needs to be considered because it adds to the mixture concentration. This implies that all compounds contribute to the mixture toxicity in direct proportion to their concentration in the mixture and their individual potency. Whether the individual concentrations in the mixture are above or below the corresponding effect thresholds does not matter. This phenomenon has been termed “something from nothing”. It has been demonstrated repeatedly for a broad range of mixtures in toxicological and ecotoxicological studies. There is unanimous agreement across all disciplines that, in the case of mixtures of similar compounds, combination effects require special consideration.

Theory predicts that mixtures composed of agents with diverse modes of action, where the concept of *independent action* is applicable, should not yield a combination effect as long as all components are present at levels associated with zero responses. If doses or concentrations used as points of departure can be equated with zero-effect-levels, this would mean that mixtures of dissimilarly acting chemicals are safe, as long as exposure to each component does not exceed its individual point of departure. With reference to the apparent diversity of chemical exposures in the “real world”, *independent action* is sometimes taken as the default assessment concept in human toxicology, when the

similarity criteria of *dose (concentration) addition* appear to be violated. Consequently, possible mixture effects are regarded as negligible for chemicals risk assessment.

In contradiction to that line of argumentation, there is decisive evidence that mixtures composed of chemicals with diverse modes of action also exhibit mixture effects when each component is present at doses equal to, or below points of departure (Part 1, 6.6). This evidence is derived from studies relevant to human toxicology and to ecotoxicology.

The apparent conflict with theory expectations can be resolved by considering that doses or concentrations used as points of departure in risk assessment must not be equated with zero-effect-levels. Instead, they describe a grey area, where the presence of effects can neither be proven, nor ruled out with confidence. Depending on the variation of the biological endpoint under consideration, responses associated with NOAELs or NOECs can be as high as 20% (toxicology) or even nearly 40% (ecotoxicology) (Part 1, 6.6).

Hence, mixture effects cannot be ruled out, even when all components of a mixture of substances with diverse modes of action are present at their individual NOAELs or NOECs. Especially when exposure is to only a certain fraction of a chemicals' NOAEL/NOECs, whether mixture effects become significant depends on the number of mixture components, the precision of the experimental data and the steepness of the individual concentration-response curves.

Whether or not risks arise from combined exposures can only be decided on the basis of better information about relevant combined exposures of human populations and wild life. This information is currently missing, and this presents a major challenge to risk assessment. Regarding uncertainty factors used in chemical-by-chemical risk assessment there are indications that they offer insufficient room to allow for mixture effects for all possible realistic mixtures (see Part 1, 7.1). The issue is linked to the wider question as to whether the commonly applied uncertainty factor of 100 is sufficiently protective even without considering mixture effects. There is no unanimous view on the subject, and the issue requires further clarification.

Is it necessary to test every conceivable combination of chemicals or is it possible to predict the effects of a mixture?

One of the key aspirations of mixture (eco)toxicology has been to anticipate quantitatively the effects of mixtures of chemicals from knowledge about the toxicity of their individual components. This can be achieved by making the assumption that the chemicals in the mixture act in concert by exerting their effects without diminishing or enhancing each others toxicity, the so-called non-interaction or additivity assumption. *Dose (concentration) addition* and *independent action* are the two concepts available for formulating the null hypothesis of additivity. Synergisms or antagonisms can then be defined in relation to this additivity assumption as upwards or downwards deviations, respectively.

There is good evidence that both *dose (concentration) addition* and *independent action* provide reasonable approximations for the prediction of combination effects when the toxicities of individual mixture components are known (Part 1, 4.2, 4.4, 4.8, 5.2). Deviations from predicted additivity, indicative of synergisms or antagonisms, are comparatively rare, relatively small and largely confined to mixtures with only a few compounds (Part 1, 4.8, 5.2). It should be specifically noted that this pattern is found in both toxicological as well as ecotoxicological studies, although the bigger part of the studies with endpoints relevant to human and mammalian toxicology focus on the issue of endocrine disruption. There is strong evidence that it is possible to predict the toxicity of chemical mixtures with reasonable accuracy and precision. There is no need for the experimental testing of each and every conceivable mixture, which would indeed make risk assessment unmanageable.

However, the use of both concepts is limited to mixtures of known chemical composition. Complex environmental samples (sludge, water, soil) or biological tissue (blood or fat tissue) of unknown composition are often subject to dedicated biotesting. But even here *dose (concentration) addition* and *independent action* can play a vital role when used in concert with advanced chemical-analytical techniques in order to pinpoint the most important pollutants, which can then guide further investigations and/or risk management steps.

Which of the two assessment and prediction concepts, dose addition or independent action, should be utilized in practice?

A question of considerable importance to risk assessment and regulation is which of the two concepts, *dose (concentration) addition* or *independent action*, should be chosen for the interpretation of empirical data, or for anticipating mixture effects of untested combinations. Although both *dose (concentration) addition* and *independent action* often provide good approximations of mixture effects, the issue of distinguishing between these concepts becomes important when the two concepts predict quantitatively different mixture toxicities.

Dose (concentration) addition is thought to be applicable to mixtures composed of chemicals with a similar mode of action. Conversely, *independent action* is applied to chemicals with diverse modes of action. The practical relevance of *independent action* for the assessment of mixture effects has been called into question on the basis of considerations of biological organisation. The principle of strictly independent events may only rarely be relevant due to converging signalling pathways and inter-linked subsystems. For these reasons, *dose (concentration) addition* has been deemed more broadly applicable, and has even been termed the “general solution” for mixture toxicity assessment.

Only a few studies have evaluated the two concepts comparatively, side-by-side in the same experimental system. In the majority of these cases, *dose (concentration) addition* provided more conservative mixture toxicity estimates, although the predictions derived from both concepts produced dose (or concentration) estimates that differed by no more

than a factor of 5. In several instances, the predictions yielded by *dose (concentration) addition* and *independent action* were even identical (Part 1, 5.2).

A few examples exist of studies where *independent action* has provided a better prediction of the observed mixture effects than *dose (concentration) addition*. These studies derive from comparative evaluations of both concepts with mixtures designed rigorously to include chemicals with different mechanisms of action. They are of fundamental importance because they directly contradict the idea of *dose (concentration) addition* as the "general solution". However, in all the examples in which *independent action* provided a more accurate prediction, *dose (concentration) addition* slightly overestimated the actual mixture toxicity (Part 1, 5.2), which suggests that the use of this concept for risk assessment purposes is sufficiently protective.

According to *dose (concentration) addition*, the EC50 of a mixture can be predicted based on the EC50 values of the individual components. Because such values are statistically highly reliable measures, usually documented in published ecotoxicological studies and/or compiled in publically available databases, the calculation of an EC50 for a mixture derived from *dose (concentration) addition* usually does not pose particular problems in ecotoxicology. Although ED50 values are often not available in human and mammalian toxicology, it is possible to conduct mixtures risk assessments by utilizing points of departure (benchmark doses, NOAELs) in the Hazard Index or the Point of Departure Index approaches (see below). In contrast, the use of *independent action* requires knowledge about the precise effects that each component would provoke if present individually at the concentration found in the mixture. This information is not readily available.

Taken together both the currently available scientific evidence as well as pragmatic considerations support the idea of adopting *dose (concentration) addition* as the preliminary default concept for the assessment and prediction of mixture effects. This is borne out by current practice in many regulatory bodies in the EU, USA and by recommendations of international bodies (see Part 3, 4.1 – 4.5, 5.1 – 5.3 and Part 4, 5.1 – 5.3).

Which chemicals should be subjected to mixtures risk assessment?

Supposing that the need for considering mixture effects in chemical risk assessment and regulation is accepted, regulators are faced with the problem of which chemicals to subject to joint assessment and regulation. In order to prevent the regulatory risk assessment of chemical mixtures from becoming impractical and unwieldy, several issues have to be taken into account.

A mixture risk assessment is not necessary for each conceivable mixture that can be constructed from the totality of the compounds that are used on the European market. It is called for only if there is a possibility that compounds actually occur as a chemical mixture. This is primarily the case for compounds that are part of a chemical product (i.e. an intentionally produced chemical mixture), compounds that are produced and emitted

together from an industrial process or that occur together in the same environmental compartment or the human body. Unfortunately, there are still considerable knowledge gaps concerning the mixture of chemicals present in human tissues. Although there are elaborate monitoring programmes in place for individual substances of concern, dedicated exposure studies that focus on chemical mixtures are largely missing.

The issue of grouping chemicals for mixture risk assessment is handled in different ways in human toxicology and ecotoxicology (Part 1, 7.3). In human toxicology, chemicals thought to exhibit their effects through common mechanisms are often grouped together. For example, pesticides and other chemicals are considered to qualify for inclusion in a common group when their mechanism of toxicity shows similarities in both nature and sequence of major biochemical events. Current debates focus on what precisely should constitute a "common mode of action". There is a precedent in employing grouping criteria based on similarities in chemical structure or derived from mechanistic considerations. There is agreement that such chemicals should be subjected to mixtures risk assessment. Recently however, it has been argued that grouping criteria based solely on chemical similarity or similar mechanisms may lead to unrealistically narrow groupings, with the exclusion of chemicals that also might contribute to combination effects. Alternative proposals therefore recommend a move towards establishing grouping criteria by focusing on common adverse outcomes, with less emphasis on similarity of mechanisms. This is in recognition of emerging evidence that biological effects can be similar, although the molecular details of toxicological mechanisms - including metabolism, distribution and elimination – may differ profoundly in many respects. A consensus is currently not in sight, but progress is likely to be made by considering groups of chemicals relevant to specific endpoints, rather than attempting to derive general grouping criteria for all endpoints and mixtures.

In contrast, ecotoxicological studies often employ broad, integrating endpoints such as mortality or reproduction. There is a consensus, that if a compound affects such endpoints, it is considered to be of relevance from a mixture perspective and *dose (concentration) addition*, *independent action* or a combination of both is applied. The use of grouping criteria based on mechanistic considerations to decide whether a compound has to be considered at all play a far less prominent role than in human toxicology.

How should mixture effect assessment concepts be applied in practice?

There are various risk assessment methods for evaluating combined exposures in practice. Without exception, these methods are derived from the *dose (concentration) addition* concept. Perhaps the best-known of these is the toxic equivalent factor (TEF) approach, widely used for the assessment of dioxin mixtures. Other applications of *dose (concentration) addition* include Toxic Unit Summation (TUS), the Hazard Index (HI) and the Point of Departure Index (PODI) (Part 1, 7.4 – 7.5).

To deal with data gaps and to take account of differing data quality (data-rich vs. data-poor situations), tiered approaches to mixture risk assessment have been proposed. Mixture risk assessment may begin with the question of whether combined exposures are

in fact likely, and at the lowest tier it may become apparent that the situation to be evaluated does not in fact present an issue for mixtures risk assessment.

In the next higher tier, data about mixed exposures may not be present, but it may be deemed desirable to safeguard against the possibility of joint effects by adopting a specific mixtures assessment factor. In a subsequent tier, sufficient data may be available to satisfy the assumptions of *dose (concentration) addition* throughout, in which case risk assessment methods that derive from this concept could be applied (HI, PODI etc.). In more data rich situations sufficient information about various modes of action may be available, such that mixed mixture assessment models (*dose (concentration) addition* within groups of compounds perceived to follow simple similar action, followed by *independent action* across groups) can be applied. Finally, in the highest tier it might be possible to address both issues of modes of action and differences in the vulnerability of various species or risk receptors.

What knowledge gaps hamper the consideration of mixture toxicology and ecotoxicology in chemical risk assessment?

The available empirical evidence of low-dose mixture studies suggests that a disregard for mixture effects may lead to underestimations of real existing risks. However, in itself, this body of evidence is not decisive when it comes to deciding whether or not risks are present in "real world" exposure settings. The crucial factor for such risks to occur is in the number of chemicals, and their concentrations: only if sufficient numbers of chemicals of sufficient potency and at sufficiently high exposure levels are present, are combination effects to be expected. Whether or not risks arise from combined exposures can only be decided on the basis of better information about relevant combined exposures of human populations and wild life. That information is currently missing, and this presents a major challenge to risk assessment.

Although *dose (concentration) addition* (and, to a limited extent, *independent action*) have proven surprisingly powerful in predicting and assessing mixture toxicities, there are also clear cases of synergisms (i.e. higher than expected mixture toxicities). Such cases are very specific for certain mixtures (compound types, their concentrations and mixture ratios), particular organisms and endpoints. Hence they cannot be incorporated into a general risk assessment scheme, but must be treated on a case-by-case basis. Therefore, any regulatory strategy must include a certain element of flexibility that allows adequate provisions for such exceptional cases. When it comes to pinpointing the causes for synergisms or antagonisms, there are substantial knowledge gaps in our current scientific understanding. There is an urgent need to define the conditions that might lead to synergistic mixture toxicities, and to establish how large synergisms are likely to be.

Dose (concentration) addition assumes that all components in a mixture contribute to the joint effect, in proportion to their prevalence and individual potency. *Independent action* assumes that the only concentrations that matter are those associated with effects after exposure to the single chemical. Thus, for each compound one by one it is necessary to evaluate whether it is present below or above such a threshold. Thus, both concepts

require that the composition of the mixture of interest is known. In reality, however, this is almost never the case. For all practical purposes, mixtures will usually not be known to their very last compound. Criteria are therefore needed to define the "relevant" components of a mixture. It is obvious, that such criteria cannot rely simply on the concentrations of the compounds in the mixture, but must also take note of the expected contribution to toxicity. However, the precise methodologies and the cut off-values that should be employed for this purpose are currently unclear.

The scientific state of the art of mixture toxicology is sufficiently advanced to make mixture risk assessment possible in a wide range of settings relevant to human toxicology and ecotoxicology. A multitude of risk assessment methods with proven practicability exists and is in use by international bodies and competent authorities within EU member states.

3. The regulatory state of the art of mixture toxicology

In this report, the regulatory state of the art in the arena of mixture toxicology has been analyzed from three different angles:

- In accordance with the tender specifications, 21 existing EU Directives and Regulations were assessed with respect to their scope of dealing with mixtures and combined exposures (**Part 2** of this report).

- Current approaches to handling mixtures and combined exposures in risk assessment and regulation by competent authorities in EU member states were compiled and assessed (**Part 3** of this report).

- Approaches to mixture toxicity assessment used in competing economies (USA, Japan) or international agencies were described (**Part 4** of this report).

As before, salient points can be summed up by using pertinent questions from a risk assessment perspective as the organising principle:

Is mixtures risk assessment not widely practiced in the European Union, because many commercial products are in effect mixtures of chemicals?

Many products that are the subject of EU Directives and Regulations are in fact mixtures of chemicals, as are the commercial preparations that reach the market. Regulatory toxicity assessments of such commercial mixtures are based on safety assessments of individual ingredients, on whole mixture testing, or on component-based approaches which assume *dose (concentration) addition* or the simple summation of the amounts of individual toxic chemicals in the preparation. Which of these approaches is applied depends on the type and use of products and the relevant pieces of legislation.

However, assessments of cumulative risks to humans and the environment resulting from simultaneous or sequential exposure to multiple chemicals from different sources via multiple routes are outside the scope of the Regulations that were examined in this project (Part 2, 2.1 – 2.21).

Which EU Directives and Regulations deal explicitly with the effects of simultaneous exposure to multiple chemicals?

Four out of the 21 pieces of legislation that were examined in this project appear to be particularly noteworthy from a mixture toxicity perspective (Part 2):

- Although Regulation (EC) No 1907/2006 (REACH) mainly focuses on individual chemicals it provides guidance on how substances that are in fact mixtures (isomeric mixtures, MCS (multi-constituent substance) and UVCB (substance of Unknown or Variable composition) such as petroleum products or surfactants) are to be assessed for their PBT/vPvB properties.

- Regulation 1272/2008 on the classification, labeling and packaging of substances and mixtures makes detailed prescriptions for the toxicity assessment of intentionally prepared commercial mixtures. The approaches prescribed are (i) whole mixture testing (ii) *dose (concentration) addition* (iii) the summation method, which is the toxicity-weighted summation of the relevant mixture components and the subsequent analysis whether or not the relative amount of relevant components is above or below a pre-defined threshold.

- Regulation (EC) No 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin provides incentives for the development of methodologies for mixture risk assessment. The task of developing viable assessment methods has been assigned to EFSA.

- Directive 2008/1/EC concerning integrated pollution prevention and control (IPPC) refers to the directive on waste incineration as a complementary piece of legislation, and this in turn includes emission limit values for mixtures of dioxins and furans that are based on the toxicological concept of Toxic Equivalence Factors (TEF).

What approaches are used by competent authorities in EU member states?

Many environmental authorities and collaborating research institutions in EU member states have extensive experience with whole mixture testing approaches. In particular these approaches are used for toxicity assessments of waste water, and waste water treatment plant effluents, for the control of emission permits under IPPC. They are also applied to practically all other types of environmental samples for the purpose of general environmental monitoring, risk assessment of contaminated sites, priority setting for risk reduction measures, and the control of remediation works and their success (Part 3, 4.2 – 4.5).

The TEF concept for the assessment of mixtures of dioxins, furans, and dioxin-like PCBs is a component-based approach in routine application. Uses of other component-based approaches or the application of the TEF approach to other groups of compounds are typically confined to special compound groups such as phenols, PAHs, and estrogens. Certain national research institutions actively engaged in the field of mixture toxicology directly support their environmental authorities. These institutions have experience with practically all types of approaches to mixture testing and assessment and they apply those flexibly to specific issues. Examples are the National Institute of Public Health and the Environment (RIVM) in the Netherlands or INIA, Division of Ecotoxicology and Environmental Risk Assessment, in Spain.

How do EU practices in mixtures risk assessment and regulation compare with approaches taken in major competing economies, especially the USA and Japan?

Of the major competing economies of the EU, the United States of America employs the most advanced approaches to mixture risk assessment and regulation, whereas the activities in Japan are rather limited. In relation to the USA and Japan, the EU takes a middle position (Part 4, 3.1 – 3.4, 4.1 – 4.3).

A major driver for mixture risk assessment in the USA has been the authorization under the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) which covers incidents with hazardous materials and mandates the US Environmental Protection Agency (US EPA) Superfund programme to clean up the highest priority sites contaminated with chemicals. The exposure scenarios normally encountered at such sites require consideration of scores of chemicals that reach exposed subjects by a variety of uptake routes, and potentially result in more than one adverse health outcome.

A second major stimulus for the practice of cumulative risk assessment in the USA has been the passing of the Food Quality Protection Act in 1996. The act mandates the assessment of risks from mixtures of pesticides with common modes of action, from any source. In response, US EPA has developed sophisticated guidelines to help decide which pesticides should qualify for inclusion in common mechanism groups. The agency has acknowledged the weaknesses of this approach which it identifies as including the omission of other chemicals that might also induce the effect of interest, although by different mechanisms.

To adequately respond to the challenges posed by such complex exposure scenarios, it is the declared intention of cumulative risk assessment in the USA to develop approaches that allow evaluations of the effects of multiple chemicals:

- Via multiple routes,
- over multiple time frames,
- which give rise to multiple adverse health outcomes.

This contrasts with the situation in the EU, where the term “cumulative risk assessment” is often applied to multiple exposure routes of single chemicals, but not to mixtures of chemicals.

In comparison with the EU and the USA, the Japanese Government is considerably less active in the area of mixtures risk assessment. No guidance documents relevant to the issue could be located. However, various governmental organizations acknowledge the need for developing test assays that allow the assessment of risks from complex environmental mixtures, in a whole mixtures approach (Part 4).

Is there guidance from major international bodies in terms of approaches to applying mixtures risk assessment and regulation in practice?

The World Health Organisation (WHO) and its allied International Programme on Chemical Safety (IPCS) are the main drivers behind the development and refinement of the TEF approach for the assessment of mixtures of dioxin-like chemicals. The equivalency factors which are constantly updated have a major impact on the practice of national governments when it comes to the risk assessment of dioxins and related chemicals.

Very recently, IPCS issued a workshop report aimed at developing a framework for consideration of risks from combined exposures (Part 1, 5.1). The application of this framework is intended as an iterative process which involves step-wise consideration of exposures and hazards in several tiers, depending on the data available to support the analysis. The analysis begins with a consideration of the potential for cumulative exposure, before any assessment of hazards take place. In its earliest tier, the IPCS report recommends adopting *dose (concentration) addition* if there is no evidence for synergisms or antagonisms. Chemicals to be subjected to this procedure should be grouped according to their chemical structure, similarity of target tissue and/or similarity in the manifestation of toxicity.

Should the combined risks turn out not to be acceptable, the assessment should be refined further by additional considerations of temporal aspects of the common toxic effect, the presence of a common metabolite, analysis of key biological targets and consideration of information about environmentally relevant mixture ratios and exposure levels.

The UN Globally Harmonised System for Classification and Labelling of Chemicals (GHS) provides detailed guidance on the classification of commercial mixtures for human health and the environment. It is the basis for the European Regulation on classification, labeling and packaging of substances and mixtures (CLP). Mixture toxicity assessments under both systems are virtually identical.

4. Recommendations

European guidelines for the assessment of chemical mixtures

Scientific research has repeatedly demonstrated that the effects of mixtures are considerably more pronounced than the effect of each of its individual components and that environmental pollution is from chemical mixtures and not from individual substances. This clearly underlines the need for dedicated regulatory considerations of the problem of chemical mixtures in the environment. For this purpose, guidelines for the assessment of chemical mixtures, which are not available today, would prove extremely helpful for application throughout the entire European Union.

Current mixture guidelines, as for example those issued by the US EPA or the recently suggested WHO guidelines, are limited to the assessment of potential human health risks from chemical mixtures. In contrast, the European regulatory system considers the protection of the environment as being equally important. A future European guideline for the assessment of chemical mixtures therefore should go beyond the reach of currently existing regulatory approaches and should extend its scope to the protection of ecosystem structure and function from the detrimental effects of chemical mixtures.

The review of the scientific state of the art shows strong similarities between the results gained from human toxicology and from ecotoxicology. Hence, a future European guideline could be built around a core of common, integrative tools, methodologies and approaches, which then branch out towards the specific consideration of sectorial issues, specific endpoints and specific environmental compartments. Mixture assessment guidelines that integrate human health effects and ecosystem integrity are a novelty, and the EU is uniquely placed to become a world leader in this area.

Strengthening the legal mandate for mixtures risk assessment in the European Union

The analysis of the scientific state of the art of mixture toxicology (Part 1) in this report shows that there is both the need as well as sufficient know-how, to assess the risks that may result from the combined exposure of humans and the environment to multiple chemicals. The question as to how this scientific knowledge might be best transferred into appropriate regulatory approaches is, however, not at all trivial.

The development of appropriate procedures and methodologies that are adequate in a specific legal context may require considerable additional efforts. As detailed in Part 4 of this study, the US EPA for instance spent many years on the development of its guidelines for the health risk assessment of chemical mixtures, and this would not have happened without an explicit legal mandate that required the agency to do so. In Europe, since 2006, EFSA has been working on a methodology for assessing cumulative risks that may result from human exposure to combinations of pesticide residues, taking advantage of the work previously carried out in the US. Multiple pesticide residues in food had been an issue of concern and debate over many years before, but the targeted development of corresponding risk assessment methods for regulatory use did not start before a clear

legal incentive was given in the pesticide residues regulation upon the initiative of the European Parliament in 2005. A lesson to be drawn from these events is that consistent and clear mandates are needed for taking mixture toxicity into account in the numerous pieces of legislation that contribute to the protection of human health and the environment from chemical risks. This seems to be an essential prerequisite for better dealing with the challenging issue of potential "cocktail effects".

Exploring options for the assessment of combined exposures within media oriented pieces of environmental legislation

Most of the 21 Directives and Regulations examined in Part 2 of this report are substance- or product-oriented pieces of legislation. They control single and multi-constituent substances, preparations of chemicals and products containing chemicals that are intentionally produced and placed on the market. Typically, they assess hazards and risks of these substances and products as if they were present in isolation. The assessment of complex exposure situations of humans and the environment resulting from multiple substances and products is out of their scope and difficult to integrate.

Mixture risk assessments require a definition of the mixture of concern. Substance- and product-oriented regulations are therefore appropriate for assessing mixtures that are already present in such substances or products. Process-oriented pieces of environmental legislation that control emissions from production, transportation, and recycling processes, such as the IPPC, provide a basis for assessing mixtures of chemicals released from a definite source. The best starting point for assessing those mixtures that finally occur in environmental media, in biota, and in humans, however, should be given by corresponding media-, site-, or population-oriented elements of legislation, such as for instance the Water Framework Directive, the Marine Strategy Directive, or the proposed Soil Directive. These types of legislation were outside the scope of this report. Options for the advancement of these pieces of legislation with the aim of taking account of, and improving, risk assessments of realistic complex exposure scenarios should be explored.

Application of concentration (dose) addition as the default assessment concept for mixture effects in tiered approaches

A particularly important commonality of toxicological and ecotoxicological studies is the high predictive power of *dose (concentration) addition* for a considerable range of endpoints, organisms and chemicals. As *dose (concentration) addition* is also typically the more conservative concept, it is recommended to employ this evaluation method as a default first tier approach for the assessment of chemical mixtures in general. Depending on the available knowledge, the resources at hand and the specific protection goals in a particular setting, finer and perhaps more realistic instruments with a higher demand in terms of input data can then be applied in subsequent tiers. Such a tiered approach seems to be generally compatible with the new GHS system, with the recently suggested IPCS approaches.

Ensuring that the generation of toxicity data is amenable to future mixture effect evaluations

Regulatory efforts on chemical mixtures that go beyond the mere testing of tissues or complex environmental samples depend on results from single substances assessments such as those conducted under REACH. It is therefore imperative to ensure that single substance studies and assessments are properly documented in a coherent and uniform way, independent of the specific regulatory area in which they were conducted. Only then will it be possible to exploit our knowledge of the toxicity and ecotoxicity of individual substances for subsequent mixture risk assessments. Furthermore, this dual-use of single substance data should already be considered when designing and implementing studies for the risk assessment of individual chemicals. Specifically, this calls for the use of benchmark doses instead of using NOAELs or NOECs as the preferred method for defining thresholds of regulatory concern and points of departure. The main reason for this demand lies in the characteristics of NOAELs and NOECs: unlike benchmark doses, they are not fixed values, but highly dependent on the experimental design employed during toxicity studies. Furthermore, NOAELs and NOECs are associated with varying effects, depending on the statistical resolving power of the underlying experimental studies, and this makes their use as input data for *dose (concentration) addition* questionable.

Research needs

More information on typical exposure situations with respect to chemical mixture needs to be compiled and systematized. Beyond the lists of priority chemicals that are currently defined in certain areas, we need to know priority chemical mixtures that are present in the environment and might have an impact on human health and ecosystems. Furthermore, our understanding of the determinants of synergistic effects needs to be improved scientifically, with a view of being able to anticipate synergisms in the future.

Overall conclusions

The scientific state of the art of mixture toxicology has been advanced significantly, not least as a result of EU-funded research. It shows that mixture risk assessment in the EU is not only necessary, but also feasible. It is necessary in order to avoid underestimations of risks that might occur under the current paradigm of considering substances on a chemical-by-chemical basis. It is feasible, as demonstrated by the practice in the USA and other countries. Because the protection of human health and the environment are goals of equal importance in EU regulations, Europe is uniquely placed to set the agenda world-wide for a truly integrated mixture risk assessment, provided there is the political will.

State of the Art Report on Mixture Toxicity

Final Report

Part 1: The state of the art of mixture toxicology - a critical appraisal of published scientific literature

22 December 2009

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Contractor	**The School of Pharmacy** **University of London (ULSOP)**
Sub-Contractors	**Göteborg University (UGOT)** **Faust & Backhaus** **Environmental Consulting GbR (FBEC)**
Responsible Scientists	**Prof. Dr. Andreas Kortenkamp (ULSOP)** **Assoc.-Prof. Dr. Thomas Backhaus (UGOT)** **Dr. Michael Faust (FBEC)**

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1. Summary

Based on the literature searches conducted during the previous reporting period, with additional information derived from an analysis of EU projects, conference publications and opinions of relevant EU Scientific Committees, a critical review was prepared with the aim of summarizing the current state of the art of mixture toxicology and ecotoxicology.

1.1 Basis of the report

The report is based on empirical findings of the effects of combinations of chemicals after simultaneous or sequential exposure, relevant to human and mammalian toxicology, and to ecotoxicology.

In the section on human and mammalian toxicology, the available evidence was grouped according to major endpoints of toxicological relevance, including carcinogenicity, mutagenicity, genotoxicity, respiratory toxicity, reproductive toxicity, endocrine disruption, immunotoxicity and neurotoxicity. Publications focusing on specific groups of chemicals were also reviewed. These included mixture experiments with metal compounds and with dioxin-like chemicals.

The available evidence on the ecotoxicology of chemical mixtures was collected for a range of different environmentally relevant chemicals: simple industrial chemicals, surfactants and pesticides as major potential pollutants of freshwater aquatic ecosystems, heavy metals as important soil contaminants, and antifouling biocides as an emerging group of compounds with a potentially substantial impact on marine ecosystems. Additionally, the joint ecotoxicity of pharmaceuticals was evaluated because – although the individual concentrations of those compounds can often be considered too low to cause any environmental risk – a broad range of different medicinal compounds is always simultaneously used and emitted in any given area. Hence, potential mixture effects of those compounds were considered of special importance.

1.2 The state of the art of mixture toxicology

During the last ten years, mixture toxicology has undergone a remarkable and productive development. While the earlier experimental studies focused mainly on mixtures composed of only two chemicals, the planning, conduct and assessment of multi-component mixtures is now possible, with clear results. This has extended from *in vitro* assays to *in vivo* studies and even to analyses of mixture effects on species communities.

Published mixture studies were mainly conducted with one of the two following aims:

- to evaluate and quantify the overall toxicity of complex environmental samples (whole mixture approach), or
- to explain the joint action of selected pure compounds in terms of their individual effects (component-based approach).

1.3 Prediction and assessment of mixture effects

One of the key aspirations of mixture toxicology has always been to anticipate quantitatively the effects of mixtures of chemicals from knowledge about the toxicity of its individual components. This can be achieved by making the assumption that several chemicals act in concert by exerting their effects without diminishing or enhancing each others toxicity, the so-called non-interaction or additivity assumption. Two concepts are available for the formulation of the null hypothesis of additivity: *concentration (or dose) addition* (CA, DA) and *independent action* (IA).

The study of mixtures composed of chemically pure agents, in laboratory settings, has yielded a considerable body of evidence showing that CA provides a sound approximation of experimentally observed additive combination effects. Comparatively few examples exist where IA produced valid additivity expectations. No case could be identified where IA predicted mixture effects that were larger than those derived from CA, and were at the same time in agreement with experimental observations.

Determinants of additive joint action of chemicals are fairly well established. Factors that might lead to deviations from expected additive effects, indicative of synergisms or antagonisms, are also quite well understood, although the magnitude of such deviations cannot be predicted quantitatively. Toxicokinetic interactions are one established cause of deviations from additivity.

1.4 Mixture studies relevant to human and mammalian toxicology

Scores of experimental mixture studies with a focus on human and mammalian toxicology have been carried out without explicit additivity expectations. It was often implicitly assumed that the joint effects of a mixture should be equal to the arithmetic sum of the effects of its components (effect summation). However, this approach is unreliable when the underlying dose-response relationships are non-linear. Frequently, the design of these studies has made it difficult to judge whether the observed effects were in line with CA or IA, or deviated substantially from expected additivity, suggesting synergism or antagonism. There are examples of claims of synergistic effects, but the observed effects may well have been in line with dose addition or independent action. Mixture studies with carcinogens, mutagens and genotoxic agents, immunotoxic chemicals, respiratory toxins and neurotoxins have frequently employed concepts of synergism that are not compatible with evaluations of combination effects in terms of stronger than additive effects according to CA or IA. In these fields, the term "synergism" is often used simply to describe that chemicals work together in mixtures. This concept of synergism does not make quantitative judgements in relation to additivity expectations. The word "synergism" is also often used to describe the phenomenon where combination effects arise although each individual substance is present at doses which do not exert responses. Although many of the studies emanating from these fields preclude assessments of mixture effects in terms of agreement with CA or IA, there is a large body of evidence to show that chemicals usually act together when present as mixtures.

Furthermore, significant combination effects have often been demonstrated to occur when the single mixture components were present at doses without observable effects.

There are numerous mixture studies involving reproductive toxicants and endocrine disrupters, including dioxin-like chemicals, which allow identification of combination effects in terms of additivity, synergisms or antagonisms. Multi-component mixture experiments have been conducted, involving over ten components, often applying the fixed mixture ratio design. In vitro assays and in vivo studies have been utilized.

Dose addition has often provided good approximations of the experimentally observed mixture effects. However, notable is a lack of studies where independent action could have expected to yield reliable additivity expectations. Very rarely have both concepts been evaluated side-by-side, in one and the same study.

There are some recent examples of studies aimed at examining mixtures modelled on exposures found in scenarios relevant to humans.

Cases where the observed mixture toxicity deviated significantly from expected additivity, indicating synergisms or antagonisms, are rare. Where these occurred, few attempts have been made to explain the observed deviations in terms of mode of action or mechanisms.

1.5 Mixture studies relevant to ecotoxicology

The majority of the studies with an ecotoxicology focus have analysed defined mixtures composed of only two compounds. Comparatively few studies analysed the joint action of more than two chemicals (up to 50 compounds in one case).

Most reviewed studies were conducted with simple aquatic bioassays with bacteria, algae or daphnids. Several studies used fish, fewer worked with terrestrial invertebrates such as earthworms or collembola. Studies with other groups of invertebrates such as molluscs or insects were confined to whole mixture studies. Mixture experiments with natural or artificial biocoenoses looked at aquatic communities only.

Mixture components were usually selected from within a specific class of compounds, defined either chemically, pharmacologically or on the basis of their use pattern. Studies with mixtures composed of compounds with different mechanisms of action or from different types of chemicals are extremely rare.

Typically, the observed mixture toxicity was either implicitly or explicitly compared to the expected mixture toxicity according to concentration addition. Only a few studies compared the observed toxicity of the mixture with predictions derived from independent action.

When both concepts were comparatively evaluated in the same study, concentration addition provided a slightly more conservative mixture toxicity estimate in the vast

majority of cases. The mixture EC50 predicted by concentration addition was usually by a factor of not more than 5 lower than the EC50 predicted by independent action.

Claims of synergisms or antagonisms were frequently explicitly or implicitly made in the sense of "more (or less) toxic than expected by concentration addition". The pharmacological and/or physiological reasons for the observed deviations were usually not investigated. Two different types of comparisons were found in the literature: either the predicted and observed *mixture effects* were compared, or predicted and observed *mixture effect concentrations* such as EC50 values were contrasted with each other. Claims of "strong" or "remarkable" synergisms were only made on the basis of the first type of comparison, the evaluation of predicted and observed effects. These were restricted to mixtures with 2-3 compounds only. When effect concentrations were compared, most deviations (synergisms and/or antagonisms) were within a factor of 3 of the EC50 predicted by Concentration Addition. In none of the documented multi-component mixtures, mixture toxicities higher than predicted by concentration addition were found.

Typical mixture designs were isobolographics, point-, fixed-ratio and surface designs. The latter design approach has only been applied to binary mixtures. Full or fractional factorial designs were rarely used.

Comparatively few studies have bridged the gap between evaluating complex environmental mixtures and component-based approaches by applying a hybrid of both methods for stressor diagnosis in mammalian toxicology and in aquatic or terrestrial ecosystems.

1.6 Mixture effects at low doses of mixture components

Already in the 80's the first ecotoxicological studies with fish and daphnids demonstrated that low concentrations of industrial chemicals associated with negligble or no discernible effects when applied singly may add to severe mixture effects. A range of follow-up studies have corroborated and extended these early findings to mixtures of specifically similarly acting pesticides and biocides and to bioassays with other aquatic organisms. Subsequently, it could be shown that this phenomenon is not restricted to combinations of similarly acting chemicals. Multi-component mixtures of dissimilarly acting substances where each component was present at its no-observed-effect-concentration (NOEC) were not without effect and even concentrations well below the individual NOECs may lead to clear ecotoxicological effects when they act simultaneously on exposed organisms.

Subsequently, several human-health oriented, toxicological mixture studies have been designed to assess whether combination effects occur when chemicals are combined at low doses – sufficiently low to be without observable effects when tested on their own. Often, these doses were in the range of those commonly used to derive estimates of safe human exposures (so-called points of departure, usually no-observed-adverse-effect-levels, NOAELs, or benchmark doses).

For combinations composed of chemicals that interact with the same sub-system of an organism, there is good evidence that mixture effects can arise at doses around, or below, points of departure. Considering the main assumptions underlying the concept of dose addition, this is to be expected.

There is good evidence that the same is true for combinations composed of chemicals with diverse modes of action, where independent action produced valid additivity expectations. This is at variance with the widely held view that mixtures of dissimilarly acting chemicals are safe, as long as exposure to each component does not exceed its individual point of departure.

1.7 Implications for regulation and risk assessment

The empirical evidence in human toxicology as well as ecological toxicology strongly supports the need to take mixture effects into consideration during the estimation of acceptable human and environmental exposures. Mixture effects were repeatedly demonstrated with combinations at doses or concentrations around points of departure, including NOECs.

With CA (DA) and IA two concepts have been developed in the scientific literature and both have been proven to predict certain types of mixtures very well (see above). However, both concepts assume artifical situations – mixtures composed entirely of similarly, respectively entirely of dissimilar substances – that might not be fullfilled by most real-life mixtures. Hence, two basic options exist for the application of CA (DA) and IA for regulatory purposes: (a) the *a priori* choice of one concept as a default approach and (b) a case by case selection of the most appropriate concept for each mixture. For implementing mixture toxicity assessments into regulation, it is of paramount importance, to analyse whether and how these options are applicable.

It has been suggested to use CA (DA) as a first, pragmatic default approach for describing the joint action of chemicals for regulatory purposes in risk assessments. In view of the available evidence, this proposal appears well founded. It should be noted, that this *modus operandi* does not deny the existence of significant synergistic or antagonistic interactions between certain mixture components, nor does it claim that the joint action of all mixtures can be precisely described by CA (DA). Biology is certainly far too complex and dynamic to be reduced to such a simple concept as concentration addition, especially when considering the reaction to an exposure toward compounds that act on dissimilar receptors, processes and physiological pathways. But deviations from expected mixture toxicities seem to be quite rare, comparatively small (usually within a factor of not more than 3 when predicted and observed EC50 values are compared) and seem to be largely limited to mixtures with only a few compounds.

Strongly connected to the issue of making choices about evaluation concepts for mixture effects is the question which chemicals should be grouped together for purposes of combined risk assessment, and which criteria should used to decide on groupings.

"Toxicological similarity" of chemicals is the criterion for grouping proposed by U.S. EPA (2000) and other international bodies. Extensive guidance exists about how this should be implemented. For example, pesticides and other chemicals are considered to qualify for inclusion in a common group when their mechanism of toxicity shows similarities in both nature and sequence of major biochemical events. The use of toxicological similarity based on mechanisms, however, may lead to overly narrow groupings. Recent alternative proposals therefore recommend adoption of a broader based move towards establishing grouping criteria by focusing on common adverse outcomes, with less emphasis on similarity of mechanisms. This is in recognition of emerging evidence that biological effects can be similar, although the molecular details of toxic mechanisms - including metabolism, distribution and elimination – may differ profoundly in many respects.

Numerous mixture risk assessment methods are available, including the Hazard Index (HI), Toxic Unit Summation (TUS), Point of Departure Index (PODI), Relative Potency Factors (RPF) and Toxicity Equivalency Factors (TEF). To take account of differing data quality (data rich vs. data poor situations), tiered approaches to mixture risk assessment have been proposed.

1.8 Knowledge gaps

Concentration addition and independent action were conceptually developed and validated for chemical mixtures. Although several recent studies were published that employed these concepts also for describing the joint action of chemical and physical stressors, such as oxygen depletion or drought, the conceptual basis and implications of such studies are far from clear.

Mixtures in the environment are usually composed of multiple components from a range of sources with dissimilar chemical structures and modes of action. Unfortunately, this is exactly the type of mixture that has been least frequently studied. Hence, more empirical evidence on the joint action of environmentally realistic mixtures, composed of agents from different chemical and functional classes are needed in order to further substantiate the conjecture that concentration or dose addition might be applicable as a general "rule of thumb" for describing the joint action chemical mixtures and to explore its limitations.

In this context, it would be especially valuable to obtain further insights into the question as to whether low, individually non-toxic concentrations of dissimilar compounds might lead to a significant mixture effect. This question is of major importance, because of its direct relevance for the question of environmental quality standards. However, only two studies, both from of aquatic toxicology and both using unicellular organisms and specifically designed "artificial" mixtures are documented in the literature.

Organisms are not only exposed to mixtures of chemicals simultaneously, but also sequentially to pulses of contaminants that enter an ecosystem e.g. after run-off events or

pesticide application. Concepts and approaches to dealing with sequential exposures are in their infancy, and very few examples of experimental studies in this area are available.

A bottleneck of major relevance is in the absence of exposure assessment strategies that take account of multiple exposures.

2. Organisation of the review

With an estimated 70,000 industrial chemicals marketed in the European Union alone, it can be anticipated that very large numbers of substances occur together in ecosystems, food webs and human tissues, all at quite low levels. Multiple exposures may result from the intended use of chemicals in personal care products, as pesticides or pharmaceuticals, or from unintentionally contaminated media, e.g. residues in food and feed or pollutants in groundwater. Growing recognition of the dynamic nature of chemical exposures has prompted considerable scientific interest in investigating the consequences of combined exposures to chemicals. There are also increasing calls that chemicals risk assessment and regulation should be modified to take account of simultaneous exposures to several chemicals. The justification often given for considering mixture effects in risk assessment is the concern that the effects of a mixture might be greater than those of each of its components alone.

This review was carried out with the intention of providing a critical appraisal of the experimental evidence for mixture effects of chemicals. In dealing with the empirical findings published in the literature, attention was given to three key topics:

- Can reliable predictions of the effects of mixtures be derived from data of the toxicity of individual components?
- Are risks to be expected from exposure to multiple chemicals at low doses?
- Is there evidence that chemicals exacerbate each others effects, leading to synergisms, and which factors determine the potential for synergisms?

These three issues define a framework for the structuring of the scientific evidence in this critical appraisal. The review is organised into the following sections:

In Section 3 of this Part, key terms of mixture toxicology are defined and approaches to investigating toxicological mixtures outlined. This sets the stage for dealing with the concepts that are used to derive predictions of mixture effects on the basis of the toxicity of individual components. A description of the features of these concepts is given, together with an analysis of their implications, particularly in relation to toxicological modes of action and mixture effects at low doses.

Section 4 provides an analysis of empirical findings of mixture effects with relevance to human and mammalian toxicology. The material is organised according to toxicological effects and endpoints, and, where relevant, according to certain groups of chemicals or compound classes that make up mixtures.

Section 5 summarizes experimental findings with relevance to ecotoxicology. It is organised in ways similar to the preceding section.

Section 6 is an appraisal of mixture studies where chemicals were combined at low doses, close to those used in regulatory toxicology for the establishment of human exposure levels.

The implications of the scientific evidence on mixture toxicology for toxicological risk assessment and regulation are discussed in Section 7

.

Finally, Section 8 identifies knowledge gaps relevant to risk assessment and regulation and gives an outline of research needs.

3. Concepts, designs and experimental strategies for investigating the joint toxicity of chemical mixtures in toxicology and ecotoxcology

3.1 Definition of key terms

Key terms in mixture toxicology are often used in different ways, with varying meanings. In the interest of clarity, we define the meaning of frequently used expressions and concepts, as used in this review.

Mixture: A mixture is a combination of several chemicals with which organisms come into contact, either simultaneously, or sequentially. A binary mixture is a combination of two agents. The term "complex mixture" is used to denote a mixture of unknown composition, isolated from environmental media or other sources. "Complex mixture" is sometimes used to describe combinations composed of three or more chemicals, but for the purposes of this review, the term "multi-component mixture" is preferred.

Mixture effect, combination effect, joint effects: The response of a biological system to several chemicals, either after simultaneous or sequential exposure. The terms are used synonymously.

Additivity: In the context of mixture toxicology, additivity cannot be equated with "additivity" in the mathematical sense. It refers to a situation, termed "non-interaction", where the toxicity of a mixture resembles the effects expected to occur when all mixture components act without diminishing or enhancing their effects. Additivity expectations for mixtures can be derived from the concepts of dose addition and independent action (see 4.1 and 4.2). In certain situations, valid expectations for additive combination effects can also be calculated by building the arithmetric sum of the individual effects of all mixture components ("effect summation").

Non-interaction, Interaction: Non-interaction is thought to occur when the observed effects of a mixture is the result of all components exerting their effects without interfering with the way all other chemicals act. The case of non-interaction is usually described by the additivity expectation of a suitable prediction concept (dose addition, independent action or effect summation). Interaction is thought to have arisen when the observed mixture effects deviate from what was expected. In this case, one or several compounds are likely to have interacted with each other, e.g. by facilitating or diminishing each others uptake, transport, metabolism or excretion. "Interaction" is the umbrella term for synergisms (mixture effects greater than expected) and antagonisms (mixture effects smaller than expected).

Potentiation: A situation where one chemical greatly exacerbates the effect of another agent, without itself producing the effect of interest.

Mechanism of action: Molecular sequence of events that produce a specific biological outcome.

Mode of action: A plausible hypothesis about measurable key events by which a chemical exerts its biological effects. Mode of action is not intended to build a comprehensive model of a chemical's actions. It is often confused with mechanism of action, or used in overlapping ways. Mode of action can include mechanisms of action, but is considered to be broader.

Mechanism-free approaches to evaluating mixture effects: When constructing additivity expections (either according to dose addition, independent action or effect summation), the input values are data about the dose-response relationships of individual mixture components. At this level of analysis, mechanistic considerations are of no relevance. Berenbaum (1981) has used the term "mechanism-free approaches" to emphasize this fact, with the intention of distinguishing other approaches, where attempts are made to understand the effects of a chemical (and of mixtures) by adopting modeling from first principles.

Doses and concentrations: The dose of a compound or mixture is understood as the amount that is taken up by an organism (derived from the Greek word which means "that what is given"). Dosage is dose per unit body weight over a defined period of time. Certain dosages result in certain concentrations of substances at or near their target site, i.e. within the body of the exposed organism. The term "concentration" is understood in a more general way and can refer to the amount per unit volume of the test chemical(s) at the target site, the sourrounding medium (water, air, soil) or the food that a test organism ingests.

Point of Departure: The point of departure (POD) is a dosage or concentration of a single chemical used in regulatory (eco)toxicology for estimating tolerable exposures to humans or ecosystems. Often, no-observed-adverse-effects (NOAELs) or no-observed-effect-concentrations (NOECs) 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 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.

3.2 Whole mixture approaches

In general, methods for mixture studies can be divided into 2 major classes, "whole mixture approaches" and "component based" approaches. Methods that use a whole mixture approach are based on the direct toxicological assessment of a given chemical mixture, such as a complex environmental sample, an engine exhaust or a human blood sample. They closely resemble the assessment of individual chemicals and do not require new, mixture-specific methodologies. Furthermore, as the whole mixture is bio-assessed, the effects of all compounds that are present in a complex sample are accounted for. Any synergistic or antagonistic interactions between the compounds are inherently captured in the observed responses of the exposed organisms. Hence, whole mixture approaches are often applied in situations where only a fragmented knowledge on the chemical composition is at hand, e.g. because no chemical-analytical methods for the involved

compounds are developed or because the available resources in terms of finances and time that can be devoted to a particular sample are limited. Whole mixture approaches have found widespread application in the area of whole effluent testing (e.g. La Point & Waller 2000; Antunes, Pereira, & Goncalves 2007; Thorpe et al. 2006; see also the review by Chapman 2000). In this context, whole mixture approaches are also incorporated in the BRF (best available technique reference) documents for the IPPC-directive (2008/1/EC) for large volume organic chemicals and waste water.

Whole mixture approaches have several appealing characteristics, but also severe limitations. Obviously, the mixture itself has to be available for a direct experimentation, which makes this approach largely unsuitable for prospective approaches such as the setting of environmental quality standards. Furthermore, the obtained results are strictly speaking only applicable to the actually investigated mixture; extrapolations to different exposure situations, especially from high to low doses, pose a range of difficulties (Gennings et al. 2000). As the exposure situation in the environment is highly dynamic, whole mixture approaches thus require frequent re-testing.

A closely related approach is to draw conclusions from documented analyses of similar mixtures. For example, the US EPA uses this methodology for estimating the risk for different combustion processes (Teuschler & Hertzberg 1995). Employing this approach implies that reliable data for a mixture are at hand that is judged to be sufficiently similar in its chemical composition and consequently in its (eco)toxicological properties to the mixture of interest. This situation is rare and hence argumentation by analogies is often not an option. Furthermore, there is a considerable dynamic in the number of pollutants and their concentrations and thus a virtually unlimited number of different mixtures, which further hampers the application of this approach for the assessment of environmentally relevant mixtures.

A means to gain further insight into the behaviour of a chemical mixture is based on physiolocally based pharmacokinetic/pharmakodynamic modelling (PBPK/PD) modelling. As the name implies, this methodology strives to model the uptake and distribution of chemicals in an organism. Therefore these models are highly specific for a particular animal and require detailed knowledge on its physiology, such as for example the exposed skin surface or the alveolar ventilation rate. Also specific data on the involved mixture components are needed, such as blood/air, blood/tissue partition coefficients and metabolic rate constants. (Krishnan et al. 1994) lists some 45 parameters that build up these models. In view of these huge knowledge demands, this approach has been restricted to toxicological studies with particular animal test systems and selected mixtures (Krishnan, Andersen, Clewell, & Yang 1994; Verhaar et al. 1997; Yang et al. 1995). Due to their strong mechanistic foundation, PBPK/PD modelling approaches lend themselves to a detailed mathematical description of the interactions between chemical mixtures and exposed biota and have therefore put forward for the development of an in-silico toxicology of chemical mixtures (Mayeno, Yang, & Reisfeld 2005; Yang et al. 2004).

3.3 Component based approaches

Many of the limitations of whole mixture approaches can be overcome by making inferences from the effects of the mixture components to their joint action. This can be done purely empirically, i.e. by testing all possible combinations of the components in a mixture.

The relationship between the concentrations of the individual toxicants and the intensity or frequency of effects that they provoke as a mixture results in a *n+1* dimensional hyperplane, with *n* being the number of mixture components. For binary combinations this hyperplane is a 3-dimensional concentration-response surface (Figure 3.1). The hyperplane of multi-component mixtures is beyond simple visualisation.

Simply exploring this hyperplane by experimentation does not require the assumption of a specific relationship between single substance and mixture effects, but needs a fairly exhaustive experimental effort especially for multi-component mixtures. A straightforward experimental approach would be to record experimental data that are evenly distributed so that the complete response surface of the mixture is accounted for. Using appropriate interpolation techniques, an empirical model can then be developed that allows estimating the mixture effect as a function of the amounts of the individual components (see below). Such approaches have a long standing in the optimisation of industrial processes and chemical products such as food products, pharmaceuticals or pesticide preparations, as the number of components to be considered is typically comparatively small and can be kept constant (Cornell 2002). They are used in a purely empirical way, i.e. without any expectation on the topology of the resulting concentration-response surface. They allow the analysis of a broad range of mixture ratios and effect levels, but conclusions are restricted to a given set of components. This seriously hampers the applicability of such purely empirical approaches for environmental hazard and risk assessment with its multi-component mixtures of varying compositions.

3.3.1 Two fundamental mixture concepts

A conceptually sound approach that links the individual components with the effects of the mixture by assuming additivity would allow predictions of mixture toxicities, without the need to systematically test a (sometimes overwhelmingly large) number of mixture ratios and mixture concentrations. It would also make it possible to draw more general conclusions about the relationship between single substances and mixture toxicities. Numerous methods and approaches for this purpose have been described in the literature.

Often, it is implicitly assumed that the anticipated combination effect is accessible by calculating the simple arithmetic sum of the individual effects of all chemicals. However, the fallacy of this expectation becomes obvious when the case of 10 agents is considered that each provoke, say, 15% of a certain response. The expectation that the resulting joint effect should be 10 x 15% = 150% turns out to be biologically impossible, if the maximally inducible effect is only 100%. This “Effect Summation” approach is hence

considered invalid for most biological mixture (eco)toxicity studies and therefore disregarded in the following.

The various aproaches can be traced back to two different fundamental concepts (Boedeker et al. 1992), which are often called concentration addition (CA, also termed "Dose Addition") and independent action (IA, also known as "Response Addition", "Effect Multiplication" or "Abotts Formula"), see also Table 3.1 for a comparative overview. Both concepts can also been found under various other names (Faust et al. 2001) and are implemented in a diverse set of models for predicting or assessing mixture toxicities, see compilations in (Altenburger et al. 1993; Berenbaum 1989; Boedeker et al. 1990; Grimme et al. 1994; Kodell & Pounds 1991). CA and IA describe a quantitative relationship between single substance toxicities and the toxicity of a mixture composed of these chemicals. These concepts are based on two entirely different ideas about how the joint action of chemicals can be perceived.

3.3.1.1 Concentration (dose) addition (CA, DA)[1]

CA is based on the idea that all components in the mixture behave as if they are simple dilutions of one another, which is often taken to mean that CA describes the joint action of compounds with an identical mechanism of action. It has been successfully applied to mixtures of organophosphorus pesticides, photosynthesis-inhibiting herbicides and polychlorinated dioxins and furans, and also estrogenic agents, to name but a few. When these chemicals interact with an identical, well-defined molecular target, it is thought that one chemical can be replaced totally or in part by an equal fraction of an equi-effective concentration (e.g. an EC50) of another, without changing the overall combined effect. If the assumption of dose addition holds true, these fractions of equi-effective single substances concentrations – also called toxic units – simply sum up to an overall toxic unit of the mixture. Therefore CA is also known as "Toxic Unit Summation". The concept can be mathematically formulated as:

$$ECx_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad \text{(eq. 1)}$$

with n denoting the number of mixture components, p_i being the relative fraction of chemical i in the mixture, and x is a common effect level, which is provoked by an exposure to a single substance or mixture concentration ECx_{Mix} resp. ECx_i.

In general, no explicit formulation of the CA-expected mixture effect $E(c_{Mix})$ is possible, direct calculations are restricted to the level of effect concentrations (ECx-values) (Faust et al. 2001). Only in the so-called "simple similar action" case, CA-expected mixture effects can be directly calculated. Simple similar action is a special case of CA (Hewlett & Plackett 1959) which assumes that the individual curves of the components are dose-

[1] The term "Concentration Addition" is mainly used in an ecotoxicological context while "Dose Addition" often refers to toxicological studies in which the actual dose (concentration of the test compounds at or near their site of action) is known. However, both terms are used synonymously in the following, as their conceptual basis is identical.

parallel, i.e. there is an effect-level independent constant potency factor between the individual concentration-response curves. On this condition, the CA-expected mixture effect can be explicitly formulated as:

$$E(c_{mix}) = f(\alpha, c_1 + \sum_{i=2}^{n} g_i c_i) \qquad \text{(eq. 2)}$$

where f is an appropriate concentration-response model, a a vector of model parameters and c_i the concentrations of the $i=1,\ldots,n$ chemicals in the mixture and g_i the potency factor mentioned before. The effect of a mixture that contains n components at concentrations $c_1, c_2,\ldots,c_n$ is assumed to be identical to the effect of e.g. the first compound at a concentration $c_1 + \sum_{i=2}^{n} g_i c_i$. All components behave as if they were simple dilutions by a factor g of this first chemical, hence all concentrations of component $2\ldots n$ can be re-scaled to the first chemical, independent of the considered effect level. A widely used application of this approach is the "toxic equivalence factor" (TEF) concept for the assessment of mixtures of polychlorinated dioxins and furans (PCDD/F) (van den Berg et al. 1998). Here, doses of specific PCDD/F isomers are all expressed in terms of the dose of a reference chemical, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), needed to induce the same effect ("equivalent" or "equi-effective" dose). The assessment of the resulting combined effect is obtained simply by adding up all equivalent TCDD doses.

Dose parallelism has often been used as a decision criterion on whether to apply CA to a mixture or not. But it should be pointed out here, that the general formulation of CA in eq. 1 does neither assume a specific shape of each concentration-response curve of the components, nor a specific relationship between the curves. Even if all chemicals in a mixture share an identical receptor binding site, differences e.g. in the toxicokinetic behaviour of the substances might lead to concentration-response curves that are not dose-parallel, if the responses of the exposed animals are observed on a higher, integrating level (e.g. reproduction). Also the biometrical description of the individual concentration-response data might exert an influence on the parallelism of the concentration-response curves. If all components curves are described by only one, inflexible model (such as the classic Probit model), the resulting curves might be more dose-parallel as compared to a biometrical analysis that uses more flexible models or even different models for different components (Scholze et al. 2001).

CA implies that every toxicant in the mixtures contributes in proportion to its toxic unit (i.e. its concentration and individual potency) to the mixture toxicity. Whether the individual doses are also effective alone does not matter. Thus, combination effects should also result from toxicants at or below effect thresholds, provided sufficiently large numbers of components sum up to a sufficiently high total dose. In view of the exposure situation in many environmental compartments, the verification or falsification of this conclusion has been a major topic in recent mixture toxicity studies. An overview of mixture studies that focused on this issue is given by Kortenkamp and co-workers (Kortenkamp et al. 2007).

From a mathematical perspective (see eq. 1), CA simply represents the weighted harmonic mean of the individual *ECx*-values, with the weights just being the fractions p_i

of the components in the mixture. This has important consequences for the statistical uncertainty of the CA-predicted joint toxicity. As the statistical uncertainties of the CA-predicted *ECx* is a result of averaging the uncertainties of the single substance *ECx*-values, the stochastic uncertainty of the CA-prediction is always smaller than the highest uncertainty found in all individual *ECx*-values. Perhaps contrary to intuition, the consideration of mixtures actually reduces the overall stochastic uncertainty, which is a result of the increased number of input data.

3.3.1.2 Independent Action

Independent action (sometimes also termed Effect Addition, Effect Multiplication or Abbotts Rule) conceptualises mixture effects in a different way. It assumes that the resulting combined effect can be calculated from the effects caused by the individual mixture components by following the statistical concept of independent random events (Bliss, 1939). This can be mathematically expressed as:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad \text{(eq. 3a)}$$

if the effect increases with increasing concentrations (e.g. when mortality data are considered) and

$$E(c_{mix}) = \prod_{i=1}^{n} E(c_i) \qquad \text{(eq. 3b)}$$

when the effect decreases with increasing concentrations (when e.g. survival rates are observed). In both equations $E(c_{Mix})$ denotes the effect provoked by the total mixture at a concentration $c_{Mix} = \sum_{i=1}^{n} c_i$. $E(c_i)$ are the effects that the individual components would cause if applied singly at that concentration at which they are present in the mixture.

Due to this probabilistic background, IA assumes strictly monotonic concentration-response curves of the individual mixture components and an euclidian-type effect parameter scaled to an effect range of 0-1 (0-100%).

As IA uses individual effects of the mixture components to calculate the expected mixture effect, this concept implies that agents present at doses below their individual effect thresholds (i.e. at zero effect levels) will not contribute to the joint effect of the mixture. Hence if this condition is fulfilled for all components there will be no combination effect. This central tenet of IA is commonly taken to mean that exposed subjects are protected from mixture effects as long as the doses of all agents in the combination do not exceed their no-observed-effect-levels or –concentrations (NOEL or NOECs).

NOELs and NOECs are the direct condensation of the results of an experimental study and denote the highest test concentration that did not provoke any statistically significant effect. This measure is therefore highly sensitive to experimental design issues such as the number of replicates and the dose spacing. It might be regarded as a major shortcoming that NOELs and NOECs are based on the failure to detect a statistically

significant effect in a given design and biotest – which of course does not prove that there is no effect in reality. Therefore, they do not describe a "safe" concentration and differ fundamentally from true No Effect Concentrations, although they are frequently equated with the latter. In fact, NOELs might correspond to effects as high as 5% on average (Allen et al. 1994). In ecotoxicological studies NOECs might correspond to effects from around 8% to as high as 38% (fish growth test) (Crane and Newman 2000). IA-compliant mixture effects are thus to be expected, even if all components of a mixture of dissimilarly acting substances are present only at their individual NOELs. If only a certain fraction of the individual NOELs or NOECs is present, it depends on the number of mixture components, the precision of the experimental data and the steepnesses of the individual concentration-response curves whether the resulting mixture effects might become significant. Furthermore, the fundamental assumption of IA, namely that completely independently acting chemicals do not influence each others toxicity, will never be completely fulfilled in real biological systems.

The general notion that mixtures of dissimilarly acting chemicals do not pose elevated risks, as long as each individual risk quotient are < 1 (Feron & Groten 2002) hence seems problematic. A detailed discussion of this issue can be found in Section 6 of this report.

Neither CA nor IA make any assumption about the targeted biological system nor do they consider any specific properties of mixture components beyond their pharmacological (dis)similarity. This is both a strength and a weakness of the concepts. On the one hand, this simplicity allows establishment of general rules for mixture toxicity assessment, which is essential for considering the joint action of chemicals in regulatory guidelines. On the other hand, it cannot be assumed that these concepts actually describe biological realities, except perhaps in very simple systems. Even if all components of a mixture are similarly or dissimilarly acting, respectively, additional (unspecific) binding sites, differences in toxicokinetics and/or biotransformation pathways will interfere. Hence, with appropriate experimental power in terms of accuracy and precision, differences between CA- or IA-expectations and the actually observed mixture toxicity will always become apparent. The crucial question therefore might not be whether deviations between simple concepts and complex biological realities can be observed, but whether CA and/or IA are over-simplistic, i.e. whether their predictive power is sufficient for a certain purpose.

3.4 The choice between concentration addition and independent action

When faced with the task of evaluating a particular mixture, the question arises as to which of the two concepts is appropriate for the mixture in question and therefore should be chosen as a basis for formulating a quantitative idea about mixture toxicity according to expected additive effects (additvity expectation). This issue becomes especially important, as the concepts often predict different mixture toxicities. In an approach to deal with these decision problems, the assumptions that underpin dose addition and independent action have been allied to broad mechanism of combination toxicity, as follows:

Dose addition is thought to be applicable to mixtures composed of chemicals that act through a similar or common mode of action (US EPA 1986, 1999, 2000). Although the original paper by Loewe and Muischneck (1926) contains little that roots dose addition in mechanistic considerations, the idea of similar action probably derives from the "dilution" principle which forms the basis of this concept. Because chemicals are viewed as dilutions of each other, it is implicitly assumed that they must act via common or similar mechanisms.

That view is borne out by empirical evidence to show CA produces reliable estimates of combined effects, if the components share either a strictly identical molecular mechanism of action, e.g. (Altenburger et al. 2000; Backhaus, Scholze, & Grimme 1999; Brian et al. 2005b; Faust et al. 2001; Kortenkamp 2007; Rajapakse, Silva, & Kortenkamp 2002b; Silva, Rajapakse, & Kortenkamp 2002b); or belong to the group of so-called baseline toxicants, e.g. (Hermens et al. 1984; Könemann 1981a). Further examples of the predictive power of CA for specific mixtures are given in the specific reviews on current evidence of mixture toxicity in human toxicology and ecotoxicology.

Conversely, IA is widely held to be appropriate for mixtures of agents with diverse or "dissimilar" modes of action. Although rarely stated explicitly, this presumably stems from the stochastic principles that underpin this concept. The idea that chemicals act independently is equated with the notion of action through different mechanisms. By activating differing effector chains, so the argument, every component of a mixture of dissimilarly acting chemicals provokes effects independent of all other agents that might also be present, and this feature appears to lend itself to statistical concepts of independent events. However, theoretically, the stochastic principles of IA are also valid when one and the same agent is administered sequentially and non-reversible events such as mortality are investigated.. Because organisms cannot die twice, the probabilism expressed in equation 3 applies, although the precise mechanisms that underlie the toxic action of the chemical are identical. In the case of simultaneous administration of many chemicals however, the principle of independent events can only be realised under the additional assumption of strictly independent, dissimilar mechanisms.

IA has always been acknowledged as being the theoretical counterpart to CA, but doubt has been cast on its practical relevance for (eco)toxicology (EIFAC 1987). One particular reason for this is that organisms are structured entities with highly interwoven physiological processes. It has therefore been assumed that IA is a rather unlikely type of joint action at complex effect levels (such as death or inhibition of reproduction) (Broderius, Kahl, & Hoglund 1995; Plackett & Hewlett 1967), mainly because the principle of strictly independent events is rarely realised in such biological systems, due to converging signalling pathways and interwoven subsystems. CA has therefore even been termed the "General Solution" for mixture toxicity assessment (Berenbaum 1985). But the few studies that were specifically designed for a comparative evaluation of both concepts for mixtures composed of strictly dissimilarly acting substances, could demonstrate that IA provides a better prediction of the observed mixture toxicities (Backhaus et al. 2000; Faust et al. 2002).

CA and IA may be regarded as special cases that provide a framework of reference, in the sense that they define extremes of possible additivity expectations. Mixtures of heterogeneous pollutants that do not only include strictly dissimilarly acting substances, but also multi-site inhibitors and non-specifically acting substances, may be expected to exert an intermediate toxicity within the window of mixture toxicity whose extremes are defined by CA and IA. Recent experimental results are consistent with this hypothesis (Walter, et al., 2002). Also, classical studies, that referred to CA only, showed a slight overestimation of the observed mixture toxicity (Broderius 1991; Hermens & Leeuwangh 1982; Könemann 1981b; Parrott & Sprague 1993) and therefore are consistent with this view.

Hence, two basic options exist for the predictive assessment of pollutant mixtures: (a) a case by case selection of the most appropriate concept, or (b) the *a priori* choice of one concept as a default approach. For implementing mixture toxicity assessments into regulation, it is of paramount importance to analyse whether and how these options are applicable.

3.4.1 Case-by-case selection or a default approach?

All existing experimental evidence clearly shows that the similarity or dissimilarity of the molecular mechanisms of action is a useful guiding principle for selecting the appropriate concept for a given mixture. It is thus a major obstacle that for many environmentally relevant mixtures knowledge about the mechanisms of action of the mixture components is scarce or even completely absent. Additionally, the mechanisms of action of the mixture components might change drastically, depending on the particular species that is considered. For ecotoxicological investigations that consider different species this might be a major hurdle.

Thus, case-by-case decisions and groupings of the compounds in a mixture according to their (dis)similarity requires substantial efforts and detailed guidance is only available for human toxicology, particularly from the US EPA who discuss the issue in greater detail in their guidance documents for mixture toxicity evaluation (US EPA 1986, 1999, 2000).

The *a priori* choice of one concept as a pragmatic default approach seems like a possible shortcut in order to avoid lengthy, disputable decisions about the similarity or dissimilarity for each mixture of interest. However, this would only be justifiable if on average only minor errors can be expected to occur by choosing the "wrong" concept (e.g. estimating the toxicity of a mixture of dissimilarly acting substances with CA or *vice versa*). Also, with the precautionary principle in mind, a concept should be selected as a default approach, that does not lead to an *under*estimation of the mixture toxicity, even when applied wrongly.

Mathematical analyses have proven that considerable differences between the concepts (> one order of magnitude) occur only with large numbers of individual mixture components (>10) and rather steep or very flat concentration-response relationships

(Faust 1999; Junghans et al. 2006). Simulation studies that evaluated the average relationship between the mixture ecotoxicities predicted by CA and IA were conducted with data from various research facilities (University of Bremen and UFZ Centre for Environmental Research) and regulatory authorities (Danish Environmental Protection Agency, German Federal Environmental Agency; US Environmental Protection Agency) in the European R&D project BEAM (Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation, EVK1-CT-1999-00012). Results of these studies clearly showed that in the vast majority of conceivable mixtures, CA predicts slightly lower mixture effect concentrations (i.e. higher mixture effects) than IA. In particular it could be demonstrated that the ratios between the EC50 values predicted by IA and CA were between 8.3 and 0.4 for the set of all possible mixtures that can be produced from a set of 106 environmental chemicals. Unfortunately, similar studies are missing in the field of human toxicology.

In stark contrast to ecotoxicology, IA is often held to be the default assessment concept in human toxicology when strict similarity criteria of dose addition appear to be violated or if specific evidence for the compounds of a given mixture is lacking (COT 2002). Implicitly taking "dissimilar action" as the simple negation of "similar action" it is then assumed that IA must hold, even without further proof that the underlying mechanisms indeed satisfy any explicit dissimilarity criterion. Actually, a major difficulty seems to lie in defining reliable criteria for "similar modes of action". Often, the induction of the same phenomenological effect is deemed sufficient for accepting similar action. However, this could be inappropriate for certain combinations of chemicals that operate by distinct molecular mechanisms. At the other extreme of the spectrum of opinions, an identical molecular mechanism, involving the same active intermediate is required to fulfill the similarity assumption. This position, with its very strict similarity criterion, may mean that only very few chemicals qualify for inclusion into mixture effects assessments, leaving out a large number of others that also provoke the same response. In effect, this would provide an unrealistically narrow perspective on real existing mixtures. A middle position is occupied by the view that interactions with the same site, tissue or target organ should qualify for similarity (Mileson et al. 1998).

In summary, it can be concluded that in the field of ecotoxicology current scientific evidence seems to support the choice of CA as a pragmatic default approach of mixture toxicity prediction for regulatory purposes. However, only a few very specific guidelines that account for the ecotoxicology of chemical mixtures and that go beyond simple whole mixture testing have been put into place (mainly the hydrocarbon block method in the Technical Guidance Documents (European Commission Joint Research Centre 2003) and the application of CA for classification and labelling purposes with respect to aquatic toxicity in the recently adopted GHS system (EU Commission & EU 2007). In contrast, a detailed framework for the regulatory consideration of human health effects of chemical mixtures has been developed by the US EPA, which strongly favours a case-by-case decision of the most appropriate concept for each mixture of interest, a view that is also supported by recent reports of European panels of scientific experts (COT 2002). Missing experimental evidence on the pharmacological (dis)similarity of the mixture components leads to the application of IA as a default approach (COT 2002), although it is currently

unclear whether this might run the risk of a systematic underestimation of mixture toxicities.

It finally should be mentioned here, that the development and justification of a rational on how to select between CA and IA is a fundamentally different issue than the analysis on whether CA and IA are appropriate concepts in the first place – or whether and to what extent interactive effects (synergisms, antagonisms) might render both, CA and IA inappropriate. Interactions that go beyond mere chemical incompatibilities of the mixture components are specific for the exposed organisms and considered groups of chemicals. A detailed discussion is therefore provided in the review of current empirical evidence in mixture toxicology and ecotoxicology.

3.5 Input requirements for using Concentration Addition and Independent Action

In order to put the predictive power of the concepts to the test, their input requirements (Table 3.1) have to be critically assessed. Both concepts rely on quantitative input data, either in terms of effects or effect concentrations. It follows, that biological variation, reproducibility and repeatability play an important role in deciding which of the two concepts should be applied.

Both concepts are applicable only to mixtures of known composition because they require knowledge about the toxicities of each mixture component. But they operate on different levels. IA uses single substance effects, $E(c_i)$, for predicting a mixture effect (eq 3 a, b), while CA is based on effect concentrations (*ECx*-values)and predicts an effect concentration of the mixture (eq 1). *ECx*-values are accessible through concentration-response analyses. Hence, for the application of CA a considerable part of the concentration-response curves for all mixture components needs to be recorded. Such curves also allow calculation of individual $E(c_i)$-values and therefore in principle also provide the necessary input data for an application of the IA-concept. But in contrast to CA, IA does not rely on concentration-response curves. It can also make use of single experimentally observed effect values as input data, although the variability of those values then has to be critically assessed.

As both concepts make use of toxicity data of each individual substance, the overall input requirements obviously increase with an increasing number of mixture components. But a major advantage of the CA concept is that the information needed for each component is constant and does neither depend on the mixture ratio nor on the number of chemicals in the mixture. If, for example, the EC50 of a mixture is to be predicted, the EC50 for each component has to be determined. These values are the necessary and sufficient input values, independently of whether a mixture of 2 or of 50 components is to be analysed. This is in sharp contrast to IA, for which the needed input information changes with the number of mixture components as well as the mixture ratio. For example, in a binary mixture a 30% effect of each individual component leads to a 50% effect of the combination. In a 10-component mixture, each component needs to be present only at a concentration that would give rise to a 6.7% individual effect. Hence, the more

compounds in a mixture, the lower the individual $E(c_i)$'s that are needed for estimating a 50% mixture effect. That lower and lower $E(c_i)$-values for each component are needed for actually calculating IA-predictions is a serious drawback of IA, as this increases experimental demands considerably.

Toxicological studies often report their findings in terms of EC50 and/or NOEL/NOEC values. As outlined, EC50 values are of only limited value for the application of IA. CA on the other hand can make use of those values for assessing whether the total concentration in an exposure scenario is above or below its anticipated EC50. Without any further knowledge about the individual concentration-response curves, no statement regarding the expected mixture effect can be made, though. The ability of CA to allow limited mixture toxicity assessments by using only EC50's also allows it to make use of QSAR-based estimates on the components toxicity, rather than of experimentally determined values.

Point estimates such as NOELs or NOECs are not directly suited as input data for either concept. This becomes apparent when reviewing the mathematical formulations of the concepts in equations 1 and 3. NOELs and NOECs neither represent effect concentrations (EC_x values as required for CA) nor effect levels ($E(c_i)$ as required by IA). In the context of both concepts, NOELs and NOECs can only be used indirectly, i.e. by attributing a certain effect level to this concentration, using an appropriate concentration-response curve. It might be tempting to substitute the individual *ECx*-values in the CA-equation with NOELs or NOECs in order to predict a mixture NOEL resp. NOEC. But this would imply that all NOELs / NOECs provoke the same, insignificant effect, i.e. that all have been determined in an identical experimental setup (in terms of replicates, spacing of test concentrations, variance structure), which is hardly ever the case. Nevertheless, a range of methods such as TEFs, TEQs or the HI for mixtures make use of a CA-like approach and sum up NOEL-based hazard quotients. This introduces an additional source of uncertainty in the assessment, because those NOELs often do not represent equi-effective doses, and therefore violate a basic requirement of CA (see equation 1). This issue is of fundamental importance and has to be distinguished from the question as to whether CA is an appropriate concept for the mixture of interest.

Due to the probabilistic assumptions that underlie IA, all input data have to be re-scaled to a range of 0-100% relative effect. This implies an effect parameter with euclidian properties and that hormetic effects (U-shaped concentration response curves) are beyond the scope of this concept, see discussion by Backhaus and coworkers (Backhaus, Arrhenius, & Blanck 2004). It also requires suitable controls in the experiments. For endpoints that are naturally confined, such as mortality, negative controls might suffice. For other endpoints appropriate positive controls are also necessary (see e.g. the vitellogenin induction studies in (Brian et al. 2005a; Thorpe et al. 2001; Thorpe et al. 2003). Although this re-scaling is not strictly required for the application of CA, it also offers a convenient way of pooling data from independent experimental runs as the absolute performance of the test organisms might slightly change from run to run, but their sensitivity can be assumed constant.

In principle, the predictive power of both concepts can only be as good as the quality of the input data, which can be attributed to two interlinked factors, the quality of the experimental raw data and the appropriateness of their biometrical description. One option for biometrical data analysis is to handle every new set of experimental data independently, e.g. by following the so-called "best-fit" approach (Scholze et al. 2001) in which a whole set of different concentration-response models is applied to each data set in order to maximise the overall fitting quality. This is especially important, if low-effect concentrations are explored, as differences between different biometrical models become most prominent here. Also, extrapolations outside the range of actually tested concentrations/doses should be avoided as they are extremely dependent on the chosen biometrical model.

3.6 Experimental designs

Two fundamentally different experimental situations can be distinguished: Either the complete analysis is run in one experiment, or the experiment is blocked, i.e. different parts of the study are run at different times. Especially in experiments with multi-component mixtures, single substance data are often gathered over longer time periods and the mixture experiments only follow after completion of the single substance experiments. As confounding factors such as variabilities between different stocks of test organisms, seasonal influences etc. are minimised, unblocked experiments are often tailored towards proof of principles, i.e. exploring the fundamental predictive power of either concept for a certain set of chemicals, organism and/or endpoints. More than unblocked studies, blocked studies are influenced by confounders and care should be taken to minimise systematic differences between the single substance and mixture experiments.

Several specific design approaches have been described in the literature for analysing the degree of deviation between CA and/or IA predictions and experimental data – each with specific advantages or disadvantages.

3.6.1 Surface designs

A straightforward experimental approach for describing the mixture hyperplane would be to decide on the number of test concentrations per component and then simply test all possible combinations. Such a "full factorial" or "surface" design leads to an even coverage of the hyperplane with experimental data. A polynomial of the form

$$f\left(E(c_{mix})\right) = \beta_0 + \sum_{i=1}^{n}\beta_0 c_i + \sum_{i=1}^{n}\sum_{\substack{j=1 \\ i<j}}^{n}\beta_{ij}c_i c_j + \sum_{i=1}^{n}\sum_{j=1}^{n}\sum_{\substack{k=1 \\ i<j<k}}^{n}\beta_{ijk}c_i c_j c_k + \ldots\beta_{12\ldots n}c_1 c_2 c_3 \ldots c_n \quad \text{eq. 4}$$

would then provide a mathematical description of the mixture hyperplane (Cornell 2002; Gennings & Schwartz 1998). In equation 4, f is a function of the mean effect, $c_1, c_2, \ldots c_n$ are the concentrations of chemicals $1,2,\ldots,n$ in the mixture and the ß's denote the regression coefficients. For the link function f the classical Weibull, Logit or Probit models are typically used. This assumes that the single substance concentration-response

relationship for all mixture components can be adequately described by one of these models.

Equation 4 also accommodates a convenient way to statistically test for deviations from CA-expected mixture toxicities by testing whether the higher order terms significantly improve the fit of the polynomial to the experimental data (Meadows et al. 2002b).

Unfortunately, the experimental effort that is required for providing enough data for estimating all ß's in equation 4 increases exponentially with the number of components in the mixture. Even if only 2 concentrations are devoted for each compound (a two-level factorial design), the number of test groups needed is still 2^n. For example, an 8 compound mixture requires 256 test groups, not considering the need for any replicates or positive/negative controls. Practically, the application of full factorial designs is thus restricted to combinations of just a few chemicals.

For multi-component mixtures, so-called "fractionated factorial designs" ("screening designs") are an option. Here, only an adequately chosen fraction of the possible treatment combinations that can be established from a given pool of components is selected for testing. Obviously, the lower the fraction of actually tested combinations, the lower is the resolution of the experiment. The major challenge is to identify the most important combinations to be actually tested and to leave out those that are considered less important. Therefore, a design that optimally balances the required experimental effort versus the achievable knowledge gain is specific for each study and study goal. Common designs include Plackett-Burman-, Cotter-, and Box-Behnken-designs and the various types of central-composite designs (Cornell 2002).

A particular surface design has been suggested by Jonker and his co-wokers (Jonker et al. 2005). In this approach an extended version of CA is fitted to the experimental data. Depending on whether the additional parameters in the CA equation improve the fit significantly, conclusions about the prevalence of dose- or ratio-dependent deviations can be drawn. The approach has so far only been applied to the evaluation of CA and only for binary mixtures.

As factorial and response-surface designs scatter their experimental power over the whole mixture hyperplane in order to get a broad overview on the behaviour of the mixture, these designs are not suitable for analysing the contribution of low doses (low-effect concentrations) to the joint action of chemicals. They are also typically used in a purely descriptive setting (see 3.3.) or are applied for analysing the predictive power of CA only**.**

3.6.2 Isobole-Design

Isoboles describe lines in the mixture hyperplane, that are defined by all combinations of $c_1, c_2,\ldots,c_n$ that provoke an identical mixture effect. The intriguing feature of CA-predicted isoboles is their strict linearity. Classical isobole designs aim at experimentally describing one or several points on an isobole, with the aim of comparing them to the predictions derived from dose addition (e.g. Kortenkamp & Altenburger 1998; Sühnel

1992). Depending on the number of points on the isobole that are investigated, isobole oriented approaches can become rather laborious. A fairly complete mixture concentration-response experiment is necessary for each investigated point on the isobole. Assuming that the single substance concentration-response curves are determined in the course of the same experiment(s), $k*(n+j)$ test groups are needed in total (n = number of mixture components, k = number of test concentrations per concentration-response curve, j = number of points that are to be investigated on the isobole). If only 1 point on the isobole is investigated, the design reduces to a fixed-ratio design as described below. The major advantage of isobole designs is their ability to detect mixture-ratio dependent deviations between predictions and observations, that are then often interpreted as interactions between the mixture components. In order to minimise k, isobole-related experiments and subsequent data evaluations often focus on one particular effect level, typically 50%. Under these circumstances, the possibility of determining effect level dependent interactions might be limited. Designs that overcome this limitation and make use of multiple complete fixed-ratio experiments have been put forward e.g. by (Casey et al. 2005).

Due to its ease of understanding and the visual clarity, isoboles are perhaps *the* standard design for analysing binary mixtures and for comparing the observed mixture toxicities to the prediction by CA (e.g. Altenburger et al. 1990; Kortenkamp & Altenburger 1998), see also the specific reviews on empirical evidence on mixture toxicology and ecotoxicology.

3.6.3 "Fixed ratio" or "Ray" designs

Using the so-called "fixed-ratio" or "ray" design, the mixture of interest is analysed at a constant ratio of its components, while the total concentration of the mixture is systematically varied. Hence, a concentration-response curve (a "ray" in the mixture hyperplane, see Figure 3.1) of the mixture is recorded, which can then be analysed in the same way as the concentration-response curve of a single chemical. On the basis of the concentration-response curves of the individual components a comparison with both, CA- and IA-predictions can then be carried out, which requires $k*(n+1)$ test groups in total. Fixed ratio designs especially allow a convenient visualisation and interpretation of experimental results, even for mixtures with many compounds. Effect-level dependent deviations between predictions and observations will become visible (Crofton et al. 2005). An obvious drawback of this design is that no statement on mixture-ratio dependent deviations from the conceptual expectations can be made. An illustration of a fixed-ratio mixture study in an algal growth inhibition assay is presented in Figure 3.2, other examples can be found in (Casey et al. 2005; Meadows et al. 2002a; Payne et al. 2000; Payne, Scholze, & Kortenkamp 2001; Rajapakse et al. 2004; Rajapakse, Silva, & Kortenkamp 2002a; Silva, Rajapakse, & Kortenkamp 2002a; Thorpe et al. 2001), see also the specific reviews on current empirical evidence on mixture toxicology and ecotoxicology.

3.6.4 "Chemical A in the presence of fixed levels of chemical B"

An approach that is restricted to binary mixtures is to analyse the shift of the concentration-response curve of the first agent that is caused by a fixed "background" concentration of a second chemical. If at least *k*2+1* test groups are tested (the concentration-response curve of the single compound, the concentration-response curve of the compound plus the fixed background and the fixed background alone) it can be assessed whether the increase in toxicity of the first chemical that is caused by the background concentration is in compliance with IA-expectations. For a comparison with CA, also the concentration-response curve of the second chemical needs to be recorded, the extended design then requires at least *k*3* test groups.

3.6.5 Point Design

In a frequently used approach, which might be called a "point design", only one mixture concentration is actually tested and its effects are compared to the effects that the individual components provoke if applied singly at that concentration at which they are present in the mixture. In principle, this design only requires *n+1* test groups, not counting any controls. Nevertheless, visible deviations between observed and predicted effects are not necessarily of relevance, as the experimental variability of effect data is sometimes considerable. Especially the steepness of the concentration-response curves might have a considerable influence. In the case of steep concentration-response curves, small, experimentally unavoidable shifts in the applied concentrations might lead to comparatively huge shifts in experimentally observed effects. An extension of the point design is therefore to record the concentration-response curves of all components and the mixture and use effect data that are the result of a complete concentration-response analysis. One particular application of the point design is to analyse a situation in which all the components are present in a concentration that is assumedly below a pre-defined threshold and to see, whether the mixture still provokes clear effects (see Figures 5.5 and 5.6 for examples of this design). If the concentration-response curves of the mixture components are not available, this design does not allow comparisons of the observed mixture effect with the CA-prediction, as it does not allow the estimation of the necessary *ECx*-values. But as it provides the $E(c_i)$-values for all components, this design type allows to assess whether the observed mixture effect is in compliance with IA.

3.7 Quality criteria for the assessment of experimental mixture studies

On the basis of the considerations in the preceding sections of this report, several quality criteria for the evaluation of published mixture studies suggest themselves. A minimum demand in component-based analyses is that the experimentally observed responses should be evaluated against an explicitly stated mixture toxicity expectation that signifies additivity. If effect summation is employed, evidence of linear concentration-response relationships is necessary, otherwise, this method is deemed unreliable.

Most of the design approaches discussed above require concentration-response analyses of the individual mixture components. In the absence of single chemical concentration-response data, the observed mixture effects are indeterminate in terms of CA, IA,, synergism or antagonism. Hence, a fundamental quality criterion for the dissemination of a mixture study that evaluates an observed mixture toxicity in this context is the precise documentation of all the underlying concentration-response data (at least the used models and the estimated parameters). As almost all scientific journals in toxicology and ecotoxicology these days accept supporting information, space restraints are no longer an argument for merely reporting (eco)toxicity data as EC50 values.

Concentration-response data also provide estimations of the relative potency of mixture components. In mixture experiments that employ the fixed mixture ratio design with the aim of analysing whether a particular mixture follows CA- and/or IA-expectations, this is instrumental for deciding about the mixture ratio. It is important to choose mixture ratios in a way that avoids that one or a few components dominate the overall mixture toxicity.

3.8. Describing and assessing deviations from the mixture toxicity predictions by CA and IA

Both concepts assume that neither pharmacokinetic nor pharmacodynamic interactions are present in the analysed mixture. Any such interaction leads to a mixture toxicity that is either lower or higher than predicted by CA, by IA or by both concepts. A range of distance measures has been suggested in the literature in order to describe and quantify such deviations between mixture toxicity predictions and observations at a predefined effect level, usually 50%. Most of these measures relate only to the predictions by CA and the most common of this group might be the Toxic Unit Summation (Sprague, 1965). Another such measure is the Additivity Index (Marking, 1977). The Mixture Toxicity Index (MTI) as suggested by Koeneman (Koeneman, 1980) refers to two different reference situations: CA and the so-called “No Addition” situation, the latter being a limiting case of IA. The Index on Prediction Quality is a related measure which builds on the ratio of observed to predicted mixture toxicity and has the advantage of being applicable to predictions by CA as well as IA (Grimme, 1994; Altenburger, 1996).

Several methods are described in the literature for testing whether observed deviations from CA are significant in a statistical sense, but to our knowledge no analogous method has been suggested for testing the deviation from the prediction by IA. Sörensen and his co-workers propose an isobole-based method in which an additional parameter is introduced while fitting the CA-isoble to the experimental mixture data. The significance of this parameter is then tested using a common F-test (Sörensen, 2007). Jonkers and his colleagues suggested a χ2-likelihood ratio test for the same purpose (Jonkers, 2005). The main drawback of these approaches is the use of one global parameter to estimate the compliance or non-compliance between an observed and the CA-predicted mixture toxicity. That is, the result of having a significant deviation does not allow to infer whether the deviation is restricted to a certain effect level only, which may be limited to the extreme end of the concentration-response curve. It has hence been suggested to use

bootstrap-based methods for providing the approximate confidence intervals for the predictions and the concentration-response fit of the experimental mixture data and base the decision on whether or not significant deviations are present on whether or not the confidence belts overlap (Grimme, 1998). This approach allows for an effect-level dependent assessment of synergistic or antagonistic deviations of the experimental results from both predictions. It should be pointed out, that any significance criterion is of only limited value unless a deviation between observation and prediction is also quantified, e.g. by using one or more of the above mentioned distance measures.

Table 3.1: Fundamental properties of Concentration Addition and Independent Action

	Concentration Addition (Dose Addition)	**Independent Action (Response Addition)**
Pharmacological Assumptions	▪ All components can be replaced by an equi-effective fraction of another ▪ No further pharmacokinetic or pharmacodynamic interactions between the mixture components	▪ All components exert their action according to stochastic principles ▪ No further pharmacokinetic or pharmacodynamic interactions between the mixture components
Input Requirements	▪ Knowledge on the qualitative as well as quantitative mixture composition ▪ Effect concentrations of all components, relating to an identical effect level, biological system (bioassay) and endpoint	▪ Knowledge on the qualitative as well as quantitative mixture composition ▪ Relative effects (0-100%) that the components would provoke, if applied singly at that concentration at which they are present in the mixture, referring to the same biological assay and endpoint.
Implications	▪ Only applicable to mixtures with a known chemical composition. ▪ No specific assumptions on the biotest are needed, nor considered by the concept ▪ Calculation of mixture *ECx* values is limited to those effect concentrations that are known for all components. ▪ Only components that are also effective if applied singly have an impact on the toxicity of the mixture. ▪ All effective components contribute to the toxicity of the mixture, i.e. individual thresholds are meaningless. ▪ The calculated *ECx* of the mixture always falls into the span of *ECx* values of the individual components. That is: $\max_{i\in(1,\ldots,n)}\{ECx_i\} > ECx_{Mix} > \min_{i\in(1,\ldots,n)}\{ECx$	▪ Only applicable to mixtures with a known chemical composition ▪ No specific assumptions on the biotest are needed, nor considered by the concept ▪ Prediction is limited to 0-100% relative effect ▪ Only components that are also effective if applied singly have an impact on the toxicity of the mixture. ▪ Only those components contribute to the mixture toxicity that are present in concentrations that would also provoke an effect if applied singly. Components that are present below their individual threshold do not contribute to the mixture toxicity. ▪ The calculated mixture effect is always higher than the highest single substance effect. That is $E(c_{Mix}) > \max_{i\in(1,\ldots,n)}\{E(c_i)\}$

	▪ The number of components in the mixture does not influence the input requirements per component. Especially, the considered effect level *x* is only dependend on the mixture *ECx*, not on the number of mixture components.	▪ The more mixture components, the lower the single substance effects $E(c_i)$ that are required for the calculation of a given mixture effect. The following equation holds: $\min_{i\in(1,\ldots,n)}\{E(c_i)\} \leq 1-\sqrt[n]{1-E(c_M$
	▪ The stochastic error in the CA-calculated mixture toxicity never exceeds the maximum error of the single substance *ECx*-values.	
	▪ NOEL/NOECs are unsuitable as input data. Especially: a mixture NOEL cannot be calculated	▪ NOELs are unsuitable as input data. Mixture NOELs cannot be calculated.
	▪ Direct calculation of the mixture EC*x*-concentration	▪ Direct calculation of the effect that is expected to occur from a given mixture concentration.
	▪ For the calculation of an effect that is expected to occur from a given mixture concentration iterative procedures have to be applied. This assumes the availability of the concentration-response curves for all mixture components.	▪ For the calculation of a mixture EC*x* (i.e. a concentration that is assumed to provoke a predefined effect *x*), an iterative approach has to be used. This assumes the availability of the concentration-response curves for all mixture components.

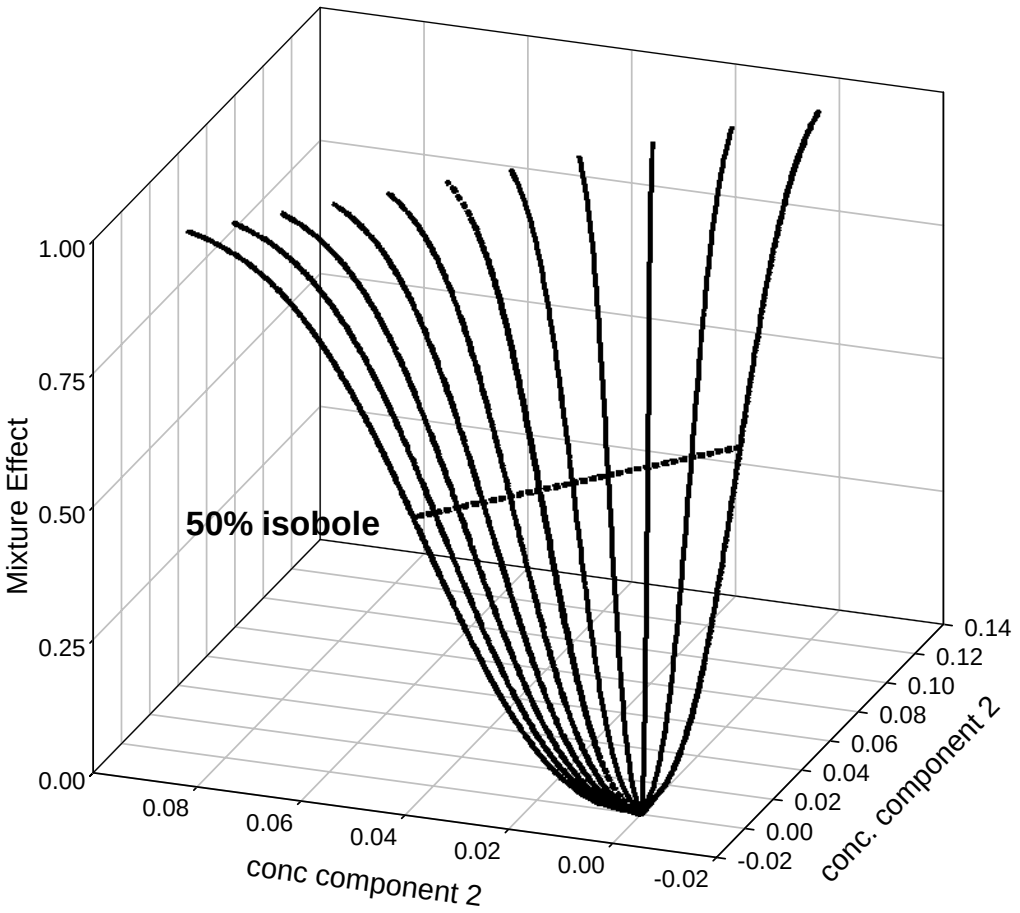

Figure 3.1: Concentration-response surface of a binary mixture

Black solid lines are so-called "mixture rays". Each one represents the concentration-response curve of a mixture in which all components are present at a constant mixture ratio. Together, they describe the whole mixture concentration-response surface of a binary mixture. The shape of this surface is specific for each mixture and depends on the shape of the individual concentration-response curves and their type of joint action. In the figure a surface according to Concentration Addition is shown. The indicated 50% isobole is the line connecting all combinations c_1+c_2 of the components that provoke 50% effect. It should be noted, that the surface is plotted using a linear scale for the concentration axes.

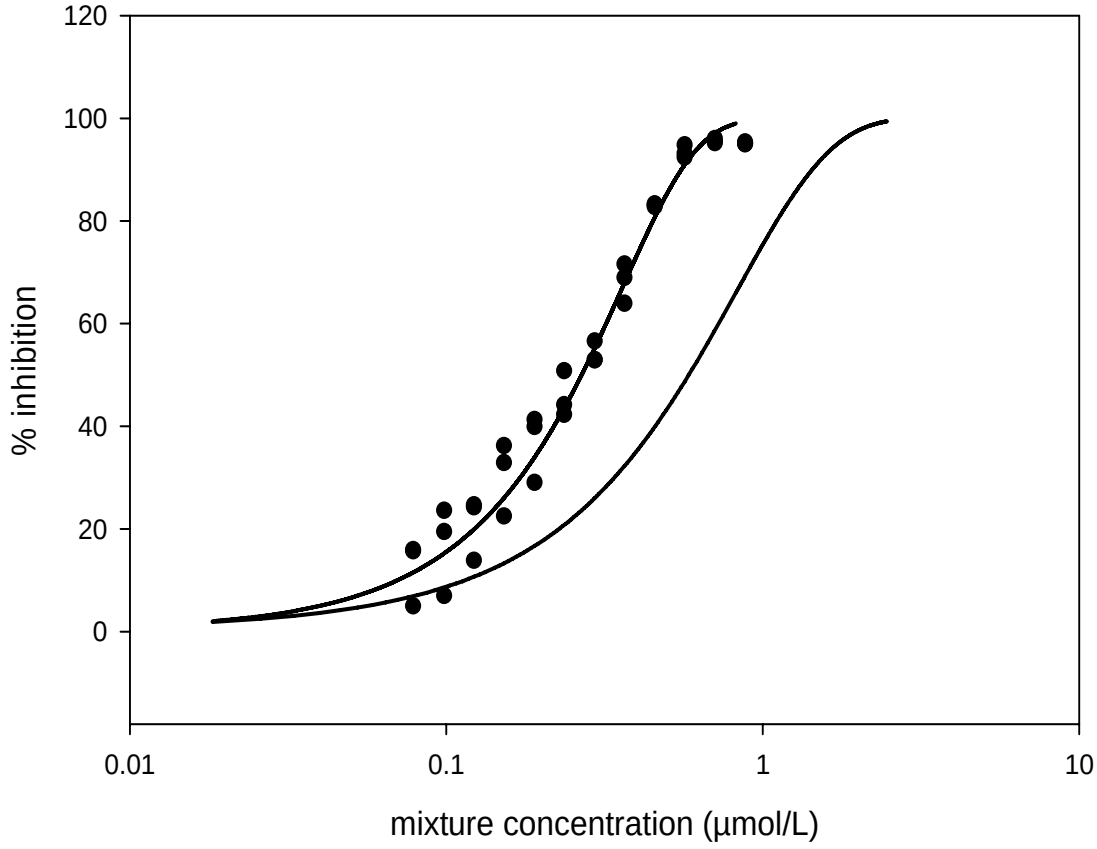

Figure 3.2: Predicted and observed toxicity of a mixture of 30 PSII-inhibiting herbicides (s-triazines and phenylureas)

Observed mixture effects are black dots. The line close to the observed effects is the CA prediction for a mixture with fixed mixture ratio of 30 PSII inhibiting herbicides. The curve to the right is the prediction yielded by IA. From the final report of BEAM (Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation, EVK1-CT-1999-00012)

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4. The effects of mixtures of chemicals: Human and mammalian toxicology studies

This section provides a critical review of papers on the combined effects of chemicals relevant to human and mammalian toxicology. Emphasis is placed on experimental studies that strive to understand mixture effects in terms of the toxicity of its individual components. Where appropriate, reference is also made to studies that have employed the so-called whole mixture approach. In the whole mixture approach combinations of chemicals are administered as if they were one single chemical, but without making any attempt to analyze the resulting effects in terms of synergisms, additivity or antagonisms.

In reviewing the published evidence, recourse was made to the quality criteria mapped out in Section 3.7. Briefly, well-designed mixture experiments assess observed effects against an explicitly stated additivity assumption, derived from the concepts of dose addition or independent action. These additivity expectations should be calculated on the basis of dose-response data of the individual chemicals in the mixture. The mixture ratio employed in the combination experiment should ensure that all (or most) of the components contribute significantly to an overall mixture effect, if at all present. Situations where several agents were administered, but where in fact only one chemical determined the resulting effect, should be avoided. Exceptions to this requirement are studies that evaluate the effects of mixtures composed according to exposure scenarios identified in specific settings.

4.1 Carcinogenicity, mutagenicity and genotoxicity

In their 1989 report on toxicants in drinking water, the US National Academy of Sciences (NRC 1989) recommended the use of independent action for the estimation of risks from mixtures of carcinogens, with the implication that carcinogenesis is a stochastic process, fulfilling the basic assumptions behind independent action. However, it is complicated to assess whether there is empirical support for this idea in the scientific literature describing the joint effects of several carcinogens, mutagens and other genotoxic chemicals. To a large degree, these difficulties can be traced to the various definitions, concepts and terms that have evolved in this field for the purpose of assessing combination effects of carcinogens. Not all of these approaches are compatible with the concepts and terms in other areas of mixture toxicology. The way in which the problem has been framed in the carcinogens literature has had an impact on the experimental design of many key studies, particularly with respect to expected combination effects in the case of additivity. In many cases, assessments of the type of combination effect in terms of dose additivity or additivity according to independent action are not possible, because of a lack of data about dose-response relationships for individual carcinogens.

4.1.1 Carcinogenicity

4.1.1.1 Definitions, terms and concepts

The researchers engaged in studying mixture effects of carcinogens have used the term "synergism" in ways that differ from the perspective taken in other areas of mixture toxicology:

"Synergism" is mostly applied to carcinogenic responses that are judged to be more than additive, with the implicit assumption that additivity means the summation of effects ("1 + 1 = 2"). Thus, synergistic effects occur in the case of "1 + 1 > 2" (Hecker 1976). The process that leads to such synergisms is called syncarcinogenesis.

Synergism (and consequently syncarcinogenesis) is sometimes also used in the sense of several carcinogens "working together", according to the original Greek meaning of the word. Here, the term does not carry implicit quantitative judgements in relation to additivity expectations. It includes additive effects. For that reason, synergisms as in "more than additive effects" are referred to as "overadditive synergisms" (Berger et al. 1987).

A somewhat different perspective on "syncarcinogenesis" is also often taken, with considerable impact on the design of experimental studies. This definition frames the phenomenon in terms of an "augmentational" action of several carcinogens, where carcinogenic effects arise although each individual substance is present at doses which do not exert effects (Shirai et al. 2006; Hecker 1976). It should be emphasized, however, that such "augmentational" or "synergistic" effects can be consistent with additive responses according to DA or IA (see Section 6).

Hecker (1976) pointed out that syncarcinogenesis can be the result of two distinct processes which he termed "pluricarcinogensis" and "co-carcinogenesis". Pluricarcinogenesis (a rarely used term) is the process that leads to cancers as a result of sequential or simultaneous exposure to several chemicals, all of which are capable of inducing cancer when given alone at suitable doses. In "co-carcinogenesis", the cancer-causing effects of one of several agents are exacerbated by the presence of other chemicals, which by themselves are non-carcinogens.

The following section summarizes pertinent studies with carcinogenic agents. Where possible, attempts were made to re-analyze the published data, with the aim of assessing whether the observed effects are in quantitative agreement with additivity expectations according to DA or IA.

4.1.1.2 Long-term carcinogenesis bioassays

Tumours of the skin
Summation (in the sense of effect summation) of the carcinogenic action of 4-nitroquinoline-N-oxide and 3-methylcholanthrene was observed by Nakahara and

Fukuoka (1960) in skin carcinogenesis studies in mice. Both chemicals were capable of inducing skin cancers on their own.

Cavalieri et al. (1983) observed syncarcinogenic (augmentational) effects in mouse skin painting studies with cyclo-penteno-[cd]-pyrene (CPEP) and benzo-[a]-pyrene. The most pronounced effect was found with a combination of 6.6 nmol benzo-[a]-pyrene and 66.6 nmol CPEP, which resulted in a tumour incidence of 69%. Administration of the single agents at these doses induced tumour incidences of 7% each. Had the chemicals acted additively according to IA, incidences of only $1 – (1 – 0.07)^2 = 0.13 = 13\%$ would have been expected. Somewhat smaller effects (incidences of ca. 30%) occurred with mixtures where the doses of either chemical were lowered (6.6 nmol benzo-[a]-pyrene plus 22.2 nmol CPEP; 2.2 nmol benzo-[a]-pyrene plus 66.6 nmol CPEP), but these were still larger than incidence of 13% predicted by IA. This re-analysis of Cavalieri's data shows that the observed effects were truly synergistic in relation to IA. Because the paper contains some data that allow rudimentary dose-response analysis for the single agents, an assessment of agreement with DA is also possible by calculating sums of toxic units, as follows: The combination of 6.6 nmol benzo-[a]-pyrene and 66.6 nmol CPEP yielded an incidence of 69%. By interpolation of the dose-response data for the single chemicals it is possible to estimate that 23 nmol benzo-[a]-pyrene and 170 nmol CPEP on their own should have produced a similar incidence. The toxic unit for benzo-[a]-pyrene is therefore 6.6/23 = 0.28, and that for CPEP 66/170 = 0.39, which sums to a value of 0.67, indicating a weak synergism in relation to DA. The doses estimated to provoke an incidence of 30% are 10 nmol for benzo-[a]-pyrene and 100 nmol for CPEP. Accordingly, the sums of toxic units for the other two combinations are 0.88, sufficiently close to 1 to suggest agreement with the DA additvity expectation.

Syncarcinogenesis was also demonstrated in skin painting studies with polycyclic aromatic hydrocarbons by Schmidt et al. (1976) and Schmähl et al. (1977).

Urinary bladder tumours

Tsuda et al. (1977) studied the effects of multiple urinary bladder carcinogens, N-butyl-n-(4-hydroxybutyl)nitrosamine (BBN), N-(4-(5-nitro-2-furyl)-2-thiaazolyl)formamide (FANFT), N-2-fluorenylacetamide (2-AAF) and 3,3'-dichlorobenzidine (3,3'-DCB). Combinations of two or three of these chemicals were given to rats at doses that were themselves not carcinogenic. Combinations of BBN, FANFT and 2-AAF; and BBN, FANFT and DCB induced urinary bladder tumours. With reference to the concept of an "augmentational" action of these agents, the authors judged these effects as "synergistically elevated". The strongest augmentational action occurred with BBN plus FANFT.

Tumours of the airways and the lung

Two different carcinogens with different target organs were found to exacerbate each others action (Montesano et al. 1974). When given together with benzo-[a]-pyrene, diethylnitrosamine which alone induces nasal cavity carcinomas in hamsters, produced more carcinomas of the trachea, bronchi and lungs. With reference to effect summation, the authors judged this effect to be synergistic.

Kimizuka et al. (1987) administered asbestos fibers and benzo-[a]-pyrene to Syrian hamsters by intra-tracheal instillation to study their joint effects after sequential exposure. Lung hyperplasia and malignant lung tumours were examined after up to 19 months. When asbestos was given first, followed by benzo-[a]-pyrene, the fraction of animals with hyperplasia was lower than that seen with benzo-[a]-pyrene alone. A reversal of the order of administration (benzo-[a]-pyrene first, then asbestos) induced a slightly higher incidence of hyperplasia, although still lower than after benzo-[a]-pyrene treatment alone. With malignant lung tumours as the endpoint of investigation, the results were more clear-cut: while no tumours were observed after application of asbestos or benzo-[a]-pyrene alone, malignancies were only observed when the two agents were combined, independent of the order of administration. However, an interpretation of these results is complicated by the fact that the authors examined the animals after differing periods of time. Nevertheless, these data are indicative of syncarcinogenesis by asbestos and benzo-[a]-pyrene.

These weaknesses were dealt with in a subsequent study by the same authors (Kimizuka and Hayashi 1993). A similar experimental set-up was used, but this time, the animals were investigated after 18 or 24 months. Benzo-[a]-pyrene was combined with either of two types of asbestos, chrysotil or amosit. At the dosages used, none of the individual agents induced malignant lung tumours after 18 or 24 months. Strikingly, all combinations (benzo-[a]-pyrene plus chrysotil and benzo-[a]-pyrene plus amsit) provoked tumours in 100% of the treated animals, a clear demonstration of syncarcinogenesis in the sense of augmentational effects.

Nesnow et al. (Nesnow et al. 1998) analysed mixture effects of five poly-cyclic 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.

Liver cancer

Berger et al. (1987) conducted combination experiments with very low doses of three genotoxic nitrosamines, N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine (NPYR) and N-nitrodiethanoloamine (NDEIA), which all produced liver tumours in the rat. The experiment was designed on the basis of dose-response data for the individual nitrosamines. A combination of 0.032 mg/kg/d NDEA, 0.13 mg/kg/d NPYR and 0.63 mg/kg/d NDEIA was given over the entire life span of the animals. It produced an incidence of malignant liver tumours of 13%. Berger et al. judged these data to show additivity, in line with an idea of syncarcinogenesis as chemicals "working together" (see definitions above). Apart from the liver, tumours were also observed in the urinary bladder, the gastrointestinal tract and in the hematopoetic and lymphatic tissues, at incidences above those found in control animals. In contrast to liver tumours, however, the incidences of those malignancies did not rise with increasing doses.

Because the combination experiment (endpoint: liver malignancies) was supported by dose-response analyses of the individual mixture components, it is possible to re-analyze the data with the aim of assessing quantitative agreement with IA or DA additivity expectations: Based on the individual dose-response curves for NDEA, NPYR and NDEIA it can be estimated that the nitrosamines on their own, at the doses present in the mixture, produced tumour incidences of around 2%. Thus, according to IA, a joint incidence of $1 - (1 - 0.02)^3 = 0.06 = 6\%$ is to be expected, falling short of the carcinogenic effect observed with the mixture. This suggests synergistic effects in relation to IA. From the individual dose-response relationships each of the chemicals on its own can be expected to produce a 13% incidence at ca. 0.06 mg/kg/d (NDEA), 0.4 mg/kg/d (NPYR) and 10 mg/kg/d (NDEIA). By using the doses present in the mixture, it is possible to derive toxic units, as follows (all dose units in mg/kg/d): 0.032/0.06 for NDEA, 0.13/0.4 for NPYR and 0.63/10 for NDEIA. The sum of these toxic units is 0.92, sufficiently close to 1 to suggest agreement with DA.

Elashoff et al. (1987) investigated a mixture of carcinogens that target the same organ, the liver. Binary combinations of the hepatocarcinogens cycad flower, lasiocarpine, aflatoxin and dipentylnitrosamine (DPN) were tested in male and female F344 rats. The authors used a 4x4 factorial design, with doses of the single carcinogens that were sufficiently high to cause liver tumours. Carcinogenicity was measured in terms of time to death and time to death with tumours. Although the authors assessed some observed mixture effects as synergistic (e.g. binary combinations of cycad flower and lasiocarpine at certain doses, or lasiocarpine and DPN), the lack of dose-response data for the single chemicals precludes clear identifications of the type of combination effects.

The same group (Fears, Elashoff, & Schneiderman 1988) looked at binary mixtures of carcinogens that act on different organ systems. Binary combinations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-butanol-butylnitrosamine (NBBN), nitrilotriacetic acid and DPN were given to F344 rats. With some mixtures containing nitrilotriacetic acid, antagonisms were detected, but doubts remain as to the validity of these conclusions, due to the study design.

Miscellaneous tumours

Takayama et al. (1989) conducted a 2 year study with male rats to study the joint carcinogenic effects of a mixture composed of 40 carcinogens with a wide variety of chemical structures. The chemicals were given via the diet at doses equivalent of 1/50 of their individual TD50. Significantly elevated tumour incidences relative to untreated controls were seen in the liver and the thyroid. However, the experimental design of this study makes it difficult to judge whether the liver and thyroid tumour incidences were additive or synergistic. It is noteworthy that the incidence of liver tumours was not elevated relative to the incidences seen after administration of any carcinogen singly.

Hirose et al. (1998) analyzed the carcinogenicity of antioxidants known to cause forestomach tumours in rodents. In a 104 week feeding study with butylated hydroxyanisol, caffeic acid, sesamol, 4-methoxyphenol and catechol, increases in forestomach papillomas were observed in F344 rats which the authors interpreted as

synergisms. This is difficult to assess because dose-response analyses with the single chemicals were not conducted. The same mixture was used in a 28 week exposure multi-organ carcinogenesis model with F344 rats, after initiation with several carcinogens. The effects of a high dose combination were smaller than anticipated by dose addition.

Walker et al. (2005) employed a two year rodent cancer bioassays with female Harlan Sprague-Dawley rats given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB-126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or a mixture of the three compounds. The three chemicals, both singly and in combination induced hepatic, lung, and oral mucosal neoplasms. The dose response for the mixture could be predicted from a combination of the potency-adjusted doses of the individual compounds, derived on the basis of World Health Organization (WHO) dioxin TCDD equivalency factor (TEF) values. This method assumes dose-additive effects. Kortenkamp (unpublished) conducted a re-analysis of the data, without utilizing the WHO TEF values, but by employing the concept of dose addition directly. In this analysis, the experimentally observed tumour incidences fell short of those anticipated by dose addition.

Taken together, the empirical findings support the idea that syncarcinogenesis (including additive effects in the sense of "working together", as well as "augmentational" effects) is likely to occur with combinations of substances that target the same organs or tissues. This applies to the skin (Nakahara and Fukuoka 1960, Schmidt et al. 1976, Schmähl et al. 1977, Cavalieri et al. 1983), the urinary bladder (Tsuda et al. 1977), the airways and the lung (Kimizuka et al 1987, 1993, Nesnow et al. 1998) and the liver (Berger et al. 1987, Elashoff et al. 1987, Fears et al. 1988). Syncarcinogenesis does not require similarity in chemical structures, nor does it matter whether administration is sequential or simultaneous. However, syncarcinogenesis is not observed when carcinogenic substances are combined that target different organs.

4.1.1.3 Short-term animal models

The studies discussed thus far were carcinogenesis bioassays that covered almost the entire life time of the treated animals. The long duration of these studies, combined with their high costs has stimulated the search for short-term animal models. The following section gives an overview of mixture experiments with short-term assays.

Hasegawa et al. (1991) used a short-term initiation-promotion model with glutathione-S-transferase-positive hepatic foci to investigate the effects of a five-component mixture of different heterocyclic aromatic amines, after initiation with diethylnitrosamine (DEN). At the highest tested doses, all chemicals individually produced foci. When combined at 1/5 of these doses, but not at 1/25, combination effects in excess of the arithmetic sum of effects of the single chemicals were observed, which the authors interpreted as synergisms. This study indicates that the heterocyclic aromatic amines can produce hepatic foci when combined at doses where each single agent is without statistically significant effect, in line with the idea of syncarcinogenesis as "augmentational" effects.

Ito et al. (1991) employed a similar protocol to evaluate potential synergisms between five heterocyclic amines at low doses. F344 male rats were given a single dose of DEN, followed by 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-f]quinoline (MeIQ), or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), singly or as a five-component mixture. Groups were given each chemical at the carcinogenic dose, or 1/5 or 1/25 of this. At the highest doses, all these heterocyclic amines significantly increased the number and area of glutathione-S-transferase-positive hepatic foci. Trp-P-1, IQ and MeIQ also gave responses at the 1/5 dose level. When the five chemicals were administered together at both the 1/5 and 1/25 dose levels, the number of foci was higher than the arithmetic sum of the individual chemicals, at the 1/5 or 1/25 dose groups. Similarly results were communicated by the same group (Hasegawa et al. 1994a; Hasegawa et al. 1994b) with combinations of five and 10 heterocyclic aromatic amines.

In a variation of the above approaches, Hasegawa et al. (1994b) used a combination of five nitrosamines and nitrosoureas for the initiation stage, followed by application of five heterocyclic aromatic amines to F344 rats to study the promotion of intestinal tumours. Combinations of the single heterocyclic amines that on their own were without significant effects led to enhancements of tumorigenesis, demonstrating syncarcinogenesis in the sense of "augmentational" effects.

Ito et al. (1995a) adopted a short-term initiation-promotion model to study the effects of 19 organophosphates and one organochlorine chemical on the formation of glutathione-S-transferase-positve hepatocyte foci as a preneoplastic lesion marker in rats. After initiation with the direct-acting, DNA damaging carcinogen diethylnitrosamine DEN, young rats were dosed with the test chemicals for 6 weeks via their diet. The 20 chemicals were combined at doses equivalent to their acceptable daily intakes (ADI), and to 100 times their ADI. There were increased preneoplastic lesions with the 100-times ADI mixture, but the ADI mixture did not induce observable effects. None of the selected chemicals were tested individually and the doses in this study were based on ADI values proposed by the Japanese Government reflecting a diversity of endpoints. These results can be interpreted as "syncarcinogenic" in the sense of augmentational effects.

In a further study from this group (Ito et al. 1995b; Ito et al. 1996), tumours were initiated by five carcinogens in combination. By using a multi-term, multi-organ protocol in the rat, a mixture of 40 pesticides combined at their ADI's was administered for 28 days. The same protocol was also used to evaluate a different mixture of 20 pesticides, all suspected carcinogens. None of the mixtures produced enhancements of tumour formation. However, this study is difficult to interpret, mainly because the experiment might have been under-powered, explaining the absence of effect.

4.1.1.4 Evidence of antagonistic effects between several carcinogens

Ruediger (2006) has reviewed evidence for antagonistic effects of carcinogenic agents, with emphasis on agents relevant to occupational settings. In principle, such effects can

arise when one chemical leads to the induction of detoxifying enzyme system that then eliminate a second mixture component at elevated rates. An example is 3-methylcholanthrene (3-MC), an agent capable of inducing cancers of the mammary gland and the skin. 3-MC is also a potent inducer of CYP 1A monoxygenases, and can therefore be expected to suppress the carcinogenicity of chemicals that are metabolized by this pathway. Accordingly, 3-MC which does not produce liver tumours on its own can inhibit liver tumours produced by 3-methyl-4-dimethylaminoazobenzene, a strong liver carcinogen (see references in Ruediger 2006).

Polychlorinated dioxins and furans (PCCD/F) as well as polychlorinated biphenyls (PCBs) are well-known inducers of liver tumours and cholangiocarcinomas in the rat. When administered together with other liver carcinogens such as 3-methyl-4-dimethylaminoazobenzene or diethylnitrosamine, reductions in tumour incidences occur. However, this suppressive effect is dependent on the timing and order of administration: Only when the inhibitory substance was given prior to administration of the other liver carcinogen, was a reduction in tumorigenicity observed. Enhanced tumour formation occurred when e.g. PCBs were administered after treatment with the other liver carcinogens (Ruediger 2006).

Aberrant crypt foci are preneoplastic lesions that are regarded as intermediate biomarkers for colon cancer. This endpoint was used by Steffensen et al. (1995) for investigations of different classes of colon carcinogens, 1,2-dimethylhydrazine (DMH), its metabolite azoxymethane (AOM) and 3,2'-dimethyl-4-aminobiphenylhydrochloride (DMAB). The chemicals require metabolic activation via CYP (DMAB through CYP 1A, DMH through CYP 2E1) to form ultimately active genotoxic intermediates. F344 and Lewis rats were treated with each carcinogen alone, or in combinations sequentially. Strikingly, the combinations produced reductions in the number of aberrant crypt foci when compared with the numbers seen after single administration. The authors were unable to offer an explanation for these effects, but ruled out metabolic interactions as the reason for these suppressive effects.

In summarizing the evidence, Ruediger (2006) listed various mechanisms that might lead to attenuations of carcinogenic effects of chemicals. Mechanisms for which there is good experimental evidence include: inhibitions of metabolic activations of procarcinogens, induction of metabolic inactivation, slow-down of cell cycle progression, and induction of apoptosis. He pointed out that all these proposed mechanisms of cancer suppressing effects of a chemical are not linked to the carcinogenicity of a substance. Rather, an inhibitory effect appears to be possible only if the suppressing component has a weaker carcinogenic potency than the other chemical in the combination.

4.1.1.5 Summary carcinogencity studies

There is overwhelming evidence that carcinogens work together to exert tumorigenic responses after sequential or simultaneous exposure. Joint carcinogenic action occurs when carcinogens are combined at doses that individually are without observable effects. It is to be expected with combinations of carcinogens that target the same organ or tissue.

Suppressions of carcinogenic effects have also been described, and such effects are highly likely when the inhibitory agent is either not carcinogenic to the tissue in question, or has a much lower potency than the second carcinogen.

Due to the specific ways in which the question of "syncarcinogenesis" has been framed, there are very few studies that allow assessments of combination effects in terms of additivity expectations derived from DA or IA. The few examples that permit such evaluations indicate agreement with DA or IA. This suggests that many "syncarcinogenic" effects do not represent true synergisms in terms of responses greater than expected according to DA or IA, but rather are consistent with those additivity concepts.

4.1.2 Mutagenicity and genotoxicity

For the purposes of this appraisal, combination effects observed with mutagenic chemicals and those that induce genotoxicity are discussed together. Mutagenicity in the narrow sense of the word can be defined as the induction of heritable changes in the DNA sequence of the affected organism, whereas genotoxicity is often used in an overlapping, but wider sense, including chromosome mutations, chromosomal aberrations and sister chromatid exchanges. The induction of micronuclei is also judged to be a genotoxic effect.

There is a fair amount of data available on the combined effects of mixtures of chemicals that induce mutagenic and genotoxic effects, but again only a limited number of these studies are informative with respect to the type of combination effect (CA or IA).

Lutz et al. (2002) tested a mixture of benzo[a]pyrene, benz[a]anthracene, and dibenz[a,c]anthracene in the Ames test, using Salmonella typhimurium TA100 and rat liver S9 fraction. Based on experiments with the individual chemicals, low effect doses were established that produced a doubling of revertants in the Ames assay. Lutz et al. termed these doses lowest observable effect level (LOEL). The three chemicals were then combined at 1/3 of their individual LOEL, with the expectation that the mixture should also not produce more than a doubling of the number of revertants. This expectation is in line with dose addition. Combined treatment produced responses in good agreement with dose additivity.

Lutz et al (2002) also investigated the induction of micronuclei in vitro with ionizing radiation from a Cs-137 source and ethyl methanesulfonate. Mouse lymphoma L5178Y cells revealed a significantly higher than dose additive effect in an experiment based on three independent replicates for controls and single and combination treatments. However, this synergism was dependent on the cell line used. When alternative cell lines were employed (human lymphoblastoid cell lines TK6 and WTK1, human primary fibroblasts from fetal lung) dose additive effects were observed.

Kligerman et al. (1993) used analytical data about chemicals found in US groundwaters to reconstitute laboratory mixtures, with the aim of assessing cytogenetic toxicity in rodents. This study was not motivated by establishing the type of combination effect (in terms of additivity, synergism, antagonism), but rather by investigating whether mixture effects would be observed at environmentally relevant concentrations of pollutants. Mixtures representative of measured ground water concentrations were administered to F344 rats and B6C3F1 mice; 10- and 100-fold higher concentrations were also tested. After 71 days of continuous dosing of rats, and 91 days of mice, lymphocytes were harvested, cultured and analyzed for sister chromatid exchanges, chromosome aberrations and micronuclei. Induction of sister chromatid exchanges was seen at all concentrations in the rat, but with chromosome aberrations and micronuclei as endpoints of evaluation, effects did not become apparent. Mice did not show any effects. Considering the pollutant profile in these mixtures, 1,2 dichloropropane, 1,2 dibromo 3-chloropropane and ethylene dibromide can be thought of as candidate chemicals responsible for these effects. However, single chemical studies were not conducted, making it difficult to attribute the observed effects in the rat to any specific combinations of chemicals.

Dolara and colleagues have presented a series of genotoxicity studies in cultured human lymphocytes where chemicals were combined at concentrations that individually did not produce any discernible effects. These studies do not permit identification of the type of combination effect involved, nor were they designed to support such assessments. Dolara et al. (1992) tested dimethoate and omethoate (two organophosphate pesticides), deltamethirn, and benomyl and observed dose-related increases in the frequency of sister chromatic exchanges. The four chemicals were combined at effect doses that produced sister chromatid exchanges in the range of untreated controls. Statistically significant effects were observed with the mixture. This study demonstrates that mixture effects can occur when concentrations associated with non-detectable effects are combined.

Based on analyses of common food items in Italy, Dolara et al. (1993) prepared a mixture of 15 pesticides which they tested for bacterial mutagencity, induction of sister chromatid exchanges in cultured human lymphocytes and micronuclei in the bone marrow of rats. Small effects were observed in the human lymphocyte assay, but the other test systems did not reveal any effects.

In a further report on a mixture of 15 pesticides, combined in proportion to the levels found in food, Dolara et al (1994) detected concentration-dependent increases in the number of non-synchronous centromeric separations in cultured human lymphocytes. Other cytogenetic effects were not observed, and the effect disappeared when benomyl was removed from the mixture.

Staal et al. (2007a) used human hepatoma cells to assess whether the effects of binary PAH mixtures on gene expression, DNA adduct formation, apoptosis and cell cycle are additive compared with the effects of the individual compounds. Equimolar and equitoxic mixtures of benzo[a]pyrene (B[a]P) with either dibenzo[a,l]pyrene (DB[a,l]P), dibenzo[a,h]anthracene (DB[a,h]A), benzo[b]fluoranthene (B[b]F), fluoranthene (FA) or 1-methylphenanthrene (1-MPA) were studied. DB[a,l]P, B[a]P, DB[a,h]A and B[b]F

dose-dependently increased apoptosis and blocked cells cycle in S-phase. Binary PAH mixtures showed an additive effect on apoptosis and on cell cycle blockage, but this evaluation was based on the idea of effect summation. DNA adduct formation in mixtures was higher than expected according to simple effect summation, which the authors interpreted as a synergistic effect.

The same group also investigated gene expression and DNA adduct formation in liver slices (Staal et al. 2007b). The effects of benzo[a]pyrene or dibenzo[a,h]anthracene (DB[a,h]A) alone and in binary mixtures with another PAH (DB[a,h]A, benzo[b]fluoranthene, fluoranthene or dibenzo[a,l]pyrene) were analyzed. All mixtures showed a response on total gene expression profiles that fell short of what the authors expected based on effect summation. In contradiction to the findings communicated earlier by this group with DNA adduct formation as the endpoint (Staal et al. 2007), the binary mixtures generally also caused effects smaller than expected according to effect summation.

4.1.2.1 Summary of mutagenicity and genotoxicity mixture studies

As with the carcinogenicity studies discussed earlier, there is a dearth of mixture experiments with mutagencity and genotoxicity as the endpoints for evaluation that allow clear assessments of the usefulness of CA or IA as prediction concepts. Some publications however show that genotoxic and mutagenic agents, combined in sufficient numbers, can work together at very low concentrations to produce mixture effects.

4.2 Reproductive and developmental toxicity, teratogenicity

A series of papers on the effects of combinations of anti-androgens on male offspring exposed during development *in utero* has been published and will be discussed in more detail in Section 4.4.2.3. of this report. These publications will be briefly summarized here. In all these studies, explicit additivity expectations formed the basis for mixture effect assessments.

Hass et al. (2007) and Metzdorff et al. (2007) found dose additive effects with a mixture of androgen receptor antagonists (vinclozolin, flutamide, and procymidone), when disruption of hallmarks of male sexual differentiation (changes in anogenital distance, retained nipples, reproductive organ weights, androgen-related gene expression) were analyzed. The effects on nipple retention, however, were slightly stronger than expected by dose addition.

Howdeshell et al. (2007) examined the effects of a binary mixture of the phthalates dibutyl-benzyl-phthalate (DBP) and di-(2-ethylhexyl)-phthalate (DEHP) after exposure of pregnant rats. The male offspring was examined for a wide range of effects typical of disruption of male sexual differentiation. This study indicates that dose addition provides fairly good predictions of the effects typical of disruption of male sexual differentiation. Independent action often underestimated the observed responses.

Using a similar experimental model, Rider et al. (2008) conducted mixture experiments with the three phthalates BBP, DBP, and DEHP in combination with the antiandrogens vinclozolin, procymidone, linuron, and prochloraz. In calculating additivity expectations, the authors used historical data from their laboratory. Despite some uncertainty inevitably introduced by those assumptions, dose addition gave predictions of combination effects for the mixed-mode antiandrogens that agreed better with the observed responses than the expectations derived from independent action.

Other mixture studies with endpoints relevant to reproductive and developmental toxicity have been conducted, but little attention was paid to formulating additivity expectations. Often, the study design does not permit re-analysis of the published data in order to determine the underlying type of combination effect.

Narotsky et al. (1995) chose to investigate a mixture of trichloroethylene, DEHP and heptachlor on the development of F344 rats. All three chemicals compromised maternal weight gain, and combination effects between trichloroethylene and DEHP occurred which the authors interpreted as synergistic. With the same endpoint, there was antagonism between DEHP and heptachlor. Without further justification, the authors expected that the joint effect of the chemicals should be equal to the arithmetic sum of their individual effects, and additivity expectations according to dose addition were not calculated. Consequently, the synergistic mixture effects could have been in line with dose addition. Similar considerations apply to the apparent synergism between trichloroethylene and DEHP on prenatal loss, and some other interactions described int his paper.

Calciu et al. (1997) investigated the teratogenic effects of camphechlor, two of its congeners T2 and T12, and combinations of T2 and T12 in cultured rat embryos. Morphological scores, crown-rump length and head length were all affected by all treatments, including single chemicals and mixtures. The mixture of T2 and T12 exhibited what the authors interpreted as synergism on decreasing crwon-rump and head lengths, but this evaluation was conducted implicitly assuming simple effect summation.

You et al. (2002) presented a study of the effects of the phytoestrogen genistein on the developmental toxicity of the pesticide methoxychlor in the rat. Effect outcomes considered were accelerated vaginal opening and delayed preputial separation in female and male offspring, respectively. The joint effect of the two chemicals was greater than each individual effect, but there was insufficient dose-response information to assess the type of underlying combination effect.

Lee et al. (2006) analyzed the joint effects of coadministration of cadmium and retinoic acid on developing limbs in C57BL/6 mice. Pregnant mice were treated with different doses of cadmium chloride and/or RA on gestational day (GD) 9.5. The chemicals were administered by intraperitoneal injection, a mode of delivery that is regarded as problematic because it may lead to disturbances during gestation. The fetuses were collected on GD 18 and double stained for examination of skeletal defects. Retinoic acid

and cadmium together induced a significant increase in the incidence and severity of forelimb ectrodactyly, relative to the effects seen with retinoic acid or cadmium alone. When mice were exposed to what the authors refer to as "subthreshold doses" of both cadmium (0.5 mg/kg) and retinoic acid (1 mg/kg), the combined treatment led to observable effects, with forelimb ectrodactyly in 19% of the fetuses. At higher doses, the two chemicals showed what the authors interpreted as synergistic effects, that is effects far exceeding the simple arithmetic sum of the chemicals' single responses. However, this could also have been a dose additive effect, but for lack of dose response information in this paper, this idea cannot be investigated.

Very recently, Christiansen et al. (2009) communicated the results of a mixture experiment with di-ethylhexyl-phthalate, vinclozolin, finasteride and prochloaz in a reproductive toxicology model for the evaluation of disruption of male sexual differentiation. With respect to changes in anogenital distance in the male offspring of exposed pregnant rats, there was dose additvity. Similar effects were observed when other hallmarks of male sexual development, including retained nipples and organ weight of sex organs and accessory glands, were evaluated. Strikingly, there was a pronounced synergistic effect with penile malformations, exceeding the responses expected on the basis of both DA and IA.

4.3 Respiratory toxicity

Comparatively few studies have been conducted with mixtures of toxicants affecting the respiratory system. Noteworthy are the results of model experiments published by Cassee and colleagues (Cassee et al. 1996; Cassee, Groten, & Feron 1996) on the effects of combinations of formaldehyde, acetaldehyde and acrolein on the respiratory system of the rat.

Various vapours and gaseous compounds can induce irritation of the nasal mucosa. The exposed animals react to this toxic insult by reducing their respiratory rate, an effect associated with direct stimulation of the trigeminal nerve endings in the nasal mucosa. Cassee et al. (1996a) have used this endpoint to study the irritant effects of formaldehyde, acetaldehyde and acrolein in Wistar rats. Dose-response relationships for the individual chemicals were established, and mixtures of all three compounds with varying mixture ratios tested. The observed effects were compared with additivity expectations derived from simple addition of the effects of the single aldehydes (effect summation), and by using a competitive agonism model. The observed responses were stronger than those predicted by effect summation, but agreed reasonably well with the agonism model.

A similar system was used by Cassee et al. (1996b) to assess histopathological and proliferative changes of the nasal epithelium after exposure to formaldehyde, acrolein and acetaldehyde, and their mixtures. Formaldehyde and acrolein produced adverse changes of the nasal epithelia, while the effects of acetaldehyde were classed as being of doubtful toxicological relevance. When the animals were exposed to a mixture of all three chemicals at high doses, changes in the epithelia and the olfactory region of the nose

were observed that were more severe than those with the single chemicals. Binary mixtures of formaldehyde and acrolein were also studied, and the authors assessed these effects as additive. Acrolein and/or formaldehyde seemed to potentiate the effects of acetaldehyde. At low doses of all three chemicals, changes were observed that were very similar to those of acrolein at the doses present in the mixture, suggesting that a true combination effect was not induced.

Schlesinger et al. (1992) exposed rabbits to sulfuric acid vapours in combination with ozone. The animals were sacrificed and the lungs lavaged in order to obtain various cells of the immune system, including macrophages. What the authors evaluated as an antagonistic effect was observed when phagocytic activity of macrophages was analyzed. Similar antagonisms were observed with superoxide production by stimulated macrophages as the endpoint. In contrast, combination effects assessed by the authors as synergistic were seen with tumour necrosis factor-induced cytotoxicity as the endpoint of evaluation. However, this study used simple effect summation as the basis for these evaluations, with no supporting dose-response analyses. The type of combination effect is therefore indeterminate.

It is quite well established that ultrafine particulate matter can exacerbate the respiratory toxicity of corrosive gases, but experimental studies that recapitulated such combined effects in animals could not be located, and have not been carried out to our knowledge.

4.4 Endocrine disruption

In studying endocrine disrupter mixtures, many researchers have followed what has been called a "whole mixture approach" where a combination of many chemicals is investigated as if it were a single agent, without assessing the individual effects of all the components. This type of experiment is useful for studying complex mixtures, or on a case-by-case basis, but leads to difficulties in extrapolating from one mixture to the other because small variations in composition may lead to significant changes in its toxic effects. But whole mixture approaches do not answer whether chemicals act in an additive, antagonistic or synergistic fashion. However, one of the major difficulties in assessing endocrine disrupters is uncertainty about their potential to act together in an additive or synergistic manner. To address these concerns the review focuses on studies that have assessed endocrine disrupter mixtures in terms of additivity, antagonism or synergy. Typically, such studies attempt to predict additive combination effects on the basis of information about the effects of all components in the mixture.

In the following, work with the three most frequently studied hormone receptors, the estrogen, androgen and thyroid receptors, will be considered. There is a rich literature concerning the Ah-receptor (AhR), which will be reviewed in Section 4.8., but interactions between AhR agonists and other endocrine disrupters will be dealt with here. Section 4.4. is an extended and updated version of an earlier review by Kortenkamp et al (2007).

4.4.1 Mixtures of estrogenic chemicals

Estrogenic chemicals have been the focus of most of the work on endocrine disrupters. While the earlier efforts have mainly employed binary mixtures (reviewed in (Kortenkamp & Altenburger 1998), work carried out since 1998 has made significant contributions to the analysis of multi-component mixtures containing three, often five and up to 12 estrogenic chemicals.

"Estrogenicity" can be defined in various ways. At the functional, physiological level, the term denotes the ability of a chemical to evoke responses similar to 17β-estradiol (E2), such as cornification of the vaginal epithelium, and uterine cell proliferation. Of toxicological concern is the role of estrogens in breast and ovarian cancer, and 17β-estradiol and synthetic estrogens are recognised human carcinogens. Advances in the understanding of the mode of action of estrogens have led to further definitions which refer to specific steps at various molecular levels, and this suggests itself as a way to structure the evidence on estrogen mixtures: Thus, "estrogenicity" can mean affinity to the estrogen receptor (ERα or β) (although this does not distinguish agonists from antagonists), the ability to activate expression of estrogen-dependent genes, or stimulation of cell proliferation of ER-competent cells. At the time of writing, no post-1998 multi-component study with ER binding as the endpoint was available.

4.4.1.1 Estrogen receptor activation

Payne et al. (2000) studied combinations of two, three and four estrogenic chemicals in the yeast estrogen screen (YES), an ERα-based gene reporter system. Individual dose-response curves for *o,p'*-DDT, genistein, 4-nonylphenol and 4-n-octylphenol were recorded and this information was used to successfully predict the joint effects of *o,p'*-DDT, genistein, 4-nonylphenol and 4-n-octylphenol for mixtures with a fixed ratio. Rajapakse et al. (2002) and Silva et al. (2002) have extended this approach to the analysis of mixtures involving eight and twelve estrogenic agents, respectively. In both cases, the mixture responses seen with the YES agreed excellently with the effects predicted by concentration addition. In an attempt to verify the assumption that concentration addition is an appropriate model for estrogen mixtures, the observed mixture effects were also compared with additivity predictions calculated using independent action. In the paper by Payne et al. (2000) both concepts produced very similar predictions. However, Silva et al. (2002) and Rajapakse et al. (2002) found that independent action underestimated the observed mixture effects by a large margin.

Examinations of the effects of ternary mixtures of estrogenic chemicals in an ERα gene reporter system based on MCF7 cells were carried out by Charles et al. (2002a). All mixtures were examined in a factorial design involving 64 treatment groups, and response surfaces constructed. Combinations of E2, 17α-ethynyl estradiol (EE2) and diethylstilbestrol showed concentration additive effects when all components were present at levels that fell within the linear range of their individual dose-response curves. At higher concentrations, however, the combined effect of the three estrogens fell short of expected additivity, a phenomenon which the authors attributed to saturation effects. In

a second paper, the same group investigated ternary combinations of further estrogenic chemicals. While combinations of benzo-[a]-pyrene, 1,2-benzanthracene and chrysene, and of methoxychlor, *o,p'*-DDT and dieldrin showed concentration additivity over a wide range of mixture ratios, the joint effects of E2, genistein and *o,p'*-DDT were antagonistic both in the low and the high concentration range (Charles et al. 2002b).

Activation of ERα was monitored by measuring expression of the *TFF1* gene (coding for the pS2 protein) to study the effects of combinations of estrogenic UV filter substances (Heneweer et al. 2005). Binary mixtures of 2-hydroxy-4-methoxy-benzophenone and its metabolite 2,4-dihydroxybenzophenone showed concentration additive effects, as did a combination of these two chemicals with octyl methoxycinnamate and 3-(4-methylbenzylidene) camphor. In a TEQ approach the authors expressed effect concentrations of the test chemicals in terms of 17β-estradiol equivalents. Le Page et al. (2006) developed a reporter gene assay based on glial cells (U251-MG) transfected with three zebrafish ER subtypes and the brain aromatase promoter linked to luciferase. This system was used to study a mixture of E2, EE2, estrone, genistein and α-zeralenol, with effects well in agreement with concentration addition.

Fent et al. (2006) reported on the effects of various pharmaceuticals in the YES assay and found that combinations of furosemide and E2, and of furosemide and phenazone were dose additive.

Kunz and Fent (2006) studied the effects of combinations of two, four and eight UV filter substances, with and without E2, and observed synergistic effects in the YES assay.

Van Meeuwen et al. (2007) investigated combinations of endogenous estrogens, such as E2 and various phytoestrogens and synthetic estrogens, with upregulation of the pS2 gene in MCF-7 BUS cells as the endpoint. Transcription of the pS2 gene is controlled by the ERα. Phytoestrogens (coumestrol, genistein, naringenin, catechin, epicatechin, quercetin) or synthetic estrogens (4-nonylphenol, octylphenol, beta-hexachlorocyclohexane, bisphenol A, methoxychlor, dibutyl phthalate) were mixed either in concentrations reflecting human serum concentrations or at equipotent concentrations for estrogenicity. Observed combination effects were assessed against additivity expectations derived from an application of the TEF approach, by using "estrogen equivalency factors". No departures from additivity were observed.

Charles et al. (2007) used an in vitro human estrogen receptor (ER) transcriptional activation assay to evaluate a mixture of six synthetic estrogens, methoxyclor, o,p-DDT, octylphenol, bisphenol A, β-hexachlorocyclohexane and 2,3-bis(4-hydroxypbenyl)-propionitrile. Dose-response curves were characterized for each of these chemicals, which were then combined at equipotent mixture ratios. Small deviations from expected concentration additivity (in the direction of an antagonism) were observed with this mixture. It is unclear whether these small deviations were due to true interactions, or whether they were a consequence of the effect of regression modeling for the individual chemicals. Fixed concentrations of the mixture of the six synthetic estrogens were also tested in the presence of varying levels of the two phytoestrogens genistein and daidzein.

Low concentrations of the synthetic estrogen mixture failed to increase estrogenic responses relative to those induced by phytoestrogens alone. However, significant increases in response occurred when each chemical in the synthetic estrogens mixture was near or above its individual response threshold. The authors evaluated the mixture effect between high doses of synthetic estrogens and phytoestrogens as greater than additive, but this evaluation was based on the assumption that departures from concentration additivity can be recognized when the dose response curve of the synthetic estrogen/phytoestrogen mixture shows a gradient different from that of the phytoestrogen combination. This is only the case if all chemicals in the mixture show parallel dose response curves, a pre-condition not fulfilled with the tested chemicals.

4.4.1.2 Cell proliferation

The effects of *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and β-HCH on the proliferation of estrogen dependent MCF7 cells (E-Screen assay) were found to be concentration additive at two different mixture ratios, but the observed responses were equally well predicted by independent action (Payne, Scholze, & Kortenkamp 2001).

Suzuki et al. (2001) tested binary mixtures of natural and synthetic estrogenic chemicals including E2, estrone, bisphenol A, butyl benzylphthalate, endosulfan, methoxychlor and pentachlorophenol for proliferative effects in MCF7 cells. Using an effect multiplication method to construct contour plots, the authors observed apparent synergisms with E2 and bisphenol A, while the remaining eight binary combinations gave additive, antagonistic or weakly synergistic effects. However, the interpretation of these results is complicated by the fact that additivity expectations were calculated by multiplication of unscaled effect measures, a method inconsistent with independent action.

Rajapakse et al.(2004) analysed mixtures containing E2, EE2, genistein, bisphenol A, 4-nonylphenol and 4 *tert*-octylphenol in the E-Screen assay. A small deviation from concentration additivity was observed. Interestingly, the omission of genistein produced an even more pronounced antagonism. However, a three-component mixture composed of E2, EE2, and genistein produced excellent agreement with predicted concentration additivity, and the same was observed for a four-component mixture with E2, EE2, genistein and bisphenol A. The presence of 4-nonylphenol and 4 *tert*-octylphenol appeared to be associated with the observed antagonisms. It is conceivable that differential activation of metabolising enzymes (e.g. cytochrome P450) or efflux pumps by mixture components has led to removal of other constituents, but this hypothesis awaits experimental confirmation.

Schmidt et al. (2005) studied combinations of various phytoestrogens and E2, with the aim of measuring joint effects on cell proliferation and apoptosis. Combination effects were not detected. This study lacks an explicit additvity expectation, and observed effects of mixtures were not evaluated.

Van Meeuwen et al. (2007) investigated combinations of endogenous estrogens, such as E2 and various phytoestrogens and synthetic estrogens, with cell proliferation in MCF-7

BUS cells (E-Screen) as the endpoint. Phytoestrogens (coumestrol, genistein, naringenin, catechin, epicatechin, quercetin) or synthetic estrogens (4-nonylphenol, octylphenol, beta-hexachlorocyclohexane, bisphenol A, methoxychlor, dibutyl phthalate) were mixed either in concentrations reflecting human serum concentrations or at equipotent concentrations for estrogenicity. Observed combination effects were assessed against additivity expectations derived from an application of the TEF approach, by using "estrogen equivalency factors". No departures from additivity were observed.

4.4.1.3 Uterotrophic assays

Charles et al. (2002a) were the first to confirm the additive effect of combinations of E2, ethynyl estradiol and diethylstilbestrol using uterine proliferation in immature CD-1 mice as the endpoint. Response surfaces constructed for permutations of each chemical at three dose levels demonstrated that the combined effects of all agents were additive.

Tinwell and Ashby (2004) have presented a study involving eight estrogenic chemicals using the uterotrophic assay with immature rats, but in this study no explicit additivity expectation was derived. The combined effect of all chemicals was always larger than the responses observed with individual components.

Diehl et al. (2006) conducted studies of combinations of genistein and E2 in the uterotrophic assay with ovariectomised Wistar rats. Dose-response studies for the individual chemicals were not conducted, and this study operates without any explicit additivity expectation. The authors seem to have fallen into the trap of effect summation, with the implicit observation that the effects seen with single chemicals should add up arithmetically. Sometimes, combination effects smaller than, or equal to the effects of the single chemicals were observed, but due to the lack of dose-response information in this study, these effects cannot be evaluated in terms of type of combination effect.

Charles et al. (2007) studied a mixture of six synthetic estrogenic chemicals (methoxyclor, o,p-DDT, octylphenol, bisphenol A, -hexachlorocyclohexane, 2,3-bis(4-hydroxypbenyl)-propionitrile)), together with a combination of the phytoestrogens genistein and daidzein in the uterotrophic assay with immature rats. The mixture responses were consistent with dose additivity.

4.4.1.4 Summary: estrogen mixtures

The available evidence shows clearly that dose (concentration) addition proved to be a valid tool for the prediction and assessment of combination effects of estrogen mixtures. Independent action led to underestimations of the observed effects.

4.4.2 Mixtures of androgen receptor antagonists and other anti-androgens

Androgens are key regulators of male sexual differentiation during the *in utero* and early postnatal development. Chemicals that counteract androgen action at some stage in this

period can lead to malformations of the reproductive tract. Changes in the anogenital distance, retained nipples and alterations in the weight of sexual organs and accessory glands are frequently studied endpoints. These effects can arise through antagonism of androgens at the steroid receptor level and/or *via* suppression of testosterone synthesis in Leydig cells. Thus, anti-androgens can be defined narrowly as androgen receptor (AR) antagonists, but a broader definition in terms of counteracting the effects of androgens in a functional sense (which would include inhibition of uptake of testosterone precursors, and of testosterone synthesis steps) has also been proposed.

Anti-androgens can disrupt male sexual differentiation in different ways. In fetal life, testosterone is a key driver of the differentiation of the Wolffian duct system into the vas deferens, epididymis, and seminal vesicles. Phthalates with a certain ester side-chain length can lower testosterone levels by interfering with the uptake of cholesterol precursors into fetal Leydig cells, where testicular androgen production takes place. In the rat, malformations of internal reproductive organs (epididymis, testes) are the consequence. Because dihydrotestosterone (DHT) is derived from testosterone through enzymatic conversion by aromatase, lower testosterone concentrations also affect the development of tissues that rely on DHT (prostate and external genitalia). DHT is further required for the regression of nipple anlagen in male rats and for the growth of the perineum to produce the normal male anogenital distance (AGD) which is longer than in females. Due to reduced DHT levels in the wake of suppressed testosterone synthesis, retained nipples and feminised AGDs are also seen in male rats exposed to phthalates in fetal life. AR antagonists impact more directly on the development of DHT-dependent tissues by blocking the androgen receptor (AR). Disruption of the enzymatic conversion of testosterone to DHT through inhibition of aromatase induces an effect spectrum similar to AR antagonists.

4.4.2.1 Androgen receptor antagonism

By applying the isobole method it was found that procymidone and vinclozolin, both AR antagonists, additively inhibited testosterone binding to the AR (Nellemann et al. 2003). Administration of a 1:1 mixture of both fungizides to castrated, testosterone-treated male rats led to dose additive alterations in reproductive organs weights, androgen levels and androgen receptor-dependent gene expression.

Birkhoj et al. (2004) have extended the use of the isobole method to three-component mixtures of the pesticides deltamethrin, methiocarb and prochloraz. An equimolar mixture of the three pesticides additively suppressed AR activation *in vitro*. When a combination of these three chemicals with simazin and tribenuron-methyl was given to castrated testosterone-treated rats, weight changes of the adrenal gland and the levator ani, as well as alterations in gene expression of AR-associated genes were observed. The combination of all five chemicals showed effects that were not found for the individual pesticides, but whether these responses were additive could not be assessed.

A mixture of the AR antagonists procymidone and vinclozolin was evaluated in the Hershberger assay where they acted additively in reducing ventral prostate and levator ani weights (Gray et al. 2001).

4.4.2.2 Suppression of testosterone synthesis in vivo

Recently, Howdeshell et al. (2008) presented the results of a mixture study with five phthalates, in which suppressions of fetal testosterone production at gestational day 18 were measured as a result of exposure of pregnant Sprague-Dawley rats. Butyl-benzyl phthalate (BBP), di-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), di-(isobutyl) phthalate (DiBP), and di-propyl phthalate (DPP) were combined at a fixed mixture ratio. Over a large range of effect levels, the observed reductions in testosterone production agreed well with the responses anticipated by dose addition. The study provides good evidence that for mixtures of phthalates capable of suppressing fetal testosterone synthesis dose addition provides a better prediction of joint effects than independent action.

4.4.2.3 Demasculinisation in male offspring exposed in utero

Wolf et al. (2004) observed that vinclozolin and testosterone proprionate, two chemicals with opposing effects on male sexual differentiation, antagonized one another during sexual development of the male rat.

Hotchkiss et al. (Hotchkiss et al. 2004) investigated a mixture of BBP and linuron, an antiandrogen capable of antagonizing the androgen receptor and of disrupting steroid synthesis. The combination induced decreased testosterone production and caused alterations of androgen-organized tissues and malformations of external genitalia. Quantitative additivity expectations based on the effects of the single chemicals were not calculated in this study, and therefore assessments concerning agreement with dose addition or independent action are not possible. However, the combination of BBP and linuron always produced stronger effects than each chemical on its own.

Jarfelt et al. (2005) studied changes in anogenital distance and retained nipples of male offspring of female rats treated with DEHP and di-(2-ethylhexyl)adipate (DEHA), but the effects of the mixture were not different from those of the single chemicals.

Hass et al. (2007) examined a mixture of three androgen receptor antagonists (vinclozolin, flutamide, and procymidone) in an extended developmental toxicity model in the rat. Disruption of sexual differentiation in male offspring was studied with changes in anogenital distance (AGD) and retained nipples (NR) as endpoints. Based on AGD changes, the joint effect of the three chemicals was predicted well by dose addition, but with NR the observed effects were slightly stronger than those anticipated by dose addition.

Metzdorff et al. (2007) analyzed further the material from the Hass et al. (2007) study by following effects typical of antiandrogen action through different levels of biologic

complexity. Changes in reproductive organ weights and of androgen-regulated gene expression in prostates from male rat pups were chosen as endpoints for extensive dose-response studies. With all the endpoints, the joint effects of the three anti-androgens were dose-additive.

Howdeshell et al. (2007) examined a binary mixture of the phthalates dibutyl-benzyl-phthalate (DBP) and di-(2-ethylhexyl)-phthalate (DEHP). Female pregnant Sprague-Dawley rats were exposed to the phthalates during gestational days 14 to 18 at a dose of 500 mg/kg-day each, both singly and in combination. The male offspring was examined for a wide range of effects typical of disruption of male sexual differentiation, including altered fetal testosterone production, changes in anogenital distance, epididymal agenesis, retained nipples, gubernacular agenesis, hypospadias, and total number of animals with malformations. Dose addition generally predicted larger effects than independent action, although for certain endpoints, both concepts anticipated equal effects. Unfortunately, it is not possible to recapitulate the dose-addition predictions given by the authors, because they were based on unpublished dose-response data for the individual phthalates. However, the authors observed that the responses generally agreed well with dose addition and were higher than the additivity expectations derived from independent action for changes in anogenital distances, epididymal agenesis, and total number of malformed males. This study indicates that dose addition provides fairly good predictions of the effects typical of disruption of male sexual differentiation. Independent action often underestimated the observed responses.

Recently, Howdeshell et al. (2008) presented the results of a mixture study of five phthalates in which suppression of fetal testosterone production at gestational day 18 was measured as a result of exposure of pregnant Sprague-Dawley rats. BBP, DBP, DEHP, DIBP, and DPP were combined in a fixed ratio. Over a large range of effect levels, the observed reductions in testosterone production agreed well with the responses predicted by dose addition.

Rider et al. (2008) conducted mixture experiments with the three phthalates BBP, DBP, and DEHP in combination with the antiandrogens vinclozolin, procymidone, linuron, and prochloraz. The mixture was given to pregnant rats with the aim of examining the male offspring for a variety of developmental effects typical of antiandrogens. This mixture contains components that act by a variety of antiandrogenic modes of action. Vinclozolin and procymidone are androgen-receptor antagonists, and linuron and prochloraz exhibit a mixed mechanism of action by inhibiting steroid synthesis and antagonizing the steroid receptor. In calculating additivity expectations, the authors used historical data from their laboratory; however, the studies sometimes had employed dosing regimens that differed from those employed in the mixture experiments. Data about the effects of some individual phthalates were not available. To bridge that data gap for the purpose of computing additivity expectations, it was assumed that the three phthalates were equipotent. Despite a certain degree of uncertainty inevitably introduced by those assumptions, dose addition gave predictions of combination effects for the mixed-mode antiandrogens that agreed better with the observed responses than the expectations derived from independent action. For a number of endpoints, including seminal vesicle

weights, epididymal agenesis, and retained nipples, there was reasonable agreement with dose addition. For others, such as hypospadias, the observed effects exceeded the dose addition expectation. In all cases, independent action led to considerable underestimations of the observed combined effects.

Very recently, Christiansen et al. (2009) communicated investigations of the consequences of simultaneous exposure to anti-androgens that exert their actions by differing molecular mechanisms. Mixtures of DEHP, two fungicides present in food, vinclozolin and prochloraz, and a pharmaceutical, finasteride were administered to pregnant rats and their effects on landmarks of sexual development in male offspring were analyzed, including changes in anogenital distance, retained nipples, sex organ weights and malformations of genitalia. Strikingly, the effect of combined exposure to the selected chemicals on malformations of external sex organs was synergistic, and the observed responses were greater than would be predicted from the toxicities of the individual chemicals. A dose of the mixture predicted by DA to elicit only marginal incidences of malformations produced effects in nearly all the animals. These observations substantiate earlier indications reported by Rider et al. (2008) of synergisms with hypospadias. However, the molecular mechanisms that might explain this synergism remain elusive. In relation to other hallmarks of disrupted male sexual development, including changes in anogenital distance, retained nipples, and sex organ weights, the combined effects were dose additive. When the four chemicals were combined at doses equal to no-observed-adverse-effect levels estimated for nipple retention, significant reductions in anogenital distance were observed in male offspring.

4.4.2.4 Summary: anti-androgen mixtures

In general, mixtures of anti-androgens followed dose addition, for a variety of endpoints typical of disruption of androgen action. This held true even for mixtures composed of anti-androgens that display a variety of mechanisms of action. No example could be identified, where independent action provided a mixture effect prediction that was more conservative than dose addition, and at the same time proved to be in good agreement with experimental data.

4.4.3 Mixtures of thyroid-disrupting chemicals

Compared with estrogens and anti-androgens, thyroid-disrupting chemicals are the least well studied endocrine disrupters. It is therefore not surprising, that few mixture studies exist using this kind of agents.

Thyroid-disrupting chemicals can alter structure and function of the thyroid gland, as well as the homeostasis of thyroid hormones by interfering with associated regulatory enzymes. Changes in the circulating levels of thyroid hormones are often the consequence. A wide variety of chemicals are able to affect thyroid hormone levels in differing ways. PCDDs, PCDFs and PCBs are thought to suppress circulating thyroid hormone levels by up-regulating hepatic enzymes that glucuronidate thyroxin (T4). Most

of the studies of thyroid disrupting effects have analysed the effects of mixtures without recording responses induced by individual mixture components, and this complicates assessment of combination effects in terms of additivity, synergism or antagonism. Wade et al. (Wade et al. 2002) exposed rats to a combination of organochlorines and two heavy metals and analysed effects on thyroid histopathology. Desaulniers et al. (Desaulniers et al. 2003) used the TCDD equivalents method and found that the effects of 16 polychlorinated biphenyls, dioxins and furans on circulating thyroxin levels could be predicted well.

Crofton et al. (2005) have presented an in-depth study of a mixture of 18 polyhalogenated hydrocarbons (2 PCDDs, 4 PCDFs and 12 co-planar and non-coplanar PCBs) to investigate the hypothesis that their joint effect on reducing T4 levels is dose-additive. Young female rats were treated for four days with individual mixture components and dose-response relationships with altered T4 levels as the endpoint recorded. This information was used to predict the dose-additive response to a mixture of all 18 chemicals. The mixture ratio was chosen to be proportional to the levels of the chemicals reported in breast milk, fish and other human food sources. The dose additivity model yielded anticipated effect doses that were higher by a factor of 2-3 than the observed responses. This deviation was statistically significant, and the joint effect of all polyhalogenated pollutants in this model can therefore be classed as synergistic. Nevertheless, the extent of underestimation of observed effects was small.

4.4.4 Summary of mixture studies with endocrine disrupters of the same class

Taken together, there is good evidence that endocrine disrupting chemicals produce combination effects in a dose additive manner. This applies to a wide range of endpoints reflecting various hierarchical levels of hormone action in a variety of organisms. Where deviations from expected additivity occurred (Charles et al. 2002ab; Crofton et al. 2005; Rajapakse et al. 2004) the differences between anticipated and observed effects were small. Thus, it is safe to say that for regulatory purposes the concept of dose addition is sufficiently accurate for predicting combination effects of groups of endocrine disrupters with similar effects.

The reported deviations are nevertheless interesting from a conceptual view point. Toxicokinetic interactions such as differential activations of metabolising enzymes in the mixtures may have played a role, and this requires further experimental study. For example, some estrogenic organochlorines may induce specific subsets of cytochrome P450 enzymes involved in steroid metabolism thus leading to increased removal of steroidal estrogens from the mixture, with a certain loss of activity. This may explain the slightly lower than expected combination effects observed in the E-Screen by (Rajapakse et al. 2004). Similar considerations may apply to the mixture of thyroid disrupting chemicals analysed by Crofton et al. (2005) where many diverse mechanisms are at play leading to reductions in circulating thyroxin levels.

4.4.5 Combination effects between different classes of endocrine disrupters

Comparatively little work has been carried out with mixtures of different classes of endocrine disrupters, such as estrogenic agents combined with anti-estrogenic chemicals, or endocrine disrupters combined with other toxicants. In terms of design and data assessment, these studies differ from those discussed so far, because not all components present in the mixture may induce the effect chosen for analysis. In these cases, a "modulatory" influence of toxicants on the effects of other chemicals is studied. It is important to realise that the magnitude of such effect modulations cannot be predicted by adopting additivity concepts such as concentration addition or independent action.

Perhaps the best-known example of "effect modulation" is the inhibitory effect of AhR agonists, such as polychlorinated dioxins and co-planar polychlorinated biphenyls, on the action of estrogenic chemicals. Themselves not estrogenic, AhR agonists are reported to suppress some E2-induced responses not by antagonising hormone binding to the ER, but by down-regulation of ER expression, induction of steroid-metabolising enzyme systems such as CYP 1A1 and 1A2, and by inhibiting various growth factors and cell cycle regulators (Chen et al. 2001; Reen, Cadwallader, & Perdew 2002; Safe 1998).

Somewhat misleadingly, the action of AhR agonists has been called "anti-estrogenic", when it is perhaps more appropriate to view them as disrupters of estrogen signalling. The dioxin TCDD was reported to inhibit the estrogen-induced proliferation of uterine tissue in immature mice (Gallo et al. 1986) and to lead to diminuitions of ER levels in the liver and the uterus. Modulations of ER levels by TCDD were also described in rats (Astroff & Safe 1988; Romkes, Piskorskapliszczynska, & Safe 1987; Romkes & Safe 1988). While down-regulation of ER expression by AhR agonists in cell models is not controversial, difficulties with reproducing the effects in rodents have led to questions about the relevance of "anti-estrogenic" effects of AhR *in vivo*. White et al. (White et al. 1995) examined the impact of TCDD on the keratinisation of the vaginal epithelium and uterine proliferation in Sprague-Dawley rats induced by E2, but failed to observe any inhibitory effects of TCDD. Uterine ER and progesterone receptor levels were also not affected, although toxicity typical of TCDD (reductions in thymus weight, induction of hepatic CYP 1A1) occurred. Similarly, Desaulniers et al. (2003) did not observe an influence of a mixture of 16 AhR agonists (various polychlorinated dioxins, furans and bipenyls) on uterine growth stimulated by EE2 in pre-pubertal female Sprague-Dawley rats. Although the reasons for these contradictory findings remain to be fully elucidated, Desaulniers et al. (2003) pointed to reports by Petroff et al. (2001) and Sarkar et al. (2000) of enhancements of TCDD-induced AhR expression and CYP 1A1 induction in the presence of E2. This could explain the lack of "anti-estrogencity" of AhR agonists in their hands. White et al. (1995) even questioned the validity of ascribing a specific "anti-estrogenic" property to TCDD in the rat. They pointed out that inhibitory actions of TCDD on E2-induced effects reported by Safe and associates occurred at TCDD doses similar to the LD50 for the Sprague-Dawley and Long-Evan strains. Since TCDD induces a well-known wasting syndrome, it is conceivable that the "anti-estrogenicity" of TCDD is in fact the result of such systemic toxicity, rather than due to specific effects opposing the action of the hormone. Thus, more work is required to evaluate whether

disruption of estrogen signalling by AhR agonists occurs at realistic doses, and whether doses shown to interfere with estrogen-mediated biochemical effects, such as changes in gene expression, also lead to suppression of estrogen action with more apical endpoints such as cell proliferation.

Epidermal growth factor (EGF) and insulin-like growth factor (IGF) are able to enhance estrogen signalling by inducing ER phosphorylation and other signalling events (Aronica & Katzenellenbogen 1993; Ignar-Trowbridge et al. 1996). These observations prompted Charles et al. (2002a) to study the impact of EGF and IGF on E2-induced activation of ER in a MCF7 cell-based reporter gene system. Several combinations of all three agents were investigated and response surfaces recorded. Although EGF and IGF on their own did not promote gene transcription in this model, there were enhancements of the effects of E2, mostly due to EGF. These results indicate that the presence of growth factors may sensitise ER-competent cells to the action of the hormone, with significant consequences in terms of lowered effect thresholds. It remains to be seen whether similar effects also occur with estrogen-like environmental pollutants. Without a doubt, the potential for greater than additive interactions through interference with interacting signalling pathways deserves further attention and should be investigated systematically.

4.5 Immunotoxicity

Very few immunotoxicological studies have been conducted with mixtures, and even fewer allow assessments of mixture effects in terms of synergism, additivity or antagonism. Because toxic responses to the immune system entail a variety of different effects, this section is sub-divided into studies indicative of direct toxic effects and investigations of allergic responses.

4.5.1 Direct toxicity to the immune system

In a whole mixture design, Germolec et al. (1989) monitored immune function in female B6C3F1 mice exposed to a mixture of 25 common groundwater contaminants. Mice exposed to the highest dose of this mixture for 14 or 90 days showed immune function changes which could be related to rapidly proliferating cells, including suppression of hematopoietic stem cells and of antigen-induced antibody-forming cells. Altered resistance to challenge with an infectious agent also occurred in mice given the highest concentration, which correlated with the immune function changes.

Schlesinger et al. (1992) exposed rabbits to sulfuric acid vapours in combination with ozone. The animals were sacrificed and the lungs lavaged in order to obtain various cells of the immune system, including macrophages. What the authors evaluated as an antagonistic effect was observed when phagocytic activity of macrophages was analyzed. Similar antagonisms were observed with superoxide production by stimulated macrophages as the endpoint. In contrast, combination effects assessed by the authors as synergistic were seen with tumour necrosis factor-induced cytotoxicity as the endpoint of evaluation. However, this study used simple effect summation as the basis for these

evaluations, with no supporting dose-response analyses. The type of combination effect is therefore indeterminate.

4.5.2 Allergies and patch testing

Skin contact with certain chemicals can induce contact sensitization, which, once established, can persist over the entire life time. Patch testing in humans is widely used to investigate contact sensitization. When performed with commercial preparations, such as cosmetic products, positive results were often seen, although the individual components in these preparations did not produce effects when tested as chemically pure agents. Clinicians have often interpreted this phenomenon as evidence of false-positive outcomes of the patch test. However, the occurrence of positive patch tests could also be the result of interactions between the chemicals in producing contact sensitization. There is limited evidence that this might indeed be the case.

Johansen et al (1998) studied groups of eczema patients suffering from contact sensitization to two fragrance substances, and a second group who were allergic against only one of the substances of the first patient group. The single chemicals and their binary mixtures were applied to the upper back. The assessment of reactions was carried out on day 3, and the extent of the reaction was measured in millimetres. The data were analysed by logistic dose-response models. The combination of two allergens in individuals allergic to both substances had a synergistic effect. Equimolar mixtures of the two allergens elicited responses as if the doses were three to four times higher than those actually used, a greater response than expected if an additive effect had been present. The authors concluded that the synergistic effect demonstrated is likely to apply to other contact allergens and should be taken into account in designing diagnostic tests and performing safety assessments.

4.6 Neurotoxicity

The nervous system is particularly vulnerable to the effects of chemicals when exposure occurs during development, when there is extensive interaction between the brain and other organs. With a few exceptions, the majority of studies conducted with mixtures of neurotoxicants preclude any definitive conclusions about the type of combination effect. Well-designed experiments will be considered first.

Rebert et al. (1995) investigated the ototoxic effects of mixtures of organic solvents on Long Evans rats. Dose-response curves for the individual chemicals were recorded after inhalative exposure, with the aim of establishing equi-effective exposures. Isobolographics (dose addition) were employed to assess the joint effects of the following binary combinations: trichloroethylene plus toluene, mixed xylenes plus trichloroethylene, mixed xylenes plus chlorobenzene, and chlorobenzene plus toluene. The binary mixtures produced responses well in line with dose additvity predictions. Similar results were obtained in experiments with binary combinations of styrene and trichloroethylene (Rebert et al. 1993).

Very recently, Wolanski et al. (2009) published the results of experiments with pyrethroid mixtures designed to test the idea that combined neurotoxic effects in the rat follow DA. A fixed mixture ratio design was adopted, with a mixture ratio in proportion to the ED30 of each individual pyrethroid. The highest dose of each pyrethroid in the mixture was below its threshold for inducing behavioural effects. Significant dose-related decreases in motor activity of the treated rats were observed, and these effects followed the predictions derived from DA. This study is important because it is the first in vivo evidence to show that the default assumption of DA is corrected when dealing with pyrethroid mixtures, independent of the mixture dosing protocol used.

A few studies could be identified whose outcome can be interpreted as indicative of potentiations although additivity expectations were not employed.

Oskarsson and Lind (1985) administered various carbamates to rats dosed with lead, with the aim of investigating the effects of the pesticides on blood and brain lead levels. Co-administration of thiram, disulfiram, diethyldithiocarbamate or dimethyldithiocarbamate led to increased lead levels in blood and brain of the rats. The resulting lead brain levels were similar to those assumed to cause serious CNS damage in humans. Similar results were obtained in combination experiments with other metals, such as mercury, cadmium, copper, zinc and nickel, and dithiocarbamates (Aaseth, Alexander, & Wannag 1981; Aaseth, Soli, & Forre 1979; Cantilena, Jr. & Klaassen 1982; Oskarsson & Tjalve 1980).

The following studies show deficiencies in their design which preclude identification of the type of mixture effect:

Nagymajtenyi et al. (1998) studied neurophysiological changes caused by combinations of lead and the pesticide dimethoate as a consequence of exposure of Wistar rats during gestation and lactation. At the age of 12 weeks, electrophysiological parameters were measured among F1 male rats. Both spontaneous and evoked electrophysiological phenomena showed dose-, treatment- and combination-dependent changes (e.g. significantly decreased mean amplitude and increased frequency of the electrocorticogram, lengthened latency and duration of the somatosensory, visual and auditory evoked potentials). These changes seemed to be more pronounced in the groups treated with the combination of lead and dimethoate than in the groups given lead or dimethoate alone. It is not possible to assess these effects in terms of synergisms, additivity or antagonisms.

Thiruchelvam et al. (2000a; 2000b) studied the role of the herbicide paraquat and the dithiocarbamate maneb on idiopathic Parkinson's disease in C57BL/6 mice. Effects on locomotor activity, density of tyrosine hydroxylase-positive neurons and levels of dopamine were measured. With all investigated endpoints, a binary combination of paraquat and maneb produced responses that were larger than those observed with the individual chemicals.

4.7 Metals

A few reports with combinations of metals have been published that used endpoints relevant to human and mammalian toxicity.

Riley et al. (2003) prepared binary combinations of zinc with other metals, such as copper, vanadium, nickel and iron, and exposed cultures of rat lung epithelial cells to these mixtures. In the mixture experiments, the effect of the metal compounds on decreases in mitochondrial succinate dehydrogenase was measured. Most binary combinations showed effects that fell short of expected additivity. Mixtures of nickel and zinc were the exception: at low concentrations of both metals, effects smaller than expected occurred, but with nickel concentrations in the range between 0.04 and 0.08 mM, combined with zinc at 0.05 mM, stronger than additive effects became apparent. However, this publication lacks detail in terms of the precise method used to calculate additivity expectations.

Pounds et al. (2004) conducted series of mixture experiments with binary combinations of cadmium and mercury, methyl mercury and methyl tin by using monkey kidney cells. The effects of a sham combination of mercury with itself were also investigated. Cytotoxicity was evaluated, with excretion of lactate dehydrogenase as the endpoint of assessment. Detailed dose-response analyses with the individual metal compounds were conducted, and these data were used to derive additivity expectations according to various methods (isobole method, other approaches consistent with dose addition, independent action, and a non-interactive model). With the aim of estimating effect doses, the single chemical dose-response data were subjected to linearization methods, but also used for non-linear regression analysis.

The joint toxicity of mercury with itself was investigated as a test case for dose additivity. Strikingly, larger than expected effects were observed when the data were assessed in relation to all utilized additivity concepts. The authors speculated that this surprising finding might be due to saturation of transport processes. A binary combination of cadmium with mercury was chosen as a test case for putative independent action, and the observed mixture effects fell between the range of combination effects predicted by dose addition and independent action. Finally, a binary combination of methyl mercury and methyl tin hydroxide was studied as an example of a mixture containing only one toxic metal compound. There were difficulties with the modeling of the dose response data of the individual chemicals, but the isobolographic method suggested dose additive effects.

Nampoothiri et al. (2007) dosed Charles River rats with lead and cadmium acetate for 15 days. After this period, the animals were sacrificed, their ovaries removed, and ovarian cells cultured, with the aim of assessing various parameters related to oxidative status, including the levels of reduced glutathione and of lipid peroxides. The uptake of the metal into the ovaries was also measured. The animals received lead and cadmium at a dose of 0.05 mg/kg/d. The binary mixture contained lead and cadmium at 0.025 mg/kg/d each. It was observed that the lead/cadmium combination led to reductions in the levels of lead and cadmium in the ovaries, respectively. The authors did not carry out

supporting dose-response analyses, nor did they operate on the basis of an explicit additivity expectation. Consequently, the data are indeterminate with respect to the type of combination effect.

4.8 Dioxins, polychlorinated biphenyls and other chlorinated hydrocarbons

Polychlorinated dioxins cause a broad spectrum of toxic effects, ranging from lethality, immunotoxicity, cancer, liver toxicity, reproductive toxicity and many more. The most potent congener of this class of compounds is 2,3,7,8-TCDD, here referred to as TCDD. Most of the effects of TCDD are thought to involve interaction with the Ah receptor. To bring about toxic responses, this interaction is necessary, but not sufficient, and the specific effects of TCDD are determined by other, often tissue-specific, factors. With increasing knowledge about the function of the Ah receptor there is an emerging consensus that chemicals other than dioxins should also be considered as "dioxin-like". Classically, this group includes polyhalogenated dibenzofurans, and also co-planar polychlorinated biphenyls (PCBs), and collectively these chemicals are referred to as "dioxin-like compounds" (DLCs).

DLCs always occur as mixtures of various congeners, never as single chemicals. Driven by the need to evaluate the effects of such mixed exposures, an interim approach has been adopted internationally that assigns relative potency factors to this class of chemicals, compared to TCDD. Relative potency factors (REPs) form the basis of the toxic equivalency factor (TEF) concept for assessing mixtures of DLCs. However, the nominal value of REPs does not necessarily equal a TEF; TEFs are decided upon by WHO committees, using REPs as guiding principles (van den Berg et al. 2006). By using a TEF, the toxicity of a specific DLC can be expressed as "TCDD equivalents" (TEQ). The concept has evolved during the last 20 years for arriving at estimates of combination effects, but it is worth remembering that in its original conception it was intended to be used to estimate the potency of **untested** congeners.

4.8.1 The toxic equivalency factor (TEF) concept for evaluating mixtures of DLCs – an application of dose addition

The TEF concept rests on the assumption that all compounds produce effects via a similar mechanism (binding to the Ah receptor), and that their potency can be expressed in relation to a reference chemical (2,3,7,8 TCDD). Based on relative effect potency (REP) values that are determined for specific DLCs in relation to specific endpoints, a TEF is assigned to that DLC congener. The combined effect of a mixture of DLC is estimated by adding their TCDD "equivalent" doses (or concentrations) (TEQs). Thus, the TEF concept is an application of dose addition.

In assigning a global TEF to a specific DLC, the assumption is made that maximal response and shape of the dose-response curves, especially their slopes, should be similar for DLC congener and the reference chemical TCDD. Essentially, the curves should be parallel. If the demand for parallel curves is not met, the REPs (and consequently the

TEFs) should change according to the effect level that is chosen for analysis. In practice, this would make the entire concept unworkable, and global TEFs could not be assigned. With these complications in mind, a WHO study group has defined criteria for an "ideal" REP study design (van den Berg et al 2006):

- A full dose-response for the congener and 2,3,7,8 TCDD should be determined.
- REP values should be derived based on effect doses corresponding to median effects (ED50).

In practice however, these requirements are not always fulfilled. In particular, the demand of parallelity of dose-response curves is often not met, with the consequence that the value of REPs depends on the effect level chosen for potency comparisons (Devito et al. 2000). A refined database of mammalian REPs for DLCs has recently been developed (Haws et al. 2006). This database has facilitated comparisons of the ranges of REPs for individual DLC congeners in relation to various evaluation endpoints: these can vary by a factor of between 10 and 10,000. A TEF is then often chosen to represent the midpoint of ranges of REPs.

4.8.2 Validation of the TEF concept through experimental mixture studies with DLC mixtures

Many attempts have been made to anticipate the toxic effect of a DLC mixture by calculating its TEQ, with TEFs and DLC doses (or concentrations) as input values. These anticipated TEQs were then compared with experimentally measured toxicities. For many PCDD/PCDF mixtures it turned out that the TEQs calculated in this way agreed reasonably well with the experimentally determined TEQs (Desaulniers et al. 2003; Hamm, Chen, & Birnbaum 2003, Safe 1998; van den Berg et al. 2006). However, it is important to realize that this approach is only an indirect test of the validity of the implicit dose additivity assumption, because the "official" TEFs that were used in these assessments might not have been an accurate reflection of the relative potency of various DLCs, for the endpoints chosen. Often, relative potencies were not measured in these studies. An additional complication was that the underlying dose-response curves for the individual DLC congeners might not have been parallel (this was rarely tested directly), thus skewing the calculation of the expected TEQs.

Relatively few studies have examined the validity of the dose additivity assumption for mixtures of DLC directly, by using the dose addition concept, without applying TEFs.

In a series of papers Petersen and coworkers have tested the toxicity of binary DLC mixtures on trout early life stages. By using the isobole method, Zabel et al. (1995) found concentration additivity on rainbow trout early life stages with binary mixtures of 1,2,3,7,8 PeCDD and TCDD; 2,3,4,7,8 PeCDF and 1,2,3,7,8 PeCDD; PCB77 and PCB126; PCB105 and TCDD; and PCB105 and PCB126. However, synergisms were identified with PCB126 in combination with TCDD and with PCB77 and TCDD. The latter mixture also exhibited antagonisms, depending on the mixture ratio. However, with

lake trout eggs, the toxicity of PCB126 and TCDD turned out to be concentration additive (Zabel, Cook, & Peterson 1995).

However, there were notable exceptions: A mixture composed of 2,3,7,8 TCDD, 3,3',4,4',5 PCB and 2,3,4,7,8 PCDF was evaluated for induction of cytochrome P450 1A1 and 1A2 (Toyoshiba et al. 2004) and for carcinogenicity (Walker et al 2005) in rats. Dose-response curves for the individual congeners were not parallel, and the dose additivity assumption of the TEF approach was not fulfilled.

4.8.3 Synergistic and antagonistic effects between DLCs and non-coplanar PCBs

It is generally accepted that co-planar PCBs are DLCs and should be evaluated together with other PCDDs and PCDFs, by using the TEF concept. A debate has concerned the question has to whether non-coplanar PCBs should also be included. In reviewing this topic, van den Berg et al. (1998) have highlighted various examples of synergistic or antagonistic effects that were observed with combinations of non-coplanar PCBs and DLCs. Antagonistic effects were observed with respect to inductions of EROD activity in chicken embryo hepatocytes, spleen responses to sheep erythrocytes in mice, induction of cleft palates in fetal mice, and malformations in chicken embryos.

There were also synergistic effects non-coplanar PCBs and dioxins in the development of porphyria in rats, CYP1A1 induction, changes in thyroid hormone levels and associated enzyme activities. Van den Berg et al. (1998) emphasized that these deviations from additivity require further investigation in order to assess the extent to which they undermine the usefulness of the TEF concept.

4.9 Deviations from expected additivity suggestive of synergisms or antagonisms

Deviations from expected additivity were observed quite rarely. Notable are the observations of Nesnow et al. (1998) who analysed mixture effects of five poly-cyclic 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 an effect surface analysis. However, the observed deviations were rather small.

Another example is the study by Walker et al (2005) who employed a two year rodent cancer bioassays with female Harlan Sprague-Dawley rats given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB-126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or a mixture of the three compounds. The three chemicals, both singly and in combination induced hepatic, lung, and oral mucosal neoplasms. A re-analysis of the data, without utilizing the WHO TEF values, but by employing the concept of dose addition directly showed that the experimentally observed tumour incidences fell short of those anticipated by dose addition (Kortenkamp, umpublished).

There are a few examples from the area of endocrine disruption that indicated antagonisms in the joint effects of estrogenic agents (Rajapakse et al. 2004, Charles et al. 2007), 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.

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5. The ecotoxicology of chemical mixtures

As there are tens of thousands of publications in the scientific literature that deal with the toxicity of chemical mixtures from one perspective or another, it is impossible to specifically list and discuss each and every individual publication. This part of the review therefore focuses on studies that provide either empirical or conceptual input on the *ecotoxicology* of chemical mixtures. It examines and discusses the key publications and gives selected examples from publications that provide empirical details about the specific behaviour of chemical mixtures that contain certain environmentally relevant chemicals (industrial chemicals, surfactants, heavy metals, pharmaceuticals, biocides or pesticides) or about specific approaches that are relevant for the field. The joint effects of endocrine disrupters have been very recently re-viewed thoroughly by Kortenkamp (2007). Those compounds were therefore not included in this review.

The aim of this review is to analyse (a) how good the classical concepts of concentration addition (CA) and independent action (IA) are for describing and predicting the toxicity of mixtures, how often and to what extent synergistic or antagonistic mixture effects occur, whether there are particularities among the individual groups of substances, and which experimental designs and bioassays were predominantly used.

5.1 Summary

The documented mixture studies can be grouped into two broad categories, depending on the aims and approaches: Component Based Approaches (CBA) and Direct Toxicity Estimation (DTE). CBAs are typically based on the classical mixture toxicity concepts, concentration addition (CA) and independent action (IA), while DTE experiments are either conducted hypothesis-free (that is, they empirically describe the toxicity of a mixture without any implicit or explicit assumption on a particular behaviour) or they also refer to CA and (rarely) IA in order to unravel the causing agents of the analysed mixture (Toxicity Identification and Evaluation (TIE) and Effect Directed Analysis (EDA)). PBPK/PB models are rarely applied, as they are generally considered to data-demanding and the available biological knowledge does not allow their application. Please refer to section 3 of the review for an in-depth introduction to the concepts, their different names, and their power and limitations.

The results of this part of the review can be summarised as follows:

1. Documented mixture studies were mainly conducted with one of the following aims: (a) to evaluate and quantify the overall toxicity of complex environmental samples using whole mixture approaches, or (b) to describe the (non)-interaction between selected pure compounds.
2. In the later type of study, concentration addition was usually employed implicitly or explicitly for formulating the null hypothesis for non-interactive mixture toxicity. Only a few studies compared the observed toxicity of the mixture instead or also with predictions by independent action.

3. When both concepts are comparatively evaluated in the same study concentration addition provided a slightly more conservative mixture toxicity estimate in the vast majority of cases. The mixture EC50 predicted by concentration addition is usually not more than a factor of 5 lower than the EC50 predicted by independent action.
4. Concentration addition has a high predictive power, especially for mixtures composed of simple industrial chemicals, (xeno)estrogens and many pharmaceuticals and pesticides. However, mixtures of heavy metals and antifouling biocides seem to have a notable tendency to deviate from the mixture toxicity predictions made by CA.
5. Claims of synergisms or antagonisms are most of the time explicitly or implicitly made in the sense of "more (or less) toxic than expected by concentration addition". The pharmacological and/or physiological reasons for the observed deviations are usually not specifically investigated. Two different types of comparisons were found in the literature: either the predicted and observed *mixture effects* are compared, or predicted and observed *mixture effect concentrations* such as EC50 values are contrasted with each other. Claims of "strong" or "remarkable" synergisms are only made on the basis of the first type of comparison, the evaluation of predicted and observed effects, and they are restricted to mixtures with 2-3 compounds only. When effect concentrations are compared, most deviations (synergisms and/or antagonisms) are within a factor of 3 of the EC50 predicted by concentration addition. In none of the documented multi-component mixtures mixture toxicities higher than predicted by concentration addition were found.
6. Most analysed studies were conducted using simple aquatic bioassays with bacteria, algae or daphnids. Several studies used fish, fewer worked with terrestrial invertebrates such as earthworms or collembola. Studies with other groups of invertebrates such as molluscs or insects are confined to whole mixture studies. Mixture experiments with natural or artificial biocoenoses are confined to aquatic communities.

Comparatively few studies aimed to bridge the gap between whole mixture studies and component based approaches and applied a combination of whole mixture studies and component based approaches for stressor diagnosis in aquatic or terrestrial ecosystems.

The majority of the studies that analysed the joint action of defined mixtures were combinations of only 2 compounds. Comparatively few studies analysed the joint action of more than 2 chemicals (up to 50 compounds in one case). Mixture components were usually selected from within a specific class of compounds only (defined either chemically, pharmacologically or on the basis of their use pattern). Studies with mixtures composed of compounds with different mechanisms of action or from different chemical and use classes are extremely rare.
Typical mixture designs are isobolographics, point-, fixed-ratio and surface designs. The latter design has only been applied to binary mixtures. Full or fractional factorial designs are rarely used.

It has been suggested to use concentration addition as a first, pragmatic default approach for describing the joint action of chemicals for regulatory purposes in environmental risk assessments (Backhaus et al. 2000; Boedeker et al. 1993; Faust et al. 2003; Junghans et al. 2006). In view of the available evidence, this claim still seems to be defendable for most types of mixtures. However, this notion does not deny the principal existence of statistically and/or biologically significant synergistic or antagonistic interactions between certain mixture components, nor does it imply that the joint action of all mixtures can be precisely described by concentration addition. Biology is certainly far too complex and dynamic to be reduced to such as simple concept as concentration addition, especially when considering the reaction to an exposure toward compounds that act on dissimilar receptors, processes and physiological pathways. But deviations from CA-expected mixture toxicities seem to be in general *antagonistic* (i.e. the observed mixture toxicity is smaller than predicted), rather *rare*, comparatively *small* (within a factor of maximum 3-5 when predicted and observed EC50 values are compared, with only a very few exceptions in which a factor of 10 lay between predicted and observed mixture toxicities, see the collection of evidence from biocide mixtures below) and seem to be largely limited to *mixtures with only a few compounds*.

Given a bioassay with a sufficient capacity and low variability, it might be hardly surprising to discover a deviation between simple concepts and complex biological reality, especially as only the very fewest of the tested mixtures fully comply with the idea of a completely similar or completely dissimilar mode of action of all the components in the tested mixture. In this context it might not be sufficient to point out deviations between predictions and observations based only on a statistical test (deviation from prediction "yes" or "no"), as long as the *quantity* of the deviations is not discussed.

Figure 5.1 illustrates two particularities of the application of CA for the judgement of a "synergistic" or "antagonistic" effect. The mixture toxicity of the depicted 16 compound mixture was precisely predicted by IA, which simultaneously led to a misjudgement (overestimation) by CA. Following the most common mixture design, the sole comparision with CA, the observed mixture toxicity would be classified as a clear "antagonism" (The same holds true, if a mixture of similarly acting substances would be solely compared to the prediction by IA). Hence, the choice of a prediction concept and a (valid) classification of the (dis)similarity of the mixture components, which requires a solid basis of pharmacological knowledge prior to the interpretation of the mixture experiment, are of paramount importance for the assessment of a mixture as "synergistic" or "antagonistic).

There was only a factor of 2 between the EC50 values predicted by both concepts, which could be distinguished experimentally, given the experimental capacity and low variability of the employed algal growth inhibition assay – but still is comparatively small when considering e.g. the typical inter-laboratory variability or the general reproducibility of mixture toxicity experiments (Cedergreen et al. 2007). On the other hand there was a difference of 65% in the predicted effect level at a concentration of 14.8 µmol/L, where the observed mixture effect was 18.4 % while CA predicted 75% effect (vertical arrow in Figure 5.1), which seems to suggest a far bigger deviation than the

mere factor of 2 between the predicted EC50 values. As mentioned in point 4 above, the claim of synergistic (higher than predicted) or antagonistic (lower than predicted) mixture toxicities is therefore often done using the seemingly larger differences of predicted and observed mixture effects. From a risk assessment perspective, though, this approach is somewhat problematic as risk quotients (PEC/PNEC ratios) are based on the comparison of effect concentrations.

The following main empirical and conceptual knowledge gaps were identified:

1. Mixtures in the environment are usually composed of multiple components from a range of different sources from different chemical classes having dissimilar modes of action. Unfortunately, this is exactly the type of mixture that has been least frequently studied. Hence, more empirical evidence on the joint action of environmentally realistic mixtures, composed of members from different chemical and functional classes is needed in order to further substantiate the above statement that concentration addition might be applicable as a general "rule of thumb" for describing the joint action chemical mixtures and to explore its limitations.
2. In this context, it would be especially valuable to get further insight into the question as to whether low, individually non-toxic concentrations of dissimilar compounds might lead to a significant mixture effect. This question is of major importance, because of its direct relevance for the question of environmental quality standards. However, to our knowledge only two studies, both from of aquatic toxicology and both using unicellular organisms and specifically designed "artificial" mixtures are documented in the literature.
3. The empirical evidence on the joint action of chemicals in the marine environment is, compared to the available data for freshwater and terrestrial system, limited. It is hence unclear, whether the special chemistry of natural marine waters in combination with the type of pollutants typically found in the marine environment has an impact on the predictive power of either concept.
4. Organisms are not only exposed to mixtures of chemicals simultaneously, but also sequentially to pulses of contaminants that enter an ecosystem e.g. after run-off events or pesticide application. Although the first approaches have been published that start bringing forward a conceptual framework for modelling of such dynamic exposure situations (Ashauer et al. 2006), this work has only just started.
5. Both, concentration addition and independent action work well if the concentration-response curves of the individual toxicants have the typical sigmoidal shape on a log-scaled concentration (or dose) axis. However, if some of the components show biphasic response patterns ("hormesis", such as it is often observed for essential metals), independent action cannot be applied as the probabilistic background of the concept implies a response scaled from 0% to 100%. Also the application of concentration addition is limited, as the concept assumes a principally similar shape of the concentration-response curve of all components, due to the idea that all components behave as if they were simple dilutions from each other. Hence both concepts cannot be applied to mixtures of

e.g. nutrients and toxicants, although such an exposure situation might certainly be considered environmentally relevant.

6. Concentration addition and independent action have been conceptually developed and validated for chemical mixtures. Although the joint action of chemical and physical stressors, such as oxygen depletion or drought, is a very typical environmental scenario, it is far from being clear on what conceptual grounds Concentration addition and Independent action could be applied also for these circumstances.

Whole mixture studies that go beyond the mere quantification of the toxicity of a complex environmental sample from water, air or soil aim at the identification of the key toxicants and the quantification of their contribution to the overall toxicity of the sample. For this purpose they combine an array of biological and chemical analyses with physicochemical manipulation and fractionation techniques. Three different but in principal similar methods can be applied. Whole effluent toxicity testing (WETT) and toxicity identification and evaluation (TIE) both originate from the toxicity evaluation of effluents by the US EPA. Here they are important parts of the US EPA programs to assess and finally reduce the impact of pollutants in the environment and to support the Clean Water Act. Since their original development TIE methods have also been developed in most other environmental compartments. Effect-based assessment (EDA) is a closely related, but more general, scientific approach developed by analytical chemists to identify unknown hazardous compounds in various environmental or technical matrices. A major difference between TIE and EDA might be that the former usually only employs *in vivo* tests with whole organisms, while EDA uses a broader suite of test systems, including e.g. *in vitro* receptor activation assays such as the yeast estrogen screen.

The first step in any such an analysis is to separate the toxicants from the matrix, which is usually a selective extraction of organic compounds. Hence, metals, although a group of major environmental pollutants, are typically excluded from the very beginning of the study. The subsequent fractionation of the samples might also affect the bioavailability of the toxicants in the sample, which might result in an over- or underestimation of the actual environmental hazard of the sample. Subsequent steps then involve a series of biotest-directed fractionations of the sample into smaller and smaller subfractions, until their chemical composition is simple enough to allow a causal link between their toxicity and the presence of identified classes of compounds or even individual substances to be made. A later confirmation step then aims at comparing the amounts and individual toxicities of the identified individual toxicants (or groups of toxicants) with the total toxicity of the original sample.

Due to the different compounds that might be present in any complex environmental sample a battery of complementary biotests that cover different molecular receptors, pyhsiological pathways, organism groups and levels of biological complexity might be the best option for any TIE or EDA study. However, current investigations are often restricted to the use of simple, acute microbial assays such as the Microtox test with the bioluminescent bacterium *Vibrio fischeri*. If so, a range of compounds that are potentially

highly toxic to other organisms might go undetected. Also the chemical identification and quantification of individual components is limited, due to the multitude of chemicals in the environment for which no adequate standards are at hand.

The final confirmation step aims to provide evidence that the toxicants that were identified during the fractionation and testing steps are actually the drivers for the total toxicity in the environmental sample or even *in situ*. This can be done by a re-combination of fractions of the original sample or by a component-based approach which is usually employing CA for providing a causal link between the toxicity of the sample and the presence of the individual toxicants. However, most mixtures found in environmental samples are obviously not composed of similarly acting substances only (one of the major assumptions of the CA concept, see section 3). It has hence been suggested to extend this approach and also include the competing concept of IA, but this might drastically increase the data demands of a EDA/TIE study (see the input requirements for the application of IA as put forward in section 3).

5.2 Empirical evidence on the mixture ecotoxicology of selected groups of chemicals

5.2.1 Industrial Chemicals

The toxicity of mixtures of simply industrial mixtures was first investigated and published in a series of publications from the Netherlands in the 80's (Deneer et al. 1988a; Deneer et al. 1988b; Hermens et al. 1984b; Hermens et al. 1984a; Hermens et al. 1985b; Hermens et al. 1985a; Hermens & Leeuwangh 1982; Hermens et al. 1984c; Wolf et al. 1988). In all studies CA provided excellent to good approximations of the toxicity of the test mixtures. In a study that was published in 1984 by Hermens and his colleagues, mixtures of 4 groups of chemicals (chloroanilines, chlorophenols, aldehydes and non-reactive so called "narcotic" chemicals) were tested. The observed mixture toxicity was close to additivity or slightly below. As the competing concept of IA was not applied to the test data it is difficult to assess whether the slight overestimation by CA can be considered a sign of a more independent action of the mixture components from different chemical classes.

The notion that the mixture toxicity of simple organic chemicals is very precisely predictable by concentration addition, or is very close to it, has since then been confirmed in a number of studies (Altenburger et al. 2000; Broderius & Kahl 1985; Dawson et al. 2006; Lu et al. 2007; Merino-Garcia et al. 2003; Nirmalakhandan et al. 1994; Nirmalakhandan et al. 1997; Shirazi & Linder 1991; Xu & Nirmalakhandan 1998). However, it should be pointed out that in several cases when the mixtures was not entirely composed of compounds for the same chemical class, toxicities lower than predicted by CA were observed that – depending on the used statistical methods and the characteristics of the applied biotest – were sometimes judged to be statistically significant (Dawson et al. 2006).

Deviations from concentration additivity were observed by Tichy and his coworkers for the effects of a binary mixture of benzene and ethanol in a short term assay with *Tubifex* (Tichy et al. 2002). However, the maximum observed deviation was by only a factor of 1.5, between a predicted EC50 of 0.4 mol/L and an observed EC50 of 0.6 mol/L and no estimates of the uncertainty around the prediction or the observations were prodvided. Unfortunately, the authors did not calculate the prediction according to Independent action for this mixture, hence an analysis of whether the mixture of those two chemically dissimilar compounds is better described by IA is not possible.

Already in 1991 Broderius concluded that "for most complex organic mixtures the joint acute action of toxicants is either strictly additive or slightly less than strictly additive and that antagonistic or more than additive effects are not prevalent" (Broderius 1991). This view was explicitly confirmed by a recent data compilation by ECETOC (ECETOC 2001) and seems to be also confirmed by the studies published afterwards.

The good predictive power of CA for this type of chemicals led to the development and application of Quantitative-Structure-Activity Relationships (QSARs) that aim to replace the need for an experimental EC50 values for each compound in the mixture by QSAR-modelled values (Escher & Hermens 2002; Hermens et al. 1984a; Hermens et al. 1985a; Lu et al. 2007; Xu & Nirmalakhandan 1998).

5.2.2 Heavy metals

Heavy metals are ubiquitous pollutants in the aquatic as well as the terrestrial environment, hence the assessment of their environmental risks is an active field of research which currently undergoes a transition phase from a focus on persistence and bioaccumulation to more environmentally realistic approaches that also take bioavailability, tolerance development and natural occurrences into account (Chapman 2008). In contrast to e.g. the previously discussed organic pollutants, a range of metals are also essential elements, such as for example copper or zinc. Toxic effects are usually associated with the concentration of the free bioavailable metal ions, although recently discussions about the potential impacts of metal nanoparticles have begun in the scientific literature (Navarro et al. 2008). Because free-ion activity depends on the water chemistry, metal toxicity is intricately connected to water chemistry (pH, water hardness, concentration of dissolved organic matter, concentration of divalent cations such as Calcium or Magnesium). In general, greater water hardness, higher dissolved organic matter or anorganic cations, and lower pH all reduce the toxicity of metals.

An extensive review of the effects of metal mixtures in aquatic organisms has been provided by Norwood and co-workers (Norwood et al. 2003). Data of more than 77 species were evaluated, covering the whole range of aquatic organisms, including algae, bacteria, crustaceans, insects, fish, protozoans, and macrophytes at various life stages (egg, embryo, larval, juvenile, fry, and adult). Analysed endpoints included mortality, growth, phosphorescence, enzyme production, metallothionein production, feeding rates, cough response and bioaccumulation. 191 experimental mixture studies were evaluated,

156 of those investigated binary metal mixtures, 18 ternary mixtures and only 17 studies investigated mixtures with more than three metals.

In 191 cases the data provided by the respective authors were sufficient to allow Norwood and his colleagues to actually analyze the predictive power of CA. They concluded that in 27% of the cases the mixtures followed strict additivity, in 30% of the cases a higher than additive joint toxicity was observed and in 43 % the mixtures were less than additive (see also Figure 5.2). As binary mixtures were the most commonly studied type of mixture, a detailed analysis of the outcome of the available studies is given in Figure 5.3. As can be seen, the majority of the studies focused on binary mixtures with zinc, copper, cadmium, mercury, nickel and lead. The interactions between binary combinations of these metals are given in Figure 5.4.

No clear pattern is discernible from the analysis of Norwood and his colleagues. Even the same mixtures are judged to be additive, less than additive or more than additive, even if only studies with fish are considered (see the discussion in (Kamo & Nagai 2008)). If a pattern actually exists, current limits of empirical evidence and the inconsistencies in study design blur the picture beyond recognition. In his recent review Chapman concluded that "it is presently not possible to accurately predict interactions among metals" (Chapman 2008). This follows earlier reviews that concluded that "interactions between heavy metals appear to be without pattern" (Wang 1987).

However, it should be noted that the conclusion "more than additive" or "less than additive" alone does not allow any assessment on the quantity of the observed deviations and hence its relevance for any practical purposes. Depending on the used statistical methodology, the variability in the employed bioassay and assessment criteria of each study, even minute deviations from the CA expectation might become visible – or coarse deviations might go unnoticed. Furthermore, most studies only compared the observed toxicity with the expectations according to CA (which assumes a similar mode of action), although conceptually IA would perhaps be more appropriate. Considering the rather distinct interactions that each metal undergoes with the exposed biota, it might not be surprising that the CA-predicted mixture toxicity deviates somewhat from the observed mixture toxicity.

The Biotic Ligand Model (BLM) can be considered ***the*** model for describing the relationship between water chemistry and metal toxicity. It computes the expected amount of metals binding to organisms and hence aims to predict the toxicity of a given metal, taking into consideration its speciation which in turn is dependent on the chemistry of the surrounding matrix. Recently, suggestions have been put forward on how to extend the application of the BLM to the bioaccumulation and interactions of mixtures of heavy metals (Borgmann et al. 2008; Kamo & Nagai 2008), see also comments in (Chapman 2008). Currently the suggested BLM-extensions are largely a theoretical construct, but if successfully validated it might provide a powerful tool for unraveling the mixture toxicity pattern of heavy metals, especially for finding (and predicting) specific interactions. First experimental studies include the work by Hatano and coworkers who successfully applied the BLM to binary mixtures of copper and cadmium in studies with the duckweed

Lemna minor (Hatano & Shoji 2008). Other efforts to consider the influence of water chemistry on the toxicity of metal mixtures include the study by Yim and coworker on the effects of water hardness on the toxicity of selected metal mixtures (Yim et al. 2006).

An additional way of improving our current understanding of the toxicity of metal mixtures would be to include the potential interactions between genetic variability and environmental factors, as put forward by Barata et al (Barata et al. 2002).

For terrestrial systems especially the interaction of metals on the level of sorption to soil has been discussed as a reason for interactions. However, experimental results that aimed to confirm this idea concluded that besides the influence on their sorption, other as yet unknown factors also play a role (Jonker et al. 2004).

Recent experimental studies of the toxicity of heavy metals in terrestrial systems include the study by Chaperon et al (Chaperon & Sauve 2007; Chaperon & Sauve 2008), who analysed the effects of mixtures of copper, lead and cadmium on the activity of soil urease and hydrogenase. The predominant outcome was an antagonism in comparison to both, CA and IA. On an effect level the maximum deviation was nearly 100% (i.e. the observed effects were 100% lower than predicted by the concepts). It should be mentioned that several of the tested metals and their mixtures actually stimulated the enzyme activity, which is conceptually problematic, especially for the application of IA (Backhaus et al. 2004). An and coworkers investigated the power of CA for describing the toxicity of binary and one ternary mixture of heavy metals (copper, cadmium, lead) on the growth of cucumber plants (An et al. 2004). Again, concentration-additive, less-than-CA and higher-than-CA mixture toxicities were observed. The authors discuss bioaccumulation as a main factor for describing the joint action of the heavy metals but acknowledge that other as yet unknown factors seem to contribute to the deviation from CA. The observed deviations again were comparatively small. The smallest sum of toxic units needed for provoking a 50% growth reduction was 0.75, i.e. the ratio between CA-predicted and observed EC50 values was 1.3. The highest sum of toxic units was 1.6 (ratio predicted/observed EC50 of 0.63), observed for a binary mixture of copper and cadmium and a ternary mixture of copper, cadmium and lead. No comparison with the predictions by IA was made. A quantitative similar deviation from the CA expected mixture toxicity was found in mixture studies with Lemna (Charles et al. 2006). 1.35 toxic units were needed in a binary mixture of copper and uranium in order to achieve 50% effect (factor of 0.74 between prediction and observation).

Vibrio fischeri, a marine bacterium was used in a series of experiments with binary mixtures by Fulladose and coworkers (Fulladosa et al. 2005). The mixture toxicity was found to be less than additive for cobalt-cadmium, cadmium-zinc, cadmium-lead, and copper-lead combinations, more than additive for copper-cobalt and zinc-lead mixtures and additive in all other investigated binary combinations (cobalt-lead, cobalt-zinc, cadmium-copper, cadmium-lead, zinc-copper). However, deviations again were within a factor of 2 of the CA-predicted EC50.

CA-expected and observed toxicities of binary mixtures of copper, zinc and cadmium to *Tetrahymena* were reported by (Gallego et al. 2007). Most mixtures proved to be slightly antagonistic (32 out of 36 reported mixtures).

Mixtures of cadmium and phenanthren were investigated by Gust in 2006. Interestingly, even sub-lethal concentrations of phenanthren shifted the EC50 for cadmium from 523 to 263 mg/kg, although phenanthren alone did not provoke any mortality in the exposed population of the freshwater amphipod *Hyalella* (Gust 2006).

Mixture studies that do not relate to any of the mixture toxicity concepts but describe effects of metal mixtures and interactions of their compounds in a purely empirical sense include the studies by (Demuynck et al. 2007), (Fortier et al. 2008).

5.2.3 Pharmaceuticals

Some of the recent ecotoxicological studies on the ecotoxicology of pharmaceuticals came to the conclusion that clearly toxic effects occur only at concentrations well above environmentally realistic levels and consequently the potential environmental risk of the investigated pharmaceuticals has been assumed to be negligible (Han et al. 2006; Stuer-Lauridsen et al. 2000). However, a broad range of different substances is used simultaneously in human and veterinary medicine in any given area, so pharmaceuticals occur as multi-component mixtures in the environment. Hence the issue of possible combination effects from low concentrations of a whole range of very different compounds is an especially important issue for this class of chemicals.

Whole mixtures of pharmaceuticals were tested in the form of complex environmental samples (Schallenberg & Armstrong 2004) as well as lab-generated mixtures (Borgmann et al. 2007; Brain et al. 2004; Christensen et al. 2006; Christensen et al. 2007; Cleuvers 2003; Cleuvers 2004; Cleuvers 2005; Eguchi et al. 2004; Escher et al. 2005; Fent et al. 2006; Han et al. 2006; Pomati et al. 2006; Richards et al. 2004; Wilson et al. 2004). Whole-mixture approaches were also often combined with component-based modelling approaches in order to verify the quality of the applied mixture concepts (see below).

A recent example of the whole-mixture approach for a mixture of pharmaceuticals is the study by Pomati and coworkers, in which the effects of a mixture of 13 human pharmaceuticals to human embryonic cells were analysed (Pomati et al. 2006). At assumed environmental exposure levels, cell growth was significantly inhibited. Results from more ecologically oriented studies can be found in a series of publications from the university of Guelph which describe the ecotoxicology of various pharmaceutical mixtures in aquatic microcosms (Brain et al. 2004; Richards et al. 2004; Wilson et al. 2004). For example, the impact of a mixture of four tetracyclines on plankton structure and function was documented by Wilson and coworkers (Wilson et al. 2004). Effects on algal communities were observed only at concentrations greater then 200 nmol/L, which is well above environmentally realistic concentrations. Zooplankton was not affected significantly at the tested concentrations. However, it should be pointed out that the effects on the bacterial populations in the microcosms were not recorded, although these

organisms are vastly more sensitive to tetracyclines than algae. For example, an EC50 of 4 nmol/L chlorotetracycline has been determined already in a simple single species assay with *Nitrosomonas* (Halling-Sörensen 2001).

Borgmann and coworkers analysed the effects of a seven compound pharmaceutical mixture on the amphipod *Hyalella* (Borgmann et al. 2007). At environmentally realistic concentrations a significant change in sex ratio as well as small, non-significant reductions in survival and number of offspring were observed. In order to maximise the number of replicates and hence the statistical power, only one mixture concentration was tested. Hence the study does not allow mixture NOECs to be estimated or any margin of safety to be determined.

If there are major changes in the mixture ratio – either due to different degradation kinetics of the individual components in experiments with prolonged exposure or due to the specific design of the experiment (e.g. (Brain et al. 2004)) – regression techniques that are normally applied for the determination of e.g. EC50 values are of only limited use. Under these circumstances the EC50 values that result from the interpolation between tested concentrations are extremely difficult to assess, as it is unclear which specific mixture composition lead to the assumed 50% effect.

The joint ecotoxicity of a complex environmental sample can in principle be assessed without any knowledge on its chemical composition. For example, a study by Schallenberg and Armstrong investigated the effects of water from a drainage area that was supposedly contaminated by a mixture of veterinary antibiotics on the bacterial community of a supposedly uncontaminated lake (Schallenberg & Armstrong 2004). The authors did see sporadic ecotoxic effects of the drainage water, but were not able to connect them with a specific exposure towards veterinary antibiotics, as the actual contamination of the different drainage water samples was not determined. The study clearly demonstrated the limits of ecotoxicological studies that investigate complex environmental samples without analytical determination of the actual exposure situation.

Comparing results from a whole-mixture study in terms of a Predicted No Effect Concentration (PNEC) with a Predicted Environmental Concentration (PEC) implicitly assumes that both PEC and PNEC refer to the same mixture, i.e. with an identical composition and mixture ratio. Otherwise, the resulting mixture risk quotient (PEC/PNEC ratio) only allows very limited conclusions (Brain et al. 2004). Furthermore, the contribution of the individual compounds to the observed mixture toxicity and their specific interactions cannot be inferred from a whole-mixture study alone. For example, in a study by Richards and coworkers strong and unexpected fish mortalities were observed after exposure to a three component mixture of fluoxetine, ibuprofen and ciprofloxacin (Richards et al. 2004). Although the authors hypothesise that it could be either an unexpected high single substance toxicity of fluoxetin or synergistic mixture effects, the actual reasons for the observed high mixture toxicity remain to be elucidated. Similarly, in the study by Pomati et al. it remained unclear how the effects of cyclophosphamide – a mixture components which actually stimulated cell growth if

applied singly – relate to the overall growth-inhibiting effects of the mixture (Pomati et al. 2006).

Current empirical knowledge convincingly shows that the toxicity of mixtures composed of pharmaceuticals for which a similar mode or mechanism of action has been described in the target organisms can be predicted by CA. Figure 5.5 gives an example for the precise predictions that CA provided for the toxicity of 10 component quinolone mixtures (Backhaus et al. 1999). A similar high predictive power of CA was also observed by Cleuvers for mixtures of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen and acetylsalicylic acid in a study with daphnids and algae (Cleuvers 2004), as well as for mixtures of the ß-blockers propranolol, atenolol and metoproplol (Cleuvers 2005). Also studies with binary mixtures of selective serotonin re-uptake inhibitors citalopram, fluoxetine, fuvoxamine, paroxetine and sertraline did not find any significant deviations from CA-expected mixture toxicities in studies with algae and daphnids (Christensen et al. 2007). Estrogenic mixture effects of furosemide and 17ß-estradiol as well as furosemide and phenazone followed CA-expectations closely in a study by Fent and workers, employing the yeast estrogen screen (Fent et al. 2006), although small effect level dependent deviations were observed. In the same studies, deviations from CA expectations were observed for several mixtures containing pharmaceuticals for which only low effects were observed. These deviations were at least partly attributable to the resulting necessity to base the CA-calculation largely on extrapolations (see discussion above and in (Fent et al. 2006)). Finally, even in a multi-species test with sewage sludge bacteria, the toxicity of a binary mixture of the two quinolone antibiotics oxolinic acid and flumequine followed the predictions made by CA (Christensen et al. 2006).

Only comparatively few studies with mixtures of dissimilar pharmaceuticals have been documented in the scientific literature. The results from the only multi-component study that we are aware of with strictly dissimilarly acting pharmaceuticals are given in Figure 5.6. IA predicted the mixture toxicity very well over the whole range of tested concentrations and mixture ratios (Backhaus et al. 2000). An algal toxicity study with the 5 dissimilar pharmaceuticals propranolol, sulfamethoxazole, ethinylestradiol (EE2), diclofenac, ibuprofen and the herbicide diuron resulted in a mixture toxicity that followed IA expectations in the lower tested concentration range and CA in the region of higher concentrations (Escher et al. 2005). As four of the components (sulfamethoxazole, EE2, diclofenac, ibuprofen) were classified as acting primarly as baseline toxicants in algae and hence sharing an identical mode of action, a two-stage prediction combining CA and IA according to (Junghans 2004) improved mixture toxicity predictions.

Studies with binary mixtures of dissimilar pharmaceuticals give a somewhat heterogeneous picture. While the toxicity of a binary mixture of clofibric acid and carbamazepine to algae was indeed predictable by IA, the effects of the same mixture to daphnids could be better described by CA (Cleuvers 2003). A mixture of diclofenac and ibuprofen was slightly more toxic to *Daphnia* than predicted by both of the concepts, while it had an intermediate toxicity to algae (Cleuvers 2003). The toxicity of binary mixtures of oxytetracycline and erythromycin to algae could be predicted by IA as well as the toxicities of oxytetracycline + florfenicol, oxytetracycline + flumequine and

flumequine + erythromycin to activated sludge microorganisms (Christensen et al. 2006). However, in the same study clear synergistic effects to algae were observed for mixtures of flumequine+erythromycin and oxytetracycline+flumequine. This heterogeneous pattern could point to misclassifications of the modes of action of the mixture components in some of the test organisms, as the assessment of the components (dis)similarity was largely based on argumentation by analogy from knowledge in the target organisms or QSAR approaches that have not been validated for pharmaceuticals so far.

However, the results could also indicate interactions between the mixture components. Chemical as well as pharmacokinetic interactions between the components can lead to higher or lower mixture effects than expected from conceptual predictions. In a multi-component mixture a plethora of interactions might occur, shifting the overall joint toxicity in both directions – and thus ultimately cancelling each other out. This might be the reason why the predictive power of CA and IA seems to be higher for multi-component mixtures than it is for mixtures of comparatively few compounds. This pattern has also been observed for mixtures of narcotic chemicals and pesticides (Warne & Hawker 1995).

It should be noted that empirical evidence of the capability of CA and IA to accurately predict the toxicities of multi-component pharmaceutical mixtures is currently extremely scarce and in the documented multi-component studies the mixture ratios were adjusted to the toxicities of the individual components. Hence no single component dominated the mixture, which might very well be the case for environmentally realistic mixtures, as has already been demonstrated for herbicide mixtures (Junghans et al. 2006). Binary interactions might then lead to deviations from the conceptual expectations, if they occur between the most important components.

A quantification of the documented deviations between CA-predicted and observed mixture toxicities is hampered by the plethora of different data analyses, aggregations, visualisations and documentation gaps in the different publications. Nevertheless it can be preliminary concluded that mixture toxicities much higher than predicted – which would be most dangerous from an environmental risk assessment perspective – have not been recorded yet. The ratio between predicted and observed effect concentrations (e.g. EC50s) seem to be always lower than a factor of 5, with the vast majority of studies showing a clearly lower ratio.

It should be pointed out, that for both archetypal cases – a mixture of strictly similarly acting pharmaceuticals (Figure 5.5) as well as a mixture of strictly dissimilarly acting pharmaceuticals (Figure 5.6) low-effect concentrations (below the individual NOECs) of the individual pharmaceuticals clearly contributed to the overall joint ecotoxicity. Especially from a mixture perspective, NOEC's thus never describe an environmentally "safe" concentration. Whether certain fractions of individual NOECs – such as PNECs, which are based on NOECs divided by an assessment factor – are environmentally acceptable from a mixture perspective, depends on the specific exposure situation and particularly on the number of involved components.

5.2.4 Pesticides

As for other compounds, most studies on the mixture toxicity of pesticides focus on the aquatic environment. In the published literature there is an unanimous agreement that the toxicity of similarly acting pesticides is accurately predictable by CA (Altenburger et al. 2003; Backhaus et al. 2002; De Zwart & Posthuma 2005; Escher & Hermens 2002). The comparatively few studies that analysed mixtures of dissimilarly acting pesticides concluded that IA is more powerful for this type of mixture, but that CA also gives at least a rough approximation of the expected joint action, with a tendency to a slight overestimation of the actually observed toxicity (Faust et al. 2003).

Already in 1994 and 1996 two large studies on the chronic algal toxicities of binary mixtures were published by Faust and Altenburger (Altenburger et al. 1996; Faust et al. 1994). 137 binary mixtures of different pesticides were studied by Altenburger with the result that CA provided the better overall prediction for the observed toxicity data. A similar result was obtained by Faust who concluded that the toxicity 66% of the tested 38 binary pesticide mixtures was predictable by CA – although all the test mixtures were composed of a herbicide and an insecticide or fungicide.

A review by Deneer (Deneer 2000) re-evaluated the results from 202 mixtures of pesticides. In total 26 studies were evaluated in which insecticides, fungicides and herbicides made up the mixtures which were studied in bioassays with fish, crustaceans, insects, molluscs and algae. Deneer came to the conclusion that in more than 90% of the cases, CA proved to be accurate within a factor of 2. It is especially important to note that this also holds true for the 85 re-evaluated mixtures that were composed of pesticides with dissimilar mechanisms of action. Strong deviations from CA were mainly observed for mixtures containing organophosphates or carbamates mixed with other organophosphates or pyrethroids. An extensive follow-up study was published by Belden (Belden et al. 2007), which confirmed these earlier findings. 207 pesticide mixture experiments in which CA was used for evaluating the outcome of the mixture experiments, and 37 experiments in which IA was applied were evaluated. The ratio between predicted and observed mixture toxicities was expressed as the model deviation ratio (MDR) (Figure 5.7). The median MDR for CA was 1, with 5% of the analysed experiments having an MDR>2 and 5% having an MDR<0.5, indicating a high average predictive power of CA. The authors conclude that "[...] results indicate that the CA model may be used as a slightly conservative, but broadly applicable model with a relatively small likelihood of underestimating effects due to interactions." (Belden et al. 2007).

Another comprehensive review of the available mixture data which is mentioned by Warne (Warne 2003) has been conducted by Ross (Ross 1996). Unfortunately, this analysis was never published in the peer-reviewed literature. According to Warne (Warne 2003) 75-80% of the re-evaluated mixtures behaved according to CA while 20-25% of the mixtures were showing deviations. For only 5% of the mixtures the CA prediction differed by more than a factor of 2.5 from the experimental observation, and for only 1%

did the deviation exceed a factor of 5. Unfortunately, no further indications were given on how those deviations distribute between over- and underestimations.

Cases of synergisms between pesticides include the recent study by Laetz and his co-workers ((Laetz et al. 2008), see also Figure 5.8. Binary mixtures of organophosphate and carbamate pesticides, already highlighted as being "special" in the review by Deneer (2000) consistently provoked more-than-concentration additive mixture effects. However, it should be kept in mind that the comparisons between CA-prediction and observations were done only in relation to the effect level. Hence a comparison of the EC50-values is not possible on the basis of the presented data and in fact, they can still be close together if the concentration-response curves of the individual compounds are steep (see discussion above and Figure 5.1).

5.2.5 Biocides

Antifoulants are a group of biocides that are used to prevent the biofouling on submerged surfaces, in particular the hulls of marine ships. The joint action of those compounds is reviewed here as a special case of mixtures of pollutants that are important for marine ecosystems. The number of very recent publications also indicates that the field of the combination ecotoxicology of these compounds is starting to grow rapidly, most likely because of the ban of TBT, which was formerly used as an "all-round" antifoulant.

Given the strong association of these compounds with the marine aquatic environment, no studies that employed soil or sediment organisms were identified. As with the other substances, the investigations of the combined action of those compounds focus to a large extent on the investigation of binary mixtures. In contrast to many of the other chemical groups that have been reviewed above, however, comparatively strong deviations from the predictions were observed frequently, with no distinguishable relation to the used test organisms or specific mixture components. Similar to the situation with mixtures of metal compounds, the factors responsible for the toxicity of antifoulant mixtures are not fully understood. In fact, copper is one of the most common antifoulants at the moment and hence a compound of many of the tested mixtures.

Strong synergistic effects between diuron and irgarol in the marine algae *Chaetoceros* were described by Koutsaftis and his co-workers (Koutsaftis & Aoyama 2006). Only 5% of the individual EC50's of both compounds were needed to provoke 50% effect, which is a factor of 10 lower than expected. In the same study, more than concentration-additive effects were also observed for mixtures of diuron and Zn-pyrithion and irgarol+Zn-pyrithion. Antagonistic combination effects were observed in binary mixtures of Zn-pyrithion+Zn and Zn-pyrithion+copper, but only to a smaller extent.

The strong synergism between diuron and irgarol that has been observed by Koutsaftis and his colleagues is surprising as all our current knowledge on the behaviour of PSII-inhibitors (to which both compounds belong) from studies in freshwater ecotoxicology indicate a strictly concentration-additive behaviour (Backhaus et al. 2002; Chevre et al. 2006; Faust et al. 2001), which is in agreement with the identical mechanism of action of

those compounds. Also a study by DeLorenzo (DeLorenzo & Serrano 2006) concluded that the mixture toxicity of irgarol and atrazine is concentration-additive. Chesworth and his co-workers investigated a binary mixture of irgarol and diuron in an assay with the seagrass *Zosteria* (Chesworth et al. 2004), but used only IA for formulating the null-hypothesis of the expected joint action. The authors showed that the observed mixture effects are either close to the predictions by IA or slightly lower.

In 2007 Koutsaftis and his co-workers published a follow-up study in which a series of binary mixtures of Zn-pyrithione, Cu-pyrithione, chlorothalonil and diuron were tested for their toxicity to *Artemia* (Koutsaftis & Aoyama 2007). The mixtures of Zn-pyrithion and Cu-pyrithione were clearly more toxic than predicted by concentration addition, independently of the investigated mixture ratio (again up to a factor of 10 was between predicted and observed mixture toxicities). The other investigated mixtures followed the CA predictions closely or were less toxic (especially chlorothalonil and Cu-pyritione). Mixture ratio-dependent deviations between predicted and observed mixture toxicities frequently became apparent with the employed isobolographic mixture design. The quaternary mixture of Cu-pyrithione, Zn-pyrithione, chlorothalonil and diuron was slightly more toxic than predicted by CA, but the ratio of predicted and observed mixture toxicity was only 2.1.

Another series of binary mixtures with the antifoulants irgarol, seanine, chlorothalonil, diuron, dichlofluanid, 2-thiocyanomethlythiobenzothiazole (TCMBT) and TBT was tested by Fernández-Alba and co-workers in assays with the freshwater green algae *Selenastrum*, the freshwater crustacean *Daphnia* and the marine bacterium *Vibrio* (Fernandez-Alba et al. 2002). Unfortunately, a range of inconsistencies in the original publication complicates the assessment of the data. For example, the authors concluded a 2-10 tenfold higher mixture EC50 than predicted, but also state that both compounds singly were not toxic enough to provoke a 50% reduction in bioluminescence. Higher than expected toxicities were also observed for the binary mixures of TCMBT+Irgarol and chlorthalonil+irgarol (only for *Vibrio* and *Selenastrum*) and the ternary mixture of irgarol+TCMTB+dichlofluanid (only *Selenastrum* and *Daphnia*).

One consistent trend seems to be prominent in a range of studies: Zn-pyrithione combinations with copper were usually noticeably more toxic than expected by concentration addition (Koutsaftis & Aoyama 2007; Mochida et al. 2006, Bao et al. 2008), (see also the visualisation in Figure 5.9), which might be traced back to the transchelation of Zn-pyritione in the presence of ionic copper to Cu-pyrithione (Dahlloff et al. 2005) which is far more toxic to most organisms.

The mixture toxicity of three antifoulants (TBT, seanine and irgarol) was investigated by Arrhenius and colleagues in natural marine algal communities and a single species algal growth inhibition assay (Arrhenius et al. 2006). Both concepts, IA and CA, were applied, but failed to accurately describe the observed mixture toxicity. Clear concentration-dependent deviations between predicted and observed mixture toxicity were recorded. CA predicted a toxicity that was up to a factor of 7 higher than observed, IA overestimated the actual toxicity by up to a factor of 4. Synergistic effects were observed

for the ternary mixture at low concentrations (a factor of 4 with respect to IA and 2.4 in comparison to CA).

Manzo and his colleagues investigated the applicability of CA and IA for mixtures of copper, irgarol and diuron with the sea urchin *Paracentrotus* (Manzo et al. 2008). Two endpoints (embryotoxicity and spermiotoxicity) were analysed and in both cases the joint action of the ternary mixture was clearly lower than predicted by both concepts.

The predictive power of both concepts was also comparatively analysed in a very recent study by Bellas with the sea urchin *Paracentrotus,* who investigated the mixture toxicity of binary and ternary mixtures of Zn-pyrithione, chlorothalonil and seanine (Bellas 2008) and came to comparable conclusions. Both concepts failed to accurately predict the mixture toxicity, but deviations were comparatively small (CA overestimated by a factor of maximum 1.6). In most cases, IA was not significantly more powerful than CA, with the exception of the mixture of Zn-pyrithione and seanine. None of the tested mixtures was more toxic than predicted by CA.

In summary, for combinations of antifoulants, the predictive power of CA seems to be notably lower than e.g. for mixture studies with pesticides in freshwater systems. Reasons for this might be found in the very nature of the investigated compounds. First of all, the compounds obviously do not follow the inherent assumption of CA of a similar mode of action. But secondly, most of the novel so-called "booster" antifoulants such as chlorothalonil or seanine are rapidly degraded in aqueous solutions. Even minute differences in the experimental protocols between the single substance tests (that were used to produce the data for the predictions) and the mixture experiment itself might hence introduce a considerable bias. Last but not least, the complex chemistry of copper in seawater might also be a complicating factor. Obviously, these hypotheses warrant further investigation.

5.3 Whole mixture studies and their use for unravelling cause-effect relationships with individual compounds in complex environmental samples

Methods that use a whole mixture approach are based on the direct ecotoxicological assessment of a given complex chemical mixture, such as the effluent from a waste water discharge. The main purpose of such an analysis is usually to assess whether the mixture causes adverse effects and to quantify their magnitude. In investigations of such an unknown chemical mixture the combined effects of all components are captured by appropriate bioassays, including any antagonistic or synergistic effects.

A potential next step is then to reveal which toxicant or group of toxicants in the sample is responsible for the observed toxicity, in order to rank and prioritise them for a possible remediation and to identify specific pollution sources. The overall aim is then to assess (with the aim of reducing) the impact of chemicals in the environment and to set environmental quality standards (such as in the context of the European Water Framework Directive).

In general three different approaches or methods are used to assess whole mixture toxicities and to identify the causative components. Effect-directed analysis (EDA) and toxicity identification and evaluation (TIE) are more general approaches, while Whole Effluent Toxicity Testing (WETT) applies specifically to the analysis of effluents (Gutierrez et al. 2008; La Point & Waller 2000). TIE originates from effluent control in a regulatory context in the USA while EDA is an approach developed mainly by analytical chemists in order to to identify unknown hazardous compounds in various environmental or technical matrices (Brack 2003). WETT is one instrument within the Clean Water Act of the USA that was enacted in 1972 with the objective of "restoring the chemical, physical, and biological integrity of the Nation's waters".

The TIE approaches that have been put forward by the US EPA are based on specific guidelines from the late 1980s onwards (US EPA 1989; US EPA 1991a; US EPA 1991b; US EPA 1992; US EPA 1993; US EPA 1996; US EPA 2007). A specific guideline on WETT approaches has also been assembled by the US EPA (US EPA 2000). OSPAR has developed a Whole Effluent Assessment (WEA) where WETT is extended to not only focus on the toxicity (T) of the chemicals but also to include the determination of persistence (P), bioaccumulation (B) i.e. the PBT-criteria that are used within OSPAR's Hazardous Substances Strategy (OSPAR Commission 2005). The difference being that the WEA are applied to the entire effluent sample instead of to the individual substances. There are currently no generally agreed guidelines available for on how to perform an EDA. It should be mentioned, that all these approaches are site-specific and as such any guidelines needs to be adjusted to the local conditions of each site.

These three methods, WETT, EDA and TIE, share the same basic principles for identifying the toxicity of a sample and its causes. The complexity of the whole chemical mixture present in a given sample is reduced step by step through various fractionation techniques. Each fraction is then assessed for its toxicity and the toxic fractions are then further dismantled, in the perfect case down to the single chemicals. This combination of biological and chemical analysis with physicochemical manipulation and fractionation techniques has been applied to the various environmental matrices (e.g. water, sediment and soil, air) and for a range of toxicological endpoints (Brack et al. 2008). TIE and WETT are based only on *in vivo* testing while EDA is applied both *in vitro* and *in vivo* in order to detect toxicologically active fractions and compounds (Brack et al. 2008).

EDA has so far mainly proven useful for detecting and identifying specifically acting toxicants close to the source of emission and at comparatively high concentrations. It is of only limited use for screening purposes in remote areas with low concentrations of individual compounds where the toxicity is relatively low compared to the unspecific effects of anthropogenic and natural compounds (Brack et al. 2008). Hence, there are currently efforts underway to improve the EDA methodology to allow the identification of potentially hazardous components in the environment even when occurring at low concentrations that do not cause acute effects. The EU-project Modelkey (www.modelkey.org) is working towards that goal.

Different types of TIEs and EDAs are set up for different environmental compartments such as the water column (e.g. effluents and receiving waters), sediments (marine and limnic), interstitial waters (pore water) and soil (Brack 2003). EDA has also been applied to air particulate matter (Brack 2003). WETT methodologies are obviously focusing mainly on effluents, but there are also few studies that focus not only on the discharged water but also made complementary field assessments as reviewed by LaPoint and Waller (La Point & Waller 2000).

Toxicity Identification and Evaluation (TIE)

TIE consists of three hierarchical phases: characterization, identification and confirmation (US EPA 2007). However, as pointed out by Burgess, before a full TIE investigation is started, a first step should be to investigate whether the sample of interest is actually toxic, in order to prevent wasting time and resources on a non-toxic sample (Burgess 2000). This very first step is crucial for the overall TIE study for several reasons. First a biotest or a biotest battery that is relevant for the environmental compartment of concern and expected pollution scenario has to be selected. For example, it would be quite obviously not sufficient to base the study on tests with daphnids if the expected main pollutants are herbicides because the sample originates from an agricultural area. Secondly, the sample in its original state might be non-toxic, however it might contain compounds that show a profound toxicity as soon as the environmental conditions change. For examples pH shifts might increase the toxicity of metals, or an increased bioturbation might alter the bioavailability of toxic compounds in the sediment. Hence, even if a sample does not show any direct toxic effects in a bioassay, this finding does only allow limited conclusions on the presences of principally toxic compounds.

The three main phases in the TIE-approach are as follows (US EPA 2007):

- Phase I – Characterization of the sample→ a suite of physical/chemical manipulations is used to build a general "profile" of the causative toxicant(s) and aims to determine the general groups of toxicant involved (e.g. metals, nonpolar organics, volatiles, ammonia)
- Phase II – Identification of toxicant(s) → more refined procedures are used to focus on the specific groups identified in Phase I, with the aim to simplify the sample for chemical analysis and usually ends with the analytical identification of the suspected toxicant
- Phase III – Confirmation of causality → corroborating data are collected to build a weight-of-evidence case and to finally establish causality between measured effects and identified toxicant(s).

Effect Directed Analysis (EDA)

The first step in the EDA is to extract or separate the toxicants from the matrix (Brack 2003). There is no single method that works for all chemical compounds, hence specific extraction techniques are used for different types of chemical and usually make use of specific physico-chemical parameters such as lipophilicity, boiling point or molecular

size or are limited to aromatic or volatile compounds. This extraction step is crucial, as only the extracted compounds have a chance to be later picked up in the bioassays and the subsequent, refined, analytical steps. A major problem, as pointed out in the review by Seiler and co-workers (Seiler et al. 2008) is that already the extraction procedures for soil and sediments have a strong influence of the bioavailability of toxic compounds and hence the "true" environmental hazard might be over- or underestimated due to biases in the extraction methods.

Only a comparatively few EDA studies with volatile compounds in water environments have been conducted so far, mainly because the extraction process for this type of compounds is far from simple (Brack 2003). In order to get a rough idea on the toxicity contribution of volatile compounds, analytical procedures normally include a stripping of volatile compounds (e.g. aerating the sample) and the toxicity is measured before and after this process.

5.3.1 Fractionation

Through the fractionation process the complexity of the mixture is gradually reduced by removing non-toxic components to enable a chemical identification of the remaining toxicants (Brack 2003). It is based on variations in the physicochemical and chemical properties of the analytes such as polarity, hydrophobicity, molecular size, planarity and presence of specific functional groups. Hence, the fractionation itself generates information about the properties of the compounds/fractions present that can be useful for the chemical identification and subsequent hazard assessment.

5.3.2 Biotesting

The toxic potency of the whole sample and of its fractions guides the further steps including the biotesting. Species fundamentally differ in their sensitivity to stressors including chemical ones (Cairns 1986; Seiler et al. 2008). Hence there is no such thing as a most sensitive species for all kind of chemicals and the choice of test species will always have a strong influence on which compounds are finally identified in an unknown sample containing a multitude of different components. If a comprehensive assessment of toxic hazard from an unknown environmental sample or chemical mixture should be assessed properly a biotest battery is therefore required. The "biological tools have to be carefully selected with respect to their ability to detect specific effects and their significance in hazard assessment" (Brack 2003).

A number of different assays have therefore been developed and applied in EDAs (Brack 2003; Seiler et al. 2008), the TIE and WETT testing are so far limited to in vivo test including many of those used in EDAs;

5.3.2.1 Aquatic organisms

EDAs and TIEs in the 1980s usually employed aquatic toxicity assays with invertebrates such as *Daphnia magna*, *Daphnia pulex*, *Ceriodaphnia dubia* and the fish *Pimephales*

promelas. However since then, the acute bioluminescence test with *Vibrio fischeri* (Microtox) has become *the* predominant test because it is highly reproducible, rapid, easy to handle and has a low sample demand (Brack 2003). However, it covers only acute effects on the bacterial energy status (ATP/ADP status) and the supporting physiological pathways (such as the respiratory chain) and logKow-dependent non-specific effects including narcosis, uncoupling and some electrophilicity-based effects. This can be considered a fundamental drawback, as many ecotoxicologically important compounds either effect molecular targets that are not present in bacteria (such as the D1-protein of plants to which all the PSII-inhibiting herbicides bind, or the mammalian estrogen receptor to which xenoestrogens bind) or they target physiological processes whose inhibition becomes only apparent after pro-longed exposures (Backhaus et al. 1997; Froehner et al. 1999). Hence the sole use of this assay runs a strong risk of a systematic toxicity underestimation.

5.3.2.2 The cellular and subcellular level

Other frequently used aquatic assays include oyster embryo assays with *Crassostrea gigas* and reproduction or growth inhibition assays with green algae, such as *Scenedesmus vacuolatus* (Brack 2003). Also assays that cover developmental toxicity are being applied in EDAs (e.g. the FETAX, the frog embryo teratogenesis assay with *Xenopus laevis* larvae).

There is a number of promising assays that are supposed to be rapid, reproducible, easy manageable and require only low sample volume, often based on specific bacterial strains or cell line-based testsystems. The resulting high throughput capacity and robustness makes them ideal candidates for EDA (Brack 2003). These assays cover toxicological endpoints such as genotoxicity, mugtagenicity and carcinogenicity that are important properties when assessing risk and hazard especially to human health. However, despite their appealing practical features, the biological or even ecological relevance of such highly simplified testsystems needs to be critically evaluated.

5.3.2.3 Cytochrome P4501A-dependent monooxygenase induction

Induction of the cytochrome P4501A-dependent monooxygenase system is commonly measured as 7-ethoxyresorufin-*O*-deethylase (EROD) activity and is often used as a surrogate measure for the identification of hazardous aromatic compounds in EDA using chick embryos, fish, rat liver cells and rainbow trout liver cells (Brack 2003).

5.3.2.4 Endocrine disruption

Xenoestrogens are one important and frequently occurring group of pollutants that can be identified by a yeast estrogen screen (YES) using *Saccharomyces cerevisisiae* with the human estrogen receptor stably integrated into the yeast genome (Brack 2003; Seiler et al. 2008). Androgenic compounds can be identified through a yeast androgen screen (YAS). The specific mixture related problems that can be encountered during the application of such assays have recently been discussed by Frische and coworkers (2009).

5.3.3 Confirmation of toxicants

Analytical confirmation is a stepwise approach that starts with a tentative identification of toxicants based on mass spectra as described by Brack et al. (Brack et al. 2008). This approach is leading to increasing evidence rather than a yes/no answer. An extensive fractionation and high chromatographic resolution prior to the recording of mass spectra are a major prerequisite for a successful chemical confirmation. Also, any GC/MS analysis is based on a comparison to a spectral library and only those that are present in the library can be correctly identified. However, for many compounds there are no analytical standards available that would allow their inclusion in a spectral library – and even if so, this does not necessarily allow full confirmation if the compounds are present in different isomers. Nuclear magnetic resonance (NMR) has been used in EDA but it is difficult to perform for many environmental trace contaminants because of high amounts and the purities of the analytes required (Brack et al. 2008). However as pointed out in the review by Brack and co-workers there are some analytical and computational techniques that can be combined to possibly identify and confirm also unknowns (i.e. those not present in reference libraries). They further point out that the detection of trace contaminants detected in EDA studies is still a challenging task and that there are no agreed guidelines and that the effort required depends on the sample complexity and composition as well as on the individual components.

5.3.4 Confirmation of causality

The toxicity confirmation aims to provide evidence that the identified compounds are actually responsible for the measured effects. Confirmation procedures in a TIE should be based on the original sample and sample manipulation should be minimised or completely avoided as far as possible (Brack et al. 2008; Seiler et al. 2008;US EPA 2007). The inclusion of a specific confirmation step into into the TIE/EDA investigation has two main reasons: (a) during the characterisation and identification of toxicity effluents/samples are manipulated in a way that may "create artefacts that lead to erroneous conclusions about the cause of toxicity" (Brack et al. 2008) and (b) a major aim for a TIE is effluent control and the confirmation should hence account for the variability of an effluent from sample to sample and from season to season (Brack et al. 2008).

Two different approaches have been suggested to confirm the identified toxicants that focuses on the identified mixture components; testing a synthetic mixture or calculation of an expected mixture toxicity (Grote et al 2005). Almost all confirmation studies in TIE and EDA have been based on the concept of concentration addition (CA) (Brack et al. 2008) assuming a similar mechanism of action of the mixture components. However, for compounds in environmental mixtures, the modes of toxic action are commonly unknown, probably not strictly similar acting and interactions might be present between the compounds in the mixture. Hence the use of CA and toxic unit summation might overestimate toxicity and consequently the hazard of the sample. However, as pointed out by Grote and co-workers (Grote et al. 2005) an overestimation of the combined effects of

a mixture is not necessarily a matter of concern, as long as this overestimation is comparatively small. However, during the confirmation step the sole application of CA could result in an underestimation of the unresolved toxicity and hence some additional components in the mixture might be overlooked and not identified. Grote and co-workers (Grote et al. 2005) therefore suggest using both CA and IA models to define a "prediction window" to describe the area between the predictions which can be used for the confirmation step.

A second limitation of the toxic unit approach is that it is usually only applied to the EC50. As concentration-response curves are known to differ in their shape and slope this may result in different conclusion on confirmation. Grote and co-workers (Grote et al. 2005) clearly showed for two different sediments that the confirmation varied between effect levels with a smaller difference between mixture toxicity and the extract toxicity for higher effect levels. Therefore they additionally suggested that the confirmation should be based on a range of different effect levels, rather than being restricted to the EC50. However, it should be pointed out that the suggestion to include also IA in the confirmation step tremendously increases the data demands for any study (see xy). Grote et al also introduced the Index of Confirmation Quality (ICQ) as a way to illustrate the quantitative measure of confirmation over a range of different effect levels.

5.3.5 Hazard confirmation in the field

The validation that the identified toxicant(s) of a sample are actually responsible for adverse effects *in situ* is the final important confirmation step. That is, in order to establish that the key toxicants identified in a complex mixture really have caused or are likely to cause damages in the field a confirmation must be performed under realistic exposure conditions or even in situ. This should include confirmation for higher biological levels such as whole organism, populations and communities (Brack et al. 2008).

Especially for solid samples such as soil and sediment such a confirmation is more difficult than for water samples because of the differences in bioavailability and difficulties to estimate this (reviewed by Seiler et al. 2008). Therefore, although the TIE approach has been applied repeatedly to sediment compartments concern has been raised because of the fragile chemistry and biology of these media (Burgess 2000). Severe changes might already occor during the collection of the sediment samples, which continues during the manipulation and storing of the samples (Burton & Nordstrom 2004a). These processes run the risk that the chemistry and bioavailability of potential toxicants may be altered. Therefore an *in situ* TIE (iTIE) has been developed for sediments to conduct initial TIE evaluations of sediment pore water with minimal sediment manipulations (Burton & Nordstrom 2004a). This method uses different kinds of resins (e.g. zeolites) that selectively extract ammonia, metals, and non-polar organics respectively and thereby can identify dominant toxicant classes of compounds and aid to identify chemicals of concern without labour-intensive extractions and a large number of tests. Current limitations of this method are the initial assembly time, the restricted

deployment to moderate flow and wadeable sites, and cross-sorption of compounds (Burton & Nordstrom 2004b).

Another way of confirming effects in the field is to sample indigenous species and analyse biochemical markers, which has been termed the "biomarker approach" (Brack et al. 2008). The biomarkers can then be correlated with concentrations of identified or expected key toxicants to provide further evidence to causality. There are several biomarkers available for invertebrates and fish that are closely linked to many of the assays frequently used in the EDA, TIE and WETT approaches. However, most of them such as e.g. cytochrome P-450 activity indicate exposure of organic pollutants in general and hence give information only on the total exposure of all mixture components that trigger the cytochrome P-450 induction. Other examples include vitellogenin in fish as a marker for exposure endocrine disruptors and DNA-damage, which mainly indicates exposure to PAHs. Biomarkers differ in their specificity and hence depending on their specificity it mainly gives information on presence of chemical groups according to their mode of action rather than on an individual toxicant. Yet, they contribute in providing complementary lines of evidence in the confirmation of key toxicants. Direct EDA and chemical analysis in tissues of benthic organisms is a straightforward way to assess bioavailability however this only considers toxicants that do bioaccumulate (Brack et al. 2008).

Approaches for establishing causality for identified toxicants and effects at the level of ecological communities (biocoenoses) are lacking to a large extent. Brack et al (Brack et al. 2008) suggests using pollution-induced-community-tolerance (PICT) as a tool to confirm impact of identified toxicants. The concept is an ecotoxicological tool based on the assumption that toxicants exert a selection pressure on the members of a community (Blanck 2002). The most sensitive organisms will become excluded and the more tolerant organisms favoured and consequently, the community will change its structure in a way that increases the community tolerance. However, although proven valid for confirming hazard due to individual compounds (Blanck & Dahl 1996; Schmitt-Jansen & Altenburger 2005) its applicability to confirm hazards posed by mixtures are limited. Currently no concept is available for using PICT for communities exposed to mixtures of toxicants (Brack et al. 2008).

WETT is a collection of useful tools for predicting the effect to individual species, yet they are not meant to directly measure natural population or community responses (La Point & Waller 2000). In situ toxicity tests or ecosystem-level testing should offer the best description to actually describe field conditions if they are conducted for long enough time (Chapman 2000). LaPoint and Waller further conclude that due to species-specific differences in sensitivity to contaminants, field assessments of effluents may be difficult to perform when more than one contaminant is present (which is the usual case). Interactions between constituents of effluents and the receiving waters could also contribute to reduce the agreement between WETT test and field assessment results. Also if a stream has several effluent discharges in close proximity mixture effects are likely and difficult to predict, especially if the effluents contain unknown pollutants or unknown

concentrations of known pollutants. Hence, getting the full picture of effluents and thereby mixture components in the receiving waters is difficult to achieve.

5.3.6 MODELKEY – a FP6 research programme

The ongoing EU-project (FP6 2005-2010) – MODELKEY ("Models for assessing and forecasting the impact of environmental key pollutants on freshwater and marine ecosystems and biodiversity", www.modelkey.org) - with 26 partners started with the inspiration from the professed aim of the Water Framework Directive to achieve a good ecological status for European river system by 2015. It is a multidisciplinary research programme aimed at "interlinking tools for an enhanced understanding of cause-effect-relationships between insufficient ecological status and environmental pollution as causative factors and for the assessment and forecasting of the risks of the key pollutants on freshwater and marine ecosystems at a river basin and adjacent marine environment scale". The subproject KEYTOX - key toxicant identification – aims to develop tools on how to apply effect-directed identification of site- and basin-specific key toxicants for the establishment of cause-effect relationship and improved risk assessment. The project aims at improving current gaps concerning analytical techniques for identifying toxicants and compare laboratory and validate available techniques (Brack 2005).

5.4 Eco-epidemiology of complex mixtures

In contrast to the heavily experimentally oriented work using EDA, WETT and TIE, eco-epidemiological studies focus on unravelling potential causes for effects observed in the field from available data on the expected and actual ecological status of an ecosystem (biodiversity, species composition) and its physico-chemical parameters. Although this application of the classical concepts of CA and IA is only in its infancy, both concepts have been successfully applied in a large-scale eco-epidemiology study in the Ohio river basin where they were instrumental in exploring the factors that are responsible for shaping fish populations at polluted and non-polluted sites (de Zwart et al. 2006, Posthuma & de Zwart 2006).

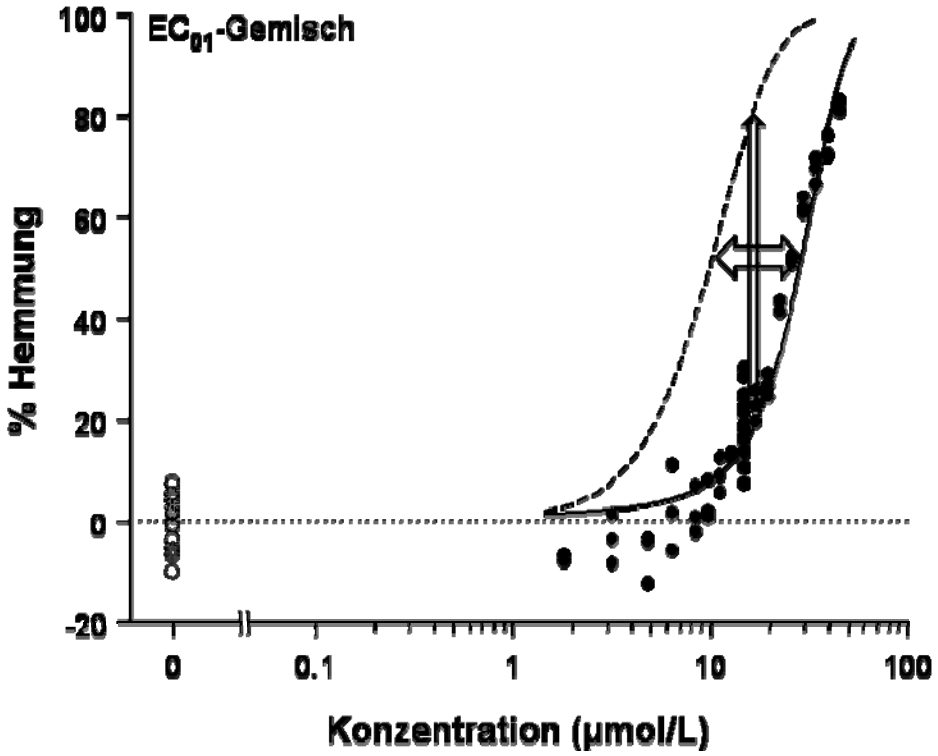

Figure 5.1: Observed (bullets) and predicted mixture toxicity of a mixture of 16 strictly dissimilarly acting toxicants.

Solid line: prediction by Concentration Addition (CA), Dashed line: prediction by Independent Action (IA). Horizontal arrow: the difference between the CA- and IA-predicted EC50 is a mere factor of 3.

Vertical arrow: the differences of CA- and IA-predicted effect levels are seemingly larger (65%).

For details see text. From (Faust et al. 2001), with permission.

In a field setting	No. of Metals in Mixture	Less Than Additive	Strictly Additive	More Than Additive	Total Tests	Could Not Test
	2	69	42	45	156	14
	3	7	6	5	18	4
	4	1	0	0	1	2
	5	3	0	3	6	2
	6	1	3	2	6	1
	7	0	0	0	0	1
	8	1	1	0	2	0
	10	0	0	1	1	1
	11	1	0	0	1	0
This	Total	83	52	56	191	25
Analysis	Percent	43.5	27.2	29.3	100.0	13.1
Author	Total	89	58	63	210	12
Interpretation	Percent	42.4	27.6	30.0	100.0	5.7

Figure 5.2: Toxicity of metal mixtures with different number of components to aquatic organisms. From (Norwood et al. 2003), with permission.

"This analysis" refers to the analysis done by Norwood and co-workers; "Author interpretation" refers to the judgements of the original authors of the experimental study. Additivity refers to CA.

		Less Than Additive	Strictly Additive	More Than Additive	Total Tests
	Zn	27	8	17	52
	Cu(II)	21	8	21	50
	Cd	24	14	15	53
	Hg	11	10	11	32
	Ni	13	2	9	24
	Pb	6	6	3	15
	Al	3	1	0	4
	Mn	6	0	2	8
	Se	7	0	2	9
	V	6	0	0	6
	Cu(I)	4	0	2	6
	As	0	3	2	5
	Mo	5	0	0	5
	Mg	1	0	0	1
This	Total	134	52	84	270
Analysis	Percent	49.6	19.3	31.1	100.0
Author	Total	145	114	100	359
Interpretation	Percent	40.4	31.8	27.9	100.0

Figure 5.3: Number of mixture studies conducted with different metals and analysis of whether their toxicities are predictable by CA. From (Norwood et al. 2003), with permission.

		Less Than Additive	Strictly Additive	More Than Additive	Total Tests
	Cu-Zn	11	1	9	21
	Cd-Zn	9	5	5	19
	Cd-Cu	1	3	4	8
	Cd-Hg	1	4	4	9
	Cu-Ni	2	1	6	9
	Pb-Zn	2	1	2	5
	Hg-Se	5	0	1	6
	Hg-Ni	2	1	2	5
	Hg-Zn	2	0	2	4
	Al-Zn	1	1	0	2
	All Others	27	9	10	46
This	Total	63	26	45	134
Analysis	Percent	47.0	19.4	33.6	100.0
Author	Total	72	60	49	181
Interpretation	Percent	39.8	33.1	27.1	100.0

Figure 5.4: Analysis of the mixture toxicity of binary metal mixtures. From (Norwood et al., 2003), with permission.

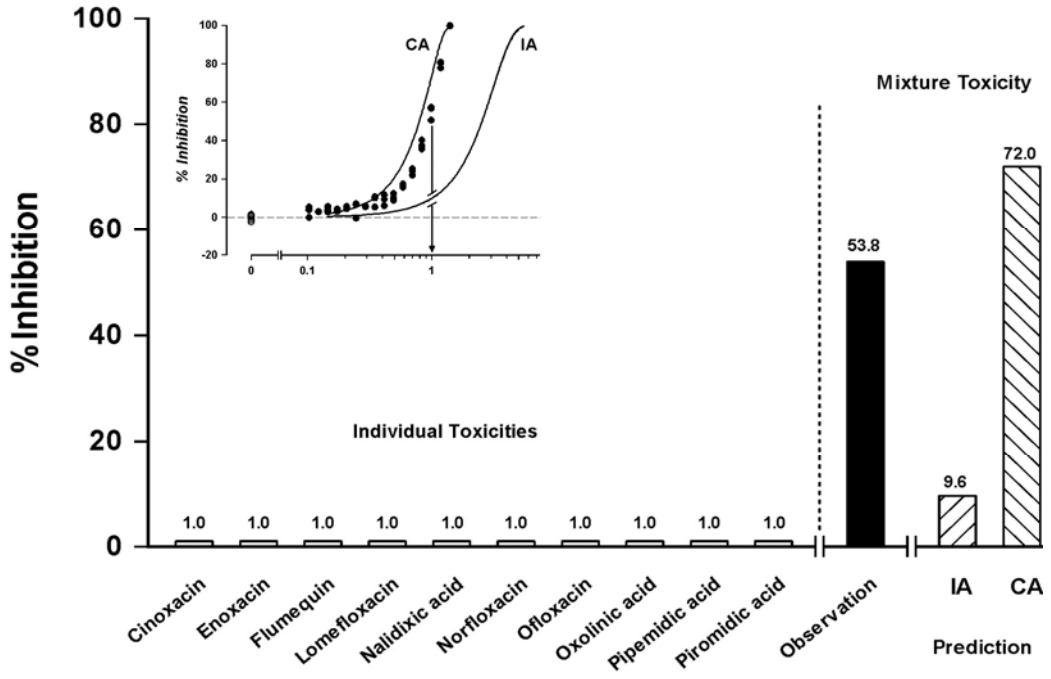

Figure 5.5: Observed and predicted mixture toxicity of a 10 component mixture of quinolone antibiotics

Experiments were conducted in a chronic bioluminescence-inhibition assay with the gram-negative bacterium *Vibrio fischeri* (for details see Backhaus et al. 1999). Mixture ratio: EC01 of the components. CA: Predicted mixture effect / mixture toxicity by Concentration Addition; IA: Predicted mixture effect / mixture toxicity by Independent Action (see later for a discussion of these concepts).

Inset: Comparison of predicted concentration response curves with experimental observations. Vertical arrow indicates the mixture concentration at which every component was present at exactly its EC_{01} (0.95 µmol/L).

Main figure: Comparison of the predicted mixture effects for this concentration with the experimentally observed mixture effect and the underlying single substance effects. It should be noted, that each individual EC_{01}-concentration is well below the corresponding NOEC.

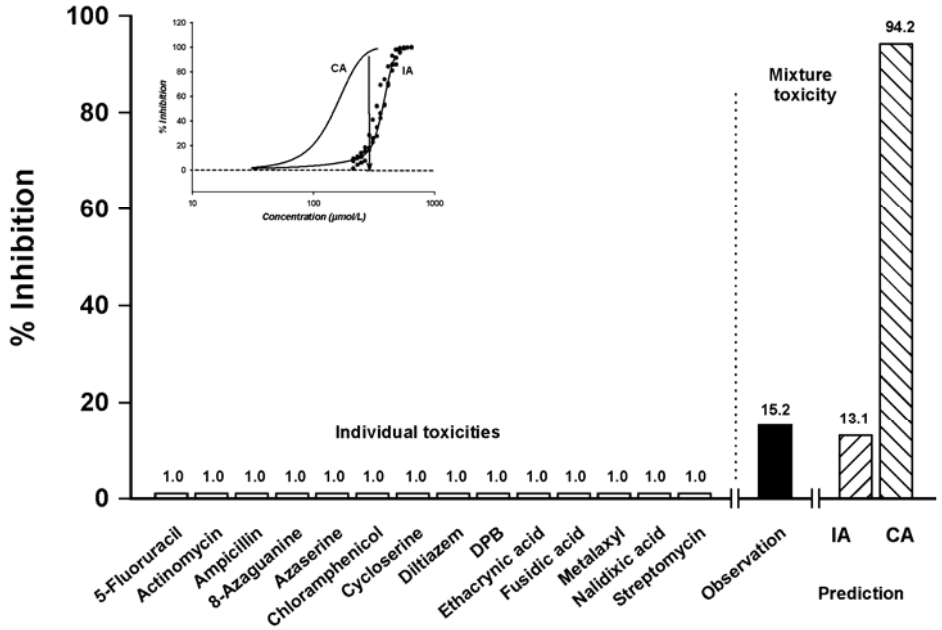

Figure 5.6: Observed and predicted mixture toxicity of a 14 compound mixture (12 pharmaceuticals + Dodecylpyridiniumbromide (DPB) and Actinomycin)

Experiments were conducted in a chronic bioluminescence-inhibition assay with the gram-negative bacterium *Vibrio fischeri* (for details see (Backhaus et al. 2000). Mixture ratio: EC01 of the components. CA: Predicted mixture effect / mixture toxicity by Concentration Addition; IA: Predicted mixture effect / mixture toxicity by Independent Action (see later for a discussion of these concepts).

Inset: Comparison of predicted concentration response curves with experimental observations. Vertical arrow indicates the mixture concentration at which every component was present at exactly its EC_{01} (0.95 µmol/L).

Main figure: Comparison of the predicted mixture effects for this concentration with the experimentally observed mixture effect and the underlying single substance effects. It should be noted, that each EC_{01}-concentration is well below the corresponding NOEC.

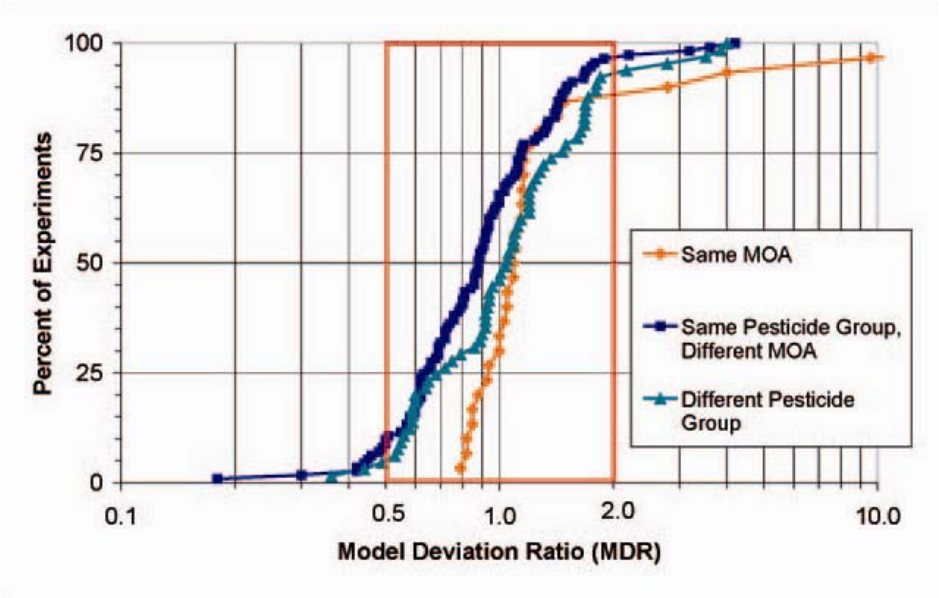

Figure 5.7: Analysis of the mixture toxicity of pesticide mixtures.

Cumulative Model-Deviation Ratio (MDR) for Concentration Addition for mixtures of pesticides with a similar mode of action (MOA), for pesticide mixtures composed from the same pesticide group but with different MOAs and for mixtures with components from different pesticide groups. The MDR gives the ratio between the CA-predicted and observed EC50 of each mixture.

From (Belden et al. 2007), with permission.

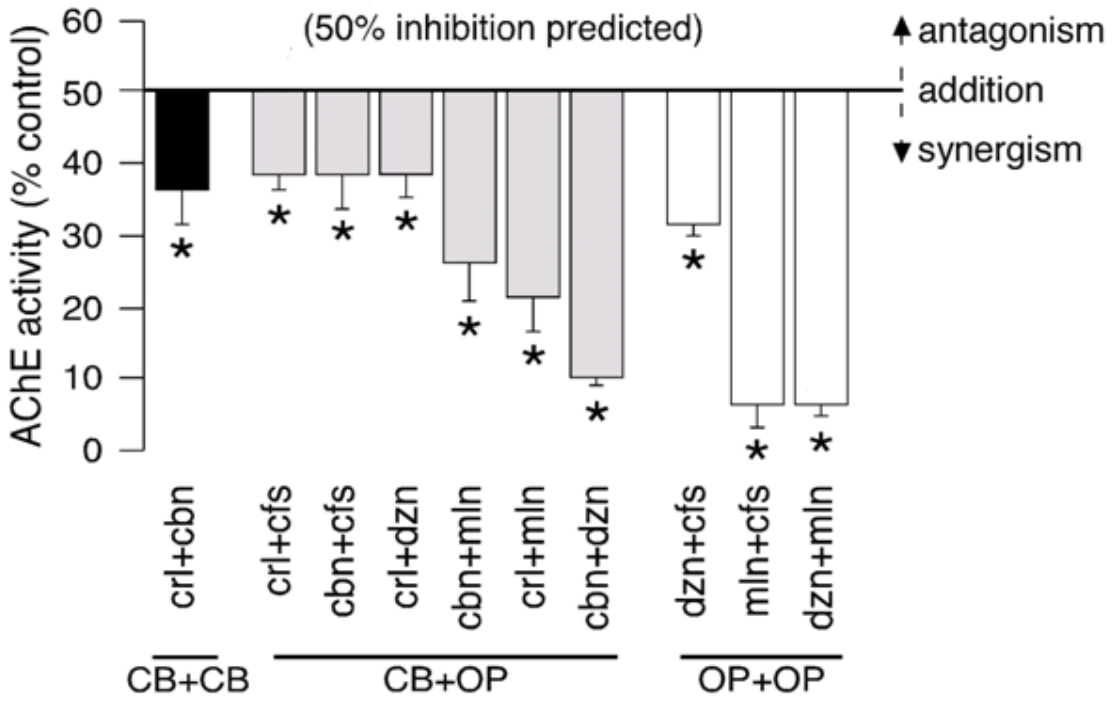

Figure 5.8.: More than concentration-additive mixture toxicity (inhibition of acetylcholine esterase activity) of binary mixtures of carbamates (CB) and organophosphate (OP) pesticides.

DZN: Diazinon; MLN: Malathion; CRL: Carbaryl, CBN: Carbofuran; CFS: Clopyrifos. From (Laetz et al. 2008), with permission.

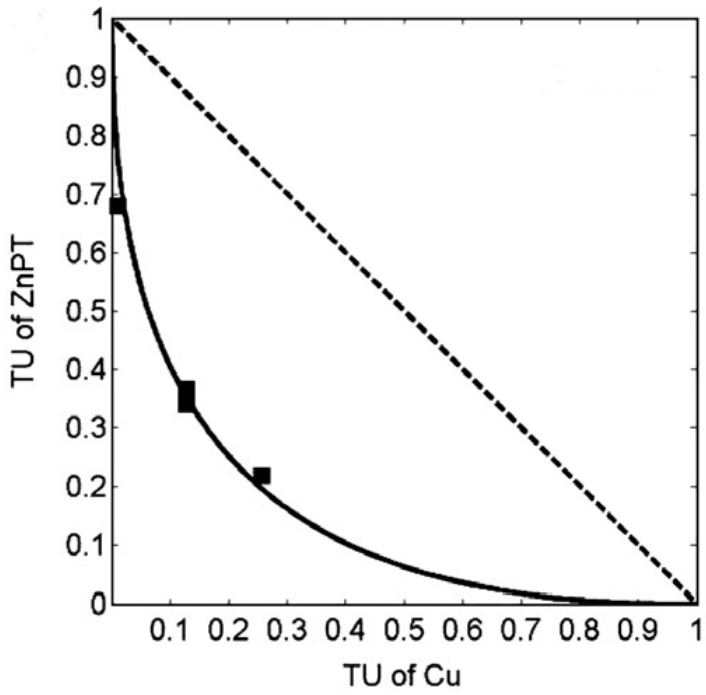

Figure 5.9: Isobolographic analysis of a binary mixture of Copper and Zn-Pyrithion.

TU= Toxic Unit, i.e. the concentration of a compound divided by its EC50. The dashed line gives the sum of toxic units for all possible mixture ratios that are needed for provoking 50% mixture effect according to Concentration Addition, which is always 1. The black squares, the experimental results, show that considerably less TU's are in fact needed to reach 50% mixture effect.

From Bao et al 2008, with permission.

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6. The effect of mixtures at low doses

As the preceding sections have shown, many published experimental mixture studies were motivated by an interest in determining the type of combination effect (for example, additive or synergistic) of the agents involved. That effort often required the administration of doses of test chemicals that were associated with measurable effects but were far removed from exposures experienced by humans. What will lend further urgency to calls to conduct mixtures risk assessment is the demonstration of combined effects at low doses of each mixture component.

Expert opinions about the likelihood of mixture effects at low doses are divided. In their recent Environment and Health Strategy, the European Commission has stated: "Even low level exposure over decades to a complex cocktail of pollutants in air, water, food, consumer products and buildings can have a significant effect on the health status of European citizens" (CEC 2003). An alternative view has been expressed by the European Crop Protection Association: "As a matter of fact, presently available data on exposure to mixtures of chemicals at doses well below the NOAELs of the individual constituents indicate that such exposure is of no health concern" (Carpy et al. 2000).

It has been argued that risks associated with low level exposure to multiple chemicals cannot be assessed without considering the mode of action of the agents that make up the "cocktail of pollutants". According to this view, recently expressed by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT 2002), and re-iterated by the Norwegian VKM (VKM 2008) a distinction should be made between similarly and dissimilarly acting agents. Similarly acting agents are assumed to show "dose additivity" over the entire dose range, including doses in the range of no-observed-adverse-effect-levels (NOAELs). This suggests that combination effects are to be expected even at doses below NOAELs. In contrast, "adverse reactions" are assumed to be unlikely with mixtures of dissimilarly acting agents, when these are combined at doses below NOAELs. In view of the diversity of "real world" mixtures composed of numerous different chemicals with a multitude of different modes of action, it is suggestive to regard dissimilar action of mixture components as the default scenario. Consequently, so the presumption, mixtures pose no health concern, as long as the doses of each component stay below NOAELs (Feron et al. 1995; COT 2002).

That view is based on two premises: First, that NOAELs are a good approximation of "safe" doses of pollutants, and second, that the distinction between "similarly" and "dissimilarly" acting chemicals in a mixture is straightforward and of relevance to the risk assessment issue at hand.

In this section, which is an extended and updated version of an earlier review by Kortenkamp et al. (2007), experimental studies that address the issue of mixture effects at low doses, for both similarly and dissimilarly acting chemicals, are reviewed. Because of the fundamental nature of the topic, we have not only reviewed studies with mammals, but have extended the scope to work with other organisms, such as fish, invertebrates and microorganisms. This approach is justified because key toxicodynamic principles that

govern the ways in which chemicals act in mixtures remain similar, regardless of organisms. On the other hand, care has to be exercised when making comparisons between *in vitro* and *in vivo* assays. To capture the effects of interacting pathways in mixture toxicology, the analysis of apical endpoints is often essential. While many assays relevant to ecotoxicology easily lend themselves to such analyses, this is more complicated in mammalian toxicology where emphasis is often on organ-specific toxicity.

6.1 Concepts and basic considerations

As outlined in section 3.3., different assumptions about the occurrence of mixture effects at low doses are implicit in the concepts of dose addition and independent action. A corollary of the dilution principle of dose addition is the expectation that every component at any dose should contribute, in proportion to its prevalence, to the overall mixture toxicity. Whether the individual doses of mixture components are effective on their own does not matter.

The idea can be illustrated by considering a dose-fractionation experiment (see Figure 6-1), where a dose of 4 x 10^{-2} arbitrary dose units produces an effect of measurable magnitude. The same effect will be obtained when the chemical is administered in 10 simultaneous portions of 4 x 10^{-3} dose units, even though the response to each one of those dose fractions is not measurable (or is exactly zero if there is a true dose threshold). If dose addition applies, the same holds when 10 portions of 10 chemicals with identical response curves are used. Thus, combined effects should also result from chemicals at doses associated with zero effect (dose thresholds) or even lower doses, provided that sufficiently large numbers of components sum to a suitably high effect dose.

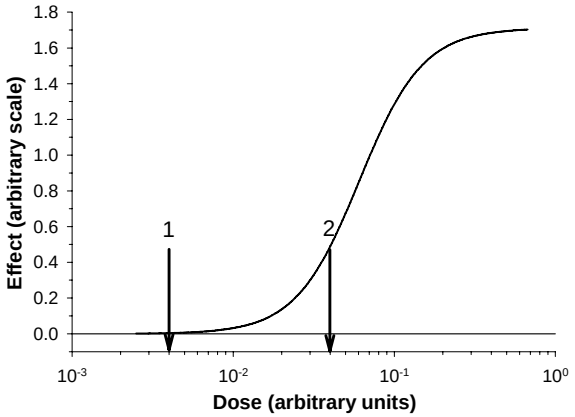

Figure 6.1 Illustration of a "sham" mixture experiment with chemicals that all exhibit the same dose-response curve.
At the low dose to the left (arrow 1, 4 x 10^{-3} dose units), the effect is hardly observable. A combination of 10 agents at that dose (arrow 2, total dose, 4 x 10^{-2} dose units) produces a significant combined effect, consistent with expectations based on dose addition.

Theoretically, the situation described above for dose addition does not apply to independent action. Under the assumption of independent action, simultaneous exposure to large numbers of chemicals at doses associated with zero effects is expected to produce a zero mixture effect. An experimental assessment of that idea, however, is complicated by the fact that true zero effect levels (dose thresholds), if they exist at doses larger than zero, are difficult to determine empirically. Especially with mixtures composed of a very large number of components, that idea forces clear distinctions between zero effects and small, albeit statistically insignificant effects. For example, under independent action, the combined effect of 100 chemicals, each of which individually provoke a response of 1%, will be 63% of a maximally inducible effect. Should each of the 100 agents produce an effect of only 0.1%, the expected combined response will be 9.5%.

To resolve the question whether combination effects can be expected from multi-component mixtures, even if all components are only present at low doses, very small effects of the individual components need to be detected. However, with most *in vivo* bioassays it is very difficult to demonstrate reliably an effect of 1%, let alone effects smaller than 1%. In environmental toxicology, usually effects of between 10 and 30% cannot be distinguished with certainty from control responses (U.S.EPA 1991; Moore and Caux 1997), and a reanalysis of developmental toxicity bioassays yielded statistical detection limits equivalent to effects of about 5-20% on average (Allen et al. 1994).

Regulatory toxicology has dealt with the uncertainties associated with estimating small responses at low doses by using uncertainty factors, with the aim of approximating zero effect levels for the purpose of estimating "safe" exposures of humans. As a starting point for establishing such "allowable," "acceptable," or "tolerable" exposures, no-observed-adverse-effect levels (NOAELs) are used. The NOAEL is the highest dose or exposure at which no statistically or biologically adverse effects can be identified (EPA 1994). It is used as a point of departure for estimating tolerable human exposures by dividing by uncertainty factors.

A number of shortcomings of NOAELs, however, have been identified, and the use of NOAELs for regulatory purposes has led to sharp criticism by the European Commission (EC 1996). There are problems with a single numerical value adequately reflecting study size and the shape of the underlying dose-response curves (Crump 1984; Slob 1999). NOAELs are not fixed attributes of toxic substances; rather, they reflect features of experimental design. Larger experimental studies will detect effects at lower exposures and thus will yield lower NOAELs (Crump 2002; Scholze and Kortenkamp 2007).

To deal with such conceptual problems, the benchmark dose (BMD) has been developed as a statistical tool to determine acceptable exposures to a chemical (Crump 1984). The BMD is a dose that causes a prescribed effect (generally within or close to the experimentally observed range) and is estimated by fitting a regression model to experimental data. Compared with NOAELs, BMDs produce lower numerical values with data of poor quality. Numerous papers have evaluated the properties of BMDs (summarized in Crump 2002). BMDs often produce numerical values similar to

NOAELs, where such comparisons are possible. For example, BMDs associated with 5% additional risk produced dose estimates similar to NOAELs (Allen et al. 1994).

In this section we will assess whether there is evidence that chemicals, when administered simultaneously, exhibit combined effects at doses that are used in risk assessment as points of departure (PODs) for estimating tolerable exposures of humans. Those PODs are typically NOAELs or lower confidence limits of BMDs (BMDLs).

6.2 Hypothesis formulation and implications for the experimental design of low dose mixture studies

In view of received expert opinion, it is necessary to examine the hypothesis that "combinations of dissimilarly acting chemicals do not show mixture effects if the doses of all its components stay below their individual NOAELs". Before reviewing the relevant literature, however, it is important to consider some requirements that must be met to ensure that experiments addressing the issue at hand are conclusive.

First, NOAELs for each mixture component should be estimated using the same assay system (and endpoint) chosen for the mixture study, under identical experimental conditions. Studies that fail to meet this requirement run the risk of administering mixture components at doses higher than NOAELs, in which case the experiment would miss the point entirely.

Alternatively, doses smaller than NOAELs may have been delivered. In this case, it is essential to consider the statistical power of the chosen experimental arrangement.

If dose addition applies, the expectation is that mixture effects may occur when the components are combined at doses equal to, or below their NOAEL. To ensure that a low-dose mixture experiment is conclusive in this case, it becomes important to ascertain that an anticipated combination effect is sufficiently large to reach statistical significance, without violating the precondition that no single mixture component should exceed its NOAEL. The magnitude of the expected combination effect depends on factors such as number of mixture components, their concentration in the mixture, and the steepness of the dose-response curves of individual components (Drescher and Boedeker 1995). It would be trivial to attempt an experiment where e.g. two agents are combined at 1/100 of their individual NOAEL. The resulting mixture effect, although existing, would be too small to be detectable.

If independent action is valid, the situation is more complicated. In this case, the hypothesis is that combination effects should not occur if all mixture components are present at levels below their NOAEL. At the same time, the assumption is made that single doses below NOAEL do not induce effects. However, it is impossible to prove that latter proposition, since apparent absence of mixture effects always leaves the doubt that small, albeit statistically insignificant, effects may have been overlooked. Therefore, an experiment that conclusively proves the hypothesis of absence of mixture effects at doses

equivalent to NOAELs is not easily designed, essentially because the hypothesis is formulated in the negative. As a matter of logic, negatives cannot be proven, and in this case, it is necessary to seek examples that falsify the hypothesis. A viable procedure in this case would be to first estimate NOAELs, and then to carry out regression analysis of the underlying dose-response function to obtain statistical estimates of effects associated with NOAELs. These can then be used to calculate an anticipated mixture effect under the independent action assumption. For the experiment to be conclusive, the expected mixture effect must reach statistical significance. Because of their greater statistical power, studies involving cell lines or microorganisms can be valuable tools to produce conclusive evidence.

6.3 Quality Criteria

Only papers published in international peer reviewed journals were considered for this review. Papers reporting joint effects of multi-component mixtures at low doses or concentrations of individual chemicals are listed in **Tables 6.1 to 6.5** and discussed in the following sections. Many of these studies were designed for an assessment of observed joint effects in terms of agreement or disagreement with predictions based on the concept of DA (Dose Addition or Concentration addition) or on the alternative concept of IA (Independent Action). Results of these comparisons are documented in the **Tables 6.1 to 6.5** as reported in the papers by using the following symbols:

" = " indicates almost perfect agreement between observation and prediction;
" ≈ "indicates that the observed joint effect differed slightly from the prediction, but under consideration of experimental errors this difference appeared to be insignificant;
" < " or " > " indicates that the observed joint effect was significantly smaller or greater than expected by the given predictive concept, respectively.

All studies were examined for compliance with a set of five different quality criteria for low dose mixture studies, which can be derived from the preceding considerations on concepts and experimental design. Quality criteria fulfilled by the studies are indicated in **Tables 6.1 to 6.5** by using the following letter code:

A – Toxicity of individual mixture components was experimentally determined under identical conditions as the mixture (otherwise estimates were derived from QSAR models or taken from the literature);
B – Stability of test concentrations under test conditions was checked by analytical methods (does not apply to animal experiments with direct dosing);
C – Uncertainty of experimentally determined effects, effect concentrations or effective doses was estimated by statistical methods;
D – Uncertainty of mixture toxicity predictions was estimated by statistical methods;
E – NOECs or NOELs were determined for every individual substance and individual concentrations or doses resulting in the given joint effect were demonstrated to be at or below these NOECs or NOELs, or insignificance of individual effects was demonstrated by other statistical approaches.

Codes given in brackets indicate that the criterion was only partly fulfilled. Where quality criteria are not listed in the tables, the corresponding information was either not available from the original paper cited or the criterion was not applicable to the specific type of study.

6.4 Studies with unspecifically acting organic chemicals

In the 1980s, a series of studies of the effects of multi-component mixtures of unspecifically acting organic chemicals on fish and other aquatic organisms was published (**Table 6.1**). Könemann (1980) combined 50 agents at concentrations of 2% of their LC50 for fish and observed a joint mortality of 50%. Evaluating a broader range of endpoints, Hermens et al. (1984, 1985) and Broderius and Kahl (1985) were able to demonstrate strong mixture effects in experiments with 21 – 50 chemicals on daphnids, fish and marine bacteria. In all these studies, a joint effect of 50% was observed when the mixture components were administered at concentrations equivalent to 2.4 – 9.6% of their individual EC50. In view of the evidence of the steepness of the concentration-response relationships of unspecifically acting organics in acute aquatic toxicity assays provided by Broderius and Kahl (1985), it seems reasonable to assume that these concentrations were below the no-observed-effect-concentration (NOEC) of each chemical. However, the validity of this assumption was confirmed by actual determinations of NOEC values in just one of these studies and for only five of the mixture components (Hermens et al. 1984). For all the other substances and studies this ultimate proof is missing. It is therefore necessary to consider mixture studies where NOEL/NOEC estimates for every mixture component were provided explicitly.

6.5 Mixtures made up of chemicals with similar specific modes of action

Table 6.2 is a compilation of low dose mixture experiments involving agents with a common specific mode of action. Jonker et al. (1996) tested the dose additivity assumption with a mixture of four nephrotoxicants, tetra-chloroethylene, trichloroethylene, hexachloro-1,3-butadiene and 1,1,2-trichloro-3,3,3-trifluoropropene, administered to female rats. All four chemicals produce kidney toxicity through a pathway involving conjugation to glutathione. Increased kidney and liver weights were observed in rats that received the agents at 25% of their individual lowest observed nephrotoxic effect level which the authors presumed to be equivalent to NOELs. This study is suggestive of combination effects at doses around NOELs, but it suffers from a lack of proof that the chosen doses were indeed NOELs.

Backhaus et al. (2000), Faust et al. (2001) and Arrhenius et al. (2004) have conducted mixture studies on marine bacteria, algae and algal communities where combinations of chemicals were selected according to very strict similarity criteria. The mixtures included 10 quinolone antibiotics (inhibitors of bacterial DNA gyrase), 18 s-triazines and 12 phenylurea herbicides (inhibitors of photosynthetic electron transport). NOECs were

estimated by using Dunnett's test, and all agents were administered at concentrations equal to, or below their individual NOECs. In all cases, significant mixture effects ranging from 28% to 99% of a maximally possible effect were observed, and these effects could be predicted quite accurately by application of the dose addition concept.

Table 6.3 lists studies with different groups of endocrine-active chemicals that show evidence for joint effects in the low dose range. Silva et al. (2002) have assessed the effects of eight xenoestrogens in a yeast reporter gene assay based on estrogen receptor alpha. All chosen chemicals were able to bind to, and activate, the estrogen receptor alpha. NOECs were estimated by using Dunnett's test (Rajapakse et al. 2002), and joint effects of up to 40% of a maximal estrogenic effect were seen at concentrations around or below NOECs. Again, the observed combined effects agreed well with the additivity expectation of dose addition. Tinwell and Ashby (2004) have analysed mixtures of eight estrogenic chemicals in the rat uterotrophic assay. Combinations of all agents at doses that gave no significant responses when tested individually produced quite strong uterotrophic effects. Mixture experiments with five estrogenic chemicals in fathead minnows (*Pimephales promelas*) presented by Brian et al. (2005) also demonstrated combination effects at concentrations that individually did not induce a significant response. The induction of the egg yolk protein vitellogenin, an estrogen receptor-mediated response, matched the dose addition expectation. Crofton et al. (2005) have conducted an in-depth study of a mixture of 18 polyhalogenated hydrocarbons (2 PCDDs, 4 PCDFs and 12 co-planar and non-coplanar PCBs) where young female rats were treated for four days. Altered serum total thyroxine levels were recorded, and the mixture ratio was chosen to be proportional to the levels of the chemicals reported in breast milk, fish and other human food sources. There was no deviation from dose additivity at the lowest tested doses of the mixture, but at higher test doses the additivity model underpredicted the empirical effects by a factor of 2-3. Significant joint effects were observed at doses of the individual mixture components equivalent to their individual NOELs, or even below.

Hass et al. (2007) studied combinations of three androgen receptor antagonists in an extended rat developmental toxicity model. The male offspring of female rats which were dosed over the entire duration of pregnancy showed significant signs of feminisation (reduced anogenital index, retained nipples) with a mixture of antiandrogens at their individual NOELs for these endpoints. Quantitatively, these effects agreed well with the responses anticipated by dose addition.

Although not designed for such purposes, the experiment by Howdeshell et al. (2008) on suppression of testosterone synthesis after developmental exposure to five phthalates indicates that phthalates are able to work together when present at individually ineffective doses. Statistically significant reductions in fetal testosterone synthesis were observed after administration of a total mixture to pregnant Sprague-Dawley rats at 260 mg/kg-d. The mixture contained DPP at 20 mg/kg-d and the other four phthalates (BBP, DBP, DEHP and DIBP at 60 mg/kg-d). DPP was tested on its own at 25 mg/kg-d, and the remaining phthalates were examined after single administration at 100 mg/kg-d. At those doses, none of the single phthalates induced effects significantly different from those

recorded in unexposed controls. The doses in the single-phthalate experiments even exceeded those in the mixture.

Table 6.1. Significant joint effects of similarly acting toxicants at low concentrations: I. Evidence from early studies on the aquatic toxicity of mixtures of non-reactive organics with an unspecific "narcotic" mode of action

Reference	Organism (Species)	Endpoint (Exposure Time)	Number of Mixture Components	Individual Concentrations	Joint Effect	Comparison with Predictions[a]	Quality Criteria Fulfilled[a]
Könemann 1980	fish (*Poecilia reticulata*)	mortality (7 or 14 d)	50	2 % of EC50	50 %	= DA	A
Hermens et al. 1984	waterfleas (*Daphnia magna*)	immobilization (48 h)	50	2.4 % of EC50[c]	50 %	≈ DA	(A)[d], B, (C, D)[e]
Hermens et al. 1984	waterfleas (*Daphnia magna*)	mortality and inhibition of reproduction (16 d)	25	6 % of EC50[c]	50 %	< DA	(A)[d], B, (C, D)[e], (E)[f]
Broderius and Kahl 1985	fish (*Pimephales promelas*)	acute mortality (96 h)	21	5.9 % of EC50[c]	50 %	≈ DA	A, B, C
Hermens et al. 1985	marine bacteria (*Vibrio fischeri*)[b]	bioluminescence inhibition (15 min)	21	9.5 % of EC50[c]	50 %	< DA	A

[a]See explanation in section 6.3. [b]Formerly *Photobacterium phosphoreum.* [c]Recalculated from the sum of toxic units reported in the paper. [d]Individual EC50 values were determined experimentally for part of the components and estimated by a QSAR model for the remaining compounds. [e]Uncertainty in the comparison of observed and predicted mixture toxicity was assessed on the basis of a fixed estimate for the error in individual effect concentrations. [f] NOECS were determined for 5 out 25 mixture components; from the data reported in the paper it can be recalculated that in case of these 5 substances 6 % of the EC50 is always a concentration that is definitely lower than the corresponding NOEC.

Table 6.2. Significant joint effects of similarly acting toxicants at low concentrations: II. Evidence from studies on groups of substances with a common specific mechanism of action in mammalians or unicellular organisms

Reference	Organism (Species)	Endpoint (Exposure Time / Route)	Mixture Components (Mechanism of Action)	Individual Doses or Concentrations	Joint Effect	Comparison with Predictions[a]	Quality Criteria Fulfilled[a]
Jonker et al. 1996	rats (female Wistar rats)	kidney toxicity examined by 40 different functional and morphological parameters (32 d / daily by oral gavage)	4 similarly acting nephrotoxicants (selective renal toxicity ascribed to a common bioactivation pathway following conjugation to glutathione)	presumed NOEL (= ¼ LOEL)	increased kidney and liver weights; (other parameters did not show significant joint effects)	(= DA)[d]	A, C
Backhaus et al. 2000	marine bacteria (*Vibrio fischeri*)	bioluminescence inhibition (24 h)	10 quinolone antibiotics (inhibition of bacterial DNA gyrase)	NOEC	99 %	= DA, > IA	A, B, C, E
Faust et al. 2001	algae (*Scenedesmus vacuolatus*)	inhibition of reproduction (24 h)	18 *s*-triazine herbicides (inhibition of photosynthetic electron transport)	4.7-60 % of NOEC[b]	47 %	≈ DA, > IA	A, B, C, E
Arrhenius et al. 2004	natural marine microalgal communities (numerous species)	photosynthesis inhibition (45 min)	12 phenylurea herbicides (inhibition of photosynthetic electron transport)	≤ NOEC[c]	28 % and 37 % (2 different communities)	≈ or < DA, > IA	A, B, C, E

[a]See explanation in section 6.3. [b]All mixture components were present at individual concentrations that were statistically estimated to exert mean individual effects of 1 % only. These individual EC1 values were demonstrated to equal 4.7 to 60 % of individual NOECs. [c]Mixture components were present at statistically estimated individual EC1 concentrations. These were demonstrated to be smaller or at most equal to individual NOECs. [d]Qualitative assessment only referring to the fact that combined exposure to individual NOELs resulted in significant joint effects. In contrast to the other studies listed, experiments were not designed for a quantitative comparison between prediction and observation in terms of intensity or frequency of joint effects.

Table 6.3. Significant joint effects of similarly acting toxicants at low concentrations: III. Evidence from studies with different groups of endocrine-active chemicals

Reference	Organism and/or Assay (Species)	Endpoint (Exposure Time / Route)	Mixture Components	Individual Doses or Concentrations	Joint Effect	Comparison with Predictions[a]	Quality Criteria Fulfilled[a]
Silva et al. 2002	YES - recombinant Yeast Estrogen Screen (*Saccharomyces cerevisiae* genetically modified to express the human estrogen receptor α)	estrogen receptor activation (72 h)	8 xenoestrogens	43-100 % of NOEC[b]	significant estrogenic activity	= DA	A, C, E
Tinwell and Ashby 2004	rats, uterotrophic assay (immature female AP rats)	uterine weight increase (3 d / daily by subcutaneous injection)	8 estrogens and xenoestrogens	≤ NOEL[c]	significant uterotrophic activity		A, C, E
Brian et al. 2005	fish (male *Pimephales promelas*)	vitellogenin induction (14 d)	5 estrogens and xenoestrogens	≤ NOEC[c]	significant vitellogenin induction (~ 50 % of maximum possible effect)	≈ DA	A, B, C, D, E
Crofton et al. 2005	rats (young female Long Evans rats)	decrease of serum total thyroxine (T4) concentrations (4d / daily by oral gavage)	18 thyroid-disrupting chemicals	≤ NOEL	significant T4 decrease	≥ DA[d]	A, C, D, E
Hass et al. 2007	Male young rats, after exposure in utero	Feminisation of anogenital distance	3 androgen receptor antagonists	≤ NOEL	Significant feminisation of anogenital distance	≈ DA	A ,C ,D, E
Howdeshell et al. 2008	rats, exposure in utero,	Suppression of fetal testosterone levels	5 phthalates	≤ NOEL	Significant suppression of testosterone levels	≈ DA	A, C, D, E

[a]See explanation in section 6.3. [b]Individual concentrations equalled 50 % of statistically estimated individual EC1 values. These concentrations were demonstrated to equal 43 to 100 % of individual NOECs. [c]Tests were not designed for conventional NOEL or NOEC determinations. However, individual doses or concentrations in the mixture were demonstrated to provoke no effects significantly different from untreated controls (i.e. they must have been ≤ NOEL or NOEC). [d]Dose-dependent additivity and synergism.

The majority of the studies discussed above were well designed to address the issue of combination effects at low doses. Taken together, there is very good empirical support for the notion that chemicals with a similar mode of action may produce combination effects at doses below NOEL/NOEC.

Noteworthy are two studies purportedly demonstrating the absence of estrogenic effects at low doses. However, these experiment are inconclusive because it was not considerede that the expected mixture effect must reach statistical significance.

Recently, van Meeuwen et al. (2007) presented the results of experiments with a combination of estradiol, phytoestrogens and synthetic estrogens in the rat uterotrophic assay. The composition of the phytoestrogen and xenoestrogen mixtures was based on data about serum levels and on human dietary intake of the chemicals. While mixtures of estradiol and phytoestrogens acted in a dose additive fashion, the combination of synthetic estrogens (4-nonyl phenol, 4-octyl phenol, bisphenol A, β-HCH, methoxychlor and dibutyl phthalate) did not lead to modulations of the effects of estradiol. This was because the xenoestrogen mixture was ineffective when administered without estradiol, even at doses equivalent to 100,000 times the human intake. However, considering the individual potency of the chosen xenoestrogens in the rat uterotrophic assay, this outcome was predictable. It can be estimated that doses equivalent to 1,000,000 should have been administered for effects to be observed with any degree of certainty – a reflection of the insensitivity of the uterotrophic assay. The authors concluded that the contribution of xenoestrogens to total estrogenicity in the human diet can probably be neglected. However, this conclusion is problematic, because the minimal criteria for low dose mixture experiments set out above were not met in this study. It is unclear what motivated the selection of the six xenoestrogens, and more agents should have been included in the mixture.

Charles et al (2007) evaluated the impact of low level exposure to a mixture of six synthetic chemicals under conditions of co-exposure to various levels of plant-derived phytoestrogen compounds. Estrogenic activity was assessed using an in vitro human estrogen receptor (ER) transcriptional activation assay and an in vivo immature rat uterotrophic assay. Initially, dose-response curves were characterized for each of the six synthetic chemicals (methoxyclor, o,p-DDT, octylphenol, bisphenol A, -hexachlorocyclohexane, 2,3-bis(4-hydroxypbenyl)-propionitrile) in each of the assays. The six synthetic estrogens were then combined at equipotent ratios and tested at 5-6 dose levels spanning from very low, sub-threshold levels, to a dose in which every chemical in the mixture was at its individual estrogenic response threshold. Both in vitro and in vivo, low concentrations of the synthetic estrogen mixture failed to increase estrogenic responses relative to those induced by phytoestrogens alone. The authors concluded from their data that chemical mixture toxicity is likely to be of concern only when the mixture components are near or above their individual response thresholds. However, this conclusion needs to be tempered in light of the fact that only six synthetic estrogens were incorporated in their mixtures.

6.6 Experimental studies providing evidence for mixture effects of dissimilarly acting chemicals at low doses

There is evidence that dissimilarly acting agents, when combined at doses below their NOAELs, may also produce significant mixture effects (**Table 6.4**).

Hermens et al. (1985) combined 33 chemicals which can be grouped into three classes with presumably differing modes of action. The mixture produced 50% mortality in fish when all components were present at 4% of their individual EC50. It was assumed that these concentrations were below NOECs, although NOECs were not estimated in this study. It is therefore conceivable that some chemicals may have been present at levels above their NOECs, and this point may be particularly relevant with compounds that exhibit shallow dose-response curves. These weaknesses have been overcome in later studies of mixture toxicity from multi-component mixtures of dissimilarly acting chemicals.

In a study utilizing a cell-proliferation assay with human breast cancer MCF-7 cells, Payne et al. (2001) tested a mixture of two estrogen receptor agonists (*o,p'*-DDT, *p,p'*-DDT), one anti-androgenic agent (*p,p'*-DDE) and a chemical that induces cell division by as yet poorly defined mechanisms (β-HCH). A significant proliferative effect was observed when these chemicals were present at concentrations equivalent to 25-100% of their individual NOECs. Independent action and dose addition predicted the observed effect equally well.

Walter et al. (2002) assessed the effect of a mixture of 11 aquatic priority pollutants on algal reproduction. The chemicals were selected for structural diversity by using chemometric methods, and their NOECs estimated by hypothesis testing methods. In this study, 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%. Based on these estimates of low effects, independent action yielded quite accurate predictions of mixture toxicity. Combined at their NOECs, the pollutants produced a joint effect of 64%.

All these studies used groups of similarly acting chemicals, where each group had a different presumed mode of action. Often, dissimilarity was inferred on the basis of diverse chemical structures, but proof of dissimilar action could not be provided because the actual mechanisms involved were unclear. There is the possibility that many of these experiments in fact utilized chemicals which at least partly acted in similar ways. Thus, there is a need to consider studies that have employed very strict criteria for dissimilar action.

A diverse mixture of 16 chemicals, all known to specifically interact with different target sites in algae, was assessed for inhibition of reproduction in algae by Faust et al. (2003). When these chemicals were combined at concentrations equivalent to 6.6 – 66% of their NOECs, a combined effect of 18% was observed. Similar to the approach taken by Walter et al. (2002), estimates of low effects, down to 1%, were produced by regression

analysis of concentration-response data of individual chemicals. These estimates were utilized to calculate mixture effect predictions according to independent action. This yielded fairly accurate predictions of the observed combination effects, while dose addition fell well short of observations. Similar results were obtained with a mixture of specifically dissimilarly acting chemicals in bacterial systems (Grimme et al. 1998).

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. However, before firm conclusions can be drawn, it is necessary to review the papers often quoted (see COT 2002, VKM 2008) in support of the notion that mixtures of dissimilarly acting chemicals are safe at doses below NOAELs. The relevant studies are listed in **Table 6.5**.

6.7 Purported absence of evidence of low-dose combination effects with dissimilarly acting agents

The first of these studies was published by Jonker et al. (1990) who prepared mixtures of 8 arbitrarily chosen chemicals which they fed to rats. Each chemical affected a different target organ, by differing modes of action. In one mixture, the agents were combined at doses equivalent to their NOAEL, and two further mixtures representing 1/3 and 1/10 NOAEL were investigated. Rats exposed to the NOAEL mixture for 4 weeks showed darkened livers, decreased haemoglobin levels and increased kidney weights. The experiment with the 1/3 NOAEL mixture yielded increased kidney weights, which the authors interpreted as "chance finding". No effects became apparent with the 1/10 NOAEL mixture. Although the authors concluded that there was "some, but no convincing evidence for an increased risk from exposure to a combination of chemicals when each chemical is administered at its own individual NOAEL", it is debatable whether the NOAEL and 1/3 NOAEL mixtures were entirely devoid of effects. However, it is important to point out that the chosen endpoint are quite difficult to quantify.

Jonker et al. (1993) also examined a mixture of toxicants that act by differing mechanisms but affect the same target organ. This mixture included four different kidney toxicants. The chemicals were combined at doses presumed to be NOAELs on the basis of range finding tests, and at ¼ of NOAELs. Rats exposed to the NOAEL combination experienced slight growth retardations, increased relative kidney weights and elevated numbers of epithelial cells in their urine. However, rats given one of the individual chemicals at doses equal to the presumed NOAEL showed similar effects. Thus, at least one dose higher than its actual NOAEL was used in the mixture experiment. The combination of ¼ of NOAEL did not provoke significant observable effects.

Ito et al. (1995) explored the effects of 19 organophosphates and one organochlorine on the formation of preneoplastic lesions in the livers of rats pre-treated with the liver carcinogen diethylnitrosamine (DEN). The 20 chemicals were combined at doses equivalent to their acceptable daily intakes (ADI) and to 100 times their ADI. There were

increased preneoplastic lesions with the 100-times ADI mixture, but the ADI mixture did not induce observable effects. None of the selected chemicals were tested individually and the doses in this study were based on ADI values proposed by the Japanese Government reflecting a diversity of endpoints. Thus, it is impossible to assess how close the doses of the chemicals in the two mixtures were to their NOAELs for preneoplastic lesions. It cannot be ruled out that the individual doses in the ADI mixture were far below their NOAELs and therefore, even in combination, significant effects might not be expectable. On the other hand, it is likely that some of the chemicals in the 100-times ADI mixture exceeded their individual NOAELs (in relation to preneoplastic lesions). This might explain why effects were seen with this mixture.

Groten et al. (1997) selected 9 chemicals with differing target organ toxicity and modes of action and exposed rats to two combinations. A mixture composed of doses equivalent to the NOAELs of each chemical produced increased relative kidney weights, hepatocellular hypertrophy and hyperplasia of nasal epithelial cells. Administered at 1/3 of their NOAELs, the 9 chemicals induced increased relative kidney weights. This study would suggest that there were effects in the low dose range. The author's conclusion that "simultaneous exposure to the nine chemicals does not constitute an evidently increased hazard (…), provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL" may have to be tempered in the light of a discussion about the toxicological relevance of the observed effects.

The effects on rats of mixtures of 18 organochlorine pesticides and environmental contaminants, including 2,3,7,8 TCDD, were analysed by Wade et al. (2002). The animals were exposed for 70 days to a combination of all agents at their respective MRL or ADI levels. This ADI mixture failed to produce observable effects. However, this experiment is difficult to interpret because none of the chemicals were tested individually and information about their NOAELs in relation to the endpoints examined is missing. Given that only 10 animals per group were used it is likely that the study was of relatively low statistical power. A combination equivalent to doses 10 times higher than those in the ADI mixture was also examined and decreases in epididymus weights were observed. However, TCDD alone, at the dose present in the mixture, produced the same effect. This indicates that the observed effects were attributable solely to TCDD, and that the contribution of all other chemicals to the overall joint effect was negligible.

While some of the studies in **Table 6.5** provide evidence for combination effects (Jonker et al. 1990; Groten et al. 1997), the apparent absence of effects in the remaining papers can be explained in terms of insufficient statistical power or flawed selections of dose levels.

Table 6.4. Significant joint effects of dissimilarly acting toxicants at or below individual NOECs

Reference	Organism or Cell Type (Species)	Endpoint (Exposure Time)	Mixture Components	Individual Concentrations	Joint Effect	Comparison with Predictions[a]	Quality Criteria Fulfilled[a]
Hermens et al. 1985	fish (*Poecilia reticulata*)	mortality (14 d)	33 aquatic pollutants from 3 groups with probably different modes of action	4% of EC50 (assumed to be below NOEC)	50 %	≈ DA or < DA[d]	A
Payne et al. 2001	MCF-7 human breast cancer cells	stimulation of cell proliferation (7 d)	4 persistent organochlorine pesticides exerting effects on cell proliferation in different ways	25-100 % of NOEC[b]	significant proliferative effect	= DA, = IA[e]	A, C, E
Walter et al. 2002	algae (*Scenedesmus vacuolatus*)	inhibition of reproduction (24 h)	11 aquatic priority pollutants selected for structural diversity by chemometric analysis	NOEC	64 %	< DA, ≈ IA	A, B, C, E
Faust et al. 2003	algae (*Scenedesmus vacuolatus*)	inhibition of reproduction (24 h)	16 toxicants known to interact with completely different molecular target sites in algae	6.6-66% of NOEC[c]	18 %	< DA, ≈ IA	A, B, C, D, E

[a]See explanation in section 6.3. [b]Recalculated from individual concentrations and NOECs reported in the study. [c]Mixture components were present at statistically estimated individual EC1 concentrations. These were demonstrated to equal 6.6-66 % of individual NOECs. [d]Observed mixture toxicity was slightly lower than predicted by DA, but significance of the difference was not assessed by statistical means. [e]Both predictive concepts, DA and IA, gave nearly identical and accurate predictions.

Table 6.5. Rat studies providing no strong evidence for significant joint effects of dissimilarly acting toxicants at or below individual NOELs

Reference	Number and Type of Rats	Endpoint (Exposure Time / Route)	Mixture Components	Individual Doses	Joint Effects	Authors Conclusions	Quality Criteria Fulfilled[a]
Jonker et al. 1990	10 male and 10 female Wistar rats per dose group	haematology, clinical chemistry, urinalysis, and pathology examined by 76 parameters (4 weeks / via diet)	8 divers chemicals, arbitrarily chosen	1/10 NOAEL 1/3 NOAEL NOAEL	no clearly treatment related effects no clearly treatment related effects slight increase in relative kidney weights and decrease of haemoglobin in males; swollen or dark livers in 3/10 of males; no other clearly treatment related effects	"some, but no convincing evidence for an increased risk from exposure to a combination of chemicals when each chemical is administered at its own individual NOAEL"	A, C, E
Jonker et al. 1993	10 male and 10 female Wistar rats per dose group	haematology, clinical chemistry, urinalysis, and pathology examined by 45 parameters (4 weeks / via diet)	4 kidney toxicants damaging epithelial cells of the proximal tubules by different mechanisms	1/4 NNEL NNEL	no clearly treatment related effects slight growth retardation in males; findings on increased relative kidney weights and epithelial cells in urine in males were inconclusive	"simultaneous administration of the four nephrotoxins at their NNEL produced only weak indications of increased toxicity"	A, C, E
Ito et al. 1995	19 or 18 male F344 rats per dose group	enhancement of liver pre-neoplastic lesion development initiated by DEN (6 weeks, via diet)	20 pesticides not classified as carcinogens and permitted for use in Japan	ADI[b] 100 x ADI[b]	no effect enhanced development of preneoplastic lesions	"the present safety factor approach is appropriate for the risk evaluation of environmental chemicals"	C
Groten et al. 1997	8 male Wistar rats per dose group	haematology, clinical chemistry, biochemistry and pathology examined by 47 parameters (4 weeks / inhalatory and via diet)	9 chemicals with diverse MoA, relevant to the general human population in terms of use pattern and exposure	1/3 NOAEL NOAEL	increase in relative kidney weights hyperplasia and metaplasia of nasal epithelium, hepatocellular hypertrophy, decreased plasma triglyceride concentrations, altered ALP enzyme activities, increased relative kidney weights	"simultaneous exposure to the nine chemicals does not constitute an evidently increased hazard (…), provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL"	A, B, C, E
Wade et al. 2002	10 sexually mature male Sprague-Dawley rats (9 controls)	general physiology, liver, reproductive organs and immune system examined by 54 parameters (70 d / by gavage daily)	18 contaminants of human reproductive tissues with diverse MoA	TCDD ≤ NOAEL[c], other 17 toxicants at MRL, RfD, TDI, or PTDI-levels	no adverse effects	"MRLs, TDIs, or RfD (…) provide adequate protection for adult male animals, for those systems examined"	C, (E)

Footnotes are given on the following page

Footnotes to Table 6.5

Abbreviations: ADI – Acceptable Daily Intake; ALP – alkaline phosphatase; ALAT – alanine aminotransferase; DEN – diethylnitrosamine; MoA – Mode(s) of Action; MRL – Minimal Risk Level estimated by ATSDR (Agency for Toxic Substances and Disease Registry of the U.S. Department of Health and Human Services); NNEL – No Nephrotoxic Effect Level; PTDI – Provisional Tolerable Daily Intake established by Health Canada; RfD – Reference Dose established by EPA (U.S. Environmental Protection Agency); TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TDI – Tolerable Daily Intake established under CEPA (Canadian Environmental Protection Act).

[a]See explanation in section Methods and Quality Criteria. [b]ADIs are based on NOAELs for non-carcinogenic effects, provided by the Japanese Ministry of Health and Welfare or taken from a FAO/WHO report. [c]NOAEL not determined in the study, but taken from the literature.

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7. Implications for dealing with chemical mixtures in toxicological risk assessment and regulation

The empirical evidence of documented mixture effects with combinations at doses around points of departure heightens the need to take mixture effects into consideration during the estimation of acceptable human exposures.

Both under the assumptions of dose addition and independent action combination effects may result from chemicals that each produces very small effects, if they are present in large numbers. A question at the centre of debates about the implications of the empirical evidence is whether current practices of chemical risk assessment and regulation are sufficiently protective to accommodate the possibility of combination effects, or whether additional steps need to be taken.

7.1 Uncertainty factors in risk assessment and standard setting – do they allow for the possibility of mixture effects?

In current regulatory practice NOAELs are combined with so-called uncertainty factors, to derive acceptable daily intakes (ADI). The uncertainty factors (UF) are intended to deal with statistical uncertainties in the estimation of NOAELs, species – species extrapolations, inter-individual variations and sometimes even extrapolations from acute to chronic effects. In human risk assessment, factors ranging from 10 to 1000 are commonly used. The claim is that the ADIs derived for single chemicals signify exposure levels that can be tolerated for a life time, without harmful effects (WHO 1978). The question is whether this claim is viable when exposure is to large numbers of chemicals, all at levels around their individual ADI.

It is sometimes argued that UF already cover the possibility of combination effects. This issue was examined during a recent expert workshop on combination effects of chemicals, organized by the Danish Environment Ministry (Kortenkamp and Hass 2009).

It appears that UF are used in two different ways: Either to assess the health risks associated with certain chemical exposures by deriving Margins of Exposure (MOE) or Margins of Safety (MOS), or with the aim of establishing recommended health-based guidance values, such as ADI, TDI, Reference Doses (RfD) and such like. Depending on context and goals, they are also referred to as Assessment Factors.

The widely used UF of 100 is obtained by multiplication of two factors, one to allow for intra-species sensitivity differences (10), the other for species-species extrapolations from animal to human (10). Additional factors may be used to compensate for uncertainties due to lack of information. For example, in the absence of data for chronic toxicity, an (additional) default factor of 10 can be employed. Similarly, if test data do not allow the estimation of a NOAEL, an additional factor of 10 may be brought into play. The various assessment factors are multiplied, and this can yield a very large overall UF. The largest reported overall UF in USEPA's Integrated Risk Information System is 10,000.

A specific factor intended to allow for possible mixture effects is not in use.

Nevertheless, the common practice of combining different types of assessment factors by multiplication has led to the idea that many overall UF's are overly conservative. By implication, this is taken to mean that mixture effects are covered. This idea appears to be based on a mistaken interpretation of the multiplication rule of probabilities for rare events. While is it clear that the occurrence of two rare independent events together tends towards zero, assessment factors cannot be equated with probabilities. A direct translation of UF's into probabilities is not possible.

There is evidence that the common practice of using a factor of 10 to deal with animal-to-human extrapolations may lead to underestimations of risk. The same applies to the factor of 10 to allow for between-human differences in sensitivity. These considerations suggest that an UF of 100 offers insufficient room to allow for mixture effects for all possible realistic mixtures.

The issue of UF's and mixture effects can be approached from a different direction by asking the question: how large would an additional assessment factor have to be to take account of mixture effects? For a combination of chemicals that follows dose addition, it can be shown that the RfD's for each individual chemical would have to be divided by the number of chemicals that contribute to an overall mixture effect. For example, if a combined effect from simultaneous exposure is due to 5 chemicals, then the RfD of every chemical has to be divided by 5, which is equivalent to saying that an additional assessment factor of 5 is needed to cover mixture effects (NRC 2008). Correspondingly larger factors are needed if more chemicals can be shown to contribute to a common adverse outcome. However, choices about sufficiently protective factors cannot be made without better information about the number of relevant chemicals, their levels and potency, and how they contribute to human exposures.

To summarize, a specific "mixtures assessment factor" is currently not employed in the traditional chemical-by-chemical risk assessment. At present there is little to suggest that commonly used UF are overly protective. There does not seem to be much "room" to allow for mixture effects. In the interest of removing some scientific uncertainty it would be of merit to analyse the issue of UF and mixture effects systematically.

7.2 Default concepts for the assessment of combined exposures?

A consensus seems to be emerging that current risk assessment practices should recognize mixture effects and should apply additional methods to take account of combined expsoures. For example, the European Scientific Committee on Toxicology, Ecotoxicology and the Environment (SCTEE 2004) pointed out that "for compounds with identical mode of action, such as oestrogenic hormones and xenoestrogens (…) the performance of individual risk assessments is problematic. …The effects may be additive, especially since these chemicals co-occur in the aquatic environment".

If it is accepted that risk assessment should consider the effects of combined exposures, it is necessary to decide which assessment concept, DA (CA) or IA should be adopted as a default for evaluating mixtures in human and ecological risk assessment.(see Section 3.4). That question becomes all the more important when the two concepts produce different predictions of mixture effects. However, in only a few cases have DA (CA) or IA been evaluated together against the same set of experimental mixture data with the aim of establishing whether either approach produces valid predictions of combined effects (see Sections 3.5 and 4 and 5).

In the opinion of U.S. EPA (2000), the empirical basis of choosing between DA and IA as a default approach for risk assessment is not strong. The decision in favor of either approach as a default for mixture risk assessment is based largely on perceptions of whether the scientific assumptions that underpin DA (CA) or IA are met. For such purposes, the two concepts have been allied to broad mechanisms of combined toxicity, with DA (CA) being applicable to mixtures composed of chemicals that have a similar or common mechanism of action, and IA for those that have diverse or dissimilar mechanisms of action (see Section 3.4.). Independent action is often held to be the default assessment concept when the similarity criteria of dose addition appear to be violated (COT 2002).

Although those ideas appear reasonable, their application to specific combinations of chemicals is far from clear-cut. One major difficulty lies in defining reliable criteria for similarity of mechanisms of action (U.S.EPA 1986, 1989; Mileson et al. 1998; Groten et al. 2001; ATSDR 2002). Often, the induction of the same phenomenologic effect is deemed sufficient for accepting similarity of action and therefore DA (CA). However, that could be inappropriate for some combinations of chemicals that operate by distinct molecular mechanisms. At the other extreme of the spectrum of opinion, the similarity assumption might require an identical molecular mechanism involving the same active intermediates. That position, with its strict similarity criterion, may mean that few chemicals qualify for inclusion in mixture-effects assessments and many others that provoke the same response are left out. In effect, that approach would provide an unrealistically narrow perspective on existing mixtures. A middle position is occupied by the view that interactions with the same site, tissue, or target organ should qualify for similarity.

Instead of using a default method, dichotomous approaches have been proposed. For example, U.S. EPA (2000, 2002) recommended using DA for the regulation of pesticides that share a common mode of action, and employing IA for dissimilar mixtures. COT (2002) have suggested to adopt IA as the default approach, and to use DA only in specific cases.

Such dichotomous approaches pose a number of problems: (i) Unambiguous criteria for what should constitute “similar” or “dissimilar” action do not exist and are currently difficult to define. Sometimes, the induction of the same phenomenological effect is deemed sufficient for similar action. At the other extreme of the spectrum of opinions, an

identical toxic mechanism, involving the same toxic intermediate is required to fulfil the similarity assumption. A middle position is occupied by the view that interactions with the same site or tissue should qualify for similarity. (ii) In most cases, the precise mechanisms of action are unknown. Exceptions are very few groups of chemicals, perhaps including some organophosphorus and carbamate pesticides, and polychlorinated dioxins and furans. Thus, it is the rule rather than the exception that agreement about similarity or dissimilarity of action cannot be reached. This situation is likely to remain unchanged in the foreseeable future. (iii) Knowledge about mechanisms is likely to change with new evidence and expectations about presumed modes of action do not necessarily match biological observation. Thus, serious doubts exist to what degree knowledge about specific molecular mechanisms can be utilized constructively in in deciding about evaluation concepts in mixtures risk assessment.

Therefore, lack of knowledge about the mode of action of mixture components should not block risk assessment because a choice between the two concepts for evaluation is difficult to make. Instead, in the absence of information, precaution should be the overriding concern. This reduces to the question which of the two assessment concept yields the more conservative mixture effect prediction?

In the ecotoxicological arena, systematic comparative studies of the mixture effect predictions produced by dose addition and independent action have shown that dose addition yielded the more conservative predictions, but that overall, the quantitative differences between both concepts were relatively small. Here, the case can be made for using dose addition as the default approach for mixture assessments. This would avoid lengthy and largely fruitless discussions about establishing modes of action. Such a modus operandi would have two advantages: First, the data requirements for proper use of DA (CA) are less stringent than those for IA (see Section 3.5.). While the former works well on the basis of effect doses, the use of IA usually requires knowledge of entire dose-response curves, particularly in the low effect range. Second, prospective mixture effect assessments should be compliant with the precautionary principle. This favours the concept that typically yields the more conservative predictions, i.e. CA (see Section 5).

While the case for CA is validated in ecotoxicology, the situation is not so clear-cut in human toxicology. Here, the relevant information is largely missing and research efforts are currently directed into conducting studies to fill these gaps. In the interim, human risk assessment could work on the basis of the rebuttable hypothesis that DA is applicable, but should rapidly modify this practice as soon as evidence to the contrary becomes available. This is in line with recent suggestions from an IPCS workshop (IPCS 2009). In proposing a framework for consideration of risk from exposure to multiple chemicals, the workshop report recommends adopting DA during the lowest assessment tier, if there is no evidence for synergisms or antagonisms.

7.3 Criteria for the grouping of chemicals to be subjected to mixtures risk assessment

Strongly connected to the issue of making choices about evaluation concepts for mixture effects is the question which chemicals should be grouped together for purposes of combined risk assessment, and which criteria should used to decide on groupings.

"Toxicological similarity" of chemicals is the criterion for grouping proposed by U.S. EPA (2000) and other international bodies (e.g. IPCS 2009). Toxicological similarity is thought to be fulfilled with substances showing similar chemical structures, similarity of target tissue and/or similarity in the manifestation of toxicity. While these are reasonable ideas, it is proving extraordinarily difficult to define workable criteria for all chemicals in a general way. There is always a danger that inappropriately narrow criteria with a detailed focus on mechanistic considerations might exclude chemicals that also produce effects related to the toxicity in question.

Organophosphate pesticdes and carbamates are examples to illustrate these difficulties. Both types of chemicals inhibit acetylcholinesterase, and this is shown to be a relevant step in the manifestation of toxicity. Because the mechanism of inhibition by carbamates is via carbamylation, and that of organophosphates by phosphorylation, and because this is judged to represent different molecular mechanisms, U.S.EPA does not assess the two types of pesticides together, but includes them in separate groupings for the purpose of mixtures risk assessment (see also Part 4). Such narrow groupings ignore that joint effects can also occur from combined exposures with other than common mechanisms.

An overly strong focus on mechanisms of toxicity may also lead into difficulties when it is applied as a grouping criterion for endocrine disrupters. A recent report by the National Research Council (NRC) of the US National Academy of Sciences has discussed this for antiandrogens, including phthalates. The NRC advised that a cumulative risk assessment should not only consider certain phthalates, but also other chemicals that could potentially cause the same health effects as phthalates (NRC 2008). It was recommended that phthalates and other chemicals that affect male reproductive development in animals, including antiandrogens, be considered in the cumulative risk assessment. Solely mechanism-based criteria may lead into a dilemma: Because there are subtle differences in the precise molecular details by which phthalates can act as endocrine disrupters, not even all antiandrogenic phthalates would be grouped together when mechanistic considerations are the sole criterion.

The NRC therefore recommended a broader based move towards establishing grouping criteria for phthalates and other antiandrogens. With this type of endocrine disrupter, a case can be made for adopting a physiological approach to analyzing toxic mechanisms of action with respect to similarity or dissimilarity. If it is recognized that the driver of male sexual differentiation during development is the effect of androgen action, it is irrelevant whether the hormones' effects are disrupted by interference with steroid synthesis, by antagonism of the androgen receptor, or by some other mechanism (for example, affecting consequences of androgen receptor activation). The resulting

biological effects with all their consequences for male sexual differentiation are similar, although the molecular details of toxic mechanisms - including metabolism, distribution and elimination - differ profoundly in many respects. Judged from such a perspective, a focus on phthalates to the exclusion of other antiandrogens not only would be artificial and lack credibility, but could imply serious underestimation of cumulative risks posed by agents for which there is simultaneous exposure

These recommendations strengthen a more holistic perspective on grouping, away from considerations which begin with toxicological mechanisms. Fortunately, the knowledge about the mechanisms that underlie the disruption of androgen action during development and its consequences for adult life are sufficiently advanced to take such an approach. However, in many other areas of relevance to mixtures risk assessment, this is currently not possible.

7.4 Mixtures risk assessment methods

The application of mixtures risk assessment methods requires clarity about the goal of the assessment. The aim can be to arrive at a risk estimate, an estimation of safe levels, of margins of exposure, or can consist in ways to prioritize certain mixtures. Estimations of safe levels or margins of exposure may be based on worst-case-assumptions, but the prioritization of mixtures (or affected sites) has to rely on fairly accurate quantitations of risk.

Considering that the empirical evidence on mixture effects showed that CA (DA) is a useful concept for the approximation of combination effects, component-based methods derived from CA suggest themselves as risk assessment approaches. These include the Hazard Index (HI), Toxic Unit Summation (TUS), Point of Departure Index (PODI), Rleative Potency Factors and the TEQ concept.

7.4.1 Hazard Index

The Hazard Index (HI) (Teuschler and Hertzberg 1995) is a regulatory approach to component-based mixture risk assessment which is based on the concept of CA and which can be generally defined by the formula

$$HI = \sum_{i=1}^{n} \frac{EL_i}{AL_i}$$

where *EL* is the exposure level, AL is the acceptable level, and *n* is the number of chemicals in the mixture. Various measures for exposure levels and expectable levels may be applied; the only constraint is that EL and AL must be expressed in the same unit. If HI > 1, the total concentration (or dose) of mixture components exceeds the level considered to be acceptable. The method offers flexibility in applying different UFs when defining AL for the individual substances.

7.4.2 Toxic Unit Summation

The method of Toxic Unit Summation (TUS) (Sprague 1970) is a direct application of the CA concept and defined by the formula

$$TUS = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i}$$

where c_i are the actual concentrations (or doses) of the individual substances in a mixture and ECx_i denote equi-effective concentrations (or doses) of these substances if present singly (e.g. $EC50_i$). The quotients c_i / ECx_i are termed Toxic Units (TU). Toxic Units rescale absolute concentrations (or doses) of substances to their different individual toxic potencies. They express the concentrations (or doses) of mixture components as fractions of equi-effective individual concentrations (or doses) ECx_i. Typically, $x = 50$ % ($EC50_i$) is chosen as the reference level, but TUS can also be calculated for any other effect level x. If TUS = 1, the mixture is expected to elicit the total effect x. If the sum of Toxic Units is smaller or larger than 1, the mixture is expected to elicit effects smaller or larger than x, respectively.

7.4.3 Point of Departure Index

The Point of Departure Index (PODI) is an approach to component-based mixture risk assessment which is similar to the HI and TUS. In contrast to the HI, however, exposure levels (EL) of chemicals in a mixture are not expressed as fractions of individually acceptable levels (AL) but as fractions of their respective points of departure (PODs) such as NOAELs or Benchmark concentrations or doses (BML) In this way, different uncertainty factors that may be included in AL values (see HI) are removed from the calculation (Wilkinson et al 2000):

$$PODI = \sum_{i=1}^{n} \frac{EL_i}{POD_i}$$

A PODI can be used to estimate margins of exposure for the mixture of interest.

7.4.4 Relative Potency Factors

The Relative Potency Factor (RPF) approach is a practical regulatory application of the CA concept for mixtures of chemical substances that are assumed to be toxicologically similar (U.S.EPA 2000). The concentrations (or doses) of mixture components are scaled relatively to the concentration (or dose) of an index compound, and then summed up. The scaling factor is called RPF. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of the index compound. In general, the mixture concentration C_m expressed in terms of the index compound for n compounds is

$$C_m = \sum_{i=1}^{n} (c_i * RPF_i)$$

where c_i is the concentration of the i^{th} mixture component, and $RPF_1 = 1$, as $i = 1$ indicates the index chemical.

7.4.5 Toxic Equivalency Factors

The Toxic Equivalence Factor (TEF) is a specific type of RPF formed through a scientific consensus procedure (EPA 2000). Based on the assumptions of a similar mechanism of action of structurally related chemicals and parallel concentration (or dose) response curves, they were first developed for dioxins. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of an index compound. The total equivalent quantity *TEQ* is estimated by summation of the concentrations (or doses) of mixture components c_i multiplied by the respective TEF_i:

$$TEQ = \sum_{i=1}^{n} (c_i * TEF_i)$$

All these methods require dose-response information of mixture components as input values. The HI sums up ratios of exposure levels and reference doses over chemicals. The reference doses can be arrived at by utilizing different UF for each mixture component. If this is perceived to be a problem, the PODI method can be used. PODI is based not on reference doses, but on points of departure (NOAELs, benchmark doses). Extrapolation issues (e.g. animal to human) are then dealt with by using one overall UF. Finally, the TEQ concept is predicated on the choice of a reference chemical and requires parallel dose-response curves for all components. Both these requirements are often not met by endocrine disrupters, but the method has been validated for dioxin-like endocrine disrupters.

7.5 Assessment frameworks and tiering

Several ways of dealing with mixtures in chemicals risk assessment have recently been proposed and discussed (NRC 2008, IPCS 2009, Kortenkamp and Hass 2009). Depending on the quality of the data that are available for mixtures risk assessment (data poor or data rich), tiering methods might be very productive to explore the problem, and utilize more sophisticated models and associated supporting data when needed.

Risk assessment may begin with the question whether combined exposures are in fact likely (IPCS 2009), and at the lowest tier (tier 0), it may become apparent that the situation to be evaluated does in fact not present an issue for mixtures risk assessment.

In the next higher tier (tier 1), termed “simple generic” (Kortenkamp and Hass 2009), data about mixed exposures may not be present, but it may be deemed desirable to safeguard against the possibility of joint effects by adopting a specific mixtures assessment factor, as discussed in 7.1.

In tier 2, “moderately simple generic”, sufficient data may be available to warrant the assumption of CA (DA) throughout, in which case risk assessment methods that derive from this concept could be applied (see 7.4.), even though IA may produce less conservative estimates.

In more data rich situations (tier 3, "complex specific") sufficient information about various modes of action may be available, such that mixed mixtures assessment models (DA within groups of compounds perceived to follow simple similar action, followed by IA across groups) can be applied.

Finally, in the highest tier 4 ("highly specific") it would be possible to address both issues of modes of action and differences in the vulnerability of various species or risk receptors.

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8. Knowledge gaps and research needs

8.1 Mixture toxicology with focus on mammalian and human toxicology

The review of relevant studies in Section 4 has shown that there is a deficit of well conducted mixture studies in the areas of carcinogenicity, mutagenicity, genotoxicity, respiratory toxicity, immunotoxicity and neurotoxicity. Many of the published studies in these areas have adopted experimental approaches that do not permit to establish the type of combination effect. Furthermore, evidence from multi-component mixture studies is underrepresented in these areas.

The choice of chemicals for mixture studies was often driven by needs to suit certain experimental conditions. This has led to a trend of mixture studies with chemicals that induce similar effects, often well predicted by dose addition. There is a dearth of studies where arguably the requirements of independent action are met. Furthermore, relatively little information exists about the ability of chemicals that themselves do not produce the effect under investigation to modulate the toxicity of other mixture components. Studies in these areas should be encouraged.

Information about relevant exposure scenarios, in terms of the nature of active chemicals, and their number, is fragmentary for most human exposure scenarios. Exposure assessment strategies that adopt a more holistic approach, instead of focusing on individual chemicals, are needed to overcome this situation.

8.2 Mixture toxicology with focus on ecotoxicology

Concentration Addition and Independent Action have been conceptually developed and validated for chemical mixtures. Although several recent studies were published that employed these concepts also for describing the joint action of chemical and physical stressors, such as oxygen depletion or drought, the conceptual basis and implications of such studies are far from clear.

Mixtures in the environment are usually composed of multiple components from a range of sources with dissimilar chemical structures and modes of action. Unfortunately, this is exactly the type of mixture that has been least frequently studied. Hence, more empirical evidence on the joint action of environmentally realistic mixtures, composed of members from different chemical and functional classes are needed in order to further challenge the above statement that concentration addition might be applicable as a general “rule of thumb” for describing the joint action chemical mixtures and to explore its limitations.
In this context, it would be especially valuable to get further insight into the question on whether low, individually non-toxic concentrations of dissimilar compounds might lead to a significant mixture effect. This question is of major importance, because of its direct relevance for the question of environmental quality standards. However, only two studies, both from of aquatic toxicology and both using unicellular organisms and specifically designed “artifial” mixtures are documented in the literature.

Organisms are not only exposed to mixtures of chemicals simultaneously, but also sequentially to pulses of contaminants that enter an ecosystem e.g. after run-off events or pesticide application. Although the first approaches addressing this point by developing the necessary conceptual framework have been published, this work is in its infancy.

State of the Art Report on Mixture Toxicity

Final Report

Part 2: Identification and description of current provisions for taking into account hazards and risks arising from mixture toxicity in 21 pieces of EU legislation

22 December 2009

Study Contract Number	**070307/2007/485103/ETU/D.1**
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Responsible Scientists	**Prof. Dr. Andreas Kortenkamp (ULSOP)** **Assoc.-Prof. Dr. Thomas Backhaus (UGOT)** **Dr. Michael Faust (FBEC)**

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1. Terms of reference, approach, and structure of the report

The objective of this part of the study was to conduct an analysis of EU risk assessment regimes, with the specific aim of assessing whether EU risk assessment regimes in 21 different EU directives and regulations take into account risks arising from mixture toxicity and if, in which way. The analysis was to result in a summary of relevant provisions, an identification of gaps, and recommendations for future improvements.

The 21 pieces of EU regulation were not selected as part of the study but they were already predefined in the Tender Specifications.

For the purpose of this analysis of existing EU regulations, the term *risk assessment* was understood in a broad and general regulatory sense, not strictly confined to current scientific concepts of risk assessment, but in particular also including *hazard*-based approaches of chemicals safety regulations. The term *mixture toxicity* was understood as unwanted adverse effects of mixtures of chemicals. Interactions of chemical factors with physical and/or biological stressors in the environment were beyond the scope of this task. The same applies to wanted beneficial effects of mixtures, such as therapeutic effects of drug combinations for instance.

For each of the 21 pieces of legislation at issue, a brief descriptive analysis was prepared. These are compiled in section 2. As a general common structure the following points are addressed in these summaries of individual legal acts, if appropriate:

- *Purpose:* – What is the general scope of the legislation? To which kind of chemicals or uses of chemicals does it apply? What are the principles established by the regulation with respect to chemicals safety?
- *Regulatory context:* – Are there any supplementary, complementary, or overarching pieces of legislation that have to be taken into account in assessing the legislations relevance for mixture toxicity assessments?
- *Regulatory status / Current revision:* – Has the act been replaced, or will it be a replaced by new pieces of legislation, or is there any Commission proposal for a revision that might be relevant in the context of mixture toxicity assessments?
- *Protection goals:* – What are the specific protection goals that shall be achieved by the legislation with respect to chemicals safety (human health, animal health, environment, etc.)?
- *Hazard and risk assessment:* – What are the principal approaches, measures, instruments or procedures for hazard and/or risk identification, assessment or reduction of chemicals that are established in the particular piece of legislation?
- *Mixture toxicity:* – Are hazards and risks arising from mixture toxicity addressed in the legislation in any way, and if so in which way?
- *Conclusion:* – Is the legal act relevant for assessing mixture toxicity?

Relevant provisions are summarized in section 3 and resulting recommendations are given in section 4.

2. Analysis of 21 pieces of EU legislation

2.1 Food additives authorisation - Directive 89/107/EEC

Act

Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption - OJ L 40, 11.2.1989, p. 27-33, as last amended by Regulation (EC) No 1882/2003 of the European Parliament and of the Council - OJ L 284, 31.10.2003, p. 1–53.

Purpose

Directive 89/107/EEC *on the approximation of laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption* came into force in 1990. In this way, all authorised food additives and their conditions of use became harmonised at European level. Only those substances included in lists adopted by the Council may be used as food additives and only under the conditions of use specified therein (Article 2, paragraph 1, in conjunction with Article 3, paragraph 2 a)). Substances shall be included in lists of approved food additives on the basis of authorisation criteria laid down in Annex II to the Directive (Article 2, paragraph 3): The use of food additives must by justified by a *reasonable technological need*, must *present no hazard to the health of the consumer*, and must *not mislead the consumer* (Annex II, paragraph 1).

Regulatory context

The Food Additives Directive 89/107/EEC defines a general scheme for authorized food additives. There is a series of specific Directives arising from this framework directive. Specific provisions for authorized colourants, sweeteners, and other additives and the conditions of their use, are laid down in the supplementary Directives 94/36/EC[1], 94/35/EC[2], and 95/2/EC[3], respectively. Furthermore, corresponding purity criteria are laid down in Directives 95/45/EC[4], 95/31/EC[5], and 96/77/EC[6].

[1] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs
[2] European Parliament and Council Directive 94/35/EC of 30 June 1994 on sweeteners for use in foodstuffs
[3] European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners
[4] Commission Directive 95/45/EC of 26 July 1995 laying down specific purity criteria concerning colours for use in foodstuffs
[5] Commission Directive 95/31/EC of 5 July 1995 laying down specific criteria of purity concerning sweeteners for use in foodstuffs
[6] Commission Directive 96/77/EC of 2 December 1996 laying down specific purity criteria on food additives other than colours and sweeteners

Since 2002, *general principles and requirements of food law* are laid down in the overarching Regulation (EC) No 178/2002[7], including general food safety requirements that apply since January 2005.

Regulatory status

Directive 89/107/EEC will be repealed by Regulation (EC) No 1333/2008[8]. from 20 January 2010. The new Regulation 1333/2008 on *food additives*, which entered into force on the 20 January 2009, will repeal all the above listed provisions in force concerning food additives and replace it by a single act. Three new parallel Regulations shall establish complementary rules for *food enzymes* (Regulation (EC) No 1332/2008)[9] and *food flavourings* (Regulation (EC) No 1334/2008)[10] and a *common authorisation procedure for food additives, food enzymes and food flavourings* ((Regulation (EC) No 1331/2008)[11].

Protection goals

The legislation on food additives shall protect human health and consumer interests. The "*environmental impact is not among the general conditions for authorising food additives*". This applies to the existing legislation and has also been explicitly confirmed in the Explanatory Memorandum to the amended proposal for the future Regulation presented by the Commission during the legislative procedure (COM(2007) 673 final, p. 5, first paragraph)[12].

However, the Commission acknowledged that the environmental impact "*is of course a legitimate factor to be considered. For instance when adverse environmental effects are identified, these can be taken into account during the authorisation…*" (COM(2007) 673 final, p. 5, first paragraph). As a consequence, the environmental aspect is mentioned three times in the new Regulation 1333/2008: Recital 7 states that the "*approval of food additives should also take into account (…) environmental factors* …", and Article 1 says that the "*Regulation lays down rules (…) with a view to ensuring the effective functioning of the internal market whilst ensuring a high level of protection of human health and a high level of consumer protection, (…), taking into account, where appropriate, the protection of the environment*". Accordingly,

[7] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

[8] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives - OJ L 354, 31.12.2008, p. 16–33

[9] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97 (Text with EEA relevance) - OJ L 354, 31.12.2008, p. 7–15

[10] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC (Text with EEA relevance) - OJ L 354, 31.12.2008, p. 34–50

[11] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings (Text with EEA relevance) - OJ L 354, 31.12.2008, p. 1–6

[12] COM/2007/673 final - Amended proposal for a Regulation of the European Parliament and of the Council on food additives (presented by the Commission pursuant to Article 250 (2) of the EC Treaty)

Article 6 specifies that food additives must meet the three general conditions (consumer safety, technological need, and no misleading of the consumer) *"and, where relevant, other legitimate factors, including environmental factors*".

Hazard and risk assessment

According to Annex II, paragraph 1 of the Food Additives Directive 89/107/EEC currently in force, "*food additives can be approved only provided that (…) they present no hazard to the health of the consumer at the level of use proposed, so far as can be judged on the scientific evidence available*". In the new Regulation 1333/2008 this condition remains basically unchanged, but the wording "*present no hazard to the health of the consumer"* has been modified into "*not (…) pose a safety concern to the health of the consumer*" (Article 6, paragraph 1 (a)).

With the commencement of the general European food law (Regulation (EC) No 178/2002), the European Food Safety Authority (EFSA) became responsible for carrying out corresponding safety evaluations. Formerly, the risk assessments were performed by the Scientific Committee for Food (SCF).

Established procedures for safety assessments of Food Additives are based on Acceptable Daily Intake values (ADI). Permitted use levels are considered to be safe when the resulting intake of additives does not exceed the corresponding ADI values.

Where necessary, maximum use levels are currently fixed in annexes to the specific supplementary legislation for colourants, sweeteners, and other additives (Directives 94/36/EC, 94/35/EC, and 95/2/EC). Under the new Regulation they will be re-examined and compiled in a common set of Annexes II and III that should be completed by 1 January 2011. During the transitional period the Annexes of Directives 94/35/EC, 94/36/EC and 95/2/EC will remain in force.

Mixture toxicity

Annex II of the Food Additives Directive 89/107/EEC currently in force provides a basis for mixture toxicity assessments. Paragraph 3 states:
To assess the possible harmful effects of a food additive or derivatives thereof, it must be subjected to appropriate toxicological testing and evaluation. The evaluation should also take into account, for example, any cumulative, synergistic or potentiating effect of its use,(…).
However, in practice this "should"-requirement might not play a significant role. Established procedures for safety assessments of food additives on the basis of ADI values for single substances do not specifically consider joint actions or interactions between additives and food consumption[13].

In the new Regulation 1333/2008 the terms *cumulative, synergistic or potentiating* do not turn up again and no other indication on the need for performing mixture toxicity assessments is included.

[13] see Groten et al. 2000. An analysis of the possibility for health implications of joint actions and interactions between food additives. Regulatory Toxicology and Pharmacology 31, 77-91.

Since 2005, overarching food safety requirements defined in Article 14 of the general European food law (Regulation (EC) No 178/2002) apply. Paragraph 4 of this Article states: "*In determining whether any food is injurious to health, regard shall be had (…) to the probable cumulative toxic effects*".

The term "*cumulative toxic effects*" is not defined in the Regulation 178/2002. In the toxicological literature it is used with different meanings. In a narrow sense it may mean toxic effects that result from repeated exposure to one and the same toxicant from the same, similar or different sources via the same or via different routes of exposure. In a wide sense it may describe toxic effects resulting from simultaneous or sequential exposure to different toxicants and thus be used as a synonym for *mixture toxicity*. In the context of general food law the term has often been used in the narrow sense of repeated doses. In the British Food Safety Act 1990 for instance, which was replaced by the European Regulation (EC) No 178/2002 cited above, the corresponding formulation was: "*In determining whether any food is injurious to health, regard shall be head (…) to the probable cumulative effect of the food of substantially the same composition on the health of a person consuming it in ordinary quantities*"[14]

Thus, without any further indications or specifications, the term "*cumulative toxic effects*" cannot be interpreted as a legal requirement for mixture toxicity assessments. Such additional indications are clearly given in case of the specific legislation on pesticide residues in food (see section 2.5), but not in case of provisions for food additives.

The new Regulation 1333/2008 on food additives provides for maximum use levels of additives that take into account "*any acceptable daily intake, or equivalent assessment, established for the food additive and the probable daily intake of it from all sources*" (Article 11, paragraph 1 (b) (i)). This formulation of the requirement avoids the term *cumulative* and all confusion that may arise from it.

Conclusion

The current European Directive 89/107/EEC on food additives indicates that aspects of mixture toxicity should be considered in scientific safety assessments. The new Regulation (EC) 1333/2008 does neither exclude a need for mixture toxicity assessments nor does it explicitly define such a need. The use of food additives must be safe for the consumer *so far as can be judged on the scientific evidence available* (Dir 89/107/EEC, Annex II, paragraph 1). The legislation builds on the assumption that *acceptable daily intakes* can be scientifically determined, but it does not prescribe how scientific risk assessments should be performed. This task is left to EFSA.

[14] cited after Penniston 2005. Changes to food hygiene legislation. Rushcliffe Borough Council. Available at http://www.rushcliffe.gov.uk/upload/public/attachments/216/foodlegislationchanges.pdf

2.2 Food contact materials - Regulation (EC) No 1935/2004

Act

Regulation (EC) No 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC - OJ L 338, 13.11.2004, p. 4–17, as amended by Regulation (EC) No 596/2009 of the European Parliament and of the Council - OJ L 188, 18.7.2009, p. 14–92.

Purpose

The purpose of Regulation (EC) No 1935/2004 *on materials and articles intended to come into contact with food* is "*to ensure functioning of the internal market*" for such materials "*whilst providing the basis for securing a high level of protection of human health and the interest of consumers*" (Article1, paragraph 1). The regulation defines general requirements for food contact materials (Article 3), specific requirements for "active" and "intelligent" food contact materials (Article 4) and it sets the frame for specific measures that may be adopted or amended for specific groups of materials or articles (Article 5). The regulation stipulates that positive lists of authorised substances may be established when drawing up specific provisions for groups of food contact materials.

Regulatory context

Regulation (EC) No 1935/2004 on food contact materials repealed and replaced Directive 89/109/EEC *on the approximation of the laws of the Member States relating to materials and articles intended to come into contact with foodstuffs*. The new legislation incorporated provisions for new materials resulting from technological progress, so called "active" and "intelligent" packaging, that were out of the scope of the previous legislation. Additionally, by means of a Regulation all provisions became directly binding and applicable in all Member States, no longer requiring transposition into national law.

The Regulation provides a framework for more specific measures. It is complemented by a list of specific supplementary acts. These define specific rules for specific food contact materials such as regenerated cellulose film (Directive 2007/42/EC)[15], ceramics (Directive 84/500/EEC)[16], plastic materials (Directive 2002/72/EC)[17], vinyl chloride (Directives 78/142/EEC[18], 80/766/EEC[19], and 81/432/EEC[20]), plasticisers in

[15] Commission Directive 2007/42/EC of 29 June 2007 relating to materials and articles made of regenerated cellulose film intended to come into contact with foodstuffs (Codified version)
[16] Council Directive 84/500/EEC of 15 October 1984 on the approximation of the laws of the Member States relating to ceramic articles intended to come into contact with foodstuffs
[17] Commission Directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs
[18] Council Directive 78/142/EEC of 30 January 1978 on the approximation of the laws of the Member States relating to materials and articles which contain vinyl chloride monomer and are intended to come into contact with foodstuffs
[19] Commission Directive 80/766/EEC of 8 July 1980 laying down the Community method of analysis for the official control of the vinyl chloride monomer level in materials and articles which are intended to come into contact with foodstuffs

gaskets in lids (Regulation (EC) No 372/2007)[21], epoxy derivatives (Regulation (EC) No 1895/2005)[22] and nitrosamines from rubber (Directive 93/11/EEC)[23] as well as good manufacturing practice (Regulation (EC) No 2023/2006)[24] and migration testing (Directives 82/711/EEC[25], and 85/572/EEC[26]).

The Regulation complements the general principles and requirements of food law laid down in the overarching Regulation (EC) No 178/2002[27].

Protection goals

The Regulation is focussed on the protection of human health; environmental risks are out of scope.

Hazard and risk assessment

As a general requirement, food contact materials and articles "*shall be manufactured (…) so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: (a) endanger human health; or (b) bring about an unacceptable change in the composition of the food; or (c) bring about a deterioration in the organoleptic characteristics thereof*" (Article 3, paragraph 1). It is the task of the European Food Safety Authority (EFSA) to assess compliance of substances with these safety criteria (Articles 7 and 10).

Mixture toxicity

Neither the recitals nor the provisions of this Regulation do explicitly or implicitly address the topic of mixture toxicity.

The general requirement to consider *probable cumulative toxic effects* in food safety assessments as laid down in the general European food law (Regulation (EC) No 178/2002) may apply. However, for the reasons explained in section 2.1 on food additives legislation, this cannot be interpreted as a mandatory requirement for mixture toxicity assessments.

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[20] Commission Directive 81/432/EEC of 29 April 1981 laying down the Community method of analysis for the official control of vinyl chloride released by materials and articles into foodstuffs

[21] Commission Regulation (EC) No 372/2007 of 2 April 2007 laying down transitional migration limits for plasticisers in gaskets in lids intended to come into contact with foods

[22] Commission Regulation (EC) No 1895/2005 of 18 November 2005 on the restriction of use of certain epoxy derivatives in materials and articles intended to come into contact with food

[23] Commission Directive 93/11/EEC of 15 March 1993 concerning the release of the N-nitrosamines and N-nitrosatable substances from elastomer or rubber teats and soothers

[24] Commission Regulation (EC) No 2023/2006 of 22 December 2006 on good manufacturing practice for materials and articles intended to come into contact with food

[25] Council Directive 82/711/EEC of 18 October 1982 laying down the basic rules necessary for testing migration of the constituents of plastic materials and articles intended to come into contact with foodstuffs

[26] Council Directive 85/572/EEC of 19 December 1985 laying down the list of simulants to be used for testing migration of constituents of plastic materials and articles intended to come into contact with foodstuffs

[27] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

Conclusion

The Regulation of food contact materials does not include provisions for taking into account hazards and risks arising from mixture toxicity. The Regulation sets the requirement that food contact materials shall not *endanger human health*, but the legislation does not prescribe how corresponding scientific risk assessments should be performed. This task is left to EFSA.

2.3 Feed additives authorisation - Regulation (EC) No 1831/2003

Act

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition - OJ L 268, 18.10.2003, p. 29–43, as last amended by Commission Regulation (EC) No 767/2009 - OJ L 229, 1.9.2009, p. 1–28.

Purpose

The purpose of Regulation (EC) No 1831/2003 *on additives for use in animal nutrition* is "*to establish a Community procedure for authorising the placing on the market and use of feed additives and to lay down rules for the supervision and labelling of feed additives and premixtures …*" (Article1, paragraph 1). To be legally placed on the market and used, feed additives must undergo a harmonised scientific safety assessment by the European Food Safety Authority (EFSA) (Recitals 4 and 14) and they must be authorised by the Community (Articles 3 and 9).

Regulatory context

The feed additives Regulation (EC) No 1831/2003 entered into force in 2003 and became applicable in 2004. The Regulation repealed and replaced the formerly applicable Directives 70/524/EEC[28] and 87/153/EEC[29] and corresponding amendments. In this way, the competence for authorising feed additives was conferred on the Commission and all rules became directly applicable in all Member States.

Detailed new rules concerning the preparation and presentation of applications and the assessment and the authorisation of feed additives have been laid down in the complementary Commission Regulation (EC) No 429/2008[30]. For applications submitted before 21 June 2008, the old guidelines that are laid down in the Annex to the repealed directive 87/153/EEC as amended by Directive 2001/79[31] (see section 2.4) continue to apply.

The Regulation complements the general principles and requirements of food law laid down in the overarching Regulation (EC) No 178/2002[32].

[28] Council Directive 70/524/EEC of 23 November 1970 concerning additives in feeding-stuffs
[29] Council Directive 87/153/EEC of 16 February 1987 fixing guidelines for the assessment of additives in animal nutrition
[30] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives - OJ L 133, 22.5.2008
[31] Commission Directive 2001/79/EC of 17 September 2001 amending Council Directive 87/153/EEC fixing guidelines for the assessment of additives in animal nutrition
[32] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

Protection goals

In contrast to some other pieces of European food safety legislation, this Regulation is not confined to the human health aspect, but the environment is explicitly included in the protection goals: The procedures and rules established by this Regulation shall "*… provide the basis for the assurance of a high level of protection of human health, animal health and welfare, environment and users' and consumers' interests in relation to feed additives, whilst ensuring the effective functioning of the internal market* (Article1, paragraph 1).

Hazard and risk assessment

Feed additives are authorised only if the applicant can demonstrate that the following requirements that are fixed in Article 5 are satisfied:

- the additive does "*not have an adverse effect on animal health, human health or the environment*",
- the additive is "*not presented in a manner which may mislead the user*",
- the additive does "*not harm the consumer by impairing the distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal products*", and
- the additive has a favourable effect on the characteristics of the feed to which it is added or on animal production.

It is the task of EFSA to "*undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5*" (Article 8, paragraph 3 (a)). In the event of an opinion in favour of authorising the feed additive, EFSA shall *inter alia* provide a proposal for the establishment of corresponding Maximum Residues Limits (MRLs) in the relevant foodstuffs (Article 8, paragraph 4 (e)). The methods and procedures to be used for these assessments are not further specified in the Regulation. It is EFSAs' general task to provide "*the best possible scientific opinions*" and to develop "*uniform risk assessment methodologies in the field falling within its mission*" (Regulation (EC) No 178/2002, Article 23 (a) and (b)).

Mixture toxicity

The feed additives Regulation also covers mixtures of additives sold to the end-user (Recital 9 and Article 1 in conjunction with Article 2), whereby "*the marketing and use of those mixtures should comply with the conditions laid down in the authorisation of each single additive*" (Recital 9).

The topic of mixture toxicity is not addressed in the Regulation, neither in the recitals nor in the provisions, neither explicitly nor implicitly.

The general requirement to consider *probable cumulative toxic effects* in food safety assessments as laid down in the general European food law (Regulation (EC) No 178/2002) may apply. However, for the reasons explained in section 2.1 on food additives legislation, this cannot be interpreted as a mandatory requirement for mixture toxicity assessments.

Conclusion

The Regulation of feed additives does not include provisions for taking into account hazards and risks arising from mixture toxicity. The Regulation sets the requirement that feed additives must *not have an adverse effect on animal health, human health or the environment*, but the legislation does not prescribe how corresponding scientific safety assessments should be performed. This task is left to EFSA.

This conclusion is reached by only checking the Regulation itself. The corresponding old *guidelines for the assessment of additives in animal nutrition* (Directive 87/153/EEC as amended by Directive 2001/79/EC) and the new implementation rules laid down in the complementary Commission Regulation (EC) No 429/2008 are separately considered in the following section 2.4.

2.4 Feed additives assessment - Directive 2001/79/EC and Regulation (EC) No 429/2008

Acts

(i) Commission Directive 2001/79/EC of 17 September 2001 amending Council Directive 87/153/EEC fixing guidelines for the assessment of additives in animal nutrition - OJ L 267, 6.10.2001, p. 1–26. (The Directive is no longer in force, but some provisions remain temporarily applicable.)

(ii) Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives (Text with EEA relevance) - OJ L 133, 22.5.2008, p. 1–65.

Purpose

The Annex *fixing guidelines for the assessment of additives in animal nutrition*, amended by Directive 2001/79 to the old Directive 87/153/EEC[33] was addressed to applicants for authorisation of a food additive. The document was "*intended as a guideline for establishing dossiers on substances and preparations being submitted for authorisation as additives in feedingstuffs or a new usage of an authorised additive. (…) The dossiers must enable an assessment to be made of the additives based on the present stage of knowledge and make it possible to ensure their compliance with the fundamental principles laid down for their authorisation*" (Annex, Part I, General Aspects, first paragraph).

Regulation (EC) No 429/2008 replaced the old Annex to Directive 87/153/EEC. The purpose remained essentially identical (see Regulation (EC) No 429/2008, Recital 2 and Annex II, *General Aspects*, 2nd paragraph).

Regulatory history, status, and context

Commission Directive 2001/79/EC of 17 September 2001 amended Council Directive 87/153/EEC of February 1987 *in the light of advances in scientific and technical knowledge* (Recital 1). In 2003, Council Directive 87/153/EEC was repealed and replaced by the new Regulation (EC) 1831/2003[34] on feed additives (see section 2.3).

The new Regulation (EC) 1831/2003 stipulated that the Commission should establish new detailed rules concerning the preparation and presentation of applications for the authorisation of feed additives (Article 7, paragraph 4). Pending the adoption and implementation of such new rules, the existing guidelines that were laid down in the Annex to the repealed Directive 87/153/EEC as amended by Directive 2001/79 remained temporarily in force (Article 7, paragraph 4, in conjunction with Article 23, paragraph 3; see also Recital 34).

[33] Council Directive 87/153/EEC of 16 February 1987 fixing guidelines for the assessment of additives in animal nutrition

[34] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition

In 2008, Commission Regulation (EC) No 429/2008 established the required new detailed rules for *the preparation and the presentation of applications and the assessment and the authorisation of feed additives*. The Regulation came into force on 21 June 2008. For applications submitted before that date, the old rules laid down in the Annex to Directive 87/153/EEC as amended by Directive 2001/79/EC continue to apply (Regulation (EC) No 429/2008, Article 4, paragraph 1). Furthermore, *for applications for authorization submitted before 11 June 2009 applicants may chose the continued application of* some sections of the old Annex to Directive 87/153/EEC as specified in Article 4, paragraph 2 of the new Regulation 429/2008.

The old guidelines for applicants, given in the Annex to Directive 87/153/EEC, were established on the basis of the *Report of the Scientific Committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition* (adopted on 22 October 1999) (Directive 2001/79/EC, Recital 12). By Regulation (EC) No 178/2002[35] on general food law, this Committee was dissolved and its tasks were assigned to the newly founded European Food Safety Authority (EFSA). The new rules laid down in Regulation (EC) 429/2008 were established after consulting EFSA, in accordance with Article 7, paragraphs 4 and 5 of Regulation (EC) 1831/2003.

Protection goals

The old guidelines in the Annex to Directive 87/153/EEC lists the physico-chemical, toxicological, and eco-toxicological data and studies that may be required compulsively or occasionally from an applicant in order to support safety assessments in relation to target species, workplace exposure to feed additives, consumers ingesting residues of feed additives or their metabolites, the environment and other special aspects.

The new rules laid down in the Annexes to Regulation (EC) No 429/2008 are basically a revised and extended version of the old guideline, amended and re-structured in the light of advanced scientific and technological knowledge, practical experiences, and complementary regulatory requirements, such as the reduction of animal testing to a necessary minimum, for instance.

Hazard and risk assessment

Guidance is given for the derivation of proposals for standard key criteria of toxicological and eco-toxicological assessments from the test results, in particular NOAEL, ADI, MRL, and PEC/PNEC values. This applies to both the old guidelines (Annex to Directive 87/153/EEC as amended by Directive 2001/79/EC) and the new rules (Annexes to Regulation (EC) No 429/2008).

Mixture toxicity

With respect to toxicological and eco-toxicological assessments, the old guidelines (Annex to Directive 87/153/EEC) entirely referred to the conventional testing and

[35] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety

assessing of single active substances and/or formulations thereof. The assessment of mixture toxicity on the basis of such single substance data or the performance of whole mixture toxicity tests were completely out of the scope of that guideline.

In contrast, the new Regulation (EC) No 429/2008 explicitly addresses hazards and risks that may arise from mixture toxicity, if feed additives placed on the market contain more than one (active) ingredient. In Annex II, section *General Aspects*, sub-section *Safety assessment*, the last paragraph establishes the following requirement:

Where an additive has multiple components, each one may be separately assessed for consumer safety and then consideration given to the cumulative effect (where it can be shown that there are no interactions between the components). Alternatively, the complete mixture shall be assessed.

No further guidance on the practical performance of *cumulative effect* assessments is included in the document.

Conclusions

The old *guidelines for the assessment of additives in animal nutrition* did not take into account hazards and risks arising from mixture toxicity.

The new rules established by Regulation (EC) No 429/2008 pay attention to the possible consequences for consumer safety that may result from feed additives with multiple components.

2.5 Pesticide residues - Regulation (EC) 396/2005

Act

Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC - OJ L 70, 16.3.2005, p. 1–16, as last amended by Commission Regulation (EC) No 822/2009 - OJ L 239, 10.9.2009, p. 5–45.

Purpose

Regulation (EC) No 396/2005 *on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin* establishes MRLs for all pesticides and all food and feed products. For products and/or pesticides for which no specific MRLs are set, a default value of 0.01 mg/kg applies (Article 18, paragraph 1(b)). MRLs established in the Regulation are directly applicable and enforceable in the Member States.

Regulatory context

The MRL Regulation (EC) No 396/2005 repeals and replaces a fragmented system of four existing Directives on pesticide residues (Recitals 1-3, and Article 48)[36] and corresponding national legislation that allowed the Member States to set their own national MRLs in the absence of Community-wide MRLs. The Regulation entered into force in March 2005, but the system of harmonised MRLs did not become applicable until these had been defined in a series of Annexes amending the Regulation (Article 50). Therefore, existing national MRLs remained temporarily in force. However, six months after the publication of the last of the Regulations establishing initial versions of Annexes I, II, III and IV[37], full applicability of the Regulation was finally achieved by 1 September 2008[38].

The Regulation complements the general principles and requirements of food law laid down in Regulation (EC) No 178/2002[39] by providing specific rules for pesticide residues (Recitals 9, 10 and 13, and Article 1). The Regulation is also directly linked to the authorisation for use of plant protection products as defined under Directive 91/414/EEC[40] (Recital 5, 12), which established data requirements for the setting of MRLs for pesticides (Article 7, paragraph 1(d)).

[36] Directives 76/895/EEC, 86/362/EEC, 86/363/EEC, and 90/642/EEC
[37] Regulations (EC) No 178/2006, 149/2008, 260/2008, 299/2008, and 839/2008
[38] http://ec.europa.eu/food/plant/protection/pesticides/index_en.htm
[39] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety
[40] Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market

Protection goals

The objective of the Regulation is to ensure that pesticide residues are not present in food and feed products "*at levels presenting an unacceptable risk to humans and, where relevant, to animals*" (Recital 5). The focus is on human risk assessment; environmental risk assessment is out of scope.

Hazard and risk assessment

MRLs established under the Regulation shall be underpinned by publicly available consumer risk assessments for which the European Food Safety Authority (EFSA) has overall responsibility (Recital 6, Articles10 and 24). In particular and *inter alia*, EFSA shall assess the risks that proposed MRLs exceed levels defined as Acceptable Daily Intake (ADI) or Acute Reference Dose (ARfD), where relevant (Article 10)[41].

Mixture toxicity

Established procedures for safety assessments of MRLs on the basis of ADI values and food consumption patterns are *per se* focused on single substance assessments. However, the Regulation explicitly addresses this as a potential weak point and sets the goal to develop advanced methodologies. Recital 6 states: "*It is also important to carry out further work to develop a methodology to take into account cumulative and synergistic effects.*" And further: MRLs should be set in "*view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health*".

As a consequence of these considerations, support measures related to harmonized pesticide MRLs that shall be established at Community level shall *inter alia* include "*studies and other measures necessary for the preparation and development of legislation and of technical guidelines on pesticide residues, aimed, in particular, at developing and using methods of assessing aggregate, cumulative and synergistic effects*" (Article 36, paragraph 1 (c)).

As a further consequence, Commission decisions concerning MRLs shall *inter alia* take account of "*the possible presence of pesticide residues arising from sources other than current plant protection uses of active substances, and their known cumulative and synergistic effects, when the methods to assess such effects are available*" (Article 14, paragraph 2 (b)).

As a step towards the implementation of these provisions, the Commission asked EFSA to develop corresponding methods[42]. In 2005, the EFSA *Panel on plant protection products and their residues* (PPR) established two working groups on *Cumulative risk assessment*, dealing with the aspects and of exposure and of toxicology, respectively. At the end of 2006, EFSA held a Scientific Colloquium on *Cumulative Risk Assessment of Pesticides to Human Health: the Way forward*[43]. The

[41] ADI and ARfD are defined in Article 3, paragraphs 2 (i) and (j) of the Regulation.

[42] http://europa.eu/rapid/pressReleasesAction.do?reference=IP/04/1485&format=HTML&aged=0&lg=en&guiLanguage=en

[43] http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620818616.htm

results were published at the end of 2007[44]. On 15 April 2008, EFSA published an opinion paper on methods for cumulative risk assessment of pesticides (EFSA 2008)[45]. The paper reviews existing methodologies, in particular those developed by the US EPA on the basis of the concept of concentration addition, such as the Hazard Index, the Point of Departure Index, and the Relative Potency Factor. EFSA combines these in a tiered approach and suggests to apply this for cumulative assessment groups (CAG) of pesticides with a common mode of action. As a next step, EFSA tested the suggested strategy for the group of conazole fungicides. The results were published in September 2009[46]. EFSA concluded that the suggested tiered approach is appropriate but cannot yet be applied on a routine basis[47]. The following issues have to be resolved first: (*i*) consensus on "cumulative assessment groups" (CGA) on a European level, (*ii)* further work on appropriate uncertainty assessments, and (*iii*) development of guidance for appropriate exposure assessments.

Conclusion

The MRL Regulation explicitly signifies the need to take mixture toxicity of pesticide residues in human food into account. To this end, the Regulation provides for the development and subsequent application of new methodology.

[44] European Food Safety Authority (EFSA) (2007) EFSA SCIENTIFIC COLLOQUIUM - Cumulative Risk Assessment of Pesticides to Human Health: The way forward. 28-29 November 2006, Parma, Italy. ISBN 978-92-9199-064-1

[45] Scientific Opinion of the Panel on Plant Protection Products and their Residues (PPR Panel) on a request from the EFSA 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 MRLs for those pesticides in the frame of regulation (EC) No 396/2005. The EFSA Journal (2008) 704, 1-85

[46] European Food Safety Authority (EFSA), Parma, Italy, EFSA Panel on Plant Protection Products and their Residues (PPR Panel) (2009) Scientific Opinion 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. EFSA Journal 2009; 7 (9); 1167, 1-187

[47] http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902880926.htm

2.6 Novel foods – Regulation (EC) No 258/97

Act

Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients - OJ L 43, 14.2.1997, p. 1–6, as last amended by Regulation (EC) No 596/2009 of the European Parliament and of the Council - OJ L 188, 18.7.2009, p. 14–92.

Purpose

Regulation (EC) No 258/97 concerning novel foods and novel food ingredients came into force on 15 May 1997. *Foods and food ingredients which have not (…) been used for human consumption to a significant degree within in the Community* before that date are *novel* in the sense of the regulation (Article 1). Placing on the market of such novel foods and food ingredients requires authorisation. By means of the regulation the authorisation procedures became harmonised at European level.

Food additives, flavourings, extraction solvents, and enzymes used in food production are exempted from the regulation and covered by other pieces of European food law (Article 2). Foods and food ingredients containing or consisting of or producted from genetically modified organisms (GMO) were originally included in the scope of the regulation. By amendments made in 2003, they were removed and subjected to a separate regulatory regime on genetically modified food and feed[48].

Novel foods and food ingredients *must not present a danger to the consumer,* not *mislead the consumer*, and not be *nutritionally disadvantageous* in comparison to such *foods or food ingredients which they are intended to replace* (Article 3).

Regulatory context

When the original act came into force in 1997, the Commission additionally published *recommendations concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports* (Commission Recommendation 97/618/EC)[49]. These recommendations had been developed by the former Scientific Committee for Food (SCF), the tasks of which have been transferred to the European Food Safety Authority (EFSA) in 2003.

Since 2002, *general principles and requirements of food law* are laid down in the overarching Regulation (EC) No 178/2002, including general food safety requirements that apply since January 2005.

[48] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed

[49] 97/618/EC: Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council. Official Journal L 253 , 16/09/1997 P. 0001 - 0036

Current revision

On 14 January 2008 the Commission presented a proposal for a revised regulation on novel foods (COM(2007) 872 final)[50]. The future Regulation shall introduce a centralised common assessment and authorisation procedure for food additives, food enzymes, food flavourings, sources of food flavourings, and novel foods (Article 19 of the proposal). It shall clarify some aspects of the definition of *novel* food, simplify the legislation and increase the efficiency of the authorisation procedure, but general principles of authorisation requirements and criteria shall remain unchanged.

On 18 December 2008 the European Parliament's Committee on the Environment, Public Health and Food Safety suggested numerous amendments to the Commission proposal, including an explicit requirement for assessing hazards and risks arising from mixtures toxicity (EP document A6-0512/2008[51]). While the Commission proposal only requires that novel food must not *pose a safety concern to the health of the consumer* (Article 6, point a), the Committee proposed to expand this formulation by adding: *…which implies that cumulative and synergistic effects (…) will be taken into account in the risk assessment* (Amendment 43).

On 25 March 2009 first plenary reading took place in the European Parliament. In the legislative resolution the plenary adopted 80 amendments, including amendment 43 on cumulative and synergistic effects as cited above (EP document P6_TA-PROV(2009)0171[52] and Council document 7990/09[53]).

On 4 June 2009 the Commission's position on the Parliament's resolution was communicated. The Commission accepted 33 amendments directly, in principal or partly, but rejected the rest, including amendment 43 on cumulative and synergistic effects (Commission document SP(2009)3060[54]).

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[50] COM/2007/0872 final: Proposal for a Regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) No XXX/XXXX [common procedure] [SEC(2008) 12] [SEC(2008) 13]

[51] European Parliament, Committee on the Environment, Public Health and Food Safety. Report on the proposal for a regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) No XXX/XXXX [common procedure] (COM(2007)0872 – C6-0027/2008 – 2008/0002(COD)). Document A6-0512/2008. Available at http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//NONSGML+REPORT+A6-2008-0512+0+DOC+PDF+V0//EN

[52] European Parliament legislative resolution of 25 March 2009 on the proposal for a regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) No XXX/XXXX [common procedure] (COM(2007)0872 – C6-0027/2008 – 2008/0002(COD)). Available at http://www.europarl.europa.eu/sides/getDoc.do?type=TA&language=EN&reference=P6-TA-2009-0171

[53] Council of the European Union, Brussels, 2 April 2009, Document 7990/09. Note from: General Secretariat of the Council to: Permanent Representatives Committee/Council, Subject: Proposal for a Regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) nº XXX/XXXX [common procedure] – Outcome of the European Parliament's first reading (Strasbourg, 23 to 26 March 2009). Available at http://register.consilium.europa.eu/pdf/en/09/st07/st07990.en09.pdf

[54] European Commission, Secretariat General, Directorate G, Relations with the European Parliament, the European Ombudsman, the European Economic and Social Committee, the Committee of the Regions and the National Parliaments, Brussels, 4 June 2009, Document SP(2009)3060. Commission communication on the action taken on opinions and resolutions adopted by Parliament at the March I

On 22 June 2009 the Council voted on a Common Position with a view to adopting the new Regulation on novel foods (Council documents 10754/09[55], 11299/09[56], 11261/09[57]). The Common Position is a political agreement that it introduces several changes in the text proposed by the Commission, some of them inspired by the amendments proposed by the European Parliament. However, it does not include the proposed amendment 43 on cumulative and synergistic effects (Council document 11261/09 ADD 1[58]).

Thus, the future legislation on novel foods will not introduce any provisions that explicitly address hazards and risks arising from mixture toxicity.

Protection goals

The novel food Regulation (EC) No 258/97 is focussed on the protection of human health. Provisions relating to the environmental risk assessment of GMOs, which were originally included in the repealed Article 9 and which are still mentioned in recital (5), became pointless with the removal of GM food from the scope of the regulation in 2003.

Hazard and risk assessment

According to Article 3 of the novel food Regulation (EC) No 258/97 currently in force, novel foods must "*not present a danger for the consumer"*. In the proposed future legislation this condition remains basically unchanged, but the wording shall be modified into "*not, on the basis of the scientific evidence available, pose a safety concern to the health of the consumer under normal consumption conditions*" ((COM(2007) 872 final, Article 6, p. 16). This version is congruent to the new Regulation 1333/2008 on food additives which shall be subjected to a common assessment and authorisation procedure as outlined above.

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and II 2009 part-sessions. Available at http://www.europarl.europa.eu/oeil/DownloadSP.do?id=16532&num_rep=7817&language=en

[55] Council of the European Union,, Brussels, 17 June 2009, Document 10754/09. Proposal for a Regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) No XXX/XXXX [common procedure] (LA) (First reading) - Political agreement. Available at http://register.consilium.europa.eu/pdf/en/09/st10/st10754.en09.pdf

[56] Council of the European Union,, Brussels, 22 June 2009, Document 11299/09. Voting result Proposal for a Regulation of the European Parliament and of the Council on novel foods and amending Regulation (EC) No XXX/XXXX [common procedure] (LA) (First reading) - Political agreement 2952nd meeting of the Council of the European Union (Agriculture) Luxembourg, 22 and 23 June 2009. Available at http://register.consilium.europa.eu/pdf/en/09/st11/st11299.en09.pdf

[57] Council of the European Union,, Brussels, 7 September 2009, Document 11261/09.Common Position with a view to adopting a Regulation of the European Parliament and of the Council on novel foods, amending Regulation (EC) No 1331/2008 and repealing Regulation (EC) No 258/97 and Commission Regulation (EC) No 1852/2001. Available at http://register.consilium.europa.eu/pdf/en/09/st11/st11261.en09.pdf

[58] Council of the European Union,, Brussels, 10 September 2009, Document 11261/09 ADD 1. Draft Statement of the Council's Reasons. Subject : Common Position adopted by the Council with a view to adopting a Regulation of the European Parliament and of the Council on novel foods, amending Regulation (EC) No 1331/2008 and repealing Regulation (EC) No 258/97 and Commission Regulation (EC) No 1852/2001. Available at http://register.consilium.europa.eu/pdf/en/09/st11/st11261-ad01.en09.pdf

Requests for authorisation of novel foods or novel food ingredients must contain the information that is necessary to demonstrate that they do not present a danger to the consumer (Article 6). Guidance on the necessary information and the assessment of this information has been given the Commission Recommendation 97/618/EC.

The recommendations outline that foods *are usually complex mixtures of macro- and microconstitutients which provide energy and nutrients and contribute to the well-being of humans*. The *assessment of the wholesomeness of foods* is considered to be a scientific challenge. *Conventional toxicological evaluation methods cannot be applied*, but an alternative strategy for *combined nutritional-toxicological testing* is needed (Recommendation 97/618/EC, section 3.1, p.5).

As a consequence, the assessment of novel food is usually based on the concept of *substantial equivalence* which has been introduced by WHO and OECD. This means that the wholesomeness of a novel food is compared to an existing food or food component. As stressed in the recommendations, the establishment of *substantial equivalence* is not a safety or nutritional assessment in itself, but an approach to compare a potential new food with its conventional counterpart (Recommendation 97/618/EC, section 3.3, p.6).

Where *substantial equivalence* can be established, there is no need for the provision of toxicological data. Otherwise toxicological requirements for the assessment of novel foods must be considered on a case-by-case basis (Recommendation 97/618/EC, section 3.7, p.7).

In the current proposal for a revised regulation on novel foods (COM(2007) 872 final), Article 10 clarifies that safety assessments of novel foods shall be performed by comparing, *where appropriate, if the food is as safe as food from a comparable food category already existing on the market in the Community or as the food that the novel food is intended to replace.* In case of food that is a traditional food in a third country, but a novelty on the EU market, *the history of safe food use* in the third country shall be taken into account. The Common Position adopted by the Council in June 2009 (see above) accepted these formulations with only very minor linguistic changes and included them in Article 13.

Mixture toxicity

Safety assessments under the novel foods regulation are performed for individual novel foods or novel food ingredients. Foods themselves usually are complex biological entities or at least complex structured mixtures of macro- and micromolecules. The wholesomeness and safety of this complex food material is usually assessed by a comparative approach, which uses the concept of *substantial equivalence* as an assessment criterion as outlined above.

Obviously, there is a certain degree of analogy between this *substantial equivalence* approach to the assessment of wholesomeness and safety of foods and the so-called *sufficiently similar mixture* approach suggested by the US EPA as an option for estimating the toxicity of a complex environmental mixture in its entirety. In this

approach, the whole mixture of concern is assessed by using available information on a mixture which is considered to be *sufficiently similar* (EPA 2000)[59].

Conclusion

Novel foods are assessed individually on a case-by-case basis, typically by comparing them to traditional counterparts. As foods typically are complex structured complex mixtures this may be considered as special type of a whole mixture approach using data on a mixture that is considered sufficiently similar.

[59] EPA (2000) Supplementary guidance for conducting health risk assessment of chemical mixtures. Risk Assessment Forum, U.S. Environmental Protection Agency, Washington, DC 20460, August 2000

2.7 GM food and feed authorisation - Regulation (EC) No 1829/2003

Act

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed - OJ L 268, 18.10.2003, p. 1–23, as last amended by Commission Regulation (EC) No 298/2008 - OJ L 97, 9.4.2008, p. 64–66.

Purpose

Regulation (EC) No 1829/2003 established single Community procedures and requirements for authorisation, supervision, and labelling of food and feed containing, consisting of, or produced from genetically modified organisms (GMOs). The objective is *to provide the basis for ensuring a high level of protection of human life and health, animal health and welfare, environment and consumer interests* (…) *whilst ensuring the effective functioning of the internal market* (Article 1 (a)).

Regulatory context

The Regulation complements the general principles and requirements of food law laid down in the overarching Regulation (EC) No 178/2002. Regulation (EC) No 1830/2003[60] and Directive 2001/18/EC[61] provide complementary provisions concerning traceability standards and permits for the cultivation of GMOs. The supplementary Regulation (EC) No 641/2004[62] details rules for the application for authorisation of genetically modified (GM) food and feed, the notification of existing products provides, and transitional measures.

Protection goals

The protection goals of the regulation are in conformity with those formulated in the regulation on feed additives (see section 2.3), and comprise human and animal health, as well as environmental protection as stated in Article 1 as cited above (see *Purpose*).

Hazard and risk assessment

GM food and feed are authorised only if the applicant can demonstrate that the following requirements that are fixed in Articles 4 and 16 are met:

- GM food or feed *must not have adverse effects on animal health, human health or the environment,*

[60] Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC

[61] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration

[62] Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation

- GM food or feed *must not mislead the consumer* or *the user*, respectively,
- GM feed *must not harm or mislead the consumer by impairing the distinctive features of the animal products*, and
- GM food or feed *must not differ from the food* or *from the feed which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer* or *for animals and humans*, respectively.

The regulation does not prescribe how corresponding scientific risk assessments should be performed. This task is left to the European Food Safety Authority (EFSA). EFSA's Scientific Panel on Genetically Modified Organisms (GMO Panel) has published corresponding guidance documents for the risk assessment of genetically modified (GM) plants and derived food and feed[63], genetically modified microorganisms (GMMs) and their derived products intended for food and feed use[64], and genetically modified plants containing stacked transformation events[65].

The risk assessment strategy explained in detail in these EFSA documents follows a comparative approach, based on the concepts of *familiarity* and *substantial equivalence*. This means that GMOs and GM food and feed are compared to their non-GM counterparts, the biology of which is well researched and which have a history of safe use, respectively. Risk assessments for GM food and feed products, which may vary from single substances over simple and complex mixtures to intact organisms, are generally performed on a case-by-case basis. Toxicological studies required in support of such a risk assessment may typically comprise safety studies of both the whole GM food/feed and individual constituents, such as newly expressed proteins, new constituents other than proteins, and natural food and feed constituents.

Mixture toxicity

Risk assessments are performed for individual GM food and feed products as a whole. Both EFSA guidelines point out the potential for *possible synergistic interactions* that may occur *between expressed proteins, new metabolites and original plant constituents* in the *case of complex genetic modifications involving transfer of multiple genes*[66]. As a consequence, the Authority may require targeted studies on modes of actions of newly expressed proteins on a case-by-case basis.

Conclusion

GM food and feed are assessed individually on a case-by-case basis by comparing them to traditional counterparts. As foods typically are complex structured complex

[63] EFSA (European Food Safety Authority) (2006) Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. The EFSA Journal 99:1-100
[64] EFSA (European Food Safety Authority) (2006) Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified microorganisms and their derived products intended for food and feed use. The EFSA Journal 374:1-115
[65] EFSA (European Food Safety Authority) (2007) Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants containing stacked transformation events. The EFSA Journal (2007) 512, 1-5
[66] The EFSA Journal 99, p. 30, and 374, p. 34

mixture or intact organisms, they are assessed as a whole. Potentials for interactions between GM food constituents are taken into account.

2.8 GM food and feed, implementation of authorisation – Regulation (EC) No 641/2004

Act

Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation - OJ L 102, 7.4.2004, p. 14–25.

Purpose and regulatory context

The Commission Regulation (EC) No 641/2004 is based on Regulation (EC) No 1829/2003 on genetically modified food and feed (see the separate section 2.7). It provides some supplementary details as regards the application procedure for the authorisation of genetically modified (GM) food and feed, the notification of existing products and some transitional measures.

Protection goals

The protection goals of the supplemented Regulation (EC) No 1829/2003 on genetically modified food and feed apply (see the separate section 2.7).

Hazard and risk assessment

As a supplement to the provisions of the parent Regulation (EC) No 1829/2003, Commission Regulation (EC) No 641/2004 provides technical details on information on detection methods for transformation events (Annex I) and on reference materials (Annex II) that shall be provided by an applicant. These details just support the assessment and authorization procedures laid down in the parent regulation (see section 2.7).

Mixture toxicity

This supplementary regulation does not provide any (additional) provisions relevant for the subject of mixture toxicity.

Conclusion

No provisions for taking mixture toxicity into account were identified in this piece of legislation.

2.9 General product safety - Directive 2001/95/EC

Act

Directive 2001/95/EC of the European Parliament and of the Council of 3 December 2001 on general product safety - OJ L 11, 15.1.2002, p. 4–17, as last amended by Regulation (EC) No 596/2009 of the European Parliament and of the Council - OJ L 188, 18.7.2009, p. 14–92.

Purpose, protection goals, and regulatory context

The purpose of Directive 2001/95/EC on *general product safety* (GPSD) is to ensure the safety of consumer products that are put on the market in any of the member states of the European Union. "*Products*" are defined here as any goods that are intended for consumers or will likely end up in their hands (Article 2, point (a)). A "*safe product"* is defined in Article 2, point (b) as "*any product which […] does not present any risk […] considered to be acceptable and consistent with a high level of protection for the safety and health of persons"*. This definition explicitly takes into account interactions with other products and the packaging and labeling of products (Article 2, points (b) (ii) and (iii)). The GPSD does consider neither the safety of nor the risks for the environment that may be caused by a product.

The GPSD covers, but is not limited to, chemicals, pesticides[67] and cosmetics[68]. Although such products are already subject to other regulations, the GPSD still applies[69]. The GPSD especially applies to risks that are not covered by the corresponding sectoral directive(s) (Recital 12), for example mechanical risks from cosmetic products that are not covered by the specific cosmetics regulations[70]. If both, the sectoral regulations and the GPSD have identical objectives, the specific sectoral regulations will apply (*lex specialis*)[71]. Hence the GPSD can be considered complementary to the sectoral regulations.

The EU Commission has published a series of guidelines on how the GPSD relates to other regulations. Unfortunately, such explicit guidance seems to be missing for most chemical products, only the relation to the cosmetics Directive and to the Directive on medicinal products are analysed in the guidance documents[72]. However, as chemicals and chemical products (preparations) are already subject to an extensive body of sectoral regulations that specifically cover chemical risks, only those risks that are not specifically covered there will be subject to the GPSD.

As the GPSD is a Directive, Member States are free to choose the specific instruments for implementing its aims and goals into national law. For a range of products, different national approaches for their risk assessment have been chosen by the

[67] Department of Trade and Industry, UK (2005) The general product safety regulations – guidance for businesses, consumers and enforcement authorities
[68] DG SANCO (Directorate General Health and Consumer Protection) (2003) Guidance document on the relationship between the General Product Safety Directive (GPSD) and certain sector directives with provisions on product safety.
[69] Department of Trade and Industry, UK (2005) *as cited above*
[70] DG SANCO (2003) *as cited above*
[71] Department of Trade and Industry, UK (2005) *as cited above*
[72] DG SANCO (2003) *as cited above*

Member States (see also RPA 2006[73]). However, EU-wide standards for the risk assessment of most chemical products exist, such as those laid down in the Technical Guidance Document[74] or the Uniform Principles (Annex VI to Directive 91/414/EEC[75]).

RAPEX

The results of the national assessments of the risks arising from the use of a product might be fed into RAPEX in case of immediate danger. RAPEX is the EU Rapid Alert System for dangerous consumer products that obliges all Member States to notify the Commission about products that pose a serious risk (Article 12). Although not explicitly stated in the GPSD itself, its current risk assessment approaches primarily focus on acute effects. It has therefore been argued, that current GPSD-based approaches are clearly limited when it comes to assessing human health risks of chemical products[76].

All products, with the exception of food, pharmaceutical and medical devices are covered by RAPEX[77]. Information on those products is exchanged through specific systems such as the RASFF (Rapid Alert System for Food and Feed). Chemical substances are specifically mentioned in the corresponding Annex II of the GPSD (*Procedures for the application of RAPEX and guidelines for notifications*, section 3, last paragraph), in which a risk assessment according to Regulation (EEC) No 793/93 and Directive 67/548/EEC is requested, in case Member States seek to limit the marketing of a product. So far, such a process has only been initiated once, in order to ban the use of Phthalates in toys due to concerns for human health[78].

Conclusions

The GPSD provides overarching definitions, aims and requirements concerning product safety for humans. Although chemical products fall into the scope of the directive, it does not explicitly target or assess chemical risks. This task is delegated to the corresponding sectoral directives. Due to its overarching and complementary nature the GPSD would, however, apply if a chemical product is assessed to be dangerous to consumers due to combination effects between chemical products from

[73] RPA (Risk & Policy Analysts Ltd, Norfolk) (2006) Establishing a comparative inventory of approaches and methods used by enforcement authorities for the assessment of the safety of consumer products covered by Directive 2001/95/EC on General Product Safety and identification of best practices. Final report prepared for DG SANCO

[74] Technical guidance document on risk assessment in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances and Directive 98/8 of the European Parliament and of the Council concerning the placing of biocidal products on the market. European Commission, Joint Research Center, Office for Official Publications of the European Communities, Luxembourg, 2nd edition, 2003

[75] Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market

[76] RPA (2006) *as cited above*

[77] RAPEX annual report 2007, Health and Consumer Protection Directorate General, 2008

[78] Directive 2005/84/EC of the European Parliament and of the Council of 14 December 2005 amending for the 22nd time Council Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (phthalates in toys and childcare articles), OJ L 344 40-43, 2005,

different sectors. However, the GPSD does not provide specific guidance on how to assess such combination effects. Moreover, it has been specifically concluded that current GPSD-related methodologies do not provide a basis for assessing any type of cumulative risks[79].

[79] RPA (2006) *as cited above*

2.10 Cosmetics - Directive 76/768/EEC

Act

Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products - OJ L 262, 27.9.1976, p. 169–200, as last amended by Commission Directive 2009/129/EC - OJ L 267, 10.10.2009, p. 18–19

Purpose and protection goal

The main objective of the *Council Directive on the Approximation of the Laws of the Member States Relating to Cosmetics Products* (short: Cosmetics Directive) is the safeguarding of the public health and protection against damage to human health that may result from the use of cosmetic products (Article 2). Member States are free to choose the instruments for incorporation into national law.

The Directive covers cosmetic products which are distinguished from medicinal and other products in terms of their intended purpose and their sites or mode of application. Cosmetic products are defined as preparations intended for use on various external parts of the human body (skin, hair, nails, lips, external genitalia) for the purpose of cleaning, perfuming, changes in appearance or in order to keep them in good condition (Article 1).

The Directive prohibits the marketing of cosmetic products that contain specified substances (Annexes II, III). For certain chemicals, positive lists are defined: For example, only specified colouring agents (Annex IV), preservatives (Annex VI) or UV filters (Annex VII) are permitted, and if cosmetics contain such types of chemicals not listed in these Annexes their marketing in the EU is prohibited.

The use of chemicals classified as carcinogenic, mutagenic or toxic for reproduction of category 1, 2 and 3 according to Annex I to Directive 67/548/EEC[80] is prohibited (Article 4b).

Other Articles of the Directive deal with animal testing and replacement tests (Article 4a), labeling rules (Article 6), requirements for keeping product information for purposes of control by relevant authorities (Article 7) and methods for analysis (Article 8).

On the basis of substantiated justifications, individual Member States may provisionally prohibit the marketing of a cosmetic product in their territories, if it is noted that a product represents a hazard to health, even though it complies with the Directive (Article12, paragraph 1).

[80] Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances

Mixture toxicity

Although the Directive contains nothing specific pertaining to the assessment of hazards that stem from several chemicals simultaneously, or from mixtures, the assessment of cumulative risk from several chemicals is compatible with the Directive. This is because it deals with cosmetic products as a whole, and not specific chemicals. The Directive stipulates mandatory consultation by Scientific Committees (Article 10), and cosmetic products fall in the remit of the Scientific Committee on Consumer Products (SCCP). The mandate of the SCCP is to provide EU institutions with scientific advice regarding risk management decisions.

Conclusion

The Cosmetics Directive deals with health risk from cosmetic products. Although chemicals risk assessment is within the scope of the directive, it does not stipulate how such risks should be determined or managed, or whether mixtures of chemicals should be considered. These tasks are delegated to the relevant Scientific Committee (SCCP).

2.11 Existing industrial chemicals - Regulation (EEC) 793/93

Act

Council Regulation (EEC) No 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances - OJ L 84, 5.4.1993, p. 1–75, as last amended by Regulation (EC) No 1882/2003 of the European Parliament and of the Council - OJ L 284, 31.10.2003, p. 1–53, replaced and repealed by Regulation (EEC) 793/93 of the European Parliament and of the Council – OJ L 396, 30.12.2006, p. 1-849.

Purpose

Council Regulation (EEC) No 793/93 on existing substances set up a program for a systematic identification, assessment, and control of the risks of existing chemical substances listed in EINECS (European Inventory of Existing Commercial Chemical Substances).

Regulatory status and context

Regulation (EEC) 793/93 has been replaced and repealed by REACH with effect from 1 June 2008 (REACH, Article 139). Transitional measures laid down in REACH shall ensure that the knowledge gained by work under the old Regulation is not lost, but effectively used under the new regime (REACH Recital 126, Articles 136 and 137, Annex I, section 0.5, and Annex XII, Introduction, 2nd paragraph).

Risk assessment and mixture toxicity

Risk assessments for priority substances under Regulation (EEC) No 793/93 were prepared by Member States Competent Authorities by applying the methodology laid down in extensive detail in the Technical Guidance Document (TGD)[81].

Guidance given by the TGD generally refers to the risk assessment of single substances. Mixture toxicity is out of scope. However, there is one exemption from the rule. This is guidance given for the risk assessment of petroleum substances, which are highly complex and variable mixtures of hydrocarbons. Different approaches are recommended for human health risk assessment and environmental risk assessment of petroleum substances. The guidance given for the human health risk assessment of petroleum substances, which is described in Appendix VII to TGD Chapter 2, favours a whole mixture approach. The corresponding environmental risk assessment part, which is given in appendix IX to TGD Chapter 3, describes the so-called Hydrocarbon Block Method (HBM), which is a component-based approach. Hydrocarbons with similar physico-chemical properties and degradation potentials are grouped or "blocked". PEC/PNEC[82] ratios are established for each block and then

[81] Technical guidance document on risk assessment in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances and Directive 98/8 of the European Parliament and of the Council concerning the placing of biocidal products on the market. European Commission, Joint Research Center, Office for Official Publications of the European Communities, Luxembourg, 2nd edition, 2003

[82] PEC – Predicted Environmental Concentration; PNEC – Predicted No Effect Concentration

summed up to a PEC/PNEC for the whole petroleum substance. This method is a pragmatic application of the concept of concentration addition.

Risk assessment reports for individual substances that were prepared under Regulation (EEC) No 793/93 took groups of related substances into account where this was considered appropriate. Thus, for instance, the environmental part of the risk assessment of zinc metal includes considerations on both exposure and effects resulting from other current EU priority zinc compounds (zinc oxide, zinc chloride, zinc distearate, zinc phosphate and zinc sulphate)[83]. The rational behind the common assessment approach is that all these *compounds dissociate to zinc ions that have the potential to cause adverse effects in biota. Differences between the compounds arise from variability in the solubility of the various compounds and in production and use that lead to differences in fates and exposure at local levels* (SCHER 2007)[84]. This approach may therefore be considered as an assessment of cumulative risks of zinc in the environment resulting from different sources and different chemical forms of the metal. The potential mixture toxicity of zinc and environmental chemicals other than zinc compounds was out of the scope of the risk assessment.

Conclusion

The former Regulation on existing substances, the corresponding Technical Guidance Document (TGD), and the resulting risk assessment reports were focused on the preparation of generic risk assessments for single substances or groups of related substances. Petroleum substances are an exemption from the rule. In this case, the TGDs recommend a whole-mixture approach and a component-based approach for human and environmental risk assessment, respectively.

[83] Risk Assessment Zinc Metal, CAS-No. 7440-66-6, EINECS-No.: 231-175-3, *Final report, May 2008,* Part 1, Environment. Rapporteur: Ministry of Housing, Spatial Planning and the Environment (VROM) in consultation with the Ministry of Social Affairs and Employment (SZW) and the Ministry of Public Health, Welfare and Sport (VWS), The Netherlands. Available at: http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/zincmetalENVreport072.pdf

[84] SCHER (Scientific Committee on Health and Environmental Risks) (2007) Risk Assessment Report on Zinc, Environmental Part. Opinion adopted on 29 November 2007. European Commission, Health & Consumer Protection Directorate General. Available at: http://ec.europa.eu/health/ph_risk/committees/04_scher/docs/scher_o_069.pdf

2.12 Dangerous substances - Directive 67/548/EEC

Act

Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances - OJ 196, 16.8.1967, p. 1–98, as last amended by Commission Directive 2009/2/EC of 15 January 2009 amending, for the purpose of its adaptation to technical progress, for the 31st time, Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances - OJ L 11, 16.1.2009, p. 6–82.

Purpose and scope

Council Directive 67/548/EEC provides a harmonized Community system for classifying dangerous substances and preparations on the basis of their intrinsic properties. For the purposes of the Directive "substances" *"means chemical elements and their compounds in the natural state or obtained by any production process, …"* (Article 2, 1.(a)). "Preparations" *"means mixtures or solutions composed of two or more substances"* (Article 2, 1.(b)).

Regulatory context

Tests on substances covered by this Directive must comply with the requirements of Council Regulation (EC) No 440/2008 on test methods and REACH (Regulation (EC) No 1907/2006).

Regulatory status

Directive 67/548/EEC will be repealed by Regulation 1272/2008 from 1 June 2015. The new Regulation (EC) 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP) entered into force on the 20 January 2009. CLP implements the Globally Harmonised System (GHS). CLP will replace Directive 67/548/EEC and also Directive 1999/45/EC (preparations) in a stepwise manner.

The following text refers to the legal status before the transition phase. The relevant provisions of the new Regulation 1272/2008 are analyzed in extensive detail in the corresponding separate section 2.15.

Hazard assessment for mixtures

Detailed rules for the classification of substances and preparations are laid down in Annex VI on *General Classification and Labelling Requirements for Dangerous Substances and Preparations* to the Directive 67/548/EEC. If it concerns data on health effects or data on ecotoxicological properties, the data required for classification and labelling may be obtained either by applying for preparations the same experimental test methods as for single substances (whole mixture approach), or by applying a so-called *conventional method* referred to in Article 6 and 7 of and Annex II and III to Directive 1999/45/EC respectively (Annex VI, point 1.6.2). The

conventional method is a component-based approach. It is an application of the concept of concentration addition. The *conventional method* is not laid down in the Directive itself, but in the complementary Directive 1999/45/EC on dangerous preparations. For the evaluation of carcinogenic, mutagenic and reproductive toxicity of preparations, the *conventional method* is the only option allowed by the Directive. For other criteria of human toxicity, the classification is carried out on the basis of the *conventional method* in the absence of experimental data, or when experimental data are available, on the basis of whole mixture testing. However, new testing of preparations in animals is restricted to cases, "*… where it can be scientifically demonstrated (…) that the toxicological properties of the preparation cannot correctly be determined by the…*" conventional method "*or on the basis of existing test results on animals, …*" (Annex VI, point 3.1.3) For the assessment of aquatic toxicity, the whole mixture testing approach and the *conventional method* may be used alternatively. Other ecotoxicological properties, such as bioaccumulation, degradability, and dangers of the ozone layer are assessed by application of the *conventional method* only.

Conclusion

Provisions on the classification of dangerous preparations take mixture toxicity into account. Both a whole mixture approach and component-based approach are applied.

2.13 Dangerous preparations - Directive 1999/45/EC

Act

Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labelling of dangerous preparations - OJ L 200, 30.7.1999, p. 1–68, as last amended by Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 - OJ L 353, 31.12.2008, p. 1–1355.

Purpose and regulatory context

Directive 1999/45/EC on dangerous preparations complements Directive 67/548/EEC on the classification of dangerous substances and extends the rules to dangerous preparations, which are mixtures of chemicals.

Regulatory status

Directive 1999/45/EC will be repealed by Regulation 1272/2008 from 1 June 2015. The new Regulation (EC) 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP) entered into force on the 20 January 2009. CLP implements the Globally harmonised System (GHS). CLP will replace Directive 1999/45/EC on preparations and also Directive 67/548/EEC (substances) in a stepwise manner.

The following text refers to the legal status before the transition phase. The relevant provisions of the new Regulation 1272/2008 are analyzed in extensive detail in the corresponding separate section 2.15.

Hazard assessment for mixtures

Detailed rules for the classification of preparations by a component-based approach are laid down in Annexes II and III to the current Regulation. The component-based approach is termed "*conventional method*". It uses the concept of concentration addition as default assumption. As laid down in Council Directive 67/548/EEC, the use of the *conventional method* is either mandatory or an alternative option to whole mixture testing, depending on the specific (eco)toxicity parameter (*as explained in section 2.12 above*).

In the future GHS based system, details will change, but the principle holds: Commercial mixtures are alternatively classified by a whole mixture approach or a component based approach.

As a consequence, the current *conventional* methodology of Directive 1999/45/EC is not described in further detail here, but an in-depth examination of the forthcoming provisions is given in section 2.15 (*see below*).

Conclusion

Provisions on the classification of dangerous preparations take mixture toxicity into account. The Directive provides for a component-based approach which uses the concept of concentration addition as default assumption. For some toxicological endpoints whole mixture testing may be applied as an alternative.

2.14 REACH - Regulation (EC) No 1907/2006

Act

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC - OJ L 396, 30.12.2006, p. 1–849, as last amended by Commission Regulation (EC) No 552/2009 of 22 June 2009 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annex XVII - OJ L 64, 26.6.2009, p. 7–31.

Purpose and scope

The new European Chemicals Legislation REACH covers the obligations of a manufacturer or importer of a substance on its own or in a mixture with respect to its chemical safety assessment (CSA) before it can be put on the European market in quantities of 1 ton/year or more.

Chemical mixtures under REACH

"*Chemical mixture*" is in this context used synonymously to "*preparation*" (European Chemicals Agency 2008d)[85], REACH, Article 3(2), that is, a combination of two or more individual substances. "*Substances within the scope of REACH (…) are typically the product of a chemical reaction in manufacture and may contain multiple distinct constituents. Substances, as defined in REACH, also include substances chemically derived or isolated from naturally occurring materials, which may comprise a single element or molecule (…) or several constituents (…)*" (ECHA 2007, p.8)[86]. *Preparations* are considered the result of a deliberate mixing of non-reacting chemicals for producing chemical products such as e.g. paints. REACH obligations with respect to registration apply to each of the individual chemicals in such a mixture, but not to the mixture itself[87]. In contrast MCS (Multi-Constituent Substances) are "*substances*" that result from a chemical reaction in which several constituents are present at > 10% and UVCB (substance of Unknown or Variable composition, Complex reaction products or Biological Materials) are mixtures that cannot be completely identified by their chemical composition. Both, MCS and UVCBs are generally treated as single substances under REACH.

A specific type of "mixture" results from a single substance that is registered by a manufacturer and enters the environment or the human body via different pathways. Such cumulative exposure is toxicologically speaking identical to a single chemical

[85] European Chemicals Agency (2008d) Guidance on information requirements and chemical safety assessments. Chapter R.20: table of terms and abbreviation
[86] European Chemicals Agency (2007) Guidance for identification and naming of substances under REACH
[87] European Chemicals Agency (2008e) Guidance on registration. Vers. 1.4

and is hence not specifically regarded in the following. However, it is important to note that a registrant is not obliged to take into account an exposure to the same substance from activities from other producers or importers (European Chemicals Agency 2008d)[88].

It should also be noted that an individual chemical (also called a "mono constituent substance") can contain up to 20% arbitrary byproducts without the need for specific consideration[89].

The overall structure of the CSA-process according to REACH is given in Figure 1. With respect to the toxicity or ecotoxicity of a compound, the main purpose of the CSA is to determine the intrinsic hazard of a compound or mixture by estimating Derived No-effect-Levels for human health (DNEL) and Predicted No-Effect-Concentrations (PNEC) for environmental assessments and to assess substance properties relating to persistence, bioaccumulation and toxicity (PBT). This information is then used for the derivation of the hazard threshold levels for human health and the environment.

Furthermore, the available toxicity information is used for the classification and labelling of the substance, a vital part of the Safety Data Sheet according to Article 31 and Annex II of the REACH regulation.

Chemical hazard assessment for human health and the environment

The procedure for the hazard assessment of a chemical is laid down in Part B of the set of REACH guidance documents (European Chemicals Agency 2008c)[90]. More detailed guidance is given in the accompanying chapters R2-R10. None of these documents contains a specific hazard assessment procedure for chemical mixtures, preparations, MCSs or UVCBs.

PBT / vPvB assessment

Section R.11.1.4.2 of the REACH guidance document R.11[91] provides detailed guidance on how to assess the PBT/vPvB properties of MCS and UVCB "substances", i.e. mixtures of partly unknown and/or variable composition. Such complex mixtures are termed PBT/vPvB substances if they contain more than 80% of a substance with PBT/vPvB properties (R.11.1.1.2)[92]). However, a specific management is "to be considered" if a PBT/vPvB compound is present in a concentration >=0.1% (R.11.1.1.2)[93], which seems to be derived from the concentration threshold of 0.1% for the need of conducting a full CSA which would consider PBT/vPvB chemicals in preparations (Article 14(2)f of the REACH Regulation).

[88] European Chemicals Agency (2008d) *as cited above*
[89] European Chemicals Agency (2008e) Guidance on registration. Vers. 1.4
[90] European Chemicals Agency (2008c) Guidance on information requirements and chemical safety assessment Part B: Hazard Assessment
[91] European Chemicals Agency (2008a) Guidance on information requirements and chemical safety assessment Chapter R.11: PBT assessment
[92] European Chemicals Agency (2008a) *as cited above*
[93] European Chemicals Agency (2008a) *as cited above*

The assessment of MCS and UVCB substances starts with a chemical characterisation, i.e. the description of the components in the mixture. As it might be often impossible to identify all components down to 0.1% relative amounts, it is advised to define "representative structures" that are later on used for describing the properties of whole blocks of the mixture. Afterwards the available information that is relevant for the PBT-assessment for each constituent, respectively each block is compiled and assessed.

If the UVCB substance consists of homologous structures and is shown to meet the ultimate ready biodegradation test criterion (>60% in 28 days), it is concluded that none of the mixture components that comprise the UVCB is falling under the P criterion (European Chemicals Agency 2008a)[94]. For UVCBs that do not consist of homologous structures, data on biodegradation are judged on a case by case basis. If the UVCB is not biodegradable or if data are lacking, a second tier of P assessment is proposed, which comprises blocking the compounds into similar groups and evaluating the P criterion (based on experimental data or QSAR estimates) for each block.

An example is given in Appendix R11-3 to chapter R 11 of the guidance document[95]. Further details for the assessment of petroleum products are provided by a series of workshop presentations and publications by CONCAWE, the oil companies European association for environment, health and safety (http://www.concawe.be). All documents are available in the virtual library of this project. It should be especially pointed out here that PETROTOX, the ecotoxicity calculator developed by CONCAWE specifically uses concentration addition (CA) for estimating the joint toxicity of the hydrocarbons in petroleum substances (HydroQual Inc. 2008)[96]. Furthermore, a presentation by Tom Parkerton from Exxon Mobil Biomedical Sciences that was given at the same workshop specifically outlines the summation of the PEC/PNEC ratios of the individual hydrocarbon blocks to estimate their joint risk quotient (Parkerton 2008)[97].

Conclusions

REACH is a typical substance-oriented regulation, providing the European framework for the hazard, exposure and risk assessment of all commercial chemicals that are not specifically covered by other sectorial regulations. REACH includes provisions for commercial chemical mixtures that result from chemical reactions or that are natural products (UVCBs and MCSs). Preparations are not subject to registration as a whole, but are specifically covered by the provisons on classification and labeling according to the GHS system (see section 2.15 below).Guidance on how to assess the hazard and

[94] European Chemicals Agency (2008a) Guidance on information requirements and chemical safety assessment Chapter R.11: PBT assessment
[95] European Chemicals Agency (2008a) *as cited above*
[96] HydroQual Inc. PETROTOX: an ecotoxicity calculator for petroleum hydrocarbon mixtures. Presentation from CONCAWE Workshop on Generic Environmental Risk Assessment of Petroleum Substances, 31. Oct 2007. 2008
[97] Parkerton, T. CONCAWE Workshop on Generic Environmental Risk Assessment of Petroleum Substances: Summary of Approach. Presentation from CONCAWE Workshop on Generic Environmental Risk Assessment of Petroleum Substances, 31. Oct 2007. 2008

risk of mixtures is specifically put forward for the PBT-assessment in R 11.1.4.2 of the guidance document on PBT assessment (European Chemicals Agency 2008a)[98].

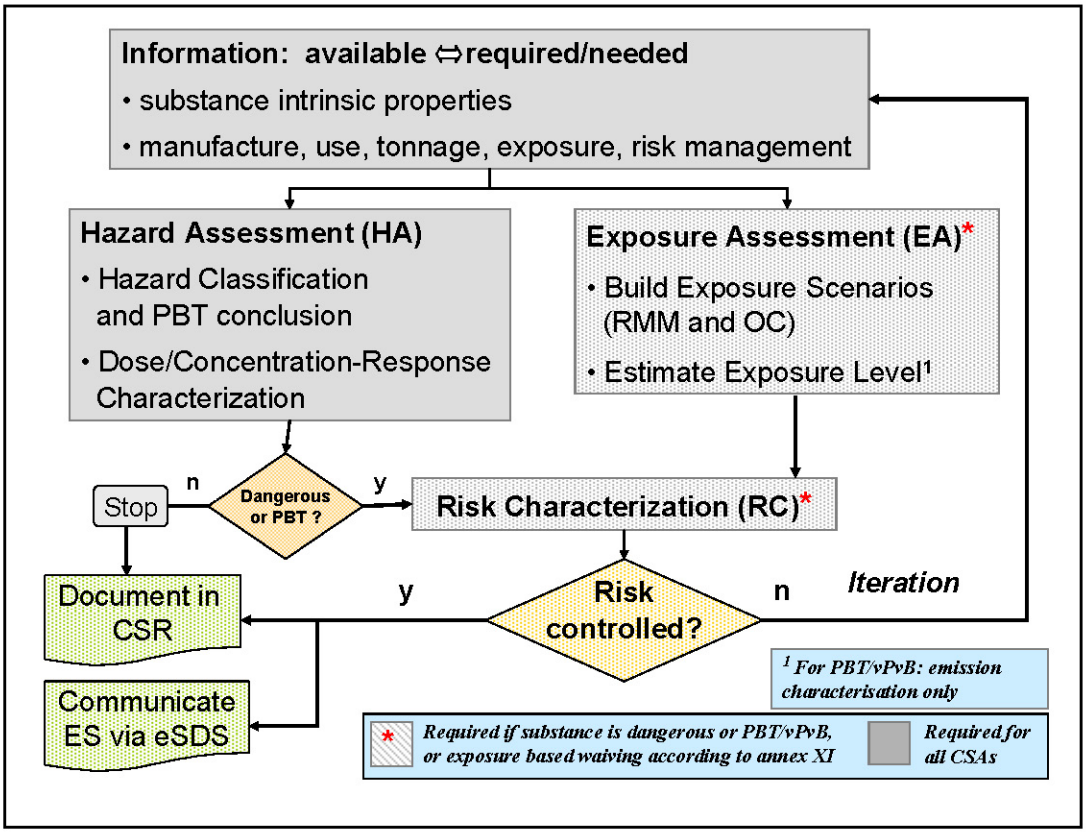


Figure 1: Outline of the chemical safety assessment according to REACH (European Chemicals Agency 2008b)[99]

[98] European Chemicals Agency (2008a) *as cited above*
[99] European Chemicals Agency (2008b) Guidance on information requirements and chemical safety assessment Part A: introduction to the guidance document

2.15 CLP – Regulation (EC) No 1272/2008

Act

Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 - OJ L 353, 31.12.2008, p. 1–1355, as amended by Commission Regulation (EC) No 790/2009 - OJ L 235, 5.9.2009, p. 1–439.

The Globally Harmonised System for the Classification and Labelling of Chemicals

Before a chemical or a mixture can be put on the market, it has to be classified by the producer or importer. This step is hence an important part of the hazard characterisation of a compound or a chemical mixture (see also Figure 1 above). The current European system is based on three key Directives:

- the Dangerous Substances Directive (67/548/EEC);
- the Dangerous Preparations Directive (1999/45/EC);
- the Safety Data Sheet Directive (91/155/EEC).

This system has been superseded by the new Regulation 1272/2008 on classification, labelling and packaging ("CLP Regulation") that follows the Globally Harmonised System for the Classification and Labelling of Chemicals (GHS) as developed by the United Nations. The third revised version of the GHS system has been published in July 2009.

The main differences between the new EC Regulation 1272/2008 and the UN GHS system concern the classification in the "lower" classes. Here the EU system uses a simpler classification scheme and did not adopt the following hazard classes: acute oral toxicity class 5, acute dermal toxicity class 5, acute inhalation toxicity class 5, skin corrosion class 3, serious eye damage class 2b, aspiration class 2, acute toxicity to the environment classes 2 and 3.

Regulation 1272/2008 entered into force on 20 January 2009 and is conceptually similar to the old regulations. Both, the old and the new system cover classification, packaging and hazard communication through labelling and safety data sheets of chemicals and chemical mixtures[100]. However, the application of Regulation 1272/2008 will most likely result in different classifications compared to the current one, which is due to changes in cut-off values and calculation methods. The classification and labelling of chemical mixtures on the European market has to be consistent with the new regulation from the first of June 2015 onwards, following single chemicals which have to be classified and labelled according to the new system from the first of December 2010 onwards.

[100] In its article 2(8) the new CLP Regulation establishes a new terminology when compared to the REACH Regulation and introduces the term "*mixture*" to what is called "*preparation*" under REACH (see also section 2.14).

The following analysis of the GHS-based classification and labelling of mixtures focuses exclusively on toxicological and ecotoxicological hazards. Physical hazards and hazards for the ozone layer were considered out of scope of this analysis.

General approach

As stated in the Recitals, it is the objective of the CLP regulation *to determine which properties of substances and mixtures should lead to a classification as hazardous, in order for the hazards of substances and mixtures to be properly identified and communicated. Such properties should include physical hazards as well as hazards to human health and to the environment, including hazards for the ozone layer* (Recital 10).

The regulation applies to all chemicals and mixtures that are put on the European market, except radioactive substances and mixtures within the scope of Council Directive 96/29/Euratom, non-isolated intermediates, substances and mixtures for scientific research, waste, medicinal products, veterinary medicinal products, cosmetic products and food and feeding stuff (Article 1, paragraphs 2-5). A mixture is defined as *a mixture or solution composed of two or more substances* (Article 2, paragraph 8) and is hence identical to the definition of the REACH regulation and it specifically includes metal alloys. Equal to individual substances, each mixture is classified into certain hazard classes, denoting the nature of the hazard and then into a hazard category, indicating the severity of the hazard within each hazard class.

It is important to note that the supplier (producer or importer) of a chemical "*should not be obliged*" to produce any new data, but he has to take into consideration all available data (Recital 20). If new information becomes available, the classification of a compound or mixture has to be adjusted accordingly (Article 15, paragraph 1). A new hazard evaluation is required when the composition of a mixture is changed (Article 15, paragraph 2) outside specified limits (see below).

Mixtures are in general assessed using the available data on the mixtures themselves (Recital 22), except for mixtures containing substances with carcinogenic, germ cell mutagenic, reproductive toxic properties or where the biodegradation and bioaccumulation properties are evaluated (only in the "*hazardous to the aquatic environment*" class). In such a case the classification of the mixtures is mainly based on the information on the mixture components.

If no test data are available for a certain mixture, so called "*bridging principles*" are applied, which allow to assess the hazard of that particular mixture using information on a similar mixture (Recital 23; Annex I). However, this requires information on the "*relevant ingredients*" of the mixture.

The classification of a mixture must take account of all available information on synergistic and antagonistic interactions among the ingredients (Article 12, paragraph 1, point (c)). Although not explicitly mentioned in the text, the terms "synergistic" and "antagonistic" seem to be defined in relation to the expectation of a concentration-additive behaviour of the compounds. In order to facilitate the application of the system in practice, generic and specific concentration limits

(Article 10) and cut-off limits (Article 11) for compounds in a mixture (including impurities present in a single chemical) are applied.

The principal aim for the classification of a mixture is to achieve a similar classification as would be achieved for an individual compound.

The hazard of a single compound or a mixture is classified with respect to the following categories:

1. Hazards for human health

    1. Acute toxicity
    2. Acute oral toxicity
    3. Acute dermal toxicity
    4. Acute inhalation toxicity (gases, vapours, dusts and mists)
    5. Skin corrosion and irritation
    6. Serious damage to the eye and eye irritation
    7. Respiratory or skin sensitisation
    8. Germ cell mutagenicity
    9. Carcinogenicity
    10. Reproductive toxicity
    11. Specific target organ toxicity – single dose
    12. Specific target organ toxicity – repeated dose
    13. Aspiration hazards

2. Environmental hazards – hazards to the aquatic environment

    1. Acute aquatic toxicity
    2. Chronic aquatic toxicity

Human health hazards

Hazard class "Acute toxicity for humans"

This hazard class is divided into three different classes, oral, dermal and inhalation toxicity, the grouping criteria for individual substances are given in Table 1. The mixture classification follows the outline given in Figure 2. If data on the mixture of interest are available, those are given priority. Only if those are not available, concentration addition (CA) is used for estimating the toxicity of the mixture. Only those components need to be considered that are present at a concentration of >= 1% (Annex I, 3.1.3.3.a, "*relevant ingredients*"), unless there are indications that the compounds are relevant for classification even at lower concentrations. This, however, seems to be in contradiction to the "*generic cut-off values*" of Table 1.1 of Annex I, which is based on Article 11 of the regulation. Here it is stated that for compounds that are classified as acute toxic, category I-3, a fraction of 0.1% is to be considered for the mixture classification.

Hazard classes "Skin corrosion and irritation" and "Serious damage to the eye and eye irritation"

The mixture classification in these two hazard classes follows the same principles as for the "acute toxicity to humans" classification. In particular, also for these hazard classes priority is given to data on the full mixture (Annex I, sections 3.2.3.1 and 3.3.3.1) and if these are not available then bridging principles are applied (Annex I, sections 3.2.3.2. and 3.3.2), including the use of a summation method for estimating the overall potency of the mixture with cut-off values given in Tables 3.2.3 and 3.3.3 of Annex I. The "*theory of additivity*" is mentioned (Annex I, sections 3.2.3.3.2 and 3.3.3.3.2), but no specific guidelines on their application is given. Ingredients are considered relevant at a fraction of 1% or more (Annex I, sections 3.2.3.3.1 and 3.3.3.1), in agreement with the generic cut-off values that are put forward in Table 1.1. of Annex I according to Article 11 of the regulation.

Hazard class "Respiratory or skin sensitisation"

According to section 3.4.3.3 in Annex I, a mixture is categorised into this class if at least one of the mixture components is classified itself into the hazard class "*respiratory or skin sensitisation*" and is present at >0.2% or >1% (Table 3.4.3 of Annex I). In contrast to the above mentioned hazard classes, CA is not applied for any classification purposes.

Hazard classes "Germ cell mutagenicity", "Carcinogenicty" and "Reproductive toxicity"

Primary classification criterion for mixtures into these hazard classes is the relative content of components that are themselves classified (summation method with cut-off criteria according to Tables 3.5.2, 3.6.2 and 3.7.2 of Annex I). Data on the mixture itself are only considered on a case-by-case basis (sections 3.5.3.2.1, 3.6.3.2.1 and 3.7.3.2.1 of Annex I) when "*demonstrating effects that have not been established from the evaluation based on the individual ingredients*". The bridging principles according to section 1.1.3 are also considered, if applicable (sections 3.5.3.3, 3.6.3.3 and 3.7.3.3). CA is not applied for any classification purposes.

Hazard classes "Specific organ toxicity – single exposure", "Specific organ toxicity – repeated exposure" and "Aspiration hazard"

Primary classification criterion is the data on the mixture itself, although specific care is advised by the guideline to ensure that "*the dose, duration, observation or analysis does not render the results inconclusive*" (sections 3.8.3.2.1 and 3.9.3.2.1 of Annex I). The application of bridging principles is put forward in sections 3.8.3.3 and 3.9.3.3 of Annex I. If no reliable data are available on the mixture itself and the bridging principles cannot be used, the mixture is classified for its specific organ toxicity using the cut-off values given in Tables 3.8.3 and 3.9.4 of Annex I. CA is not applied for any classification purposes.

The classification of a mixture into the hazard class "*aspiration hazard*" follows the same outline (section 3.10.3.1 of Annex I, use of data on the whole mixture of

interest, section 3.10.3.2 of Annex I, bridging principles, section 3.10.3.3 of Annex I, cut-off values for individual compounds).

Environmental hazards

The classification of a compound or a mixture with respect to its environmental hazard focuses exclusively on the hazard towards aquatic organisms. It should, however, be noted that the UN Sub-Committee of Experts on the GHS has requested the OECD to explore the needs for a classification with respect to hazards for the terrestrial environment. As a first step a draft report on the existing national classification systems has been published by the OECD in 2008.

Acute aquatic toxicity and chronic aquatic toxicity are considered separately, the resulting hazard classes for individual compounds are given in Table 2. In contrast to the original GHS system, as put forward by the United Nations Economic Commission for Europe, which distinguishes three hazard classes for acute toxicity (Table 3), the European system only considers one acute toxicity category (Table 2). It should be furthermore noted that the classification for chronic toxicity into the chronic categories I-IV is also based mainly on acute toxicity data (EC50 values from the standard acute bioassays). This is obviously based on an acute to chronic extrapolation, an approach which is not directly followed for mixtures, as their chemical composition is assumed to undergo significant changes over prolonged exposure times. The classification of a mixture according to their hazard for the environment follows a similar tiered approach as the classification for human hazards, which is summarised in Figure 3 and Figure 4.

Classification for acute aquatic toxicity

The classification of a mixture into acute aquatic toxicity category I is based on toxicity data for the whole mixture if those are available and is then identical to the classification of an individual compound. However, there seems to be a discrepancy between the approach that is laid down in section 4.1.3.3.1 of Annex I where it is stated that "*When the mixture as a whole has been tested to determine its aquatic toxicity, it is classified according to the criteria that have been agreed for substances, [...]*", which implies that a mixture is classified as acute category I if its EC50 is ≤ 1mg/L (see Table 2), and section 4.1.3.3.2.b, according to which a mixture with an EC50 ≤ 1mg/L and a chronic NOEC > 1 mg/L would *not* be classified for acute toxicity. That is, section 4.1.3.3.2b suddenly introduces the chronic toxicity estimate as an additional threshold criterion for the classification for acute toxicity.

If data for the mixture of concern are not available, a component-based approach, the so-called "summation method", is applied (see Figure 5): if the mixture contains equal to or more than 25% of compounds that are classified into acute toxicity category I, the whole mixture is also classified into this category. Highly toxic compounds are given increased weight for the summation by the application of so-called M-factors (Table 4.1.3. to Annex I of the Regulation). If the mixture contains compounds that are not classified but for which toxicity data are available, CA is used for the preliminary classification of that sub-mixture (section 4.1.3.5.2 of Annex I) and the result of the CA application is then fed into the application of the summation rule. Finally, if relevant compounds are present in the mixture that are not classified for

their acute toxicity and for which also no toxicity data are present, the clause "*contains x% of components with unknown hazards to the aquatic environment*" shall be included in the safety data sheet and the classification of the mixture shall be based on the assessable rest of the mixture (section 4.1.3.6.1 of Annex I).

Classification for chronic aquatic toxicity

Data on the chronic toxicity data (NOEC values) of the whole mixture are used in combination with the classification information on the individual compounds for the classification of the mixture into chronic aquatic toxicity category I-IV (Figure 3). The available chronic toxicity estimates for the whole mixture serve as a pre-filter before the application of the summation method in which information on the classification of the individual mixture components are used as input data for classifying the mixture (Figure 5) according to section 4.1.3.5.5.4 of Annex I.

The use of concentration addition for classification purposes

A prominent characteristic of the guideline with respect to the classification into the classes "*acute toxicity (humans)*" and "*acute toxicity (environment)*" is the application of concentration addition (CA) without even mentioning the competing concept of independent action (IA). In light of current scientific understanding and empirical evidence, especially the usually small quantitative differences between the mixture toxicity expectations according to CA and IA and the fact that CA is usually slightly more conservative, this seems a pragmatic and defendable approach (see also the review on empirical evidence in Part 1 of this report). Furthermore, as the classification categories usually are defined in orders of magnitude, any difference between CA- and IA-predicted mixture toxicities will most likely be negligible. Due to the specific demands of IA in terms of input data, this concept might also never been applicable to a commercial chemical mixture without producing specific sets of toxicity data for this sole purpose – which would clearly be in conflict with the professed aim to reduce the need especially for animal testing and to optimise the use of available experimental data. However, it should be mentioned that this sole use of CA is in contrast to the detailed case-by-case selection as pursued e.g. in the methods and approaches for mixture toxicity assessment for human health as put forward by the US EPA (US EPA 2000)[101].

The summation method – a critical reflection

The summation method, that is the classification of a mixture in dependence of the relative amount of already classified compounds, plays a central role for the classification of a mixture into most hazard classes. An obvious advantage of this approach is its ease of use, which will allow the rapid classification of any mixture that is produced for example by a down-stream user.

However, it has to be regarded a major disadvantage of this method that it bears a substantial risk for underestimating the toxicity of a mixture, assuming that its combination toxicology could in fact be adequately estimated by CA – an assumption

[101] US EPA. Supplementary guidance for conducting health risk assessment of chemical mixtures. EPA/600/8-90/064 . 2000

to which the regulation itself seems to agree at least for the hazard classes "*acute toxicity (human health)*" and "*acute toxicity (environment)*". Such an underestimation obviously depends on the applied cut-off values, the actual toxicity of the ingredients of the mixture, and their actual concentration in the mixture.

An example: according the summation method (see also Figure 5) a mixture is classified for "*acute toxicity (environment)*" category I if the following relationship holds (section 4.1.3.5.5.3.2 of Annex I):

$$\sum_{i=1}^{n} c_{\text{acute cat 1}} \times M \geq 25\%$$

That is, a mixture that is containing 24% of compounds with an acute toxicity classification is itself not classified for acute toxicity[102]. Assuming that the acute toxicity classification for all those compounds that make up those 24% is based on an acute EC50 of 0.11mg/L, and even if the rest of the mixture is inert, CA would predict an overall toxicity of the mixture of:

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{24}{0.11}} = 0.46 \text{ mg/L}$$

The same result would of course be obtained under the assumption that a compound with an EC50 of 0.11 mg/L would simply be diluted with water, i.e. after the application of the bridging principles in section 4.1.3.4 of Annex I. This example shows that the application of the summation method for a mixture containing 24% of compound(s) with an EC50 of 0.11 and 76% of inert (or slightly toxic) compounds could be classified as not acutely toxic (EC50> 1mg/L), while the application of CA (or a simple dilution) would result in a classification of the mixture.

The situation becomes even more aggravated if one would assume that those 76% are made of compounds that just "escaped" a classification as acute toxic, i.e. compounds with an EC50 of for example 1 mg/L. For such a mixture CA calculates a mixture toxicity of

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{24}{0.11} + \frac{76}{1}} = 0.34 \text{ mg/L}$$

The regulation provides an "*all you can catc*h" phrase in section 4.1.3.5.4, which states:

If a mixture is classified in more than one way, the method yielding the more conservative result shall be used.

[102] For the sake of keeping the examples as simple as possible, it is assumed that all classified components have a weighting factor of M=1. However, the outlined principles also hold for highly toxic compounds with weighting factors>1.

However, this would imply that indeed all possible methods for calculating an expected mixture toxicity, especially CA and the summation method, are adequately considered and comparatively assessed. It also has to be pointed out that such an "*all you can catch*" phrase is only been put forward for the classification as "*acute toxic (environment)*", but not for the classification into the class "*acute toxic (human)*".

It is interesting to analyse how big the percentage of compounds with an "*acute toxic (environment)*" classification can be, so that the summation method and CA come to the same conclusion of "*no classification for acute toxicity (environment)*". This occurs if no more than 11% of the mixture consists of compounds with an EC50 of 0.11 mg/L (i.e. compounds classified as "*acute toxic to the environment*"). Under this circumstances CA predicts

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{11}{0.11}} = 1\,\text{mg/L}$$

That is, the EC50 would now be exactly 1 and the mixture, if following the classification rule as laid down for single compounds in Table 2, would not be classified for acute aquatic toxicity.

In summary, the application of the summation rule allows a nearly 2.5 times higher concentration of acutely toxic compounds in a mixture than the application of CA or bridging principles before the mixture would be classified as acutely toxic for the environment.

Other critical issues

As the composition of any mixture will never be completely known, the definition of a "*relevant compound*", i.e. a compound that is to be considered in a compound-driven classification of the mixture, plays a vital role. For the aquatic compartment this definition is given in section 4.1.3.1 of Annex I as follows: *The "relevant components" of a mixture are those which are classified "Acute Category 1"or "Chronic Category 1" and present in a concentration of 0.1% (w/w) or greater, and those which are classified "Chronic Category 2", "Chronic Category 3" or "Chronic Category 4" and present in a concentration of 1% (w/w) or greater, unless there is a presumption (such as in the case of highly toxic components (see 4.1.3.5.5.5)) that a component present in a lower concentration can still be relevant for classifying the mixture for aquatic environmental hazards. Generally, for substances classified as "Acute Category 1" or "Chronic Category 1" the concentration to be taken into account is (0.1/M)%.*

This provides specific guidance on the concentration levels at which classified compounds are to be considered in the application of the summation method. The application of the already established system of M-factors allows for the consideration of highly toxic compounds.

Any synergistic interaction between the mixture components can be expected to be highly scenario specific, depending on the number and nature of the involved

components, the exposed organisms and analysed endpoint. Accordingly, the guideline only points to the potential importance of such interactions and the need for their adequate considerations (Art. 12, paragraph 1, point (c) and sections 3.8.3.4.4, and 3.9.3.4.4 of Annex I). However, it would have clarified the regulation if precise definitions for the used terms "*synergism*", "*antagonism*" and "*potentiation*" would have been included. We provide definitions in our review on the scientific state of the art for mixture toxicity assessment (Part 1 of this report).

As the application of the summation method and the application of CA might come to different conclusions concerning the hazard of the mixture of interest, it is unclear why section 4.1.3.5.2 of Annex I of the regulation requires the application of CA for classifying the regarded sub-mixture into an appropriate hazard category and then feeding this result into the summation method – instead of using the input data for a direct classification of each compound and using those results for the application of the summation method.

Although for the classification into acute toxicity categories specific guidance is given on how to handle data from range finding tests (Table 3.1.2 of Annex I), similar guidance is missing for the hazard class "*toxicity for the aquatic environment*".

Conclusions

The new CLP Regulation, which is based on the United Nations GHS system, provides detailed guidance on the hazard classification of commercial chemical mixtures for human health and the aquatic environment. Four different classification methods are described in the regulation: (i) test data for the whole mixture are available and are used for classifying the mixture as if it were a single substance (ii) "*bridging principle*s" are applied in order to classify a mixture on the basis of a similar mixture that is already classified, (iii) the amounts of classified individual substances in the mixture of interest are known and certain thresholds are put forward that, if exceeded, lead to a classification of the mixture ("*summation rule*"), and (iv) even though the individual components are not classified, there are toxicity data available for the mixture components; concentration addition is then used for estimating the joint toxicity of these compounds.

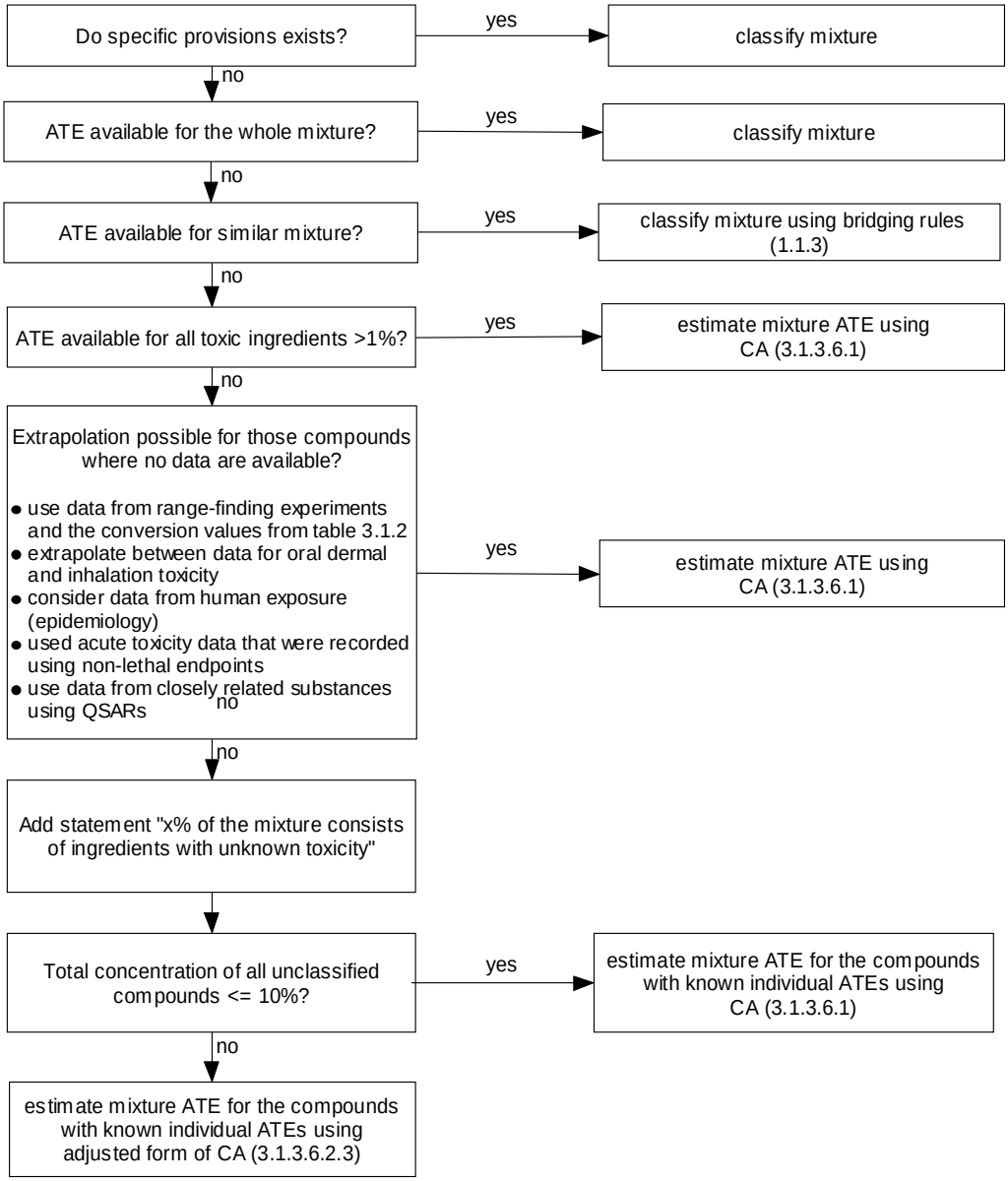
A tiered approach using the methods in the given order (i) - (iv) is applied for most hazard classes. However, for the hazard classes "*germ cell mutagenicity*", "*carcinogenicity*" and "*reproductive toxicity*" the classification is primarily based on the components in the mixture (method (iv)). Data on the mixture itself are only used on a case-by-case basis. The classification for "*chronic toxicity to the environment*" uses a combination of methods (i) - (iv).

Exposure Route	Category 1	Category 2	Category 3	Category 4
Oral (mg/kg body-weight) See Note (a)	ATE ≤ 5	5 < ATE ≤ 50	50 < ATE ≤ 300	300 < ATE ≤ 2 000
Dermal (mg/kg bodyweight) See Note (a)	ATE ≤ 50	50 < ATE ≤ 200	200 < ATE ≤ 1 000	1 000 < ATE ≤ 2 000
Gases (ppmV (1)) see: Note (a) Note (b)	ATE ≤ 100	100 < ATE ≤ 500	500 < ATE ≤ 2 500	2 500 < ATE ≤ 20 000
Vapours (mg/l) see: Note (a) Note (b) Note (c)	ATE ≤ 0,5	0,5 < ATE ≤ 2,0	2,0 < ATE ≤ 10,0	10,0 < ATE ≤ 20,0
Dusts and Mists (mg/l) see: Note (a) Note (b)	ATE ≤ 0,05	0,05 < ATE ≤ 0,5	0,5 < ATE ≤ 1,0	1,0 < ATE ≤ 5,0

(1) Gas concentrations are expressed in parts per million per volume (ppmV).

Table 1: Classification of individual compounds and mixtures according to their acute human toxicity.

From Regulation (EC) No 1272/2008. ATE=Acute toxicity estimate (LD/EC50)



3.1.3.6.1: CA for the ATE-estimation of the mixture, based on the compounds toxicity

$$\frac{100}{ATE_{Mix}} = \sum_{i=1}^{n} \frac{c_i}{ATE_i}$$

3.1.3.6.2.3: Adjusted CA for the ATE estimation of the mixture based on the compounds toxicity

$$\frac{100 - \sum_{j=1}^{q} c_j}{ATE_{Mix}} = \sum_{i=q+1}^{n} \frac{c_i}{ATE_i}$$

ATE: Acute Toxicity Estimate
n: Number of compounds in the mixture with an identified individual ATE_i
q: Number of compounds in the mixture with an unknown individual ATE_i
c: relative concentration in the mixture (in weight or volume %)

Figure 2: Tiered approach for the classification of a mixture for its acute toxicity to humans.

After Regulation (EC) No 1272/2008

Acute (short-term) aquatic hazard		
Acute Category 1	(Note 1)	
96 hr LC_{50} (for fish)	≤ 1 mg/l and/or	
48 hr EC_{50} (for crustacea)	≤ 1 mg/l and/or	
72 or 96 hr ErC_{50} (for algae or other aquatic plants)	≤ 1 mg/l.	(Note 2)
Chronic (long-term) aquatic hazard		
Chronic Category 1	(Note 1)	
96 hr LC_{50} (for fish)	≤ 1 mg/l and/or	
48 hr EC_{50} (for crustacea)	≤ 1 mg/l and/or	
72 or 96 hr ErC_{50} (for algae or other aquatic plants)	≤ 1 mg/l	(Note 2)
and the substance is not rapidly degradable and/or the experimentally determined BCF ≥ 500 (or, if absent, the log K_{ow} ≥ 4).		
Chronic Category 2		
96 hr LC_{50} (for fish)	> 1 to ≤ 10 mg/l and/or	
48 hr EC_{50} (for crustacea)	> 1 to ≤ 10 mg/l and/or	
72 or 96 hr ErC_{50} (for algae or other aquatic plants)	> 1 to ≤ 10 mg/l	(Note 2)
and the substance is not rapidly degradable and/or the experimentally determined BCF ≥ 500 (or, if absent, the log K_{ow} ≥ 4), unless the chronic toxicity NOECs are > 1 mg/l.		
Chronic Category 3		
96 hr LC_{50} (for fish)	> 10 to ≤ 100 mg/l and/or	
48 hr EC_{50} (for crustacea)	> 10 to ≤ 100 mg/l and/or	
72 or 96 hr ErC_{50} (for algae or other aquatic plants)	> 10 to ≤ 100 mg/l	(Note 2)
and the substance is not rapidly degradable and/or the experimentally determined BCF ≥ 500 (or, if absent, the log K_{ow} ≥ 4) unless the chronic toxicity NOECs are > 1 mg/l.		
'Safety net' classification		
Chronic Category 4		
Cases when data do not allow classification under the above criteria but there are nevertheless some grounds for concern. This includes, for example, poorly soluble substances for which no acute toxicity is recorded at levels up to the water solubility (note 3), and which are not rapidly degradable and have an experimentally determined BCF ≥ 500 (or, if absent, a log K_{ow} ≥ 4), indicating a potential to bioaccumulate, will be classified in this category unless other scientific evidence exists showing classification to be unnecessary. Such evidence includes chronic toxicity NOECs > water solubility or > 1 mg/l, or evidence of rapid degradation in the environment.		

Table 2: Classification of individual substances and mixtures for the hazard to the aquatic environment.

According Regulation (EC) No 1272/2008 (Table 4.1.0)

Acute toxicity

Category: Acute 1	
96 hr LC_{50} (for fish)	≤ 1 mg/l and/or
48 hr EC_{50} (for crustacea)	≤ 1 mg/l and/or
72 or 96hr ErC_{50} (for algae or other aquatic plants)	≤ 1 mg/l
Category: Acute 1 may be subdivided for some regulatory systems to include a lower band at $L(E)C_{50}$ ≤ 0.1 mg/l	
Category: Acute 2	
96 hr LC_{50} (for fish)	>1 - ≤ 10 mg/l and/or
48 hr EC_{50} (for crustacea)	>1 - ≤ 10 mg/l and/or
72 or 96hr ErC_{50} (for algae or other aquatic plants)	>1 - ≤ 10 mg/l
Category: Acute 3	
96 hr LC_{50} (for fish)	>10 - ≤ 100 mg/l and/or
48 hr EC_{50} (for crustacea)	>10 - ≤ 100 mg/l and/or
72 or 96hr ErC_{50} (for algae or other aquatic plants)	>10 - ≤ 100 mg/l
Some regulatory systems may extend this range beyond an $L(E)C_{50}$ of 100 mg/l through the introduction of another category	

Table 3: Classification for acute hazard to the aquatic environment according the UN GHS system

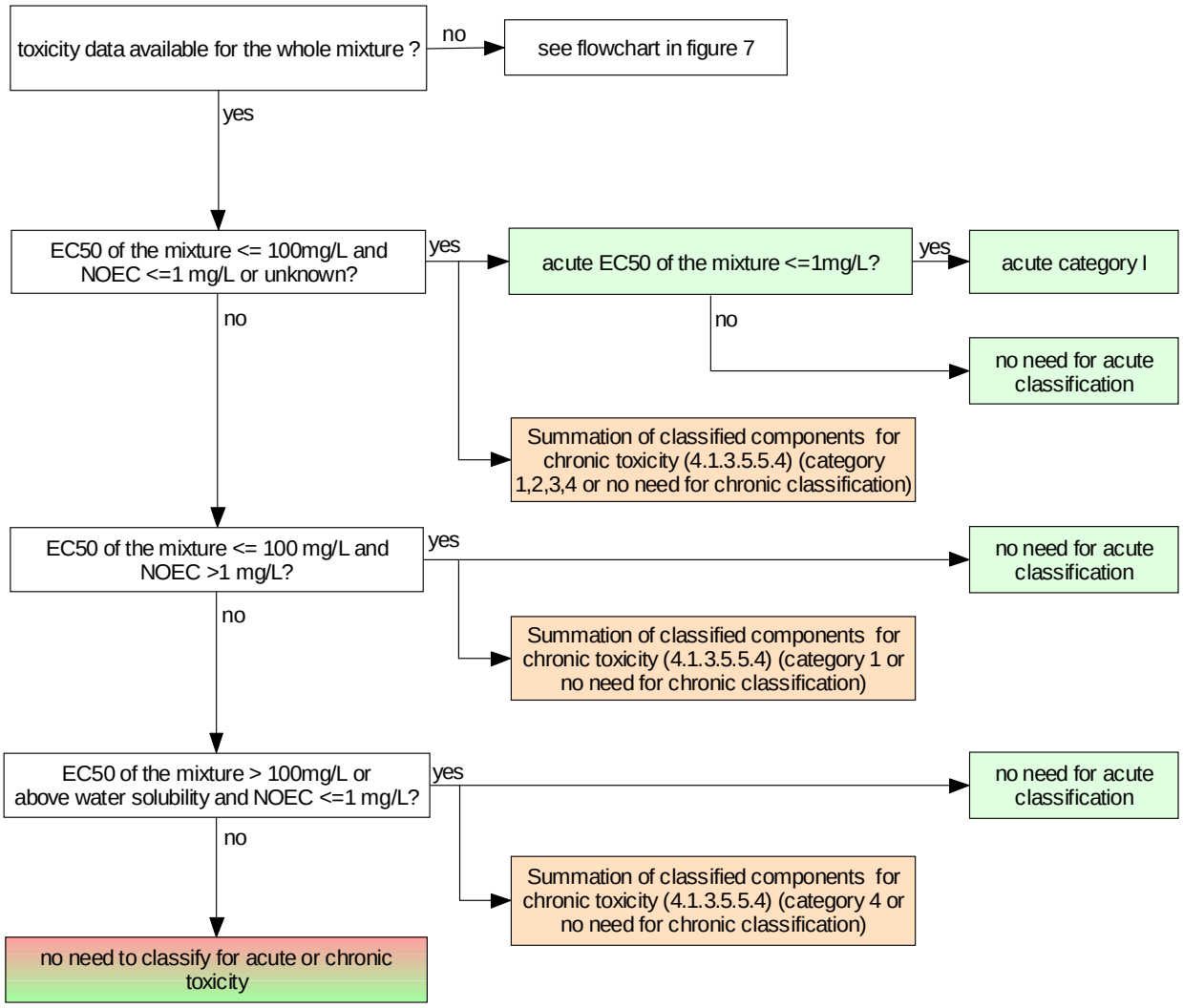


Figure 3: Classification of a mixture for aquatic hazard if acute toxicity data of the whole mixture are available.

According to Regulation (EC) No 1272/2008. NOEC=No Observed Effect Concentration, i.e. actual chronic toxicity data for the whole mixture

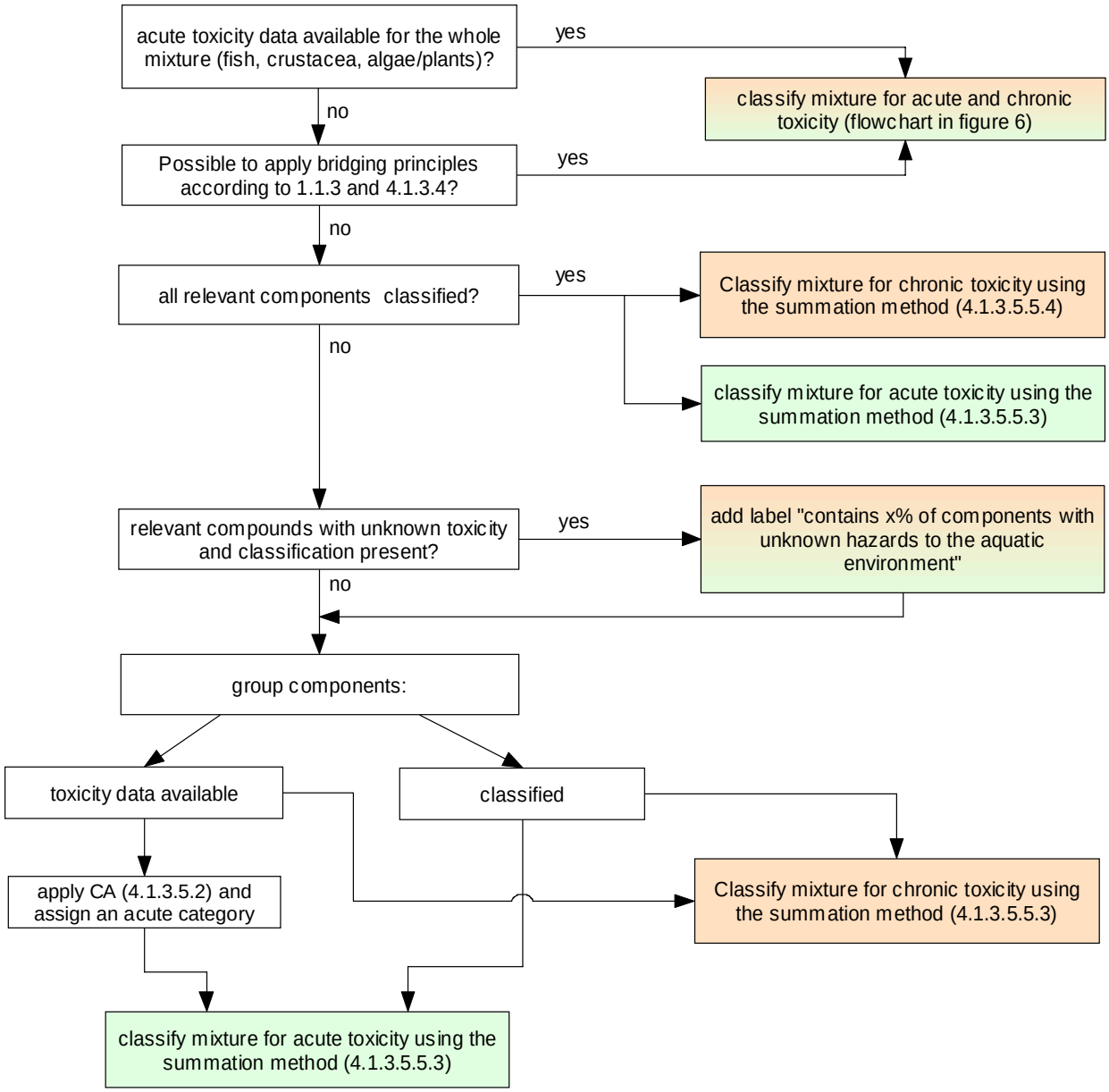


Figure 4: Tiered approach for the classification of mixtures for their acute and chronic environmental hazards if acute toxicity data are available for the mixture.

According to Regulation (EC) No 1272/2008

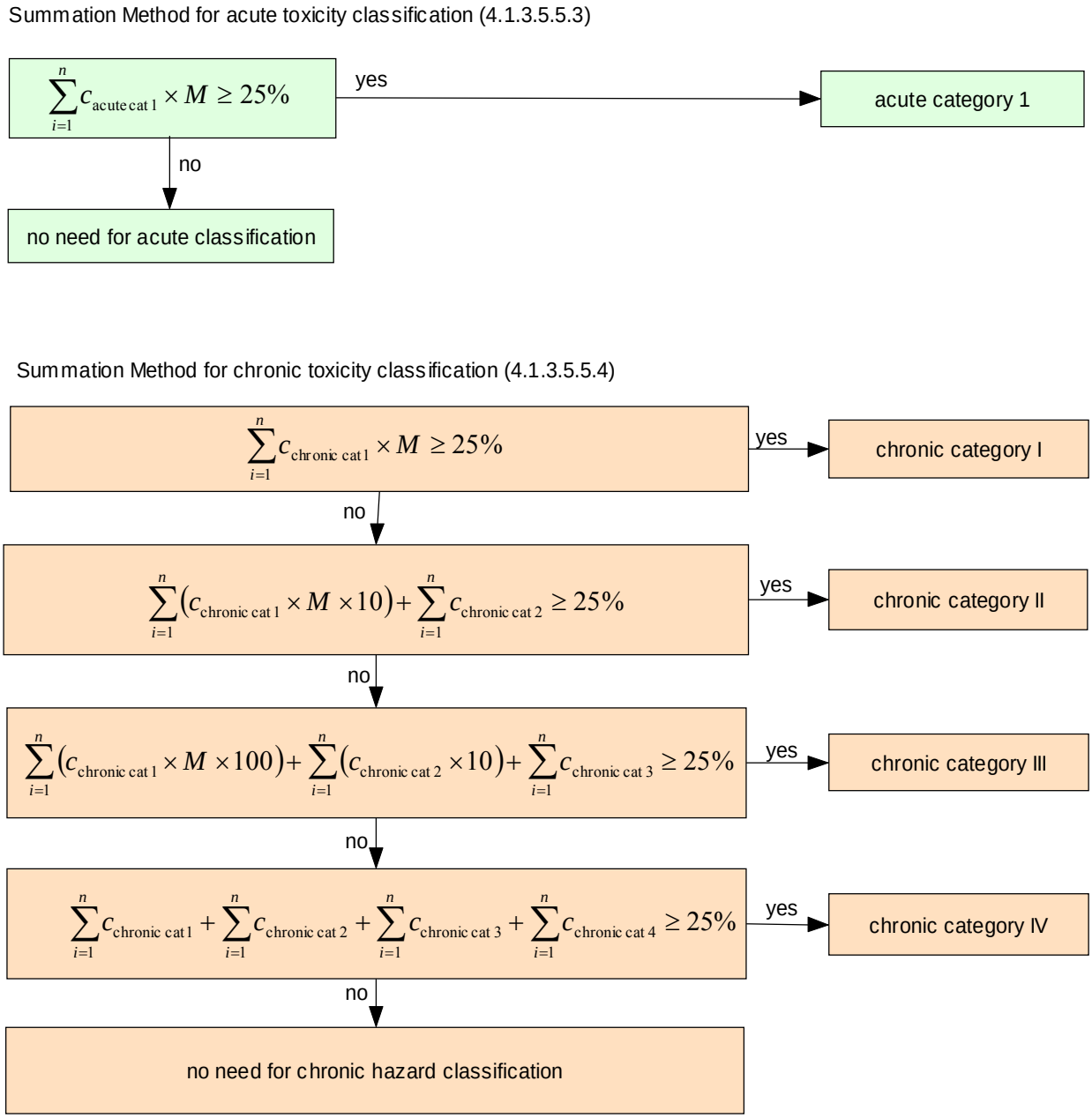


Figure 5: Summation method for mixture classification for acute and chronic hazard to the aquatic environment.

According to (Regulation (EC) No 1272/2008.

2.16 Pesticides authorisation – Directive 91/414/EEC

Act

Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market - OJ L 230, 19.8.1991, p. 1–32, as last amended by Commission Directive 2009/117/EC - OJ L 237, 9.9.2009, p. 11–14.

Purpose and regulatory context

Council Directive 91/414/EEC of 15 July 1991 lays down uniform rules on the evaluation, authorization, placing on the market and control of plant protection products in the EU. Annex I of the Directive gives a positive list of active substances that may be used in plant protection products. The use of other active substances is forbidden for this purpose.

There is a direct interplay between Directive 91/414/EEC on the authorisation of plant protection products and Regulation (EC) No 396/2005 on maximum residue levels (MRLs) of pesticides in or on food and feed (*see section 2.5*). Where appropriate, the setting of corresponding MRLs is a condition for granting authorisation to a plant protection product (Article 4, paragraph 1 (f)).

Council Directive 91/414/EEC applies without prejudice to provisions concerning the classification, packaging and labeling of dangerous substances and preparations under the Directives 67/548/EEC and 1999/45/EC, respectively (*see sections 2.12 and 2.13*).

Current revision

The Commission presented an initial proposal for a new legislation on pesticide authorisation in 2006 ((COM(2006) 388 final)[103] and a modified proposal in March 2008 (COM/2008/0093 final)[104]. On 15 September 2008, the Council adopted a Common Position (EC) No 25/2008[105] on the new legislation. On 13 January 2009 the European Parliament adopted the proposal[106]. On 24 September 2009 the new legislation was formally endorsed by the Council[107]. The legislation will soon be

[103] Proposal for a Regulation of the European Parliament and of the Council concerning the placing of plant protection products on the market {SEC(2006) 930} {SEC(2006) 931} - COM/2006/0388 final - COD 2006/0136

[104] Amended Proposal for a Regulation of the European Parliament and of the Council concerning the placing of plant protection products on the market - COM/2008/0093 final - COD 2006/0136

105 Common Position (EC) No 25/2008 adopted by the Council on 15 September 2008 with a view to the adoption of Regulation (EC) No …/2008 of the European Parliament and of the Council of … concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC – (2008/C 266 E/01)

[106] European Parliament, 13.1.2009, Position of the European Parliament adopted at second reading on 13 January 2009 with a view to the adoption of Regulation (EC) No .../2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC (EP-PE_TC2-COD(2006)0136). Available at http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//NONSGML+TC+P6-TC2-COD-2006-0136+0+DOC+PDF+V0//EN

[107] European Union, The European Parliament, The Council, Brussels, 10 September 2009, Document PE-CONS 3608/09. Regulation of the European Parliament and of the Council concerning the placing

adopted and published by the Commission. The Regulation will then apply 18 months later, so probably in spring 2011.

The new Regulation aims to reinforce the protection of public health and the environment and improve functioning of the internal market. The principles of procedures and criteria for the risk assessment of plant protection products will remain unchanged. However, the new legislation will introduce hazard-based cut-off criteria for the approval of active substances used in plant protection products. It will no longer be allowed to approve substances with certain serious, intrinsic properties. These are CMRs[108] (category 1 and 2), endocrine disrupters, POPs[109], and PBTs[110] or vBvBs[111], whereby the exact classification criteria and assessment procedures remain to be established for endocrine disrupters.

The new Regulation will introduce a clear requirement for the consideration of potential mixture effects of plant protection products and their residues on human health. Plant protection products *"…shall not have no (…) harmful effect on human health, (…), taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available;..."* (Article 4, paragraph 3, point (b)). The same applies to the residues of plant protection products (Article 4, paragraph 2, point (a)). Thereby the Regulation becomes consistent with the requirements laid down in Regulation (EC) 396/2005 on maximum residue levels of pesticides in or on food and feed (see section 2.5).

The requirement for *taking into account known cumulative and synergistic effects* will be limited to the assessment of hazards and risks for human health. With respect to environmental safety, the new Regulation sets only the general requirement that plant protection products and their residues *"… shall not have any unacceptable effect on the environment…"* (Article 4, paragraph 2, point (b), and paragraph 3, point (e). During the legislative procedure, the European Parliament's Committee on the Environment, Public Health and Food Safety recommended to amend this environmental requirement also by the phrase "..., *taking into account cumulative and synergistic effects and all relevant exposure routes to organisms in the environment; methods to assess such effects will be presented by the Authority*" (EP Document A6-0444/2008[112], Amendment 43). However, this initiative was unsuccessful.

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of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Available at http://register.consilium.europa.eu/pdf/en/09/st03/st03608.en09.pdf

[108] substances with cancerogenic, mutagenic or reproductive effects

[109] persistent organic pollutants

[110] persistent, bioaccumulating and toxic

[111] very persistent and very bioaccumulative

[112] European Parliament, Committee on the Environment, Public Health and Food Safety, 12.11.2008, Document A6-0444/2008. Recommendation for second reading on the Council common position for adopting a regulation of the European Parliament and of the Council on the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC (11119/8/2008 – C6-0326/2008 – 2006/0136(COD)). Available at http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//NONSGML+REPORT+A6-2008-0444+0+DOC+PDF+V0//EN

Protection goals and risk assessment

Plant protection products in the sense of the Directive are both (i) single *active substances* and (ii) preparations containing one or more active substances (Article 2). Plant protection products must have *no harmful effect on human or animal health, directly or indirectly (e.g. through drinking water, food or feed) or on* groundwater and they must have *no unacceptable influence on the* environment (Article 4). To achieve these protection goals, extensive toxicity testing and detailed risk assessments are carried out for both (i) individual active ingredients and there residues in food and the environment and (ii) formulated plant protection products. Details on corresponding data requirements are laid down for active substances in Annex II to the Directive and for the entire plant protection products in Annex III. Uniform Principles for Evaluation and Authorisation of Chemical Plant Protection Products are established in Annex VI to the Directive.

Mixture toxicity

Hazard and risk assessments of whole finished plant protection products, which typically are mixtures of chemicals, are preferably based on toxicity tests with the final product, which is a so-called *whole mixture approach*. However, such mixture testing and assessment is confined to the acute toxicity to humans or organisms in the environment that may come into direct contact with the product. Other hazard and risk assessments performed under the current Directive 91/414 usually refer to individual active ingredients and their residues in food and the environment. However, methodologies for the human risk assessment of multiple residues of different active ingredients in food are currently developed under the complementary Regulation (EC) No 396/2005 on maximum residue levels of pesticides in or on food and feed (*see section 2.5*). Accordingly, the forthcoming new Regulation on pesticide authorization will include an explicit requirement for *taking into account known cumulative and synergistic effects* on humans as detailed above.

Conclusion

Where mixtures of chemicals are authorised for use as a plant protection product, their joint acute toxicity is taken into consideration by a whole mixture approach. Risks of pesticide residues in food and the environment are currently assessed individually. In the future, hazards and risks arising from the joint exposure of humans to different pesticide residues shall be taken into consideration.

2.17 Biocides – Directive 98/8/EC

Act

Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market - OJ L 123, 24.4.1998, p. 1–63, as last amended by Directive 2009/107/EC of the European Parliament and of the Council of 16 September 2009 - OJ L 262, 6.10.2009, p. 40–42.

Purpose and regulatory context

Directive 98/8/EC of 16 February 1998 established a uniform regulatory framework for the placing of biocidal products on the EU market. Annexes I and IA to the Directive define a positive list of active substances that may be contained in biocidal products. Biocidal products must be authorized by Member States. Member States must not authorize products that contain active substances not listed in Annex I or IA.

The Directive does not apply to a series of product types falling under other sectorial pieces of EU product law, in particular medicinal products for human use, veterinary medicinal products, food and feed additives and food contact materials, cosmetic products, and plant protection products (*see the corresponding separate sections of this report*). In contrast, provisions concerning the classification, packaging and labeling of dangerous substances and preparations under the Directives 67/548/EEC and 1999/45/EC, respectively, may apply simultaneously (*see sections 2.12 and 2.13*).

The control of biocidal products under Directive 98/8/EC is similar to the control of plant protection products under Directive 91/414/EEC (*see section 2.16*). Both pieces of legislation share some basic features and text passages.

Current revision

On 12 June 2009, the Commission has presented a proposal for a new legislation *concerning the placing on the market and use of biocidal products* (COM(2009) 267 final)[113]. The existing Directive 98/8/EC shall be replaced by a Regulation that is directly binding and applicable in all Member States. The proposal seeks to simplify the procedures concerning the authorisation of biocidal products without reducing the high level of protection for the environment and human and animal health.

Annex VI of the proposed Regulation shall establish *Common Principals for the Evaluation of Dossiers for Biocidal Products*. These shall include the explicit requirement to assess the overall toxicity and ecotoxicity of a biocidal product that may contain various substances of concern. To this end, Point 52 of Annex VI of the commission proposal states: "*In each of the areas where risk assessments have been carried out, i.e. effects on man, animals, and the environment, the competent authorities shall combine the results for the active substance together with the results for any substance of concern to produce an overall assessment for the biocidal product itself. This should take account of any likely synergistic effects of the active*

[113] Commission of the European Communities, Brussels 12.6.2009. Proposal for a Regulation of the European Parliament and of the Council concerning the placing on the market and use of biocidal products - COM(2009) 267 final - 2009/0076 (COD)

substance(s) and substances of concern in the biocidal product". And point 53 adds: "*For biocidal products containing more than one active substance any adverse effects shall also be combined to produce an overall effect for the biocidal product itself.*"

Protection goals and risk assessment

Biocidal products in the sense of the Directive are both (i) individual *active substances* and (ii) preparations containing one or more active substances (Article 2). Biocidal products must have *no unacceptable effects* on *human health* or *animal health* or *the environment,* neither *directly* nor *indirectly*, neither themselves nor as a result of their residues (Article 5). To achieve these protection goals, extensive toxicity testing and detailed risk assessments are carried out for both (i) individual active substances and (ii) formulated biocidal products. Details on corresponding data sets required for the risk assessment and the authorisation procedure are laid down for active substances in Annexes IIA and IIIA, and for the entire biocidal products in Annexes IIB and IIIB to the Directive. Common Principles for the Evaluation of Dossiers for Biocidal Products are established in Annex VI to the Directive.

Mixture toxicity

Hazard and risk assessments of whole finished biocidal products, which typically are mixtures of chemicals, are preferably based on toxicity tests with the final product, which is a so-called *whole mixture approach*. However, such whole mixture testing is is usually confined to the acute toxicity to humans that may come into direct contact with the product. Other hazard and risk assessments performed under Directive 91/414 usually refer to individual substances.

Conclusion

Where mixtures of chemicals are authorised for use as a biocidal product, their joint acute toxicity is taken into consideration by a whole mixture approach. Other tests and assessments of toxicity and ecotoxicity under the Directive on biocides refer to individual substances, not to mixtures. The current Commission proposal for a future revised legislation includes the explicit requirement to assess the overall toxicity and ecotoxicity of the entire biocidal product and to take potential *synergistic effects* of product components into consideration.

2.18 Human medicines – Directive 2001/83/EC

Act

Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use - OJ L 311, 28.11.2001, p. 67–128, as last amended by Directive 2009/53/EC of the European Parliament and of the Council of 18 June 2009 - OJ L OJ L 168, 30.6.2009, p. 33–34.

Purpose and regulatory context

Before Directive 2001/83/EC came into force, all the Community provisions governing medicinal products were spread over a fragmented system of various Directives governing the placing on the market, production, labelling, classification, distribution and advertising of medicinal products for humans use. The Community code established by Directive 2001/83/EC brought them all together in a single legal instrument.

No medicinal product may be placed on the market of a Member State unless an authorisation has been issued by the competent authorities of that Member State or by the European Medicines Agency (EMEA).

Benefit / risk assessment and mixture toxicity

The Directive basically considers three types of risks of medicinal products (i) risks to the patients' health, (ii) risks to public health, and (iii) risks of undesirable effects on the environment. The assessment of these risks follows a *risk-benefit balance* approach. The applicant for marketing authorisation for a medicinal product shall *demonstrate that the potential risks are outweighed by the therapeutic efficacy of the product* (Recital 7). If the risk-benefit balance is not considered to be favourable, the marketing authorisation shall be refused (Article 26) or an existing authorisation may be suspended, revoked, withdrawn or varied (Article 116).

The Directive builds on the consideration that *standards and protocols for the performance of tests and trials on medicinal products are an effective means of control of these products and hence of protecting public health and can facilitate the movement of these products by laying down uniform rules applicable to tests and trials, the compilation of dossiers and the examination of applications* (Recital 8). As a consequence, *Analytical, Pharmacotoxicological, and Clinical Standards and Protocols in Respect of the Testing of Medicinal Products* are laid down in extensive detail in Annex I to the Directive.

Medicinal products may be single substances, but more typically they are combinations of substances. Toxicity studies required for the marketing authorization of medicinal products are partly performed for the active substance or the combination of active substances present in the product and partly for the finished product, as detailed in Annex I, Part I, section 4.2.3. Thus, toxicity of the entire product is taken into account.

Particular attention is paid to wanted and unwanted interactions of substances combined within a medicinal product, interactions of a medicinal product with other medicinal products administered concomitantly, and other forms of interaction which may affect the action of a medicinal product, such as interactions with alcohol, tobacco, and foodstuffs. Corresponding non-clinical and clinical studies on pharmaco-kinetic and pharmaco-dynamic interactions and reports on any relevant observations are part of the standard dossier requirements, as laid down in Part I of Annex I, in particular sections 4.2.2, 4.2.3, 5.2.3, and 5.2.4. Corresponding information for patients is an obligatory part of package leaflets (Article 59, p paragraph 1., point (c) (iii)).

Environmental risk assessments shall evaluate possible risks due to use and/or disposal of a medicinal product as laid down in Annex I, Part I, section 1.6. This *impact shall be assessed and, on a case-by-case basis, specific arrangements to limit it shall be envisaged* (Article 8, paragraph 3 (ca)). These provisions for environmental risk assessments do not specifically address any aspect of mixture toxicity.

Conclusion

Where medicinal products are chemical mixtures, their human toxicity is assessed as a whole. Additionally, interactions with other medicinal products and factors such as alcohol, tobacco, and foodstuffs are taken into account. Provisions on environmental risk assessments of medicinal products do not explicitly take mixture toxicity into account.

2.19 Veterinary medicines – Directive 2001/82/EC

Act

Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products - OJ L 311, 28.11.2001, p. 1–66, as last amended by Regulation (EC) No 596/2009 of the European Parliament and of the Council of 18 June 2009 - OJ L OJ L 188, 18.7.2009, p. 14–92.

The Community code relating to veterinary medicinal products was established in parallel to the Community code relating to medicinal products for human use (Directive 2001/83/EC, *see the previous section 2.18*) and shares some basic features and text passages.

Purpose and regulatory context

Directive 2001/82/EC *on the Community code relating to veterinary medicinal products* consolidated previously separated provisions on production, marketing, distribution and use of veterinary medicinal products in a single act.

No veterinary medicinal product may be placed on the EU market unless an authorisation has been issued. Depending on the type of the veterinary medicine a centralized authorisation by the European Medicines Agency (EMEA) may be either mandatory or optional. In cases where the centralized procedure is not mandatory, other national authorization procedures may be used alternatively.

For veterinary medicinal products intended for food producing species, marketing authorization is only granted, if the active substance or substances they contain are listed in Council Regulation (EEC) No 2377/90[114] on *maximum residue limits of veterinary medicinal products in foodstuffs of animal origin*, which has recently been replaced by the new Regulation (EC) No 470/2009[115] on *residue limits of pharmacologically active substances in foodstuffs of animal origin*. Veterinary medicinal products that are not authorized must not be administered to animals.

Benefit/risk assessment and mixture toxicity

The Directive basically considers three types of risks of veterinary medicinal products (i) risks to the animal, (ii) risks to human health, and (iii) risks of undesirable effects on the environment. In general, the assessment of these risks follows a *risk-benefit balance* approach. The applicant for marketing authorisation for a veterinary medicinal product *must demonstrate that the potential risks are outweighed by the*

[114] Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin

[115] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council - OJ L 152, 16.6.2009, p. 11–22

benefits due to efficacy. Failing such demonstration, the application must be rejected (Recital 11).

The Directive builds on the consideration that *standards and protocols for the performance of tests and trials on veterinary medicinal products are an effective means of control of these products and, hence, of protecting public health and can facilitate the movement of these products by laying down uniform rules applicable to tests and the compilation of dossiers, allowing the competent authorities to arrive at their decisions on the basis of uniform tests and by reference to uniform criteria…* (Recital 24). As a consequence, *Requirements and Analytical Protocol, Safety Tests, Pre-Clinical and Clinical for Tests of Veterinary Medicinal Products* are laid down in detail in Annex I to the Directive.

Veterinary medicinal products may be single substances, but more typically they are combinations of substances. Toxicity and ecotoxicity studies and assessments required for the marketing authorization of veterinary medicinal products are performed for the product, its active substances and relevant metabolites. They shall clarify (i) the potential toxicity of the product to the target animal, (ii) potential harmful effects of residues of the product or substance in foodstuffs to man, (iii) potential risks of the product to directly exposed humans (e.g. during administration), and (iv) potential risk to the environment resulting from the use of the product (Annex I, Title I, Part 3, Chapter I) Thus, toxicity and ecotoxicity of the entire product is taken into account.

During clinical studies, attention is paid to interactions with other medicinal products or feed additives, but in comparison to medicinal products for humans use, this point clearly plays a minor role. For ecotoxicity assessments the opposite relation is true. In comparison to medicinal products for humans use, the information requirements laid down in Annex I, Title I, Part 3, Chapter I, section 6 are clearly more specific and detailed. Mixture toxicity, however, is not addressed in these provisions for ecotoxicity assessments.

Conclusion

Where veterinary medicinal products are chemical mixtures, their toxicity to animals, humans and the environment as a whole is taken into consideration. Additionally, interactions with other veterinary medicinal products are taken into account with respect to effects in the target animals. Environmental mixture toxicity, potentially resulting from the joint occurrence of different residues of veterinary products or from the joint occurrence of pharmaceuticals and other pollutants, is not taken into account in the Directive.

2.20 Environmental impact assessment (EIA) - Directive 85/337/EEC

Act

Council Directive 85/337/EEC of 27 June 1985 on the assessment of the effects of certain public and private projects on the environment - OJ L 175, 5.7.1985, p. 40–48, as last amended by Directive 2009/31/EC of the European Parliament and of the Council of 23 April 2009 - OJ L 140, 5.6.2009, p. 114–135.

Purpose

Competent national authorities in EU member states must carry out an environmental impact assessment before giving consent to "*projects likely to have significant effects on the environment*". This rule was fixed in 1985 in Article 2 of Council Directive 85/337/EEC *on the assessment of the effects of certain public and private projects on the environment*. Disparities between national laws *"with regard to the assessment of environmental effects of public and private projects may create unfavourable competitive conditions"* (Recitals). As a consequence, the directive approximated national laws by introducing harmonized principles of the assessment of environmental effects. These principles refer to (i) the definition of project categories which should be subject to an EIA (Article 4), (ii) the content of the assessment (Article 3), (iii) the main obligations of the *developer*[116] of a project (Article 5), and (iv) the procedures to be followed, including consultation of interested parties and public participation. The directive covers a broad range of project categories with all sorts of environmental impacts, from agricultural and industrial installations to infrastructure projects such as dams, motorways, airports, power stations, etc., as indicated by Annexes I and II to the directive. This *inter alia* includes certain facilities which are or may be a source of emission of chemical pollutants during construction or operation.

Regulatory context

Categories of projects covered by the EIA directive and those covered by the IPPC directive 2008/1/EC on *integrated pollution prevention and control* (IPPC) overlap to a large degree. Member states may therefore provide for a single procedure in order to fulfill the requirements of both directives (Article 2a of the EIA directive). Furthermore, for certain projects covered by the EIA and IPPC directives, the so-called *Seveso II Directive* 96/82/EC *on the control of major-accident hazards involving dangerous substances*, may apply additionally. Additionally, there may be some interrelationship with the voluntary EMAS[117] scheme and with the SEA directive on strategic environmental assessment[118]. as has been detailed by the IMPEL NETWORK (1998)[119] and by Sheate et al (2005)[120], respectively.

[116] The *developer* means the applicant for authorization for a private project or the public authority which initiates a project (Article 1).
[117] Regulation (EEC) No 761/2001 of the European Parliament and of the Council of 19 March 2001 allowing voluntary participation by organisations in a Community eco-management and audit scheme (EMAS) (replaced Council Regulation (EEC) No 1836/93 of 29 June 1993)
[118] Directive 2001/42/EC of the European Parliament and of the Council of 27 June 2001 on the assessment of the effects of certain plans and programmes on the environment
[119] IMPEL NETWORK (1998) Interrelationships between IPPC, EIA, SEVESO Directives and EMAS Regulation. European Union Network for the Implementation and Enforcement of Environmental Law,

Current revision

Currently there is no official proposal for a revised legislation that would replace and/or repeal Directive 85/337/EEC.

Protection goals

The scope of the directive is very broad and covers any type of potential impact on man and the environment. The EIA shall "*identify, describe and assess (…) the direct and indirect effects*" on "*human beings, fauna and flora, soil, water, air, climate and the landscape, material assets and the cultural heritage*", as well as "*the interaction between the factors*" (Article 3).

Hazard and risk assessment

Unlike specific pieces of chemicals regulation, the EIA directive does not use the concepts of *hazard* and/or *risk* assessment, but operates with *impact* and *effect* assessment as key terms.

With respect to chemicals, the information required from the *developer* of a project shall, *inter alia* and if relevant and reasonable, include (i) *an estimate, by type and quantity, of expected residues and emissions (water, air and soil pollution,* …), and (ii) a *description of the likely significant effects (…) on the environment resulting from (…) the emission of pollutants, (…)*, (iii) a description of *forecasting methods used to assess the effects* (…) and a description of *measures envisaged to prevent, reduce and where possible offset any significant adverse effects* (…) (Annex IV, paragraphs 1, 4 and 5, in conjunction with Article 5, paragraph 1).

Based on this information, the EIA of the competent authority shall *identify, describe and assess* the *effects* in *an appropriate manner* (Article 3). The directive does not prescribe any specific procedures, methods, or criteria for this effect assessment. This specification of the assessment is the privilege of the competent national authorities and corresponding national legislation.

As a support to member states, the European Commission has published guideline documents for the implementation of the EIA, addressing several steps and aspects of an EIA procedure[121]. Corresponding to the very general nature of the directive, these guidelines also address procedural aspects on a very general level. They do not detail any rules for assessing the effects of pollutants.

Final report, December 1998. Available at: http://ec.europa.eu/environment/eia/eia-studies-and-reports/impel-full-text.pdf

[120] Sheate W, Byron H, Dagg S, Cooper L (2005) The relationship between the EIA and SEA Directives. Final Report to the European Commission. Imperial College London Consultants, August 2005. Available at: http://ec.europa.eu/environment/eia/pdf/final_report_0508.pdf

[121] Available at: http://ec.europa.eu/environment/eia/home.htm

Mixture toxicity

An EIA for a project shall include the *interactions* between the effects on factors, such as human beings, fauna and flora, etc. (Article 3). The *description of the likely significant effects*, which is required from the developer of a project, shall *cover the direct effects and any indirect, secondary, cumulative, short, medium and long-term, permanent and temporary, positive and negative effects of the project* (Footnote (1) to paragraph 4 of Annex IV). Furthermore, in deciding about the need for an EIA for a specific project, considerations shall inter alia have particular regard to *the cumulation with other projects* (Annex III, paragraph 1, in conjunction with Article 4).

As a consequence, *likely significant effects* from mixtures of pollutions, if relevant for specific projects, should be covered by the scope and the requirements outlined in the Directive. However, whether and how such mixture effects might be identified, described and assessed is entirely left to the competent national authorities and corresponding national legislation.

Guidelines for the Assessment of Indirect and Cumulative Impacts as well as Impact Interactions under the EIA directive have been published[122]. However, corresponding to the general nature of the directive, this guideline sets out rules and options on a general procedural level of EIA enforcement. It is of no practical relevance for mixture toxicity assessments.

Conclusion

Toxicity of pollutant mixtures to humans or to organisms in the environment can be subject of an impact assessment carried out under the EIA directive, if considered as one of the likely significant effects of residues or emissions from a project or a cumulation of projects by competent national authorities. Whether and how such effect assessments for chemical mixtures are performed as part of an EIA procedure, is an affair of competent national bodies and corresponding national legislation.

This conclusion is reached by isolated consideration of the EIA directive, not anticipating any complementary pieces of EU legislation that in case of a specific project may apply simultaneously. The IPPC directive is addressed in a separate section (2.21). Further considerations of other complementary pieces of legislation mentioned in this section were out of the scope of this study.

The analysis of the EIA directive leads to the question, whether assessments of mixture toxicity actually play any role in the practice of environmental impact assessments on the member states level, and if so, in which way this is done. The results of the survey on approaches and practical experiences in assessing the mixture toxicity of complex environmental samples in EU Member States, which are documented in Part 3 of this report, provide some indicative insights into this field.

[122] European Commission (1999) Guidelines for the Assessment of Indirect and Cumulative Impacts as well as Impact Interactions. Office for Official Publications of the European Communities, Luxembourg. Available at: http://ec.europa.eu/environment/eia/eia-studies-and-reports/guidel.pdf

2.21 Integrated pollution prevention and control (IPPC) - Directive 2008/1/EC

Act

Directive 2008/1/EC of the European Parliament and of the Council of 15 January 2008 concerning integrated pollution prevention and control (Codified version) - OJ L 24, 29.1.2008, p. 8–29, as amended by Directive 2009/31/EC of the European Parliament and of the Council of 23 April 2009 - OJ L 140, 5.6.2009, p. 114–135.

The new codified version repealed and replaced Council Directive 96/61/EC of 24 September 1996 concerning integrated pollution prevention and control - OJ L 257, 10.10.1996, p. 26–40, as last amended by Regulation (EC) No 166/2006 of the European Parliament and of the Council - OJ L 33, 4.2.2006, p. 1–17. The codified act includes all amendments to the previous Directive 96/61/EC and introduces some linguistic changes and adaptations, but the substance has not been changed.

Purpose

Industrial and agricultural activities with a high potential for pollution require a permit. This rule was fixed in 1996 in Articles 4 and 5 of Council Directive 96/61/EC concerning integrated pollution prevention and control and renewed in the corresponding new codified version 2008/1/EC. After a transitional period, the directive became fully implemented by October 2007, the deadline by which all relevant existing installations had to be brought into conformity with the requirements of the IPPC directive. The directive defines the industrial activities concerned (Article 1 and Annex I), establishes a general procedure for the authorization of such activities by member states (Articles 6-8), and sets minimum requirements to be included in all permits (Article 9 in conjunction with Articles 3 and 10). The aim is *to prevent (…) or reduce emissions in the air, water and land (…) in order to achieve a high level of protection of the environment taken as a whole* (Article 1).

Application of the *best available techniques* (BAT) is a governing principle for all measures taken against pollution under the IPPC directive (Article 3(a)). *"Best" means most effective in achieving a high general level of protection of the environment as a whole* (Article 2, paragraph 12(c)). *Emission limit values* (ELV) are a key instrument for pollution reduction under the directive. Every permit issued under the directive shall include such ELVs *for polluting substances (…) likely to be emitted from the installation concerned in significant quantities* (Article 9, paragraph 3). ELVs are based on the *best available techniques* (BAT).

Regulatory context

Industrial activities covered by the IPPC directive overlap to a large degree with project categories subject to an environmental impact assessment (EIA) under Council Directive 85/337/EEC *on the assessment of the effects of certain public and private projects on the environment* and both directives may apply side by side (Article 1 of the IPPC directive). The IPPC Directive focuses on the environmental impact of installations during operation. In contrast to an EIA, it does not cover the construction phase and it does not cover infrastructure projects. For certain installations under the IPPC directives, the so-called *Seveso II Directive* 96/82/EC *on the control of major-*

accident hazards involving dangerous substances, may apply additionally. The aim of the Seveso II Directive is the prevention of major accidents which involve dangerous substances and the limitation of their consequences for man and the environment. Additionally, there may be interrelationships of the IPPC Directive with the voluntary EMAS[123] scheme as has been detailed by the IMPEL NETWORK (1998)[124].

ELVs for a specific installation are set by competent national authorities, supported by a Community-wide exchange of technical information on BAT, catalysed by the European IPPC Bureau[125]. However, where member states do not choose to apply any stricter or more detailed rules, limit values which have been set in 14 different sectoral EU directives listed in Annex I to the IPPC directive or in any other Community legislation apply as minimum emission limit values (Article 19, paragraph 2). The list of complementary pieces of EU legislation that must be taken into consideration under the IPPC directive is further extended by Article 10 which states: *Where an environmental quality standard requires stricter conditions than those achievable by the use of the best available techniques, additional measures shall in particular be required in the permit …* The forthcoming Directive *on environmental quality standards in the field of water policy*[126] is an example for such related acts.

Based on the IPPC directive, the Commission established the European Pollutant Emission Register (EPER)[127], which was succeeded by the European Pollutant and Transfer Register (E-PRTR)[128]. These registers document information on pollutant emissions from IPPC establishments.

Current revision

In 2007, the Commission adopted a proposal for a revised legislation *on industrial emissions*[129]. The proposal recasts the IPPC directive and six related acts into a single legislative instrument. This will not affect the principles of the legislation. In particular the instrument of BAT-based ELVs and the interrelation with environmental quality standards set in other pieces of EU legislation will be unchanged. On 25 June 2009 the Council of the European Union reached political

[123] Regulation (EEC) No 761/2001 of the European Parliament and of the Council of 19 March 2001 allowing voluntary participation by organisations in a Community eco-management and audit scheme (EMAS) (replaced Council Regulation (EEC) No 1836/93 of 29 June 1993)

[124] IMPEL NETWORK (1998) Interrelationships between IPPC, EIA, SEVESO Directives and EMAS Regulation. European Union Network for the Implementation and Enforcement of Environmental Law, Final report, December 1998. Available at: http://ec.europa.eu/environment/eia/eia-studies-and-reports/impel-full-text.pdf

[125] http://eippcb.jrc.ec.europa.eu/

[126] Directive of the European Parliament and of the Council on environmental quality standards in the field of water policy, amending and subsequently repealing Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC and 86/280/EEC, and amending Directive 2006/60/EC, adopted by the Council 18 September 2008, PE-CONS 3644/08

[127] 2000/479/EC: Commission Decision of 17 July 2000 on the implementation of a European pollutant emission register (EPER) according to Article 15 of Council Directive 96/61/EC concerning integrated pollution prevention and control (IPPC)

[128] Regulation (EC) No 166/2006 of the European Parliament and of the Council of 18 January 2006 concerning the establishment of a European Pollutant Release and Transfer Register and amending Council Directives 91/689/EEC and 96/61/EC

[129] COM/2007/0844 final: Proposal for a Directive of the European Parliament and of the Council on industrial emissions (integrated pollution prevention and control) (Recast) [COM(2007) 843 final] [SEC(2007) 1679] [SEC(2007) 1682]

agreement on a revised text of the future legislation (Council document 11885/09)[130]. The following text still refers to the existing Directive 2008/1EC.

Protection goals

As stated in Article 1, the IPPC directive aims *to achieve a high level of protection of the environment taken as a whole*. To this end, the directive takes an *integrated approach*, i.e. emissions into air, water or soil are controlled in a single permit, with the aim to avoid the shifting of pollution between various environmental media, which otherwise might be encouraged by a fragmented approach (Recitals 8 and 9).

Hazard and risk assessment

Under the IPPC directive, the term *pollution* is not confined to the emission of *substances (…) which may be harmful to human health or the quality of the environment*, but also includes *vibrations, heat or noise* on the emission side, and *damage to material property, or impair or interfere with amenities and other legitimate uses of the environment* on the impact side (Article 2, paragraph 2). The IPPC aims to reduce pollution to a technical minimum as a precautionary measure. BAT-based ELVs are an important instrument of that strategy. Toxicological hazard and/or risk assessments do not play a direct role in this approach.

However, due to the outlined interweavement of the IPPC with other pieces of legislation, hazard and/or risk assessments of chemicals performed under related national and/or European law play an indirect role in the IPPC. This may not only apply to the identification of emitted substances as potentially *harmful to human health or the quality of the environment*, but also more specifically to the setting of some ELVs, and in particular to *environmental quality standards* (EQS) which have to be considered under the IPPC. EQS set under the forthcoming directive *on environmental quality standards in the field of water policy* (see above) may serve as an example for such mechanisms: A permit for emissions to waters under the IPPC regime will have to ensure compliance with this EQS directive, the character and the purpose of the EQS and the procedure for their determination in turn are set out in the Water Framework Directive (WFD)[131], the WFD in turn refers to the Technical Guidance Document (TGD) for risk assessments under the Existing Substances Regulation (EEC) No 793/93 as an agreed methodology for risk assessment (WFD, Annex V, section 1.2.6).

Mixture toxicity

The setting of ELVs by competent national authorities or by complementary EU legislation is not confined to single pollutants, *but emission limit values may also be laid down for certain groups, families or categories of substances* (Article 2, paragraph 6). In addition to the BAT principle, considerations on mixture toxicology and approaches used in mixture toxicology may play a role in the setting of such

[130] Council of the European Union, Brussels, 8 July 2009, Document 11885/09. Proposal for a directive of the European Parliament and of the Council on industrial emissions (integrated pollution prevention and control) (Recast). Available at http://register.consilium.europa.eu/pdf/en/09/st11/st11885.en09.pdf

[131] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy

group ELVs. Among the minimum ELVs defined in complementary pieces of EU legislation listed in Annex II to the IPPC directive, there is one prominent example. This is the Toxic Equivalence Factor approach (TEF) used in the setting of ELVs for dioxins and furans emitted from waste incineration plants under Directive 200/76/EC[132].

Another aspect of the IPPC that is worth considering in the context of mixture toxicity comes from the interrelation with the EPER and E-PRTR registers. These data basis may provide valuable information for modelling approaches to the assessment of cumulative exposure to multiple pollutants, one of the current bottlenecks in assessing potentially resulting cumulative risks.

Conclusion

ELVs set under the IPPC are usually based on the BAT principle without any direct considerations of toxicity. However, in case of group ELVs, considerations on mixture toxicology or use of approaches developed in mixture toxicology may play an additional role. On the level of Community legislation there is one example: the use of the TEF approach in the setting of ELVs for dioxins and furans.

This conclusion is reached by isolated consideration of the IPPC directive, including the 14 complementary acts that are directly mentioned in Annex II of the directive, not anticipating any other complementary pieces of EU legislation that in case of a specific installation or specific types of pollution may apply simultaneously. The EIA directive is addressed in a separate section (2.20). Further considerations of other complementary pieces of legislation mentioned in this section were out of the scope of this report.

The analysis of the IPPC directive leads to the question, whether mixture toxicity is an aspect actually taken into account in any permits given under the IPPC framework by competent national authorities. The results of the survey on approaches and practical experiences in assessing the mixture toxicity of complex environmental samples in EU Member States, which are documented in Part 3 of this report, provide some indicative insights into this field.

[132] Directive 2000/76/EC of the European Parliament and of the Council of 4 December 2000 on the incineration of waste

3. Summary of relevant provisions

With respect to their scope of dealing with mixture toxicity and combined exposure to multiple chemicals, the 21 examined Directives and Regulations may be summarized as follows:

Many products that are subject to the various directives and regulations are in fact mixtures of chemicals, as are the commercial preparations that reach the market. Regulatory toxicity assessments of such commercial mixtures are based on safety assessments of individual ingredients, on whole mixture testing, or on component-based approaches which assume concentration additivity or the simple summation of the amounts of individual toxic chemicals in the preparation. Which of these approaches is applied depends on the type and use of products and the relevant pieces of legislation.

However, assessments of cumulative risks to humans and the environment that may result from simultaneous or sequential exposure to multiple chemicals from different sources via multiple routes are outside the scope of the Regulations that were examined in this report.

Four out of the 21 pieces of legislation that were examined in this study appear to be particularly noteworthy from a mixture toxicity perspective:

- The REACH Regulation (EC) No 1907/2006, although mainly focusing on individual chemicals, provides guidance on how substances that are in fact mixtures are to be assessed for their PBT/vPvB properties. This applies to isomeric mixtures, multi-constituent substances (MCS), and substances of unknown or variable composition (UVCB), such as petroleum products or surfactants for instance.

- Regulation 1272/2008 on the classification, labeling and packaging of substances and mixtures makes detailed prescriptions for the toxicity assessment of intentionally prepared commercial mixtures. The approaches prescribed are (i) whole mixture testing, (ii) concentration addition, or (iii) the summation method, which is the toxicity-weighted summation of the relevant mixture components and the subsequent analysis whether or not the relative amount of relevant components is above or below a pre-defined threshold.

- Regulation (EC) No 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin provides incentives for the development of methodologies for mixture risk assessment. The task of developing viable assessment methods has been assigned to EFSA.

- Directive 2008/1/EC concerning integrated pollution prevention and control (IPPC) refers to the directive on waste incineration as a complementary piece of legislation, and this in turn includes emission limit values for mixtures of dioxins and furans that are based on the toxicological concept of Toxic Equivalence Factors (TEF).

4. Gaps and recommendations

Strengthening the legal mandate for mixture risk assessments

The analysis of the scientific state of the art of mixture toxicology in Part 1 of this report shows that there is both the need as well as sufficient know-how to assess the risks that may result from the combined exposure of humans and the environment to multiple chemicals. The question as to how this scientific knowledge might be best transferred into appropriate regulatory approaches is, however, not at all trivial.

The development of appropriate procedures and methodologies that are adequate in a specific legal context may require considerable additional efforts. As detailed in Part 4 of this report, the US EPA for instance spent many years on the development of its guidelines for the health risk assessment of chemical mixtures, and this would not have happened without an explicit legal mandate that required the agency to do so. In Europe, since 2006, EFSA has been working on a methodology for assessing cumulative risks that may result from human exposure to combinations of pesticide residues, taking advantage of the work previously carried out in the US. Multiple pesticide residues in food had been an issue of concern and debate over many years before, but the targeted development of corresponding risk assessment methods for regulatory use did not start before a clear legal incentive was given in the pesticide residues regulation upon the initiative of the European Parliament in 2005. A lesson to be drawn from these events is that consistent and clear mandates are needed for taking mixture toxicity into account in the numerous pieces of legislation that contribute to the protection of human health and the environment from chemical risks. This seems to be an essential prerequisite for better dealing with the challenging issue of potential "cocktail effects".

Exploring options for the assessment of combined exposures within media oriented pieces of environmental legislation

Most of the 21 Directives and Regulations examined in this part of the report are substance- or product-oriented pieces of legislation. They control single and multi-constituent substances, preparations of chemicals and products containing chemicals that are intentionally produced and placed on the market. Typically, they assess hazards and risks of these substances and products as if they were present in isolation. The assessment of complex exposure situations of humans and the environment resulting from multiple substances and products is out of their scope and difficult to integrate.

Mixture risk assessments require a definition of the mixture of concern. Substance- and product-oriented regulations are therefore appropriate for assessing mixtures that are already present in such substances or products. Process-oriented pieces of environmental legislation that control emissions from production, transportation, and recycling processes, such as the IPPC, provide a basis for assessing mixtures of chemicals released from a definite source. The best starting point for assessing those mixtures that finally occur in environmental media, in biota, and in humans, however, should be given by corresponding media-, site-, or population-oriented elements of legislation, such as for instance the Water Framework Directive, the Marine Strategy Directive, or the proposed Soil Directive. These types of legislation were outside the

scope of this report. Options for the advancement of these pieces of legislation with the aim of taking account of, and improving risk assessments of realistic complex exposure scenarios should be explored.

State of the Art Report on Mixture Toxicity

Final Report

Part 3: Survey on approaches and practical experiences in assessing the mixture toxicity of complex environmental samples and waste samples in EU Member States

22 December 2009

Study Contract Number	**070307/2007/485103/ETU/D.1**
Contractor	**The School of Pharmacy** **University of London (ULSOP)**
Sub-Contractors	**Göteborg University (UGOT)** **Faust & Backhaus** **Environmental Consulting GbR (FBEC)**
Responsible Scientists	**Prof. Dr. Andreas Kortenkamp (ULSOP)** **Assoc.-Prof. Dr. Thomas Backhaus (UGOT)** **Dr. Michael Faust (FBEC)**

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1. Summary

Many environmental authorities and collaborating research institutions in EU member states have extensive experience with whole mixture testing approaches. In particular these approaches are used for toxicity assessments of waste water, and waste water treatment plant effluents for the control of emission permits under IPPC. They are also applied to practically all other types of environmental samples for the purpose of general environmental monitoring, risk assessment of contaminated sites, priority setting for risk reduction measures, and the control of remediation works and their success.

The TEF (Toxic Equivalence Factor) approach for the assessment of mixtures of dioxins, furans, and dioxin-like PCBs is a component-based approach in routine application. Uses of other component-based approaches or the application of the TEF approach to other groups of compounds are typically confined to special compound groups such as phenols, PAHs, and estrogens for instance. Certain national research institutions actively engaged in the field of mixture toxicology directly support their environmental authorities. These institutions have experience with practically all types of approaches to mixture testing and assessment and they apply those flexibly to specific issues. Examples are the National Institute of Public Health and the Environment (RIVM) in the Netherlands or INIA, Division of Ecotoxicology and Environmental Risk Assessment, in Spain.

2. Terms of reference

The task for this part of the study was

- to analyze practical approaches for assessing the toxicity of
  - environmental samples (air, water, sediment, soil, biota) and/or
  - waste samples (hazardous waste, municipal and industrial wastewater)

  that are currently used in relevant EU member States.

The analysis should include both types of approaches,

- whole mixture approaches (i.e. direct toxicity testing of the mixture) and
- component-based approaches (i.e. estimating the total toxicity from information on identified components only).

3. Approach and structure of the report

The task was addressed by two different, subsequently applied means:

(i) Performance of a general survey on practical approaches used in member states by means of a **written questionnaire** addressed to environmental authorities in all EU member states.

(ii) Performance of oral **expert interviews** with representatives of three selected member states authorities with the aim to deepen the insight gained by the written survey.

The results obtained by means of the written questionnaire and the subsequent expert interviews are documented in extensive detail in the following sections 4 and 5. The questionnaire used in the written survey and a glossary of terms that accompanied the questionnaire are documented in Annexes A and B, respectively.

4. Written survey

4.1 Questions and addressees

Design of the questionnaire

The written survey was performed by means of the **questionnaire** documented in **Annex A** to this part of the study report. It was designed to capture all kinds of approaches and practical experiences that could have some relevance in the wide field of assessing the mixture toxicity of complex environmental samples and waste samples, without any restrictions, neither to specific toxicological or ecotoxicological endpoints nor to the specific purposes of the assessment.

The questionnaire comprised 12 main questions plus an optional field 13 for any general comments. The questionnaire was structured in three main parts, asking for *(i)* whole mixture approaches, *(ii)* component-based approaches, and *(iii)* general experience in the field of mixture toxicity assessment.

The questionnaire was designed to definitely deliver a base set of information by means of a multiple choice part, and to provide facultative in-depth information by means of corresponding free text options or requests. To this end, for each of the twelve questions a logically closed set of response options was offered for answering by tick marks. For each pre-defined response option, the opportunity for comments or specifications was offered. In some cases additional free text information was explicitly requested. This applies to the assignment of biotests to the sample types for which they are used (question 4), and to the assignment of component-based approaches to sample types, substance groups and endpoints to which they are applied (question 7).

The questionnaire was accompanied by a brief explanation of background and scope in an accompanying letter, and by a **glossary of terms**. This glossary is documented in **Annex B** to this part of the study report. The glossary gave exact definitions of all key terms used in the questionnaire for different types of component-based approaches, including references to the literature where these terms originate from. The necessity to include this glossary results from the unfortunate fact that a confusing variety of different terms is used in the literature to denote different concepts, models, and assumptions on the joint action of toxicants. Often these terms are used with differing, sometimes even with contradictory, and very often with insufficient definition. The aim was to avoid the almost complete confusion that may result from this fact and to insure comparability of answers to the questionnaire.

Addressees and administration of the questionnaire

The questionnaire was addressed to environmental authorities in all EU member states. It was administered to the intended recipients by DG Environment. It was distributed by Email and directly channelled to persons in the target institutions that are nominated experts in one or more of the fields of soil, water, air quality, waste, and REACH. These were asked for their support by filling in the questionnaire either themselves or by forwarding it to competent persons and organisation units. They were further asked to return the completed questionnaire directly to the study leader (Prof. Kortenkamp).

The questionnaires were sent out in January and early February 2009. The latest response was received in mid May 2009.

4.2 Responding institutions and their expertise

We received a total of 25 questionnaires, completed by different kinds of institutions from 14 out of the 27 EU member states.

Table 1 gives a complete list of the responding institutions, the countries in which they are situated, and the contact persons that kindly filled in the questionnaire and/or collated the answers within their institutions.

Table 1 also gives short code names that were assigned to each questionnaire, basically consisting of the two-letter internet code for the respective country (e.g. NL), and additionally a number if different institutions in the same country completed the questionnaire (e.g. DK1, DK2, DK3,…). **For brevity and simplicity, the code names defined in Tab. 1 are used to denote responding institutions and their answers to the questionnaire throughout the following report text and all subsequent tables**.

The 25 questionnaires represent a heterogeneous set of different types of institutions, different sizes of organisation units, and different fields of competence. Partly the questionnaires were directly completed by ministries or authorities or individual departments of such governmental organisations, partly they were forwarded to collaborating national research institutions or university departments.

The questionnaire obtained from the Netherlands (**NL**) differs from all others, because it represents an integrated reply. As was written in an accompanying letter, *the questionnaire has been sent to various Dutch research institutes and policy makers and attempts to gain an insight in the present state of play concerning mixture toxicity within the Netherlands.* The input received from all the services approached was compiled in a single consolidated questionnaire, thereby representing an answer for the whole Netherlands. This process was kindly organised by Dr Martien P. M. Janssen from the Netherland's National Institute of Public Health and the Environment (RIVM) (see also the expert interview with Dr Janssen in section 5.1).

In Denmark, the questionnaire was directly completed by the Danish Environmental Protection Agency (EPA) (**DK1**), and additionally the Danish EPA asked several relevant persons and institutions to answer the questionnaire. As a result we got a total

of six different questionnaires from Denmark (**DK1** – **DK6**). Altogether this appears to give a good insight into relevant activities and competences in the field of mixture toxicity in Denmark. However, as was written in an accompanying letter, not all persons and institutions that were approached by the Danish EPA responded to the inquiry. Thus, the picture obtained may be somewhat less complete than in the case of the Netherlands (see also the corresponding expert interview with Dr. Henrik Tyle from the Danish EPA in section 5.3).

In the cases of the other 12 member states from which responses were received, the pictures obtained might be a bit more fragmented, as the responding institutions from a single country do not necessarily cover all relevant aspects in terms of the different environmental media (water, soil, air) and waste types or in terms of human mixture toxicity and ecotoxicity of chemical mixtures. However, taken altogether the picture obtained by the written survey might be considered as a good cross section through the types of approaches used and the level of experience available in EU member states. This estimate is largely based on the knowledge about the current status gained in part 1 of the study from the analysis of the published literature, including reports from European research projects, congresses, and expert committees.

Table 2 provides a complete compilation of all responses of all responding institutions to the multiple choice part of the questionnaire. This gives a good overview on the approaches used and the experience represented by the 25 institutions in the field of mixture toxicology. Additional detailed information that results from the corresponding free text parts of the questionnaire is analyzed in the subsequent three sub-sections (4.3 – 4.5).

According to their general experience with different mixture toxicity assessment approaches (Tab. 2, questions 1, 2, 5, and 8) the 25 responding institutions may be grouped into four categories:

(i) 3 institutions that have no experience in assessing the mixture toxicity of complex samples, but assess individual substances only (HU1, IE2, SE),

(ii) 10 institutions that apply the whole mixture approach (direct toxicity testing), but not any component-based approaches (DK3, DK6, FI1, FI2, HU2, IE1, IT, SI, SK, UK),

(iii) 1 institution that applies a component –based approach, but not any whole mixture testing (BE1), and

(iv) 11 institutions applying both types of approaches, whole mixture testing and component-based modeling approaches (BE2, DK1, DK2, DK4, DK5, EE, ES, FR1, FR2, HU3, NL).

Within the 22 institutions that have experience with mixture toxicity assessments (groups *ii* to *iv*) the spectrum of sample types, methods and endpoints covered by a single institution varies considerably. It ranges from absolute specialists who use a single method for a single sample type, such as the use of the TEF for dioxin-like compounds in soil samples by BE1 for instance, to generalist in the field who have experience with virtually every type of methodology for mixture toxicity assessment, such as NL or ES (see the multiple choice responses to questions 3, 4, 6, and 7 documented in Tab. 2).

Tab. 1. Responding institutions

Questionnaire CODE	Country of Origin	Institution	Contact Person
BE1	Belgium	OVAM Public Waste Agency of Flanders Soil Management Department	Griet Van Gestel
BE2		Environment & Health Flemish Government Department of Environment, Nature and Energy	Karen Van Campenhout
DK1	Denmark	Danish Environmental Protection Agency Chemicals Unit	Flemming Ingerslev
DK2		Aarhus University National Environmental Research Institute Department of Marine Ecology	Jakob Strand
DK3		Aarhus University National Environmental Research Institute Department of Terrestrial Ecology	John Jensen
DK4		Aarhus University National Environmental Research Institute Department of Policy Analysis	Hans Sanderson Marianne Thomsen
DK5		University of Copenhagen	Nina Cedergreen
DK6		Technical University of Denmark Dept of Environmental Engineering	Kresten Ole Kusk
EE	Estonia	National Institute of Chemical Physics and Biophysics	Anne Kahru Irina Blinova
ES	Spain	Division of Ecotoxicology and Environmental Risk Assessment INIA[1] - Ministry of Science and Innovation (upon request from the Ministry of the Environment and Rural and Marine Affairs)	José V. Tarazona
FI1	Finland	Finnish Environment Institute	Eija Schultz
FI2		National Institute for Health and Welfare	Hannu Komulainen
FR1	France	French National Institute for Industrial Environment and Risks (INERIS)	Selim Ait-Aissa
FR2		INSERM (Institut national de la santé et de la recherche médicale)	Patrick Balaguer

Table continued on the following page

[1] Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

Questionnaire CODE	Country of Origin	Institution	Contact Person
HU1	Hungary	Ministry of Environment and Water Air Quality Dept.	Judit Varga
HU2		Central Directorate for Water and Environment	György István Toth
HU3		Ministry of Environment and Water Environmental Management Department	Hilda Farkas
IE1	Ireland	Enterprise Ireland Shannon Aquatic Toxicity Laboratory	Robert Hernan
IE2		Health & Safety Authority	Yvonne Mullooly
IT	Italy	Italian Environmental Protection and Research Institute (ISPRA) Environmental Department Metrology Unit	Maria Belli
NL	The Netherlands	1. Directorate-General of Public Work and Water Management / Rijkswaterstaat, Centre of Water Management 2. Ministry of Housing, Spatial Planning and the Environment / National Institute of Public Health and the Environment	1. D. A. Jonkers / G. Niebeek J.L. Maas 2. J.K.B.H. Kwisthout J.M.C. Appelman N.J. Molenaar / M.P.M. Janssen E. Verbruggen M. van Raaij E. Van der Grinten M. Mesman L. Posthuma *All responses were kindly compiled in a single questionnaire by* M.P.M. Janssen
SE	Sweden	Swedish Environmental Protection Agency	Helene Lager
SI	Slovenia	Public Health Institute Maribor Collaborator of Ministry of the Environment and Spatial Planning	Mojca Kos Durjava
SK	Slovak Republic	Water Research Institute	Lívia Tóthová
UK	England & Wales	Environment Agency	Dean Leverett

Tab. 2. Responses to the questionnaire (multiple choice part only)

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK
1. Do you have any practical experience in assessing the mixture toxicity of complex environmental samples or waste samples?																									
Yes	X	X	X	X	X*	X	X	X	X	X	X	X	X	X		X	X	X		X	X		X	X	X
No, we assess only individual components															X				X			X			
2. Do you apply the whole-mixture approach, i.e. direct toxicity testing, for any kind of complex environmental or waste samples?																									
Yes		X	X	X	X*	X	X	X	X	X	X	X	X	X		X	X	X		X	X		X	X	X
No	X														X*				X*			X*			

Table continued on the following pages

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK

3. For what kind of samples do you apply the whole-mixture approach?

air		X										X									X		X		
surface water			X	X		X	X		X	X	X			X		X	X	X		X	X		X	X	
ground water								X	X	X				X		X	X						X	X	
sediment				X		X			X	X			X	X			X	X		X	X		X	X	
soil					X				X	X	X						X	X		X	X		X		
biota				X							X										X			X	
waste					X			X	X	X	X			X		X	X	X		X				X	
waste water			X					X	X	X	X			X*			X	X			X		X	X	X
waste water treatment plant effluents			X			X		X	X	X	X			X*		X	X	X			X		X	X	
others										X	X	X		X*											

Table continued on the following pages

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK
4. What kind of biotests do you use for the whole-mixture approach?																									
sub-cellular assays (enzyme assays, immuno assays, receptor assays etc)		X		X				X			X			X							X				
cell cultures		X	X							X		X	X	X						X	X				
bacteria								X	X		X	X					X	X		X	X		X	X	
algae							X	X	X	X	X					X	X	X		X	X		X	X	X
other plants					X*		X		X	X	X						X	X		X	X			X*	
protozoa									X														X	X	
daphnids							X		X	X	X					X	X	X		X	X		X	X	X
other invertebrates				X	X		X	X	X	X	X							X		X	X		X		X
fish				X						X	X					X	X	X		X	X			X	
other vertebrates										X															
multi-species assays						X	X			X				X											
others			X						X		X					X									

Table continued on the following pages

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK

5. Do you apply a component-based approach, i.e. estimating total toxicity from information on identified components only, for any kind of complex environmental sample or waste sample?

Yes	X	X**	X	X		X	X		X	X			X	X			X				X				
No								X			X*	X			X*	X		X	X*	X		X*	X	X	X

6. For what kind of samples do you apply component-based approaches?

air		X																							
surface water			X			X	X			X				X			X				X				
ground water										X				X			X								
sediment			X			X				X			X	X							X				
soil	X		X							X							X				X				
biota			X	X																	X				
waste										X				X			X								
waste water			X						X	X				X			X				X				
waste water treatment plant effluents			X			X				X				X			X								
others		X	X							X															

Table continued on the following pages

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK

7. What kind of component-based approaches do you apply?

Approaches based on the assumption of Concentration Addition (CA)

direct application of the CA formula			X				X										X				X				
TUS (Toxic Unit Summation)			X			X			X	X							X				X				
TEF (Toxic Equivalence Factor)	X	X	X	X						X			X	X*							X				
RPF (Relative Potency Factor)			X														X				X				
PODI (Point of Departure Index)																	X								
HI (Hazard Index)										X							X								
other CA based approaches																									

Approaches based on the assumption of Independent Action (IA) (also called Response Addition)

direct application of the IA formula							X*			X											X				
other IA based approaches																					X				

any other										X											X				

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK

8. Do you apply any approach or methodology to the mixture toxicity assessment of complex environmental samples or waste samples that does not fit into either of the categories of "whole mixture approach" or "component-based approach"?

No	X		X		X	X	X	X	X		X	X	X	X		X		X		X			X	X	X
Yes		X		X						X							X				X				

9. How would you describe your level of experience in practically applying approaches for assessing the overall toxicity of complex environmental samples or waste samples?

(Different levels may apply to different approaches used)

extensive experience / frequent routine application		X		X			X		X	X	X	X		X				X		X	X		X	X	X
limited experience / occasional use only	X		X		X		X	X					X			X	X			X	X		X	X	
marginal experience / exceptional use only													X											X	
application is still in the phase of development / establishment				X			X					X					X			X	X*				
no experience															X*				X*			X*			

Table continued on the following pages

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK
10. For what purposes do you apply approaches for assessing the overall toxicity of complex environmental samples or waste samples?																									
general environmental monitoring		X		X					X	X		X	X	X		X	X			X	X			X	
control of emission permits		X	X							X						X	X	X			X		X		X
risk assessment of contaminated sites	X	X			X	X		X	X	X	X			X		X	X				X		X	X	
priority setting for risk reduction measures			X							X							X				X			X	
control of remediation works and their success		X							X	X	X	X				X	X				X		X	X	
research and development	X	X		X		X	X	X	X	X	X	X	X	X			X	X		X	X		X	X	
others			X		X*			X*						X						X			X		

Table continued on the following page

Response Option	BE1	BE2	DK1	DK2	DK3	DK4	DK5	DK6	EE	ES	FI1	FI2	FR1	FR2	HU1	HU2	HU3	IE1	IE2	IT	NL	SE	SI	SK	UK

11. Negative experience / Warnings:

Are there any approaches or methodologies for assessing the mixture toxicity of complex samples which you have used or tested in the past, but which you have abandoned due to negative experiences?

No		X		X			X		X		X	X	X	X		X					X		X	X	
Yes			X			X		X*		X							X	X		X					X

12. Positive experience / Recommendations:

Are there any approaches or methodologies for assessing the mixture toxicity of complex samples which you consider particularly valuable for specific samples, endpoints, and purposes, and which you would recommend for a more extensive use in EU member states?

No			X													X							X	X	
Yes		X				X	X	X	X	X	X	X	X	X			X	X			X				X

X* - The response option was not explicitly marked, but conclusively results from either a preceding overarching question or from the answers to corresponding detailed questions or free text responses.

X -** The opposite response option was ticked, but the answers to the subsequent detailed questions and corresponding free text comments unambiguously indicated that this occurred accidentally.

4.3 Whole mixture approaches

Sample types

Direct toxicity testing is an approach that is practically applied to any type of complex environmental samples and waste samples. This is already evident from the overview on multiple choice responses obtained for the corresponding question 3 (Tab. 2). A more detailed presentation of the corresponding responses is additionally given in Tab. 3, which lists for every sample type the institutions that apply a whole mixture testing approach and additionally any specification of the sample matrix that may apply according to the corresponding free text comments obtained.

Biotests

Virtually every type of biotest, ranging from sub-cellular receptor binding assays to multi-species test systems, is also somewhere used for whole mixture testing, except test with vertebrates other than fish and amphibians (Tab. 2, question 4, and Tab. 4).

Most of the respondents kindly assigned to every biotest in use the sample type(s) where it is applied to (Tab. 4). However, this information did not become available in all cases and some respondents complaint that they regard this as a superfluous exercise, as they are able to flexibly apply almost every biotest to every type of sample. This can be achieved by appropriate sample preparation methods and/or adaptations of the bioassay to a specific sample type, mainly depending on the specific question at issue and on the available resources. Where no allocation of biotests to sample types is given in Tab. 4, Tab. 3 must therefore be consulted for the range of samples that are assessed by a given institution and may hence be a possibility for the application of the specific biotest.

Level of experience

In general, a high level of experience with whole mixture testing methodologies is available in EU member states. 14 out of the 25 responding institutions described their level of experience in the field of mixture toxicity assessments as “extensive” (Tab. 2, question 9). From the details provided in free text form it comes out that in 13 out of the 14 cases this applies to whole mixture testing methodologies (DK2, FI1, FI2, FR2, IE1, IT, SI, SK, UK) or to both whole mixture testing and component-based assessment methods (BE2, EE, ES, NL).

Purposes

Whole mixture approaches are particularly used for toxicity assessments of waste water, and waste water treatment plant effluents for the control of emission permits under IPPC. They are also applied to practically all other types of environmental samples for purposes of general environmental monitoring, risk assessment of contaminated sites, priority setting for risk reduction measures, and the control of remediation works and their success (see Tab.2, question 10).

Comments and recommendations

In questions 11 and 12 we asked for negative and positive experiences and resulting warnings and recommendations for the use of specific methodologies. A considerable part of the responses specifically referred to the methodology of direct toxicity testing, and some of the comments received under point 13 of the questionnaire (General Comments) also relate to advantages and disadvantages of whole-mixture testing methods. These comments and recommendations are compiled and partly summarized in the following structured overview.

- *General recommendations of whole-mixture testing*

  FI1 emphasized the positive experience with direct toxicity testing: *Whole effluent assessment using biotests as complementary methods to chemical analyses have in our experience been valuable in assessing the quality of effluents, soil quality etc. Hormonal effects of wastewaters and reproduction and bioaccumulation tests of contaminated soils have been used successfully as endpoints. We consider the whole mixture toxicity approach as the most "environmentally relevant" and a cost-effective way for e.g. permits and monitoring purposes.*

- *General requirements for reliable whole mixture testing*

  UK emphasized that a *high level of QA/QC among the laboratories undertaking such analyses for regulatory purposes is essential in providing confidence in the approach for regulators AND industry* and pointed to its corresponding guidelines[2].

  IE1 also emphasized the importance of quality assurance programmes and regular participation of laboratories in inter laboratory proficiency schemes, and additionally stressed the advantages of using internationally standardized biotests for obtaining comparable results for different samples.

  FR1 pointed to a lack of quality assurance measures for cellular assays: *A growing number of environmental laboratories are using cell cultures for bio-analysis of organic contaminants in complex mixtures. However, few (or no?) inter-laboratory validation studies have been conducted on such tests for this purpose. This is a gap in their routine application for whole-sample testing approach.*

- *General problems of whole mixture testing*

  HU3 pointed to the fact that most whole mixture testing is confined to acute toxicity. Chronic tests are rarely performed due to their high costs.

[2] http://publications.environment-agency.gov.uk/pdf/SCHO0106BKDP-e-e.pdf and http://www.environment-agency.gov.uk/business/regulation/38783.aspx

DK3 considered *the need to find suitable reference materials (controls)* as *the overall major problem in assessing the effect/risk of mixtures in complex matrices like contaminated soil and sewage sludge*.

DK6 pointed to several technical problems with direct toxicity testing of environmental samples: *Complex samples are complex to test now and then due to their contents of ready biodegradable compounds (oxygen depletion), particles (interference with particle counting of algae), deviating pHs, etc.* As a possible solution to these problems **DK6** recommended: *Extraction on SPE columns allows testing on up-concentrated samples so that high concentrations can be tested (e.g. 4 L/L).*

- *Negative experiences with some special biotests*

UK and **ES** both advised against the use of the Microtox assay as an inappropriate *surrogate for higher organism toxicity* and due to a *lack of ecological relevance*.

IE1 reported: *Some species we've tested have not been particularly sensitive e.g. Artemia, Crangon crangon.*

IT informed about negative experiences with acute and chronic toxicity test with the marine Copepod Acartia tonsa: *Disadvantages are the following: a) the breeding organisms are not easy to keep under laboratory conditions, b) three algal species are requested for feeding, c) the separation between males and females, by microscope, is difficult and laborious , d) a high number of animals (from 270 to 360 adults) is used per test.*

- *Positive experiences with specific biotests and corresponding recommendations for more widespread use*

FR1 recommended the *use of in vitro estrogenic and dioxin-like activity to trace ER and AhR ligands in complex mixtures*.

FR2 also made recommendations for monitoring the activity of endocrine disrupting chemicals: *Our methodology ; use of reporter bioluminescent cells is a very useful tool to monitor nuclear or Ah receptor ligands in environmental samples. This tool is well adapted to EDCs HTS. A very interesting complementary tool is the use of xenopus or zebrafish (transgenic) larvaes for in vivo experiments. This tool is also well adapted to EDCs HTS.*

FI2 gave dedicated recommendations for the monitoring of mutagenic and genotoxic activities in drinking water: *Mutagenicity testing (e.g., using the Ames test) is a valid tool to evaluate the cancer risk of chlorinated drinking water, monitoring of the quality of the drinking water and the levels of mutagenic by-products. We have published tens of papers on this topic. The method would certainly have use and it is rather simple and easy. Chemical analysis of the known potent genotoxic by-products in drinking water can also be used for estimation of the genotoxic risk of municipal drinking water.* And further: *The Ames-test is recommended for monitoring of mutagenic*

substances in drinking water. The Comet assay could be used to evaluate the genotoxic risk of, e.g., paper and board used for food packaging. However, for the purpose it's sensitivity may be not high enough. The Comet assay could be used also for monitoring the genotoxicity of indoor and outdoor air. However, more data are needed for the final "judgement".

DK4 recommended more widely usage of *micro/mesocosms for testing complex mixtures – thus to both evaluate and to generate testable hypothesis in the lab.*

Approaches or methodologies that do not fit into either of the categories of "whole mixture approach" (WMA) or "component-based approach" (CBA).

In question 8 we asked: *Do you apply any approach or methodology to the mixture toxicity assessment of complex environmental samples or waste samples that does not fit into either of the categories of "whole mixture approach" or "component-based approach"?* In five questionnaires the response option "YES" was marked, and in four of these further details were kindly provided as a free text comment. One of these comments pointed to another type of biotests or endpoint than those listed under the question for whole mixture testing approaches, i.e. assessments *based on differential gene expression* (**BE2**). The other three comments are addressed in the following sections on component-based approaches (4.4) and integrated assessment strategies (4.5).

Tab. 3. Sample types assessed by whole mixture toxicity testing

Sample Type	Institution[3]	Sample Specification
Air	BE2	
	FI2	Particle samples collected from indoor and outdoor air.
	NL	E.g. particulate matter, dust exposure
	SI	
Surface water	DK1	Pre-concentrated samples[4]
	DK2	Freshwater from rivers
	DK4	Depth integrated water samples
	DK5	
	EE	Rivers, channels, ditches (in case of solid waste leachates)
	ES	
	FI1	
	FR2	Organic extracts
	HU2	
	HU3	Communal and industrial wastewaters inlet to receivers
	IE1	Riverine samples
	IT	
	NL	Samples of rivers, lakes, estuaries and agricultural area; freshwater and marine
	SI	
	SK	Whole samples, samples after filtration

Table continued on the following pages

[3] Key to codes given in Tab. 1

[4] DK1 gave the following additional comment: *The Chemicals Unit DK-EPA has conducted projects on hormones and endocrine disruptors in surface waters and wastewater. Here the YES-Assay was used to assess the estrogenicity of pre-concentrated samples. In general the work in the chemicals unit has focus on the chemicals rather than on the recipient in which they occur. Therefore, the project mentioned here is considered rather exceptional in comparison to the rest of the work in the unit. References: Survey of Estrogenic Activity in the Danish Aquatic Environment, part A. Environmental Project, 977. Danish Environmental Protection Agency.; Survey of Estrogenic Activity in the Danish Aquatic Environment, part B. Environmental Project, 1077. Danish Environmental Protection Agency.*

Sample Type	Institution[3]	Sample Specification
Ground water	DK6	
	EE	Ground water of Kohtla-Järve area (oil-shale region)
	ES	
	FR2	Organic extracts
	HU2	
	HU3	Water samples taken from the monitoring wells of industrial waste separators and contaminated areas
	SI	
	SK	Whole sample
Sediment	DK2	Marine sediments, bulk samples
	DK4	Depth integrated sediment samples
	EE	Various sediments (Baltic sea, rivers)
	ES	
	FR1	
	FR2	Organic extracts
	HU3	Sediments of rivers, lakes and reservoirs
	IE1	E.g. marine sediments
	IT	Interstitial water
	NL	Polluted sediments in sedimentation area, estuaries or harbours (freshwater and marine)
	SI	
	SK	Leaches, pore water, eluates
Soil	DK3	Contaminated sites
	EE	Various polluted soils
	ES	
	FI1	
	HU3	
	IE1	
	IT	Leachate of contaminated soil. Preparation of leachate for tests with water organisms by using a ratio of 1 part dry mass of soil and 10 parts water[5]
	NL	Contaminated sites: nature, residential areas, industrial area, agriculture; a formal approach is named soil quality TRIAD [6]
	SI	

[5] ,According to ISO 11465 (Soil quality – Determination of dry matter and water content on a mass basis – gravimetric method) and CEN Guideline EN 12457-2 (Characterization of waste – Leachate).
[6] See Jensen, J. and M. Mesman (2006). Ecological Risk Assessment of Contaminated Land. Decision Support for site-specific investigations. Bilhoven, The Netherlands, National Institute for Public Health and the Environment, no. 711701047.

Sample Type	Institution[3]	Sample Specification
Biota	DK2	Whole organism biological effects monitoring in marine molluscs and fish (national monitoring NOVANA)
	FI1	
	NL	Molluscs and fish – bioaccumulation studies Mollusks – genotoxicity studies Soil organisms in the field (sampled and effects determined on organism level and community level)
	SK	Leaches
Waste	DK3	Sewage sludge
	DK6	
	EE	Mostly oil shale industry solid waste (semi-coke, ashes)
	ES	
	FI1	
	FR2	Organic extracts
	HU2	
	HU3	Eluates prepared from soil samples taken from industrial waste deposits from contaminated areas
	IE1	Industrial solid wastes – generate and test eluates
	IT	Leachate of waste. Preparation of leachate for tests with water organisms by using a ratio of 1 part dry mass of waste and 10 parts water[7]
	SK	Extracts, leaches, eluates
Waste water	DK1	Pre-concentrated samples[8]
	DK6	
	EE	Mostly oil shale industry solid waste (semi-coke, ashes) leachates
	ES	
	FI1	
	FR2	Organic extracts
	HU3	Treated and untreated industrial and communal waste waters
	IE1	Effluent toxicity tests as required in EPA IPPC licence
	NL	Influents and effluents of treatment plants, hospital waste water; diffuse effluents
	SI	
	SK	Sample after filtration
	UK	Industrial effluents from sites regulated under IPPC

[7] According to CEN Guideline EN 12457-2 (Characterization of waste – Leachate) and EN 14735 (Characterization of waste – Preparation of waste samples for ecotoxicity tests)
[8] See footnote to surface water testing by DK1 above

Sample Type	Institution[3]	Sample Specification
Waste water treatment plant effluents	DK1	Pre-concentrated samples[9]
	DK4	
	DK6	
	EE	Various
	ES	
	FI1	
	FR2	Organic extracts
	HU2	
	HU3	Model experiments on the degradation of various types of waste waters using activated sludge
	IE1	Effluent testing as part of EPA IPPC licence
	NL	Samples from different industries or domestic treatment plants, treatment plants of hospitals
	SI	
	SK	Whole sample, sample after filtration
Others	ES	Mixtures from metabolic/degradation processes, fertilizers including those produced from wastes; leachates, run-off and drainage samples
	FI1	Soil extracts, waste leaching test eluates
	FI2	Drinking water samples from municipal water works Extracts from paper and board.
	FR2	Organic extracts of human adipose tissue

[9] See footnote to surface water testing by DK1 above

Tab. 4. Biotests used for whole mixture toxicity testing

Biotest Type	**Institution**[10]	***Test Specification***	***Sample Type(s)***
Sub-cellular assays (enzyme assays, immuno assays, receptor assays etc)	BE2	Ames-test	Air samples
	DK2	ER-CALUX, AR-CALUX AhR-CALUX	Passive samplers from freshwater surface waters
	DK6	YES assay	WWTP effluents
	FI1		
	FR2	Receptor binding assays; source of receptor : human cell lines (MCF-7,…) or E Coli	Pure compounds or all kind of samples
	NL	DR-Calux ER-Calux	Sediment, waste water, surface water
Cell cultures	BE2	Alveolar epithelial A549 cell line Bronchial epithelial Beas-2B cell line Macrophage THP-1 cell line Cat-tox assay	Air samples
	DK1	YES-assay[11]	Surface water, wastewater, wastewater effluent
	ES	Fish cell lines	All
	FI2	Comet assay (endpoint DNA damage)	Air samples (indoor, outdoor), extracts from paper and board
	FR1	Reporter gene cell lines for estrogenicity (MELN cells) (Anti)androgenicity (MDA-kb2 cells) Micro-EROD assay for dioxin-like activity in H4IIE and PLHC-1 cells	Organic extracts of sediments

Table continued on the following pages

[10] Key to codes given in Tab. 1

[11] DK1 gave the following additional comment: *The Chemicals Unit DK-EPA has conducted projects on hormones and endocrine disruptors in surface waters and wastewater. Here the YES-Assay was used to assess the estrogenicity of pre-concentrated samples. In general the work in the chemicals unit has focus on the chemicals rather than on the recipient in which they occur. Therefore, the project mentioned here is considered rather exceptional in comparison to the rest of the work in the unit. References: Survey of Estrogenic Activity in the Danish Aquatic Environment, part A. Environmental Project, 977. Danish Environmental Protection Agency.; Survey of Estrogenic Activity in the Danish Aquatic Environment, part B. Environmental Project, 1077. Danish Environmental Protection Agency.*

Biotest Type	**Institution**[10]	***Test Specification***	***Sample Type(s)***
Cell cultures continued	FR2	In house nuclear (ERs, AR, GR, PR, MR, PXR, CAR, PPARs, RXRs, TRs,…) and Ah receptor bioluminescent cell lines	Pure compounds or all kind of samples
	IT	Cytotoxicity: viability assay by dye uptake with fluorescein diacetate and propidium iodide or viability assay by neutral red uptake. Established fish cell line: RTG2 (Gonad rainbow trout)	
	NL	Fish cell lines Genotoxicity test (Comet assays)	Waste water mainly
Bacteria	DK6	Microtox	WWTP effluents
	EE	Vibrio fischeri luminescence inhibition assay (Microtox) Recombinant luminescent sensor bacteria for heavy metals and phenols	
	FI1		
	FI2	Bacterial reverse mutation test (the Ames test) (endpoint gene mutation)	Drinking water samples
	HU3	Azotobacter	
	IE1	Vibrio fischeri (Microtox)	
	IT	Determination of the inhibitory effect of water samples on the light emission of luminescent bacteria, according to ISO 11348-3. Vibrio fischeri (Freeze-dried bacteria)	

Table continued on the following pages

Biotest Type	**Institution**[10]	***Test Specification***	***Sample Type(s)***
Bacteria continued	NL	Microtox acute test	Surface water (concentrates), sediment pore water, waste water (as is or concentrates), pore water samples from soils
		Microtox solid phase test	Sediment (marine mainly)
		Mutatox	Sediment
		Bacterial plate test (antibiotic test)	Waste water (extracts), surface water (concentrates)
		Biolog plates (biological activity)	Pore water samples form soils
		CLPP (Community Level physiological profiling) with Biolog plates	Pore water samples form soils
		PICT (Pollution Induced Community Tolerance) with Biolog plates	Pore water samples form soils
	SI		
	SK	Vibrio fischeri based on EN ISO 11 348	Surface water, ground water, sediment, biota, waste, waste water, WWTP effluents
Algae	DK5		
	DK6	Fresh and marine algae	Eluent from solid waste
	EE	Pseudokirchneriella subcapitata growth inhibition assay	
	ES	Modified OECD algal growth test	All
	FI1		
	HU2		
	HU3	Unicellular green algae strains according to relevant rules and regulations	10:1 L/S eluates (with mineral water, or DMSO) from waste samples; surface, under ground, industrial and communal waste waters
	IE1	Freshwater (e.g. Psuedokirchneriella subcapitata) and marine (e.g. Skeletonema costatum)	

Biotest Type	Institution[10]	*Test Specification*	*Sample Type(s)*

Table continued on the following pages

Algae continued	IT	Freshwater algal growth inhibition test according to ISO 8692 Marine algal growth inhibition test according to EN ISO10253 (Pseudokirchneriella subcapitata, Phaeodactylum tricornutum, Dunaliella tertiolecta)	
	NL	Algal-growth test Pulse Amplitude Modification test – on basis of fluorescence	Surface water (concentrates) and waste water (as is or concentrates) Pore water samples from soils
	SI		
	SK	EN ISO 8692 and national Standard STN 83 8303	Surface water, ground water, sediment, biota, waste, waste water, WWTP effluents
	UK	Freshwater & marine algae	Industrial effluents
Other plants	DK3	Terrestrial plants	
	DK5		
	EE	Lemna minor growth inhibition assay Seed germination assays with various plants	
	ES	Adapted Lemna minor test	All
	FI1		
	HU3	White mustard seed (Sinapis alba)	10:1 L/S eluates (with mineral water, or DMSO) from waste samples; surface, under ground, industrial and communal waste waters
	IE1	Lemna minor	

Table continued on the following pages

Biotest Type	Institution[10]	*Test Specification*	*Sample Type(s)*
Other plants continued	IT	Seed germination / Root Elongation toxicity test according to UNICHIM method 1651:2003[12]; (Cucumis sativus L.; Lepidium sativum L.; Sorghum bicolor L. x Sorghum sudanense instead of Sorghum saccharatum)	
	NL	Latuca sativa (emergence, growth) Raphanus sativus (root elongation, root/shoot ratio, growth)	Soil
	SK	Lemna minor based on ISO 20 079 Sinapis alba based on national Standard STN 83 8303	Surface water, ground water, sediment, biota, waste, waste water, WWTP effluents
Protozoa	EE	Tetrahymena thermophila growth inhibition assay and the viability assay	
	SI		
	SK	Thamnocephalus platyurus	Surface water
Daphnids	DK5		
	EE	Acute Daphnia magna immobilization assay	
	ES	Modified OECD test	All
	FI1		
	HU2		
	HU3	Daphnia ,magna (static method)	10:1 L/S eluates (with mineral water, or DMSO) from waste samples; surface, under ground, industrial and communal waste waters
	IE1	Daphnia magna	
	IT	Inhibition of the mobility of daphnids – Acute toxicity test (24 and 48 h) according to ISO 6341 (Daphnia magna Straus)	

Table continued on the following pages

[12] "Determination of the inhibition of the seed germination and root elongation on Cucumis sativum L. (cucumber), Lepidium sativum L. (water cress), Sorghum saccharatum Moench (sorghum). (Short-chronic toxicity test)".

Biotest Type	Institution[10]	*Test Specification*	*Sample Type(s)*
Daphnids continued	NL	Daphnia acute or IQ-test Chronic reproduction test	Surface water (concentrates), waste water (as is and concentrates) Sediment pore water
	SI		
	SK	EN ISO 6341 and national Standard STN 83 8303	Surface water, ground water, sediment, biota, waste, waste water, WWTP effluents
	UK	Daphnia magna	Industrial effluents
Other invertebrates	DK2	Mussel: biomarkers; neutral red retention and vitellogenin-like proteins Amphipod: Reproductive success, biomarkers, tolerance, genetic diversity, behavior, mortality Gastropods: TBT specific effects, imposex and intersex	Marine and freshwater environments Sediment bioassays and marine environment Marine environment
	DK3	Soil invertebrates	
	DK5		
	DK6	Marine copepod Acartia tonsa	WWTP effluents
	EE	Thamnocephalus platyurus mortality assay	
	ES	Earthworms	
	FI1		
	IE1	Marine crustacean (e.g. Tisbe battagliai)	
	IT	Inhibition of mobility of Artemia sp (Anostraca – Crustacean) – Acute toxicity test (24, 48 and 96 h) and chronic test (14 days) (Artemia franciscana)	

Table continued on the following pages

Biotest Type	**Institution**[10]	***Test Specification***	***Sample Type(s)***
Other invertebrates continued	NL	Marine species (Corophium)	Sediment
		Mollusks (bioaccumulation)	Sediment/ surface water
		Crustacean species (Rotifer Brachionus, Thamnocephalus)	Surface water (concentrates)
		Folsomia candida (Springtail) (growth, reproduction, survival)	Soil
		Eisenia fetida (Earthworm) (growth, reproduction, survival)	Soil
		Enchytraes albidus (growth, reproduction, survival)	Soil
		Nematodes species (community composition)	Soil
		Earthworm species (community composition)	Soil
		Mites species (community composition)	Soil
	SI		
	UK	Tisbe; Oyster Embryo-Larval Development	Industrial effluents
Fish	DK2	Reproductive success, biomarkers, genetic diversity, intersex	Marine environment
	ES	Embryo and embryo-larval assay	All
	FI1		
	HU2		
	HU3	Static tests using international and national methods, zebra fish, guppi.	10:1 L/S elutes (with mineral water, or DMSO) of waste samples; surface, under ground, industrial and communal waste waters
	IE1	Oncorhynchus mykiss, Psetta maxima	

Table continued on the following pages

Biotest Type	**Institution**[10]	***Test Specification***	***Sample Type(s)***
Fish continued	IT	Early-life stage toxicity test: assessment of lethal and sublethal effects according to OECD guideline 210:1992 (Dicentrarchus labrax) Acute toxicity test (96 h) according to OECD guidelinev203:1992 (Juvenile of Dicentrarchus labrax and Cyprinus carpio) Prolonged toxicity test: 14-day and 28 day according to OECD guideline 204: 1984 (Juvenile of Dicentrarchus labrax and Cyprinus carpio)	
	NL	Bioaccumulation in wild populations	Surface water
	SK	Poecilia reticulata based on EN ISO 7346/1, 2 and national Standard STN 83 8303	Surface water, ground water, sediment, biota, waste, waste water, WWTP effluents
Other vertebrates	ES	Amphibians (Xenopus assay)	All
Multi-species assays	DK4	Mesocosm	
	DK5		
	ES	Two assays developed by our group: The soil microcosm MS·3 (Multi-Species Soil System) The Multi-species water/sediment system	Wastes, soil, effluents
	FR2	coregulator – receptor interaction assays	Pure compounds
Others	DK1	(*In our general work with chemical assessment, data from basically all types of biotests are relevant)*	
	EE	Recombinant luminescent sensor bacteria for heavy metals and phenols	
	FI1	Isolated hepatocytes	
	HU2	Root growth inhibition on mustard seed.	

4.4 Component-based approaches

Sample types

The application of component-based approaches for mixture toxicity assessments also covers the full spectrum of possible sample types, but the available experience is concentrated in a considerably smaller part of the responding institutions. While 21 of the responding 25 institutions apply direct toxicity testing methods, only twelve have experience with component-based assessment methodologies. This is already evident from the overview on multiple choice responses obtained for the corresponding question 5 (Tab. 2). A more detailed presentation of the corresponding responses is additionally given in Tab. 5, which lists for every sample type the institutions that apply component-based assessment methodologies to such samples, and additionally any specification of the sample matrix that may apply according to the corresponding free text comments obtained.

Concepts and models

All twelve institutions that have experience with component-based assessment approaches apply models and methods that are based on the assumption of Concentration Addition (CA), while only three of them also apply approaches based on the alternative assumption of Independent Action (IA) or mixed models that combine both assumption (DK5, ES, NL) (Tab. 2, question 4, and Tab. 6). Furthermore, the application of CA is largely confined to the direct application of the CA formula, the Toxic Unit Summation method (TUS), and the Toxic Equivalence Factor approach (TEF). The practical use of other CA-based methods, such as the Relative Potency Factor approach (RPF), the Point of Departure Index (PODI), and the Hazard Index (HI) is obviously rare in EU member states.

Part of the respondents kindly specified for every component-based approach in use (i) the sample type(s), (ii) the substance group(s), and (iii) the endpoint(s) where they apply it to (Tab. 6). Others explained that they apply such methods flexibly in a case by case manner. The practical application of CA based approaches is typically confined to groups of substances for which a similar mechanism or at least a similar mode of action is assumed. Such groups of substances which were frequently mentioned in the free text responses are: dioxines, furanes and dioxine-like PCBs, substances with estrogenic activity, PAHs, phenols, and certain groups of metals, pharmaceuticals, and pesticides, e.g. organophosphates (Tab. 6).

Level of experience

Only five of the responding institutions described their level of experience with one or more component-based approaches “extensive” (BE2, DK5, EE, ES, NL), which is in marked contrast to the widespread use and the high level of experience available for direct toxicity testing methodologies.

Purposes

The TEF (Toxic Equivalence Factor) approach for the assessment of mixtures of dioxins, furans, and dioxin-like PCBs is a component-based approach that is routinely applied for various purposes, including the control of emission limit values under IPPC. Uses of other component-based approaches or application of the TEF approach to other groups of compounds are typically confined to studies with special compound groups, but they are not restricted to special purposes. General environmental monitoring, risk assessment of contaminated sites, priority setting for risk reduction measures, and the control of remediation works and their success were all marked as purposes for which component-based approaches are used.

Comments and recommendations

In questions 11 and 12 we asked for negative and positive experiences and resulting warnings and recommendations for the use of specific methodologies. Part of the responses specifically referred to component-based approaches. Some of the comments received under point 13 of the questionnaire (General Comments) also related to advantages and disadvantages of component-based approaches, and the same applies to some comments received in response to question 8 on methodologies that do not well fit into either of the categories of "whole mixture approach" (WMA) or "component-based approach" (CBA). These comments and recommendations on the use of component-based approaches are compiled and partly summarized in the following structured overview. Overarching comments dealing with integrated strategies that make use of both types of approaches, WMA and CBA, are separately considered in section 4.6.

- *Recommendations for using approaches based on the assumption of Concentration Addition (CA)*

  DK5 stated: *In my experience CA explains most mixtures well (or at least as well as IA), in tests on whole organism growth or survival. Also for independently acting chemicals. The exception is mixed bacteria cultures (sludge bacteria), where IA seems to be a good predicting model of dissimilar acting compounds, aAnd metabolic biomarkers, where I also know of an example, where IA was the best predictor.*

  EE recommended (Concentration) *Additivity* by pointing to two papers[13] on the assessment of the toxicity of phenolic wastewaters.

  DK4 stated: *For chemicals with similar mode of action the methodology used is CA and HI. For me it would really require in depth evidence to adopt any other approach (synergism or antagonism). It´s impossible to address real life*

[13] *Kahru, M. Kurvet and I. Külm (1996) Toxicity of phenolic wastewater to luminescent bacteria Photobacterium phosphoreum. Wat. Sci. &Tech. 1996, Vol. 33, Nr. 6, 139-146.*
Kahru, A., Põllumaa, L., Reiman, R. and Rätsep, A. (1999) Predicting the toxicity of oil-shale industry wastewater by its phenolic composition. ATLA, Vol. 27, p. 359-366.

exposure profiles for their combined effects upon mixture exposure (Comment given by Marianne Thomson)[14].

DK6 who uses the whole-mixture testing approach, but does not apply component-based approaches, expressed the following view: *The information on content of chemicals is generally so limited that it is impossible to point out the significant contributors to toxicity. If we should do so I would prefer using the TU and CA concept.*

HU3 informed: *Hungarian laboratories perform examination on contaminated sites of soils and ground waters in order to establish the cleaning target values based on complete mixture and components. In Hungary CA is applied widespread, but it is not a practice to apply TUS, RPF, PODI, HI methodology.*

- *General problems with using component-based approaches*

Two comments pointed to the problem of defining groups of substances for which CA might be an appropriate assumption.

DK1 described the problem as follows: *A general problem in the regulatory work with chemicals is that while the different approaches for assessing mixtures (see question 7) are well established, more pragmatic knowledge is needed in relation to when the different approaches could be used. Often questions as the following remain unanswered: Can additivity be assumed for a mixture of substances with the same mode of action (e.g. antiandrogenic) but not the same mechanism of action (e.g. receptor-blocking and inhibition of androgenproduction)? If independent action is assumed for a mixture of substances in algal tests, under which conditions can this assumption also be assumed to be valid for other taxonomic groups?*

DK4 briefly touched on the topic by writing: *Warnings about chronic toxicity and pharmacodynamic compounds (narcosis is not a MoA), and confusion between Modes and Mechanisms of Action – focus on modes then mechanisms.*

Approaches or methodologies that do not fit into either of the categories of "whole mixture approach" (WMA) or "component-based approach" (CBA).

In question 8 we asked: *Do you apply any approach or methodology to the mixture toxicity assessment of complex environmental samples or waste samples that does not fit into either of the categories of "whole mixture approach" or "component-based approach"?* In five questionnaires the response option "YES" was marked, and in four of these further details were kindly provided as a free text comment. One of these comments pointed to another type of biotests or endpoint than those listed under the question for whole mixture testing approaches, and has therefore been reported in the corresponding previous section (4.3). One of the three remaining comments will be

[14] *The questionnaire completed by* **DK4** *included separate answers from Hans Sanderson und Marianne Thomson.*

addressed in the following section on integrated assessment strategies (4.5). The other two were the following:

NL pointed to the ecotoxicological risk assessment of petroleum substances by the so-called Hydrocarbon Block Method by commenting: *TPH (total petroleum hydrocarbons) is grouped in homogeneous blocks. These are actually not single components but groups of compounds with similar properties*. This method may hence be considered as another pragmatic extension of the CA concept.

DK2 considered *Environmental assessment criteria of contaminants (OSPAR EAC and EU water Framework Directive EQS) for assessing contaminant levels in sediment, mussels and fish (national monitoring)* as an approach that does not fit into either of the categories of "whole mixture approach" (WMA) or "component-based approach" (CBA). This approach assesses monitoring results by means of a set of criteria for an array of monitored priority substances. The further inquiry, whether this might be considered as a conventional single substance approach or as another approach for assessing the mixture toxicity of complex environmental samples, was raised in the in-depth expert interview with Dr. Tyle from the Danish EPA (section 5.3).

Tab. 5. Sample types assessed by component-based approaches

Sample Type	Institution[15]	Sample Specification
Air	BE2	
Surface water	DK1	
	DK4	
	DK5	
	ES	
	FR2	
	HU3	
	NL	Samples from agricultural areas Samples from for any relevant water body of (policy) interest Samples for assessing natural disasters which involve secondary chemical releases (for the UN: UNEP/OCHA)
Ground water	ES	
	FR2	
	HU3	Samples from contaminated areas
Sediment	DK1	
	DK4	
	ES	
	FR1	
	FR2	
	NL	Polluted sediments and dredge material Samples from any relevant sediment volume of (policy) interest
Soil	BE1	
	DK1	
	ES	
	HU3	Eluates from contaminated soils. Elutes: 10:1 L/S (with mineral water, pH=4,5 buffer, pH=2 HNO3, n-hexane)
	NL	Samples from any relevant sediment volume of (policy) interest

Table continued on the following page

[15] Key to codes given in Tab. 1

Sample Type	Institution[15]	Sample Specification
Biota	DK1	
	DK2	Mussels and fish
	NL	(Bioaccumulation data of biota)
Waste	ES	
	FR2	
	HU3	Waste extracts. Elutes: 10:1 L/S (with mineral water, pH=4,5 buffer, pH=2 HNO3, n-hexane)
Waste water	DK1	
	EE	Oil shale industry wastewaters
	ES	
	FR2	
	HU3	Various industrial wastewaters
	NL	Different
Waste water treatment plant effluents	DK1	
	DK4	
	ES	
	FR2	
	HU3	Various industrial wastewaters
Others	BE2	Human samples: serum, whole blood, urine and hair samples
	DK1	All matrices[16]
	ES	Mixtures from metabolic/degradation processes, fertilizers including those produced from wastes; leachates, run-off and drainage samples

[16] DK1 gave the following additional comment: *The chemical unit of the Danish EPA, is contributing to the development of legislation for chemicals and is also involved in the management of existing legislation. An integrated part of this work is the assessment of the hazardous properties of chemicals as a part of risk assessment, classification and establishment of quality standards. In this work basically all matrices are considered. The markings above reflect the matrices of primary interest.*

Tab. 6. Component-based approaches applied by responding institutions

Model / Method	Institution	Model Specification / Modifications	Sample Type(s) Assessed	Substance Group(s) Assessed	Toxicological Endpoint(s) Assessed
Direct application of the CA formula	DK1		*See Note 1) at the end of the Table*		
	DK5		Artificial pesticide polluted surface water	Pesticides	Growth of algae, iLemna or other aquatic macrophytes. Mobility of daphnids or other invertebrates.
	HU3		*See Note 3) at the end of the Table*		
	NL		Surface water	Metals	Acute and chronic toxicity
TUS (Toxic Unit Summation)	DK1		*See Note 1) at the end of the Table*		
	DK4		Water and sediment	Pharmaceuticals	Acute and chronic ecosystem response (cosm) / QSAR
	EE		wastewaters	Phenols[17]	Inhibition of luminescence of Vibrio fischeri
	ES		*See Note 2) at the end of the Table*		
	HU3		*See Note 3) at the end of the Table*		
	NL	TUS based on NOEC or EC50	Surface water, sediment, waste water	Pesticides, metals, PCBs, PAHs	Acute and chronic toxicity: algae, daphnids, bacteria

[17] Kahru, M. Kurvet and I. Külm (1996) Toxicity of phenolic wastewater to luminescent bacteria Photobacterium phosphoreum. Wat. Sci. &Tech. 1996, Vol. 33, Nr. 6, 139-146. Kahru, A., Põllumaa, L., Reiman, R. and Rätsep, A. (1999) Predicting the toxicity of oil-shale industry wastewater by its phenolic composition. ATLA, Vol. 27, p. 359-366.

Model / Method	Institution	Model Specification / Modifications	Sample Type(s) Assessed	Substance Group(s) Assessed	Toxicological Endpoint(s) Assessed
TEF (Toxic Equivalence Factor)	BE1		Soil	Dioxines, furanes and dioxine-like PCBs	
	BE2		Blood samples	PCBs and dioxine like substances	Calux
	DK1		*See Note 1) at the end of the Table*		
	DK2	WHO TEQ-approach	Biota	Dioxin, furans and co-planar PCBs	
	ES		*See Note 2) at the end of the Table*		
	FR1		Sediment (organic extracts)	PAHs, estrogenic steroids	Estrogenic, (anti)androgenic and dioxin-like activities (Cell cultures)
	FR2		All sample types	EDCs	Nuclear or Ah receptor activities
	NL	WHO TEF-values	Biota, sediment	Dioxins, PCBs	Dioxin like activity
RPF (Relative Potency Factor)	DK1		Surface water, wastewater, wastewater effluent	Estrogens (RPF was applied in a survey of estrogenic activity in Danish surface waters)[18]	Estrogenic effect (YES assay)
	HU3		*See Note 3) at the end of the Table*		

[18] Survey of Estrogenic Activity in the Danish Aquatic Environment, part A. Environmental Project, 977. Danish Environmental Protection Agency.; Survey of Estrogenic Activity in the Danish Aquatic Environment, part B. Environmental Project, 1077. Danish Environmental Protection Agency.

Model / Method	Institution	Model Specification / Modifications	Sample Type(s) Assessed	Substance Group(s) Assessed	Toxicological Endpoint(s) Assessed
RPF (Relative Potency Factor) continued	NL	US-EPA RPFs RPFs derived by RIVM	Surface water, waste water, sediment	Dioxins, estrogens, pesticides (e.g. organophosphates)	Dioxinlike activity, estrogenic activity, ChE-inhibition (DR-Calux, ER-Calux)
PODI (Point of Departure Index)	HU3	*Not performed yet, in progress*	*See Note 3) at the end of the Table*		
HI (Hazard Index)	ES		*See Note 2) at the end of the Table*		
	HU3	*Not performed yet, in progress*	*See Note 3) at the end of the Table*		
Direct application of the IA formula	DK5		Artificial pesticide polluted surface water	Pesticides	Growth of algae, iLemna or other aquatic macrophytes. Mobility of daphnids or other invertebrates.
	ES		*See Note 2) at the end of the Table*		
	NL	Model OMEGA; multi-species: Potential Affected Fraction (msPAF) See below, we use a mixed-model approach	Surface water, biota, sediment	Metals, PCBs, PAHs, OCBs, pesticides	Effect on ecosystem level or on species level
Other IA based approaches	NL	multi-species: Potential Affected Fraction (msPAF) mixed model approach	Soil	Metals, PAH	Effects on species level

Model / Method	Institution	Model Specification / Modifications	Sample Type(s) Assessed	Substance Group(s) Assessed	Toxicological Endpoint(s) Assessed
Any other component-based approaches	ES	We have developed an Index combining toxicity, persistence and accumulation of each fraction	*See Note 2) at the end of the Table*		
	NL	multi-species: Potential Affected Fraction (msPAF)	Surface water, soil, sediment	Metals, PCBs, PAHs, OCBs, pesticides…., limitations only by the worldwide limitation in data sources (e-toxBase of RIVM contains 188.000 entries for > 5000 compounds)	Effect on ecosystem level or on species level

Note 1) – DK1 gave the following general comment: *In addition to a specific project on estrogens* (mentioned in line RPF / DK1), *our general work uses many of the approaches from time to time (most of our work is however aimed at assessments of single substances). a) In principle we cover all sample types, but main focus is on water. b) As a general rule of thumb, the groups of substances we deal with can be considered as a group if they have a common mode of action. An example is PAH's. c) Most of our assessments take as a starting point the basis-set of test-organisms (i.e. Algae, crustaceans and fish)*

Note 2) – ES gave the following general comment: *Due to our experience in the area, we are using a case-by-case approach, selecting for each sample, on the basis of its composition and available information, an optimized assessment protocol; the marked methods are usually applied.*

Note 3) – Different models may be occasionally applied on a case by case basis, no one is used generally for a specific sample type and/or substance group (see the oral expert interview in section 5.2)

4.5 Integrated assessment strategies

Whole mixture approaches (WMA) and component-based approaches (CBA) both have their advantages and disadvantages and may best be used in a complementary manner rather than as alternative tools. Advanced assessment strategies integrate them both and apply them flexibly in different situations. This is the general recommendation that came out from some of the questionnaires, in particular those completed by the National Institute of Public Health and the Environment (RIVM) (**NL**) in the Netherlands and the INIA, Division of Ecotoxicology and Environmental Risk Assessment, in Spain (**ES**). Both indicated that they are particularly engaged in the field of mixture toxicology, not only having extensive experience with practically all established types of approaches in the field, but developing new integrated methodologies for practical purposes in support of their national environmental authorities.

The Netherlands recommendations for mixture toxicity assessment of complex samples

In question 12 we asked for approaches or methodologies for assessing the mixture toxicity of complex samples which the responding institutions consider particularly valuable, and which they would recommend for a more extensive use in EU member states. These are the positive experiences that were reported in the questionnaire completed by **NL**:

- Use of *whole mixture approaches:*
  - *in case of risk assessment of contaminated sites like polluted sediments or agricultural area polluted with pesticides,*
  - *in case of priority setting for risk reduction measures for sediment, waste water and surface water (may substances be a factor of concern if a good ecological status has not been gained).*
- Use of *component based approaches:*
  - *in case of priority setting of contaminated sites,*
  - *in case of risk assessment of contaminated sites,*
  - *in case of human risk assessment through aggregated exposure.*
- *Component-based approaches:*
  - *are useful for evaluating cost-effectiveness of different risk management scenarios, and for multi-stress analysis (diagnosis) of (bio)monitoring data. Such data are collected under e.g. the Water Framework Directive, but the over-all data set is difficult to analyze due to "over-parameterisation", that is: when all individual chemicals are monitored, the output is "non-significant" due to the curse of dimensionality". Cumulative risk assessments, e.g. by msPAF, helps out!*

- *Bioassays are highly recommended for determining the ecological and chemical quality because within the Water Framework Directive*

  - this approach *may replace the monitoring of a long list of substances by chemical measurements,*
  - *the absence or presence of an effect enables to assess the importance of chemical substances in affecting the good ecological quality (besides hydrology, morphology, species interactions, the presence of macronutrients) (This may be relevant in considering the effectiveness of BAT/BEP in taking further measures for substances or focusing on other relevant parameters such as macronutrients),*
  - this approach *includes interactions between substances,*
  - this approach *incorporates in situ bioavailability,*
  - *they are cost efficient.*

The complementary question about any negative experiences with mixture toxicity assessment methodologies (question 11) was commented as follows:

- *The only negative experience is that whole mixture toxicity is still applied on a voluntary basis or in case of investigative monitoring. There are no legal requirements to perform whole mixture toxicity tests in any case.*

In more technical terms the following point was additionally made:

- *The component based method is too general for risk assessment. This method is mostly followed by the estimation of bioavailability of metals and organic compounds like PCBs and PAHs.*

This point was further clarified in the expert interview related to the **NL** questionnaire (section 5.1).

In addition to these remarks about positive and negative experiences and resulting recommendations, **NL** made the following general comments on the issue (question 13):

- *There are still a lot of uncertainties about the chemical interactions between substances. It is clear that interactions between substances may appear both in the environment and at the site of uptake in the organism, but only limited information is available on specific substances. At present, it is not possible to estimate the effect of interactions on the toxicity level of a certain substance. Therefore, Dutch policy focus primarily on BAT/BEP principles in permitting releases of substances before applying any general environmental quality standards (EQS). The general standards are defined as maximum permissible concentrations. Policy aims to reach a lower standard, the negligible risk concentration in the long term. Derivation of environmental quality standards are in most cases based on single substance toxicity tests.*

Finally, **NL** hinted to the fact that

- *A working group under the POP Review Committee for the Stockholm Convention on POPs (UNEP) is dedicated to mixture toxicity and will report in October 2009.*

Development of integrated assessment strategies at INIA in Spain

In an accompanying letter to the questionnaire kindly completed by **ES**, Dr Tarazona from INIA explained:

- *As we are applying a large number of methods to almost all kind of samples, selecting for each sample an optimized assessment approach, it has not sense, in our case, to discriminate which methods are applied to which samples; thus I have included some published references that describe the goals and current status of our activity.*

In response to question 8 about methodologies that might not fit well into either of the categories of WMA or CMA, the integrated approach taken by **ES** was outlined:

- *We have developed an Index combining toxicity, persistence and accumulation of each fraction, which can be integrated with whole mixture approach and Toxicity Identification Evaluation methods. For wastes, we are using a combination of chemical analysis interpreted by TUS/TEF with a battery of toxicity tests (in vitro and in vivo) for characterizing the unidentified components. Both results are combined in a single Hazard Assessment of the mixture to be used in Risk Assessment.*

In response to question 12 about positive experiences and resulting recommendations, further suggestions for integrated assessment approaches were sketched:

- *The combination of a "generic toxicity" applicable to all chemicals measured by standard endpoints, plus the specific assessment for chemical of high concern (PBT-POPs; endocrine disrupters, genotoxic, immunotoxic, and other biologically active chemicals) based on chemical analysis and/or measuring mechanistic end-points, seems to be a very promising tool. The information can be combined in the risk assessment phase, when the information obtained in the different parts of the process is transformed in Risk Units. The approach is scientifically solid and compatible with current risk management practices for decision making.*

As a general comment (question 13) **ES** further explained that the *use of combined biological and chemical tools for assessing complex mixtures is a key objective* in three ongoing large research networking projects funded by the Spanish[19] and the Madrid-Region Governments[20], covering wastewater effluents, wastes and

[19] CONSOLIDER-INGENIO TRAGUA-CSD2006-00044
[20] Programas de Excelencia en I+D RESIDUOS-S-0505-AMB-0352 and EÍADES-S-0505/AMB-0296

contaminated soil and pointed to some recent key publications emanating from these and several smaller projects on the issue.[21]

[21] Pablos MV.; Fernández C.; Babín MM.; Navas JM; Carbonell, G.; Martini, F.; García-Hortigüela P and Tarazona JV. Use of a novel battery of bioassays for the biological characterisation of hazardous wastes. Ecotoxicology and Environmental Safety. Doi: 10.1016/j.ecoenv.2008.12.016.
Gutiérrez S, Fernández C, Escher BI, Tarazona JV. (2008). A new hazard index of complex mixtures integrates bioconcentration and toxicity to refine the environmental risk assessment of effluents. Environ Int. 34:773-81.
Babín MM, Cañas I, Tarazona JV (2008). Short communication. An in vitro approach for ecotoxicity testing of toxic and hazardous wastes. Spanish Journal of Agricultural Research 6 (Special Issue): 124-128.
González-Doncel M, González L, Fernández-Torija C, Navas JM, Tarazona JV. (2008). Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: studies with Prestige oil and medaka (Oryzias latipes). Aquat Toxicol. 87:280-8.
Barata C, Alañon P, Gutierrez-Alonso S, Riva MC, Fernández C, Tarazona JV. (2008). A Daphnia magna feeding bioassay as a cost effective and ecological relevant sublethal toxicity test for Environmental Risk Assessment of toxic effluents. Sci Total Environ., 405:78-86.
Fernández MD and Tarazona JV. (2008). Complementary approaches for using ecotoxicity data in soil pollution evaluation: risk based versus direct toxicity assessments. In: Soil Pollution Research Trends. Ed. Javier B. Domínguez. Pp. 1-50. Nova Science Publisher. 2008. ISBN: 978-1-60456-319-1.
Fernandez, MD, Babin M and Tarazona JV (in press). Application of bioassays for the ecotoxicity assessment of contaminated soils. In Methods in Molecular Biology, Biorremediation, Cummings, S. ed. (Totowa, USA: Humana Press)

5. Expert interviews

To deepen the insight gained by the written survey, oral expert interviews were conducted with a selection of experts from relevant member states institutions. Requests for an interview were focused on such institutions that have a particularly broad experience in the application of diverse methodologies for mixture toxicity assessment to a wide array of different types of environmental samples and waste samples. As a selection criterion, inquires were sent out to those institutions that (i) apply whole mixture testing to at least 5 out of 10 different categories of samples (see Tab. 2, response to question 3 of the written survey) and (ii) apply at least 4 out of 10 different component-based approaches (see Tab. 2, response to question 7 of the written survey). Within the available time frame, experts from three out of four institutions meeting these criteria kindly gave their consent to an interview.

The interviews followed specified catalogues of questions that were sent out to the interview partners prior to the agreed interview date. The interviews were structured into three main parts: (i) an introductory set of general questions on current mixture toxicity assessment, asking the experts to briefly express their views on needs and relevance, legal background and political drivers, as well alternative options for mixture toxicity assessments in terms of whole mixture testing and component-based modelling approaches, (ii) a main set of questions going specifically through part of the answers that were kindly provided in the written questionnaire, asking for some clarifications, explanations, and more detailed comments, and (iii) a final set of questions, again going back to the more general level, asking for the experts opinions on prospects and future developments in the field. Parts (i) and (iii) of the interview guides were largely identical for the three interview partners; parts (ii) were different.

All three interviews were conducted by phone and limited to a time of approximately 30 minutes each. All three interview partners gave their consent to an audio recording of the conversation. The recordings were first transcribed and then edited protocols were prepared. Editorial changes included slight shortenings and condensations of some interview passages with the aim of improving readability without distorting the content and meaning of the spoken words. Resulting protocols were sent out to the interview partners for final corrections and approval.

The interview protocols given in the following sections 5.1 to 5.3 include all introductory explanations and all questions that were listed in the interview guides that were prepared prior to the conduct of the interviews. Where such questions were actually skipped during the interview, this is indicated. The following formatting is used in the protocols:

- Headlines indicating the interview structure are printed in capitals.
- Questions are printed in bold.
- Answers are given in italics.

5.1 The Netherlands :

Dr Martien P.M. Janssen, National Institute of Public Health and the Environment (RIVM)

PHONE INTERVIEW ON PRACTICAL EXPERIENCES IN ASSESSING MIXTURE TOXICITY OF ENVIRONMENTAL SAMPLES AND WASTE SAMPLES IN AN EU MEMBER STATES

INTERVIEW PARTNER

Country: The Netherlands
Institution: National Institute of Public Health and the Environment (RIVM), Expert Centre for Substances
Name: Dr Martien P.M. Janssen

INTERVIEW DATE

19 June 2009

BACKGROUND AND AIMS OF THE INTERVIEW

As part of an ongoing contract study on mixture toxicity, the European Commission, DG Environment asked me to analyze the status of experience and practical approaches for assessing the toxicity of complex environmental samples and waste samples currently used in EU member states. As a first step, a survey was conducted by means of a written questionnaire sent out to competent authorities in all member states. As a second and final step, the analysis shall now be refined by means of interviews with a few selected experts from relevant member states authorities. I am very grateful for your willingness to support the study by taking part in this interview.

INTERVIEW STRUCTURE

First, you will be given time to introduce yourself and the work of your agency. Then I would like to start with a short set of general questions on current mixture toxicity assessment, asking you to briefly express your views on needs and relevance, legal background and political drivers, as well alternative options for mixture toxicity assessments in terms of whole mixture testing and component-based modelling approaches. In the subsequent main part, I would then like to continue by going specifically through the answers that you have kindly provided in the written questionnaire, asking you for some clarifications, explanations, and more detailed comments. Finally, I would like to go back to the more general level, asking for your opinion on prospects and future developments in the field.

INTRODUCTION

[Prof. Kortenkamp gives some additional introductory explanations on his research work and the study aims and then continues with the first question:]

May I ask you to start with a brief introduction of yourself and of the authority that you represent? How would you summarize your tasks and responsibilities?

I am working in the National Institute of Public Health and the Environment and partly I am based at the Ministry of Housing, Spatial Planning and the Environment. Within the National Institute I am coordinating work on standard setting. I also coordinated the completion of your written questionnaire. I got answers from different parties within in the Netherlands and collated them in one answer for the whole country.

[Prof. Kortenkamp gives his thanks for that collation work]

Now I am in a difficult position: Probably, I am not able to answer all your questions in detail, but in a number of cases I have to refer to more specialized persons.

[Dr. Janssen asks for some additional explanations about the purpose of the study and the aims of the interview. Prof. Kortenkamp gives a brief summary]

GENERAL QUESTIONS ON MIXTURE TOXICITY ASSESSMENT

NEEDS AND RELEVANCE

From your answers to our questionnaire we learned that you perform extensive assessments of the toxicity of whole complex mixtures in addition to conventional single substance risk assessments. What are the main reasons for this additional effort? Is it that you consider single substance assessments to be insufficient for your tasks and aims? What are the drivers in the Netherlands?

I think that there are various drivers. A lot of those activities are location-based. We realized that with a single component approach, you will not get a really good view on what is happening precisely. The TRIAD approach and some of our early warning systems trigger the assessment of more than one substance. This is what comes out from several of the answers to your questionnaire.

We also realized that – as I am working in standard setting - in most of the cases, standards are set for single components. But sometimes, mixtures are taken into account. For instance, for mineral oil we developed a kind of mixture approach, taking into account the toxicities of the different components of mineral oil. Although we developed it, this standard has not yet been implemented in the Netherlands legal framework.

So I think, you should distinguish between the scientific work, the application, and the legal framework. Within the legal framework actually most of our regulations now are European based. To my opinion, not many of those directives provide a basis for doing something on mixtures.

Are there any special problems of mixture toxicity of complex environmental samples or waste samples in your country? Or are there special areas of concern in terms of substance groups, polluted locations, environmental effects or diseases that are considered as actual or potential mixture effects?

Actually I am not aware of that other than the ones mentioned above.

Are there not some special problems with polluted sediments from the river Rhine?

There may be some experiments running that test whole sediments. And I know that there are experiments using complete water samples, looking what kinds of substances are causing the overall effects. So in essence you could say that those are mixture toxicity experiments. But further more, I am not really aware of things going on in this field. May be you should approach some of the experts mentioned on page I of the questionnaire.

What about substance groups? Are there particular groups of substances occurring as mixtures that you are concerned about in the Netherlands currently?

I can imagine polyaromatic hydrocarbons as such a group, and probably metals. But as I explained before, this is not my special field of work: You should speak to people that are doing the TRIAD approach and working on polluted sites.

LEGAL MANDATES AND POLITICAL DRIVERS

In your written responses to the questionnaire as well as your accompanying letter you stressed that *there are no legal requirements in the Netherlands to perform complete mixture toxicity tests* and *no plans to introduce such legal requirements*. Nevertheless, your answers show that there is extensive experience in dealing with mixtures in the Netherlands which has a leading position in the field.

Are there any special political drivers in your country that stimulate your engagement in the performance and advancement of mixture toxicity assessments?

I think, we are aware that with a one-substance-approach you will not cover all things. Although in the risk estimation we have built in some precautionary principle by means of additional assessment factors, people are still interested to know the effect of mixtures. But if you are asking for questions from politicians or something like that, I am not aware of that. I think this work is mainly driven by people working in this field and being aware that sometimes

it would be more appropriate to apply a mixture toxicity approach instead of a single substance approach.

In Denmark politicians are particularly concerned about human health effects of endocrine disrupters when they occur as mixtures. This week the Danish environment minister has come forward with a press release on the issue, announcing that he is going to take a corresponding initiative at European level. Is there anything like that going on in the Netherlands at the moment?

No, not to my knowledge.

Do you think that mixture toxicity is sufficiently taken into account within the existing legislation for the protection of human health and the environment in your country? Or do you see the need for substantial improvements?

Any legitimation for doing it is lacking, as I already said. We are doing a number of things because people working with toxic substances think that it is necessary. I don't know if the word scientific is the right word, but it is driven by people promoting this approach, because they think that it gives a more realistic picture and practical solutions. Actually there are two drivers, one is the precautionary principle and the second one is to gain scientific insights.

The US EPA has developed a very elaborate framework of documents for dealing with chemical mixtures. People from the agency clearly say that this would not have been done if they not had specific provisions in corresponding laws. What is your estimation of the situation in the Netherlands in that respect?

Officially the Dutch government has precisely the same view.

The same view that a particular clause in laws is required?

Not "required". "Required" is too strong.

Would it be "desirable"?

Yes. The Netherlands will not do more than required by the European Union. That's the official state of play. We might do some more in a number of cases, for instance where there are no regulations by the European Union and where this approach provides a good solution. Examples are the early warning systems and local risk assessments such as TRIAD.

This means that probably initiatives should come from the European Commission?

I can imagine that. I should also conclude that Denmark is a little bit more expressed considering this issue than the position the Netherlands would take.

OPTIONS: WHOLE MIXTURE TESTING VS COMPONENT-BASED MODELLING

[The following question was skipped:]

You apply both, whole mixture testing and component-based whole mixture toxicity modeling. How would you summarize the reasons why you consider these as complementary rather than alternative approaches?

SPECIFIC QUESTIONS RELATING TO YOUR WRITTEN ANSWERS TO THE QUESTIONNAIRE – CLARIFICATIONS AND FURTHER DETAILS

QUESTION 3 – THE TRIAD APPROACH TO THE ASSESSMENT OF CONTAMINATED SOILS

In question 3 we asked for the kinds of samples for which you apply the whole-mixture approach. In the case of *soil* you commented that for contaminated sites a formal approach named TRIAD is used and you kindly provided a reference to a 2006 RIVM report on a novel decision support system which includes the TRIAD approach.

How would you summarize the principles and advantages of the TRIAD approach?

Actually I cannot, because I am not really involved in that. I will provide you a name and a telephone number.

[As a consequence the following additional question on TRIAD was skipped:]

It was the intention of the report to *promote more practical experience in site-specific evaluation of ecological risk*. Do you have indications that this hope will materialize?

QUESTION 6 – APPLICATION OF COMPONENT-BASED APPROACHES TO SURFACE WATER SAMPLES IN THE CONTEXT OF UNEP/OCHA ENVIRONMENTAL EMERGENCY RESPONSES

In question 6 we asked for the kinds of samples for which you apply component-based approaches. In case of *surface water* your comment pointed to the application of such approaches *for assessing natural disasters which involve secondary chemical releases (for the UN: UNEP/OCHA)*.

Could please explain this important international engagement in some more detail? Are there any guidelines, tools or practical cases of prior importance in this context?

I am not informed about that. The answer came from the same group that can also tell you more about the TRIAD approach.

QUESTION 7 B) – APPLICATION OF CA AND TUS TO METALS AND PESTICIDES

In question 7 b) we asked you to specify *groups of substances within a complex mixture to which the use of* a specific component- based *approach might be confined*. In the case of *Direct application of the CA formula* and *TUS* you *inter alia* indicated *metals* and *pesticides*.

Does this mean that you assume concentration-additive action of all metals or all pesticides in a sample, or does this apply to certain sub-groups only for which you assume a similar toxicological mode of action (such as organophosphorous pesticides for instance, as indicated in your response relating to the RPF approach)?

The latter is correct. For instance, I know at least on example where we did this, this are the chloroanilines. We developed a standard for all three mono-chloroanilines and I think we did for some organophosphorous pesticides as well. It depends a little bit on the standard we derive, whether it is for a whole group or a single substance. I mentioned the example of mineral oil, which is a little bit different approach. There we followed the toxic unit summation approach.

QUESTION 7 – PROMOTION OF PODI

With respect to the PODI approach you commented: *Should be promoted*.

What are the driving factors and the specifically envisaged applications behind this recommendation? What are the advantages that you expect from the application of this approach?

I don't now, but I may look up who answered that question.

QUESTION 7 (AND 10) – HI AS A FIRST TIER APPROACH

You did <u>not</u> mark HI as an approach that you apply, and correspondingly you did <u>not</u> indicate any sample types, substance groups, and endpoints to which this approach may be applied. However, you commented that HI can be used as a first tier approach for evaluating site specific contaminations and in your response to question 10 you wrote that it is included as a calculation tool for local area managers in the *risicotoolboxbodem.nl*.

I would conclude that your extensive experience indeed also includes practical application of the HI approach. Is this correct?

I should refer to the remark I made earlier on TRIAD. It is one group working on TRIAD, Risicotoolbox, etc. I would recommend you to have another interview with someone from that group, because they are really involved in risk assessments for soils and they use those kinds of approaches on a local scale.

QUESTION 7 – MODEL OMEGA AS A SPECIFICATION /MODIFICATION OF THE IA FORMULA

You indicated that you apply the IA formula by means of a *model Omega*.

Could please give a brief explanation of this model? Could you give me a reference to a documentation of that model?

I have to ask for that, because that answer comes from my colleagues from the Ministry of Transport, Public Works and Water Management.

QUESTION 7B) – msPAF AS MIXED MODEL APPROACH TO ALL KINDS OF SUBSTANCES

You have developed the msPAF approach in which substances with the same *toxic mode of action* (*TMoA*) are pooled (as explained in the RIVM report that you mentioned in your response to question 3). Within such groups msPAFs are calculated according to Concentration Addition, while IA is assumed for the combined action of different groups. You state that the applicability of this mixed approach is hampered *only by the worldwide limitations in data sources*.

Which criteria do you use for deciding whether either a common or a different toxic mode of action can be assumed? How do you handle this problem in cases where there is insufficient knowledge about the modes of toxic action of substances in a mixture?

I have to give the same answer. It is the group of RIVM working on TRIAD, Risicotoolbox and msPAF. I would refer you to this group.

QUESTION 10 – RISIKOTOOLBOXBODEM.NL FOR RISK ASSESSMENT OF CONTAMINATED SITE

You mention that you have established the web-based calculation tool *risikotoolboxbodem.nl* for use by local area managers.

Is this tool well accepted and used by the target group of local area managers? Would you recommend it as a successful example for imitation by other EU member states?

Same answer as before. It is the same group.

QUESTION 11 – NEGATIVE EXPERIENCE / WARNINGS

You state that the *component based method is too general for risk assessment. This method is mostly followed by the estimation of bioavailability of metals and organic compounds like PCBs and PAHs.*

Could you please explain this point in some more detail?

I do not see precisely who answered that. But I can explain a little bit, because in standard setting and in the water framework directive we experience the same actually. What I can imagine for metals, for instance in the water framework directive, is that you may apply bioavailability in the end. I know, in risk assessment for soils it is done in the same way. You look at the total

amounts, but you don't know if the total amount is available. In most cases it is not. So, on-site you look at the availability and the final effects and you may overestimate the effects by just following the general approach.

Over-estimating the effect by not taking bioavailability into account?

Yes. The only trade-off that we now have in the discussions that we generally have with industry on whether bioavailability should be applied already in permitting, is that from the legislative side we could say no. So apply bioavailability in compliance assessment, but not in permitting. Between those two areas there is still some friction, because in permitting you would take into account precaution but in compliance assessment you might look whether the substance on-site is really a problem or not.

QUESTION 12 – POSITIVE EXPERIENCE / RECOMMENDATIONS

You state that component-based approaches *are useful (…) for multi-stress analysis (diagnosis) of (bio)monitoring data (such data are collected under e.g. Water Framework Directive, but the over-all data set is difficult to analyse due to "over-parameterisation", that is: when all individual chemicals are monitored, the output is "non-significant" due to the curse of dimensionality". Cumulative risk assessments, e.g. by msPAF, helps out!*

I am not quite sure whether I understand the argument correctly. Could you please explain the problem and how msPAf helps out of it in some more detail?

But, probable we should ask the TRIAD people there as well?

I think so. Yes.

QUESTION 13 – GENERAL COMMENTS – POP REVIEW COMMITTEE

You pointed to the mixture toxicity *working group under the POP Review Committee for the Stockholm Convention on POPs (UNEP)* which *will report in October 2009*.

Are you directly involved in this activity? Are there any preliminary outcomes already available?

I think that there are preliminary outcomes. I am participating in the regular meetings, but not of this specific working group. I can point you to the Stockholm secretariat and give you a contact person. Below the POP review committee there are several working groups, normally on the substances that are being handled and sometimes on more general scientific topics, in this case mixture toxicity. The working group was formed in October last year. I guess it has been started in January or February. Normally there is a chairman, a drafter, and a number of members which comment on the draft. We are now in June. This process normally goes on until June or July and then there will be a final draft in August, which will be translated in the six UNEP languages. So I think there should at least be a draft report and in August there will be a final draft, which will be discussed around 15 October.

It might be that someone from the Commission is in the working group, but I am not sure. I will send you some of the names.

QUESTION 13 – GENERAL COMMENTS – EQS FOR MIXTURES

You stated that *environmental quality standards* (EQS) *are in most cases based on single substance toxicity tests.*

Do you see a need for the development of EQS for mixtures?

I explained to you that for some substance groups we use a combined standard, for the monochloroanilines for instance. That really depends on the data we have and whether we treat such a group as some single substances. It is more or less on an ad hoc basis. If you want to go for standards for mixtures, you already have the problem of defining the components of that mixture, because the amount of potential combinations is tremendously large. That is one of the problems you will encounter, I think. I know some experimental studies that have been carried out with a combination of an organic with a metal or something like that. But those are really limited. On the other hand you have a tremendously large number of combinations to cover. So, in the short term, I do not see any solution to go for a standard for let's say a mixture of a certain organic and a metal, or something like that.

So you say, the changing composition is an impediment to define an EQS for a mixture?

I think it is, yes. I can imagine that you have a mixture approach for instance for PCBs, which are often a combination of substances, or for dioxins, or for mineral oil, which is another case of course.

In many cases there the composition doesn't change very much in environmental media or in human tissues because they are so bioaccumulative and persistent?

Yes.

PROSPECTS

How would you assess your know-how and experience in the field of regulatory mixture toxicity assessment in comparison with other EU member states?

What do you mean precisely by that question?

You presumably have an idea what's going on in other EU member states. How do you see this in relation to the Netherlands? Do you think you do more than they or how do you assess that?

You already answered that more or less in your introductory questions, I should say.

You can confirm that view or would you disagree with me there?

May be. In the UNEP / POPs framework we started the discussion on mixtures because we often have a debate on whether the environmental concentrations in the polar areas would cause an effect. The argument is that you should not only look at those compounds as single substances, but take into account that there are more substances accumulating in those areas. That's why we started the study on mixtures within UNEP /POPs framework. And I think the feeling also in other countries is more or less the same on this topic.

Meaning they see the need to look at mixtures?

I think so. There may be differences between the European member states, as you already stated, but that has to do with the political framework. I explained you a little bit on that, for instance why Denmark probably has a different position than the Netherlands. I think that is one of the reasons. The other reason is the scientific input. That is probably driven by the amount of money being available for this kind of work.

What, in your opinion, would be the most important steps towards improvements in the field of mixture toxicity assessment?

I think throughout our answers to the questionnaire you see that we mostly apply mixture toxicity assessments in the field situation, looking whether there really is a problem or not. And I think that is the place where you can make the most progress. I don not see so many possibilities in standard setting as I explained before, because the number of combinations is tremendous and the data are lacking.

Are there any important current or planned projects or initiatives in the field of regulatory mixture toxicity assessment in your country? If so, what is the role of your institution therein?

I don't think so. May be you should ask this also to the persons working on TRIAD, msPAFs and the Risicotoolbox.

Do you know about any important activities of other EU member states in the field apart from the ones we have mentioned?

No.

In a recent speech[22], Commissioner Stavros Dimas considered gaps in knowledge and assessment of exposure "*to a cocktail of many different substances*" as one of three major long-term challenges for chemical safety

[22] Stavros Dimas, Member of the European Commission, responsible for the Environment, Speech/09/275. REACH: Achievements and Challenges. Helsinki Chemicals Forum 2009, Helsinki, 28 May 2009.

regulation, in addition to challenges posed by endocrine disrupters and nanotechnologies. Would you share this view, or would you disagree?

I would share this view. The question I have is only on applicability. How should you include it in a regulation or how you should implement it in certain things that you develop?

What are your expectations or recommendations with respect to activities of the European Commission on the subject of "cocktail effects"?

Keep in mind that I am coming from a standard setting background. In the Netherlands we normally apply an assessment factor of ten to account for potential combination effects. What would be interesting to know is: what is the actual surplus of mixture toxicity above single substance toxicity? What does a combination add to single substance toxicity?

THANK YOU FOR THIS INTERVIEW

Prof Dr Andreas Kortenkamp

5.2 Hungary :
Dr Hilda Farkas, Ministry of Environment and Water

PHONE INTERVIEW ON PRACTICAL EXPERIENCES IN ASSESSING MIXTURE TOXICITY OF ENVIRONMENTAL SAMPLES AND WASTE SAMPLES IN AN EU MEMBER STATES

INTERVIEW PARTNER

Country: Hungary
Institution: Ministry of Environment and Water, Dept. of Environmental Management
Name: Dr Hilda Farkas
Position: Head of Department

INTERVIEW DATE

19 June 2009

BACKGROUND AND AIMS OF THE INTERVIEW

As part of an ongoing contract study on mixture toxicity, the European Commission, DG Environment asked me to analyze the status of experience and practical approaches for assessing the toxicity of complex environmental samples and waste samples currently used in EU member states. As a first step, a survey was conducted by means of a written questionnaire sent out to competent authorities in all member states. As a second and final step, the analysis shall now be refined by means of interviews with a few selected experts from relevant member states authorities. I am very grateful for your willingness to support the study by taking part in this interview.

INTERVIEW STRUCTURE

First, you will be given time to introduce yourself and the work of your agency. Then I would like to start with a short set of general questions on current mixture toxicity assessment, asking you to briefly express your views on needs and relevance, legal background and political drivers, as well alternative options for mixture toxicity assessments in terms of whole mixture testing and component-based modelling approaches. In the subsequent main part, I would then like to continue by going specifically through the answers that you have kindly provided in the written questionnaire, asking you for some clarifications, explanations, and more detailed comments. Finally, I would like to go back to the more general level, asking for your opinion on prospects and future developments in the field.

INTRODUCTION

May I ask you to start with a brief introduction of yourself and of the authority that you represent? How would you summarize your tasks and responsibilities?

My name is Hilda Farkas, I am a PhD and a chemical engineer. I work for the ministry of environment. I am the head of a department dealing with waste issues. Waste is my main working area, but of course I am also informed on issues relating to the other environmental media.

[Dr. Farkas also introduces a colleague attending the interview. He is also a PhD and a chemist with broad experience in the analyses of environmental samples.]

[Prof. Kortenkamp introduces himself too. He gives some brief additional explanations on his research work and the study aims and then continues with the next question:]

GENERAL QUESTIONS ON MIXTURE TOXICITY ASSESSMENT

NEEDS AND RELEVANCE

From your answers to our questionnaire we learned that you perform assessments of the toxicity of whole complex mixtures in addition to conventional single substance risk assessments. What are the main reasons for this additional effort? Is it that you consider single substance assessments to be insufficient for your tasks and aims?

Yes. But first I would like to explain a little bit what we do here in Hungary, because for us it was not very clear, what the reason for your questionnaire was. Probably we misunderstood each other. I don't know. It will be clear later, after this conversation, I guess.

First of all we make a chemical analysis of individual components in environmental samples, and we compare it to the single limit value. That's clear. For example we do so for metals. Secondly, sometimes we apply a kind of toxic unit summation, for example for nitrate and nitrite, especially for the drinking water samples. A third type of analysis is simple addition. When we analyse for example PAHs, or PCBs, or halogenic aliphates, we measure a group of these components, 16 PAHs or 7 PCBs for instance. We simply summarize the concentrations of these congeners, and we have a limit value for the sum. As a fourth type of analysis, for example for dioxins, we use this TEQ model, which accounts for the different toxic features of the special congeners. So, sometimes we use single substance assessment, sometimes we use different types of models for the assessment of mixtures.

Interposed question: **When you sum up the individual measurements for chemicals, for example PCBs or PAHs, you then apply a limit value for**

the sum, you said. Where do these limit values come from? Do you establish them yourself or do you apply some international values?

Since the accession we use European limit values, of course for drinking water and especially for the air. But for the soil, as we do not have European limit values, we use Hungarian national limit values, and also we use such kind of limit values for the assessment of single components of waste.

Interposed question: **And these limit values are derived on the basis of toxicological criteria?**

Yes.

But, if I can continue the explanation, there is one more type of chemical analyses. In the case of TPH[23] *the measurement is a sum and we also have a sum limit value. But the reason is the analytical method which gives a sum parameter. It is not like in the case of dioxins or PCBs where we analyze the special congeners and sum them up afterwards. So this is a little bit different. It completes the whole picture of what we measure in Hungary.*

In addition to chemical analyses, we also use a very wide range of biological tests to assess the complex effects of the environmental media or waste. This does not only include ecotoxicological effects, but also infectious properties in case of hospital waste for example, and cell toxicity assays for the assessment of hazardous waste.

Are there any special problems of mixture toxicity of complex environmental samples or waste samples in your country? Or are there special areas of concern in terms of substance groups, polluted locations, environmental effects or diseases that are considered as actual or potential mixture effects?

Not really. This methodology was developed in the very early 1980 years. For our hazardous waste legislation it is a bit older than the European one. And in fact we started to develop these biological tests, because we had learned that sometimes the chemical analyses could not reflect the environmental aspects of the waste appropriately. We have a very good tradition to characterize the features of hazardous waste on the basis of the biological tests.

LEGAL MANDATES AND POLITICAL DRIVERS

Is your work in the field of mixture toxicity assessment based on a legal mandate or requirement or is this an additional initiative with an investigative character?

Of course we have a legal background for such kinds of assessment. We have limit values, we have methods described in different national legislative pieces, and of course we have standards on how to make these measurements,

[23] Total Petroleum Hydrocarbons

on how to calculate the assessment, and on which kind of model we should apply when we assess one of the samples.

Interposed question: **Is this enshrined in any special law in Hungary that says you should, when assessing environmental quality, take mixture effects into consideration?**

Yes, of course. It is not an act, but government decrees. We have special decrees for hazardous waste, for surface water, for ground water quality, for drinking water and soil protection and for remediation, full of limit values and methods.

An act is the highest legislation level in Hungary, and the corresponding implementation rules are given in the government decree, which is the implementation law of the act. We have an environmental act, a waste management act, a water act, and of course the details of the implementation are given in the corresponding government decrees. This is the legal basis for our work

Are there any special political drivers in your country that stimulate your engagement in the performance and advancement of mixture toxicity assessments?

As I explained, in some cases we assess mixture toxicity on the legal basis. But sometimes of course the researchers are very interested to know more about these potentials, because sometimes we are facing problems when we assess single substance measurements. Who to combine them, who to weigh them, which substance is more, which is less important for the overall effect? Sometimes you get for example 40 results from one laboratory sample and experts face practical problems in giving an overall assessment on the basis of these single results, even if part of them is based on special modelling approaches.

Do you think that mixture toxicity is sufficiently taken into account within the existing legislation for the protection of human health and the environment in your country? Or do you see the need for substantial improvements?

Of course I would like to see improvements. The environmental experts feel that there is a need to move on, because, as I explained, we sometimes have a problem of how to compare different single results and how to get an overall environmental effect assessment. So that's the reason why I think that on the European level, for example, we can move on.

OPTIONS: WHOLE MIXTURE TESTING VS COMPONENT-BASED MODELLING

You apply both, whole mixture testing and component-based whole mixture toxicity modeling. What are the main reasons why you consider these as complementary rather than alternative approaches?

They are complementary, I think, because these approaches can give us different results with different consequences. In Hungary we follow the worst case scenario principle, and therefore we think that complementary information improves the basis for identifying realistic worst case scenarios.

SPECIFIC QUESTIONS RELATING TO YOUR WRITTEN ANSWERS TO THE QUESTIONNAIRE – CLARIFICATIONS AND FURTHER DETAILS

QUESTION 6 – COMMENTS ON SAMPLE TYPES

Question 6 was: *For what kind of samples do you apply component-based approaches?* You marked 6 of the 10 optional sample types and gave additional comments. In case of *Surface Water* you wrote: *Determination of the limiting concentrations of the known quantity of toxic compounds by tests listed in Para 4* (i.e. biotests used for the whole-mixture approach). For the other 5 sample types your comments were basically the same or very similar.

I am not sure whether I understand your comments correctly:

Does this mean that you assess single substances in the complex samples in a conventional substance-by-substance approach (by comparing individual concentrations against individual limit values)? Or does this mean that you experimentally determine limit values that are then used as input data for the models that you marked in your answer to the subsequent question 7 (CA and related models)?

Of course the answer depends on what the reason for assessment are and what the type of samples are. As I explained, we use different models of course. But in general, the most complex model we use is the hazard index type of approach, and this is used for the determination of cleaning limit values for the remediation of polluted sites. For other samples and assessment purposes we use the approaches that I outlined in my remarks at the beginning.

QUESTION 7 – MODEL SPECIFICATIONS / MODIFICATIONS

Question 7 was: *What kind of component-based approaches do you apply?* You marked *Direct application of the CA formula, TUS, RPF, PODI,* and *HI*. For each of these you gave additional comments in the optional field *Model Specification / Modifications*. In case of *Direct application of the CA formula,* for instance, you wrote: *The similar examination of the components of a mixture is applicable to two or more component mixtures. Application of acute tests*.

I am not sure whether I understand your comments on *Model Specification / Modifications* correctly:
My impression is that you gave additional explanations of the models rather than stating specifications or modifications of the model definitions that we gave in the glossary that accompanied the questionnaire. Is this perception correct?

The problem is that we did not really understand the question. As I tried to explain we use different models for different samples. So we could not say that the CA model, or hazard index model, or any TUS model is used widely in Hungary, or is used especially for one medium. We use a mixture of approaches. That is the reason, why it was very difficult to fill in the questionnaire, because the methods are not uniform for all media.

But now, I guess, we understand better what information you wanted to get from us. For example, I can say that the TUS model is used specifically for nitrate/nitrite assessment. Or I can say that the hazard index is used for setting remediation target values. So I can give you some examples on how we use these models in different situations, but none is used generally.

QUESTION 7 B) – GROUPING OF SUBSTANCES FOR DIRECT APPLICATION OF THE CA FORMULA AND TUS

In question 7 b) we asked you to indicate for every modelling approach used: *groups of substances within a complex mixture to which the use of the approach might be confined (e.g. pesticides, dioxins, …).* In case of *Direct application of the CA formula* and *TUS* your reply was: *Effect of individual toxic materials, e.g. modelling for request, *Unique components by individual tests, Phenol, toluene, ethyl benzene, xylols, nC6, and C6-C12 mixture*.

Do I understand you correctly, if I would state that you consider the mentioned aliphatic and aromatic hydrocarbons as a group of similar acting substances and calculate their mixture toxicity by direct application of the CA formula or TUS?

Yes.

Is it also correct that you do not apply CA or TUS for any other group of compounds?

[This second part of the question was skipped]

QUESTION 7 B) – GROUPING OF SUBSTANCES FOR APPLICATION OF RPF, PODI, AND HI

Your replies to question 7 b) (groups of substances) in case of RPF, PODI, and HI were: *Effect of similar type toxic materials, Risk based approach of several compounds*, and *Complex effect of toxic materials*, respectively.

Could you please explain this in some more detail by characterising the relevant groups of compounds and materials included in the assessment in chemical and/or toxicological terms?

[Question was skipped]

QUESTION 7 C) – (ECO)TOXICOLOGICAL ENDPOINT(S)

In question 7 c) we asked you to indicate for every modelling approach used: *the (eco)toxicological endpoint(s) assessed by means of the approach (e.g. acute fish toxicity, estrogenic activity, …).* In all cases your answer was: *Destroyment, survival, cell aspiration inhibition*.

To which type(s) of cells and/or organism(s) do these parameters refer?

For waste water, soil samples, and waste samples we use different organisms, such as bacteria, daphnids, fish, and mustard seeds. We determine root growth of mustard seeds and survival in case of the other assays, and we also determine LD50 or LC50.

QUESTIONS 7, 9, AND 10 – DEVELOPMENT OF PODI AND HI

In your answers to questions 7, 9, and 10 you stated that you do not yet practically apply the PODI and the HI approach, but that this is under elaboration.

What are the driving factors and the specifically envisaged applications behind these developments? What are the advantages that you expect from the application of these approaches?

[Question was skipped]

QUESTION 12 – RECOMMENDATION OF LIMIT AND TARGET VALUES

You recommend the establishment of contamination limit values and clean-up target values for remediation works as you have done in case of an ex Soviet airfield.

From the context of your answers to the other questions, I assume that this applies to mixtures of the hydrocarbons you mentioned in your response to question 7 b).

Is this assumption correct?

Yes. The hydrocarbon mixtures are really complex, including aliphatics and aromaics, small chain and long chain compounds, persistent components, and volatile components. So it is really a typical example of the mixture toxicity problem.

Have these values and developments been documented somewhere, and if yes, could you give a reference to such a document?

[This second part of the question was skipped]

QUESTION 13 – GENERAL COMMENTS – WIDESPREAD APPLICATION OF CA IN HU

You stress the widespread application of CA in Hungary for the examination of contaminated sites and the establishment of remediation target values.

Is this application of CA confined to the ecotoxicological assessment of hydrocarbon mixtures (as mentioned before) or do other groups of substances play a role too?

[Question was skipped]

PROSPECTS

How would you assess your know-how and experience in the field of regulatory mixture toxicity assessment in comparison with other EU member states?

I do not have a lot of information about the other member states. I know that many efforts are made in the field of remediation. National remediation programs are running and started twenty years ago. There is a wide range of activities to compare the different methods used in the field, and in particular the calculation and assessment of cleaning target values. This is the most important question in the field of remediation, and therefore there are a lot of activities aiming at the establishment of a common understanding and the harmonisation of the methodology. But if I understand correctly, this discussion did not yet come to an end. It is still ongoing.

Concerning hazardous waste or waste characterisation, I should say that the introduction of the list of waste of the European Union stopped a little bit the activity of complex toxicity measurements. As I mentioned earlier, we have a very good background and a lot of research results on who to assess the characteristics of waste by our methods, including biological methods. But after the publication of this list, many people thought that everything is clear now, that by classifying waste according to the list everything is correct, solved and perfect. But this is not true. That is why Hungarian legislation contains a special prescription. It says that if the characterisation of waste is not clearly determined by the list, the owner should make an examination of the hazardous features of the waste.

What, in your opinion, would be the most important steps towards improvements in the field?

[Question was skipped]

You are very active in the field of remediation. Are there any other important current or planned projects or initiatives in the field of regulatory mixture toxicity assessment in your country? If so, what is the role of your institution therein?

At the moment I cannot see any other activities. I am especially interested in further developments in the waste field, of course. I cannot say anything about air, but I guess that in the future it will be very important to go ahead in this complex field of assessment.

Do you know about any important activities of other EU member states in the field?

[Question was skipped]

In a recent speech[24], Commissioner Stavros Dimas considered gaps in knowledge and assessment of exposure "*to a cocktail of many different substances*" as one of three major long-term challenges for chemical safety regulation, in addition to challenges posed by endocrine disrupters and nanotechnologies. Would you share this view, or would you disagree?

Yes, of course, I absolutely agree with him.

What are your expectations or recommendations with respect to activities of the European Commission on the subject of "cocktail effects"?

It would be very useful, if the Commission could publish any recommendations on the issue. The harmonisation of the practices or methods between the member states is very important. If we want to be comparable and have equivalent market conditions, we should follow the same methods and we should establish the national regulations on the same level. That is very, very important.

THANK YOU FOR THIS INTERVIEW

Prof Dr Andreas Kortenkamp

[24] Stavros Dimas, Member of the European Commission, responsible for the Environment, Speech/09/275. REACH: Achievements and Challenges. Helsinki Chemicals Forum 2009, Helsinki, 28 May 2009.

5.3 Denmark :

Dr Henrik Tyle, Environmental Protection Agency

PHONE INTERVIEW ON PRACTICAL EXPERIENCES IN ASSESSING MIXTURE TOXICITY OF ENVIRONMENTAL SAMPLES AND WASTE SAMPLES IN AN EU MEMBER STATES

INTERVIEW PARTNER

Country: Denmark
Institution: Danish Ministry of the Environment, Danish Environmental Protection Agency, Chemicals Unit
Name: Chief advisor Henrik Tyle

INTERVIEW DATE

2 July 2009

BACKGROUND AND AIMS OF THE INTERVIEW

As part of an ongoing contract study on mixture toxicity, the European Commission, DG Environment asked me to analyze the status of experience and practical approaches for assessing the toxicity of complex environmental samples and waste samples currently used in EU member states. As a first step, a survey was conducted by means of a written questionnaire sent out to competent authorities in all member states. As a second and final step, the analysis shall now be refined by means of interviews with a few selected experts from relevant member states authorities. I am very grateful for your willingness to support the study by taking part in this interview.

INTERVIEW STRUCTURE

First, you will be given time to introduce yourself and the work of your agency. Then I would like to start with a short set of general questions on current mixture toxicity assessment, asking you to briefly express your views on needs and relevance, legal background and political drivers, as well alternative options for mixture toxicity assessments in terms of whole mixture testing and component-based modelling approaches. In the subsequent main part, I would then like to continue by going specifically through the answers that you and your colleagues have kindly provided in the written questionnaire, asking you for some clarifications, explanations, and more detailed comments. Finally, I would like to go back to the more general level, asking for your opinion on prospects and future developments in the field.

INTRODUCTION

May I ask you to start with a brief introduction of yourself and of the authority that you represent? How would you summarize your tasks and responsibilities?

I am Henrik Tyle, employed by the Danish EPA since 1982. For the last 25 years I have dealt with chemical risk and hazard assessment in various regulatory aspects.

GENERAL QUESTIONS ON MIXTURE TOXICITY ASSESSMENT

NEEDS AND RELEVANCE

From your answers to our questionnaire we learned that you perform assessments of the toxicity of whole complex mixtures in addition to conventional single substance risk assessments. What are the main reasons for this additional effort? Is it that you consider single substance assessments to be insufficient for your tasks and aims?

First of all it should be clear that I have taken over this job from a colleague. He completed your questionnaire and he also circulated it to various Danish research institutions. As I saw, he did not include some potentially relevant research groups dealing with air pollution. So the answers you have got do not cover all research groups. There are a few more.

I think your question is a little bit difficult, because you refer to single substance risk assessment without definition. However, chemical substances covered by chemicals legislation range from single chemical structures to actually chemically complex mixtures.

Different legislations deal with the safety of chemicals. They are all to a certain extent scenario specific. Either they concern one medium such as air at the workplace or surface water for instance. Or they are related to a specific use of a chemical, for example as an active ingredient in a pesticide or in a biocide. The legislation which is least scenario specific is the former existing substance regulation and the notification scheme for new chemicals, now taken over by REACH, because the idea here is to assess the safety of industrial chemicals in relation to <u>all</u> potential uses.

From the questionnaire, Denmark appears to stand out a bit because there is a lot of activity related to mixture toxicity assessment going on in your country. What are the main reasons for this?

I think the main reason is concern about endocrine disrupters, starting in the nineties. That is why many people deal with that subject. And also of course there is an academic interest in correcting simplistic scenario-based approaches which are used in regulation, a kind of critical comment or an inspiration for regulatory improvements. Recently, our environment minister acted on it by raising the issue of the combined "cocktail" effect of certain chemicals at the meeting of EU environment ministers in Luxembourg on 25 June.

That almost answers the next question: Are there any special problems of mixture toxicity of complex environmental samples or waste samples in your country? Or are there special areas of concern in terms of substance groups, polluted locations, environmental effects or diseases that are considered as actual or potential mixture effects? I guess yes, you said that already, that is endocrine disrupters. But is there anything else?

Early in the nineties, there was a guidance created on industrial effluent discharge permits, also on how to evaluate complex mixtures. This was based on the toxic unit approach and some inspiration was taken from, I think, the US and perhaps even Germany in that regard. But the whole surface water area, and all the drinking water area, is no longer placed institutionally at the Danish EPA. It has been transferred to another agency two years ago. The state regions and this agency they are working together in relation to the implementation of the water framework directive and also discharge permits of especially polluting factories.

LEGAL MANDATES AND POLITICAL DRIVERS

Is the work going on in Denmark in the field of mixture toxicity assessment based on a legal mandate or requirement or is this an additional initiative with an investigative character? What I am talking about is specific Danish law, not EU law. Is there an explicit demand to look at mixture toxicology in any Danish law?

At the EU level, you are probably aware that EFSA is starting to consider the joint assessment of pesticides with a common mechanism of action. Concerning specific national Danish law, I think there is no specific demand besides what I already said, that we have made a guidance document on the ecotoxicological evaluation of complex mixtures released from specific polluting factories.

I think we touched on the next question already too: Are there any special political drivers in your country that stimulate your engagement in the performance and advancement of mixture toxicity assessments? You have mentioned endocrine disruption. That is already a very strong stimulus. Is there anything else?

Endocrine disruption indeed is a very strong stimulus, and actually from a more academic point of view you could be a bit concerned that the problem is only raised in that regard, because mixture toxicity is a general issue, across different endpoints of course. But it is actually almost only mentioned in that context in the public debate.

Do you think that mixture toxicity is sufficiently taken into account within the existing legislation for the protection of human health and the environment in your country? Or do you see the need for substantial improvements?

When it comes to the chemicals legislation, 95% of all our regulation is not particularly Danish. It is either very much in agreement with procedures in other EU countries, or it is a direct implementation of EU legal requirements. So, it is a little bit strange to talk very much about Danish law here, because we have very few additional regulations. We are setting limit values in the outdoor air for inhalation for example. We have some environmental surveys and surveys of content of chemicals in consumer products and articles which are reported in the press with information about endocrine disrupters and other dangerous chemicals found in the wrong places. In this regard it is noted that the Danish EPA around ten years ago changed its communication strategy. The Danish EPA and the ministry of Environment is much more communicative with the public and the press now, and I think this is also why endocrine disrupters have stayed on the political agenda. The other main reason for this is that we here in Denmark have some very active research groups especially as regards endocrine disrupting chemicals. They inform continuously the public via the mass media about the outcome of their research covering epidemiology, human studies, toxicity studies and ecotoxicology. These research groups have established a network and are being supported by the Dansih EPA in various ways. The press is also getting information by the Danish EPA about interesting results from our consumer survey campaigns for instance.

OPTIONS: WHOLE MIXTURE TESTING VS COMPONENT-BASED MODELLING

You apply both, whole mixture testing and component-based whole mixture toxicity modeling. How would you summarize the reasons why you consider these as complementary rather than alternative approaches?

They have each their weakness and strength, and actually optimally you should do both.

SPECIFIC QUESTIONS RELATING TO YOUR WRITTEN ANSWERS TO THE QUESTIONNAIRE – CLARIFICATIONS AND FURTHER DETAILS

Note: From Denmark we have received six completed questionnaires, one from your agency and five from different research institutes at Danish universities, which your agency has kindly asked to contribute to our survey. If I do not explicitly state otherwise, my following questions refer to the answers that we kindly received from your agency, the Danish EPA.

QUESTIONS 2-4 – WHOLE MIXTURE APPROACH

In your response to questions 2 to 4 on whole mixture approaches, you pointed out that the focus of your unit in the Danish EPA is on substance assessment, and hence on component-based approaches. As an exception from the rule, you mentioned a *Survey on Estrogenic Activity in the Danish Aquatic Environment*.

Do other units of the Danish EPA apply any kind of whole mixture approach for regulatory purposes, as far as you know? Other EU member states, such as the UK for instance, use whole mixture testing for the

assessment of waste water effluents from industrial sites regulated under IPPC. Is this approach not used in Denmark too?

Yes, it is. And as I said, we made a guidance document and we have had that for a long time. But you should also be aware that in the EU the active ingredients of pesticides are approved at the EU level, but pesticides as formulations, they are authorised at the national levels. And there are actually testing requirements on formulations, at least on acute toxicity as far as I know.

QUESTION 7 A, B), AND C) – SAMPLE TYPES, SUBSTANCE GROUPS AND ENDPOINTS TO WHICH CA-BASED APPROACHES ARE APPLIED

In your response to question 7 you stated that you apply some approaches based on the assumption of Concentration Addition (CA): *Direct Application of the CA formula, TUS, TEF,* and *RPF*.

In the case of *RPF,* you indicated that this method has been used for assessing the activity of mixtures of estrogens in Danish waters and waste waters (presumably by means of the YES assay as you indicated in your corresponding response to question 4).

In the cases of *TUS, TEF,* and *Direct Application of the CA formula,* you did not give any indications on sample types, substance groups, and endpoints.
Are these approaches also used for estrogens? Or are they applied to other groups of compounds and endpoints?

The toxic unit concept is used in relation to acute toxicity, and even sometimes as regards longer term toxicity, in relation to aquatic organisms of complex industrial effluents in the context of industrial discharge permits. TEF is used for dioxin types of chemicals. And the direct application of the CA formula, I think, is employed more on an ad hoc basis, when relevant.

QUESTION 8 – APPROACHES NOT FITTING INTO THE CATEGORIES OF "WHOLE MIXTURE APPROACH" OR "COMPONENT-BASED APPROACH" – EQS FOR MIXTURES

In question 8 we asked for practical approaches that do not well fit into either of the categories of "whole mixture approach" or "component-based approach".

Your colleague Jacob Strand from NERI in his response pointed to OSPAR Environmental Assessment Criteria (*EAC*) and EU Environmental Quality Standards (*EQS*) which are applied in a Danish national monitoring program.

To my knowledge, *EQS* and *EAC* values in most cases refer to single substances, with the exemption of some few congeneric groups such as PAHs and PCBs.
Is this correct or do I err here? Do you see a need for the development of EQS for mixtures?

First of all, I had the same impression as you. Secondly, sometimes a trigger value or/and a marker approach is used. For example one selected PAH in a petroleum substance must not be above a certain limit value before it triggers something, for example classification and labelling. In relation to air pollution, I think, there is a tradition to sum the concentrations of certain PAHs. May be that these are types of approaches my colleague refers to.

In the context of the water framework directive and in relation to OSPAR there is a tradition "to go out and take the temperature and see if the patient is ill" (i.e. making environmental monitoring and surveys). It is a retrospective approach, and that is my concern about this. You could see the chemicals legislation as a kind of a more preventive type of regulation, because you try to assess the chemicals also on the basis of models and predictions about what could happen if they occur in the environment.

The tradition from the more media-oriented side, OSPAR and the water framework directive, is to go out and measure. But one will not get information about something, if one does not ask for that. And that is the whole problem, that in many of these regulations they are still measuring PCBs and so on, and not really the new stuff.

And then of course there is the issue of the whole biomarker approach, EROD for example. By this enzyme activity assay you can for example measure the co-planar PCBs in a smart way inside an organism. But it is in only very few cases that you have well enough established information about causality between a biomarker and an adverse effect. And that is the barrier of acceptance of all of this. For TEF you also use these kinds of enzyme activity or binding to the Ah receptor. What is the bioavailable sum of exposure to a certain type of chemicals affecting the biomarker in question? That is the idea. But there are not that many well working biomarkers which are used or can be used for regulatory purposes. This is partly because of conservatism, but also because of some inherent problems in relation to biomarkers and their causality for or link to adverse effects, I think.

QUESTION 9 – EXPERIENCE

You pointed out that the toxicity assessment of complex environmental samples is normally not in the focus of the Chemicals Unit of the Danish EPA and that your experience in this field is limited.

Do other competent units use component-based approaches for the purpose, as far as you know?

As I already said, both the whole effluent and also the component-based approaches are used for industrial wastewaters. And there are all these research activities, or survey activities, going on in relation to health issues for example. You have got some examples from those filling out the questionnaire. They are feeding into some debate about existing legislation. But I would say that there is a kind of a mismatch here between what the researchers are doing and how chemicals are assessed for regulatory purposes. There are policy issues involved here. Because, if one is interested in raising suspicion or concern, one is targeting the issue in one way. If you

are going to bring people to the court and give them a bill, or at least putting the responsibility on them, you have to make your case in another and very persuasive way, so that the guilty one is being charged. And that is the problem, I think. There is a discussion now on the use of the dose addition approach in the context of applications for granting authorisation under REACH. And it seems that, if the applicant is not himself using that, ECHA is currently of the opinion that it may be practically very difficult to apply the approach. My starting point would be, what could then the member state committee do, who is going to evaluate whether a substance is adequately controlled or not? Does the MSC not then have an obligation not just to take into account the information coming from the application, but also to make its own evaluation based on a more holistic and realistic approach? A similar problem is already been acknowledged to exist under REACH as regards how to perform and who should perform the regional assessment when environmental risk assessment is being performed. This concept was developed under the EPR[25] program but does not really exist under REACH. You are targeting each individual actor, i.e. each registrant or applicant concerning each individual substance. And that is the deficiency of the legal set-up in a way, because who and what chemical is to blame, if there are many contributors/chemicals involved in a certain problem/risk? Who should then be in charge? I.e., who is responsibe and which chemical should be affected by risk management decisions in that case? That is a policy issue.

QUESTION 10 – PURPOSES AND RELATED METHODOLOGIES

You marked *control of emission permits* and *priority setting for risk reduction measures* as purposes for which you apply methods for mixture toxicity assessment.

You already explained the *control of emission permits*. But what about *priority setting for risk reduction measures*? Do you apply both approaches, whole mixture testing and component-based approaches, for both of this purposes too? Could you explain this purpose and the corresponding methodologies in some more detail or give some examples?

With respect to priority setting for risk reduction, I think there is one interesting observation regarding REACH, and this refers to the grouping approach. Under REACH, grouping is very much underlined as an approach for minimizing new testing needs, but it is almost not at all addressed when it comes to risk assessment. If chemicals are so similar that you can read across, then it is also very likely that they have similar modes of action and or similar toxicity profiles, and therefore the total risk of the exposure to all of them should be taken into account in the risk assessment. So, why can you use the read-across approach when it comes to information requirements, but not when it comes to the assessment of the risk you are dealing with? In my view, this is a very strange logic. So, also in that regard, I think that our environment ministers' initiative is interesting.

I have not yet finalized my own thinking about this issue. The most simplistic read across approach could apply to chemicals with the same mechanism of

[25] Extended Producer Responsibility

action. Chemicals with a very similar structure may with a high probability have the same reactivity with biological tissue and share the same mechanism of action. For application of the dose addition approach, you even do not need the same mechanism of action. It is actually enough that they have the same mode of action, which is a much broader concept. There could be very many mechanisms involved in the same toxicological key property of a group of chemicals. But when I am talking about reading across, I am only talking about common mechanisms. Therefore, if we could match grouping in relation to information requirements with what we are doing in risk assessment, if we more realistically want to take account of simultaneous exposure to chemicals with similar modes of action, that would be a way forward in a more generic way, I think.

However, there could be some issues involved here, which make some adaptations necessary. It is very much depending on how you are actually reading across. Of course we need to make some good test cases. Certain types of endocrine disrupting chemicals may be very good cases. We have a good documentation of their properties and can show that the principle works.

QUESTIONS 10 – MCSS AND UVBCS

You also marked the option *other* purposes and pointed to the involvement of your unit in the European assessment of so-called multi constituent substances (MCSs) and substances of Unkown, Variable or Biological Composition (UVBCs). You stated that *"a range of approaches for assessing the mixture effects"* are applied in this context.

How would you summarize this *range of approaches*? Are there some leading principles that are consistently applied?

This may require more time than we have now. But what I can do is to give you some examples. CONCAVE, the organisation on petroleum substances, a long time ago developed the so-called hydrocarbon block method for environmental risk assessment. It is described in the TGD and it is transferred to the REACH guidance document. Basically they are reading across within categories of similar chemicals, and they are filling data gaps on very, very complex chemicals with many hundred constituents (UVCBs) by using this approach. The idea behind the hydrocarbon block method is that some petroleum streams have components which have so different fate properties that it does not make sense to make an overall assessment of the complex substance because, except just after being released into the environment, it does not appear to a similar extent (concentration) in the various environmental compartments. So what you should assess is actually the hydrocarbon blocks, that are the families of constituents which have similar environmental fate – and effect properties. This is different from an approach that CONCAVE took some years earlier, when dealing with the consequences of massive emission of oil products in spill situations. At those times, they developed the so-called water-accomodated fraction (WAF) approach, where they basically made the dosing into the aquatic test systems by ultra-sonicating a certain load of the complex substance with very low solubility components in there, and then they took the fraction that contained those components which could be brought into a stable dispersion and tested it for

acute toxicity. And that made sense in a way for giving a kind of a guestimate on what could be the consequence of massive release of that complex petroleum substance or product, but it is not really a useful concept for the assessment of longer term toxicity. The WAF approach is a whole mixture approach, only for an acute toxicity assessment in massive spill situations. For the risk assessment used under the NONS and ESR programmes CONCAWE later developed the hydrocarbon black method, which is a very different approach. For human health assessment these methods are not used. Humans may be exposed directly to these complex UVCBs. Therefore you have the tradition of a direct testing approach, I would say. I may add that in practical hazard and risk assessment this direct testing approach is often complemented by a marker approach, e.g. in the GHS for classification and labelling (i.e. the substance is classified for a certain health hazard endpoint, if the content of the chemical or product exceeds a certain trigger value – see further in my presentation at the January 2009 ED workshop).

QUESTION 11 – PROBLEMS IN THE REGULATORY WORK WITH MIXTURES

In your comment to question 11, you raised the problem that while *different approaches for assessing mixtures (…) are well established, more pragmatic knowledge is needed in relation to when the different approaches could be used* in the regulatory work. In particular, you pointed to the problem of appropriate grouping criteria for the assumption of concentration addition, and to the problem of transferring mixture toxicity assumptions across taxonomic groups.

What, in your view, might be an appropriate way for stimulating progress towards pragmatic solutions for these problems?

Grouping criteria for the assumption of concentration addition may range from being very scientifically correct for very narrow groups of chemicals, as in the case of TEFs for dioxins, to sum very general approaches such as the hazard index, where regardless of endpoints and toxicity profiles, just all those chemicals which can jointly occur in a working environment or in an industrial effluent, for instance, are summed up. Such pragmatic schemes, they are not totally scientifically justified, but they are using a common sense view: If you include every chemical that may be relevant in a certain exposure situation in your assessment, relate the exposure level of every chemical to the acceptable limit value for each, sum up all the fractions and then you come up with a value below one, then at least you can say that you do not have any significant problem.

Would that be an appropriate way for stimulating progress?

Under REACH we should argue that we are already using to a certain extent the concentration addition approach. I have dealt with nickel. There are all these nickel salts, but it is the nickel ion what they have in common and that with very few exceptions is contributing to toxicity and ecotoxicity. Thus, in the nickel risk assessment we are making an assessment of the nickel ion, regardless of the salt where it comes from. But these salts are different substances. So, by analogy as regards the assessment what is the difference of

that employed on nickel to employment of a similar approach to some of the phthalates which have the same impact on organisms (e.g. antiandrogenicity)?. Why can't we just sum up the exposure and effect of that of such phthalates? You have addressed that in your work.

But in addition to this, there is another issue. And that is that there still will be a lot of chemicals where you do not fully know the toxicity profile, and furthermore there will also be a lot of unforeseen exposure scenarios. Regardless of how well you try to develop the exposure assessment under REACH, you will not be able to deal with all aspects of being exposed to chemicals in daily life: exposure from medicine, from food, and so on and so on. We can only make certain scenarios and try to assess the risk according to those scenarios. There will remain very big problems with how well we are representing the totality of the reality and also how variable reality for different persons or environmental organisms is.

What I am saying is therefore, may be we should not make a risk assessment by only relating hazard levels for individual chemicals to the predicted or measured exposure levels. We have to be more cautious because there could be various chemicals acting in a similar way. Or may be we should simply adopt a more common sense approach and agree to adapt our assessment principles so that that we only accept to fill up the exposure level to a tenth of the hazard level?

And take account of mixtures in that way?

I would not call it an extra assessment factor, but it is in effect the same as to say that we do need an extra assessment factor of ten when we perform chemical by chemical risk assessment to take account of simultaneous exposure to similar chemicals .

Do I understand you correctly that one obstacle is also that there is no vehicle at the moment to deal with exposure to substances that come from areas that are currently covered by separate EU regulations, for example, cumulative exposure to pesticides, pharmaceuticals, household chemicals, food additives etc. etc.?

Yes. Each sector is making its own risk assessment, almost all fully neglecting that you can have contributions from the other sectors. Who is considering all the sectors? Even in REACH you are not dealing with feed additives, veterinary medicines, pesticides, biocides, human medicine, and so on and so force. Therefore, may be we should as a general approach simply say max. 10 % of the safe level for each chemical should be allowed. I have seen some interesting presentations from Thomas Backhaus from Gothenburg, Prof. Vighi from Milan, and other people even in the health area who looked on the total impact in terms of the sum of toxic units in different realistic exposure scenarios, such as wastewater treatment plant effluents, surface waters, and certain human exposure situations. Actually, the results appear to be similar. If you cover the first ten chemicals in terms of toxic units, you often cover 95 % of the total toxic load in many practical situations. And that kind of

distribution is not a big surprise for me. Based on such findings you could argue for that factor of ten I have mentioned. That could be a very pragmatic way forward, and it would not - and that is very important I think - be a great threat to industry, because for far most chemicals, exposure will not be near to that level. It is only the top priority chemicals, i.e. the chemicals where the exposure level is very close to the hazard level, which would be targeted by this. It would not be a dramatic change of everything.

PROSPECTS

You have already answered two of the next questions:

How would you assess your know-how and experience in the field of regulatory mixture toxicity assessment in comparison with other EU member states?

What, in your opinion, would be the most important steps towards improvements in the field?

[Questions were skipped]

Endocrine disrupters and the risks of cumulative exposure to endocrine disrupters are in the focus of research and policy initiatives in Denmark, you mentioned that. Are there any other important current or planned projects or initiatives in the field of regulatory mixture toxicity assessment in your country? If so, what is the role of your institution therein?

I don't think so. We especially finance endocrine disrupter research and there is a debate now on also financing some biomarker work too. But I am not aware that there should be further initiatives. What will be interesting is, when the debate crystallizes on the chemical action plan, because this will also affect the staffing at the Danish EPA and who is dealing with REACH and so on.

Do you know about any important activities of other EU member states in the field?

No.

In a recent speech[26], Commissioner Stavros Dimas considered gaps in knowledge and assessment of exposure *"to a cocktail of many different substances"* as one of three major long-term challenges for chemical safety regulation, in addition to challenges posed by endocrine disrupters and nanotechnologies. Would you share this view, or would you disagree?

These three issues are important and they are being discussed today. But there are other issues of course too. What is not discussed so much anymore are persistent chemicals. Just because the PBT concept has been reflected in

[26] Stavros Dimas, Member of the European Commission, responsible for the Environment, Speech/09/275. REACH: Achievements and Challenges. Helsinki Chemicals Forum 2009, Helsinki, 28 May 2009.

REACH does not mean that the problem of PBTs and PBT like chemicals has disappeared or that identification of those chemicals in practice is easy…

What are your expectations or recommendations with respect to activities of the European Commission on the subject of "cocktail effects"?

We are in an economic crisis, and there are a lot of initiatives to downsize the implications of REACH, and now we are coming here with something which could burden certain industries. It would be an advantage to other parts of industry, but normally, industry reacts by simply going against regulatory initiatives and actions. The whole issue of mixtures illustrates the inherent problem of regulating individual companies and individual chemicals chemical by chemical. Each company being in charge of one chemical, and not taking into account that there are other actors with similar chemicals being released too. The main obstacle to doing something effective here is illustrated by the response by ECHA in relation to whether we can assess the cumulative risk of phthalates in applications for granting authorisation. They state, in their view it can only be expected that an applicant takes into account that substance he is actually asking for permission to use. My position would be, it has to be taken into account that similar chemicals might also be used and may expose man and the environment and that an overall evaluation of the risk of the total exposure to all these chemicals should be made. If the company is not doing it for us in their application, the authorities then should do it, because adequate control does not mean that we have to accept the limited knowledge base that industry can provide us with. As authorities we have to have a broader view. But then the problem comes back that the whole idea behind REACH was self-regulation by industry. But you cannot self-regulate, if you ignore the rest of the world and other actors and other chemicals and think that you are only responsible for what you are doing and that other people are not doing something similar. That is the whole issue here. And I do not know for sure what would be the best approach to deal with that policy problem actually.

But you mapped out a pragmatic approach?

Yes. That is one attempt. It is simply to say: OK, but there are other actors too. You only get one tenth of what you think you can get, because you are not alone in the world.

THANK YOU FOR THIS INTERVIEW

Prof Dr Andreas Kortenkamp

Annex A

Questionnaire: Survey on approaches and practical experiences in assessing the mixture toxicity of complex environmental samples and waste samples in EU Member States

This questionnaire is addressed to environmental authorities in all EU Member States. **Background and scope** of this questionnaire are explained in the accompanying letter. In the attached **Glossary of Terms** please find definitions of all key concepts and terms of mixture toxicology used in this questionnaire. For any further questions please contact the study leader Prof. Dr Andreas Kortenkamp. Please return the completed questionnaire **within 6 weeks**. Contact details and return address are given in the accompanying letter. We thank you for your kind cooperation.

You may continue any question on a separate sheet as necessary.

Country of Origin	
Authority	
Contact Person	

1. Do you have any practical experience in assessing the mixture toxicity of complex environmental samples or waste samples?		
☐	**Yes**	*Please continue with the following questions*
☐	**No, we assess only individual components**	*Skip questions 2 to 12 and continue with the final point 13*

WHOLE MIXTURE APPROACH

2. Do you apply the whole-mixture approach, i.e. direct toxicity testing, for any kind of complex environmental or waste samples?		
☐	**Yes**	*Please continue with the following questions*
☐	**No**	*Skip questions 3 and 4 and continue with question 5*

3. For what kind of samples do you apply the whole-mixture approach?		
	Sample Type	*Sample Specification / Comment*
☐	**air**	
☐	**surface water**	
☐	**ground water**	
☐	**sediment**	
☐	**soil**	
☐	**biota**	
☐	**waste**	
☐	**waste water**	
☐	**waste water treatment plant effluents**	
☐	**others**	

4. What kind of biotests do you use for the whole-mixture approach?

Which biotests do you use for what kind of complex samples?

	Biotest Type	*Test Specification / Comment*	*Sample Type(s)*
☐	**sub-cellular assays** (enzyme assays, immuno assays, receptor assays etc)		
☐	**cell cultures**		
☐	**bacteria**		
☐	**algae**		
☐	**other plants**		
☐	**protozoa**		
☐	**daphnids**		
☐	**other invertebrates**		
☐	**fish**		
☐	**other vertebrates**		
☐	**multi-species assays**		
☐	**others**		

COMPONENT-BASED APPROACH

5. Do you apply a component-based approach, i.e. estimating total toxicity from information on identified components only, for any kind of complex environmental sample or waste sample?		
☐	**Yes**	*Please continue with the following questions*
☐	**No**	*Skip questions 6 and 7 and continue with question 8*

6. For what kind of samples do you apply component-based approaches?		
	Sample Type	*Sample Specification / Comment*
☐	**air**	
☐	**surface water**	
☐	**ground water**	
☐	**sediment**	
☐	**soil**	
☐	**biota**	
☐	**waste**	
☐	**waste water**	
☐	**waste water treatment plant effluents**	
☐	**others**	

7. What kind of component-based approaches do you apply?

Please specify the model(s) or method(s) used and indicate for every approach:

a) the sample type(s) to which it is applied (e.g. waste water, soil, …)

b) groups of substances within a complex mixture to which the use of the approach might be confined (e.g. pesticides, dioxins, …)

c) the (eco)toxicological endpoint(s) assessed by means of the approach (e.g. acute fish toxicity, estrogenic activity, ….)

	Model / Method	*Model Specification / Modifications*	*a) Sample Type(s)* *b) Substance Group(s)* *c) Endpoint*
Approaches based on the assumption of Concentration Addition (CA)			
☐	**direct application of the CA formula**		a) b) c)
☐	**TUS (Toxic Unit Summation)**		a) b) c)
☐	**TEF (Toxic Equivalence Factor)**		a) b) c)
☐	**RPF (Relative Potency Factor)**		a) b) c)
☐	**PODI (Point of Departure Index)**		a) b) c)

☐	**HI (Hazard Index)**		a) b) c)
☐	**other CA based approaches**		a) b) c)
	Approaches based on the assumption of Independent Action (IA) (also called Response Addition)		
☐	**direct application of the IA formula**		a) b) c)
☐	**other IA based approaches**		a) b) c)
	Any other component-based approaches		
☐	*please specify*		a) b) c)
☐	*please specify*		a) b) c)

GENERAL EXPERIENCE

8. Do you apply any approach or methodology to the mixture toxicity assessment of complex environmental samples or waste samples that does not fit into either of the categories of "whole mixture approach" or "component-based approach"?		
☐	**No**	*Please continue with the next question*
☐	**Yes**	*Please provide details*
Details		

9. How would you describe your level of experience in practically applying approaches for assessing the overall toxicity of complex environmental samples or waste samples? *Different levels may apply to different approaches you may have mentioned in your answers to questions 2 to 8. Please indicate which level applies to which approaches or methodologies.*		
	Level of Experience	*Approach / Methodology*
☐	**extensive experience / frequent routine application**	
☐	**limited experience / occasional use only**	
☐	**marginal experience / exceptional use only**	
☐	**application is still in the phase of development / establishment**	
☐	**no experience**	*all others*

10. For what purposes do you apply approaches for assessing the overall toxicity of complex environmental samples or waste samples?

Different purposes may apply to different approaches you may have mentioned in your answers to questions 2 to 8. Please indicate which purpose applies to which approaches or methodologies.

	Purpose	*Approach / Methodology*
☐	**general environmental monitoring**	
☐	**control of emission permits**	
☐	**risk assessment of contaminated sites**	
☐	**priority setting for risk reduction measures**	
☐	**control of remediation works and their success**	
☐	**research and development**	
☐	**others** *Please specify*	

11. Negative experience / Warnings:

Are there any approaches or methodologies for assessing the mixture toxicity of complex samples which you have used or tested in the past, but which you have abandoned due to negative experiences?

☐	**No**	*Please continue with the next question*
☐	**Yes**	*Please provide details*

Details

12. Positive experience / Recommendations:

Are there any approaches or methodologies for assessing the mixture toxicity of complex samples which you consider particularly valuable for specific samples, endpoints, and purposes, and which you would recommend for a more extensive use in EU member states?

☐	**No**	*Please continue with the next question*
☐	**Yes**	*Please provide details*

Details

13. General Comments

Thank you for completing this questionnaire.

Annex B

Glossary of terms (attached to the questionnaire)

Methods for hazard and risk assessment of chemical mixtures fall into two general categories: the **whole-mixture approach** and the **component-based approach**. The whole-mixture approach evaluates a mixture as a single entity, either by directly testing the mixture of concern or by using data available on a similar mixture. The component-based approach relies on toxicological data and exposure information for individual mixture constituents. The mixture toxicity is assessed in terms of expectable additive or interactive actions of mixture components. In this context a number of key terms and concepts is used which are defined in the following.

CA **Concentration Addition** (or Dose Addition)

The concept of Concentration Addition (CA) assumes a similar action of mixture components. CA was originally outlined for binary mixtures (Loewe & Muischnek 1926) but can be extended to any number of *n* mixture components (Berenbaum 1985) and is generally defined by the formula

$$\sum_{i=1}^{n} \frac{c_i^*}{ECx_i} = 1$$

where c_i^* are the individual concentrations (or doses) of the substances *1* to *n* which are present in a mixture that elicits the definite fractional effect *x* (e.g. 50 % mortality), and ECx_i denote the equivalent effect concentrations (or doses) of the single substances (e.g. $EC50_i$), i.e. those concentrations (or doses) that alone would cause the same quantitative effect *x* as the mixture. The CA formula means that a mixture component can be replaced totally or in part by an equal fraction of an equi-effective concentration (or dose) of another without changing the overall combined effect (e.g. 0.5 x EC50 of substance A can be replaced by 0.5 x EC50 of substance B in a mixture causing 50 % total effect).

HI **Hazard Index**

The Hazard Index (HI) (Teuschler & Hertzberg 1995) is a regulatory approach to component-based mixture risk assessment which is based on the concept of CA and which can be generally defined by the formula

$$HI = \sum_{i=1}^{n} \frac{EL_i}{AL_i}$$

where *EL* is the exposure level, AL is the acceptable level, and *n* is the number of chemicals in the mixture. Various measures for exposure levels and expectable levels may be applied; the only constraint is that *EL* and *AL* must be expressed in the same unit. If HI > 1, the total concentration (or dose) of mixture components exceeds the level considered to be acceptable.

IA **Independent Action** (also called Response Addition)

The concept of Independent Action (IA) assumes a dissimilar action of mixture components (Bliss 1939). The idea is that toxicants primarily

interact with different molecular target sites and lead to a common toxicological endpoint via distinct chains of reactions within an organism. Under these presumptions the fractional effects of individual mixture constituents (e.g. 50 % response) are expected to be independent from each other in a probabilistic sense. IA is commonly defined for a binary mixture by the equation

$$E(c_{mix}) = E(c_1) + E(c_2) - E(c_1) \bullet E(c_2)$$

which can be extended to any number of mixture components, giving

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i))$$

where c_i are the actual concentrations (or doses) of the individual substances *1* to *n* in a mixture. $E(c_i)$ are the fractional effects *(x* %) caused by the individual substances, if they are present alone in exactly the same concentration (or dose) that is present in the mixture, and *E(cmix)* is the total expected effect of the mixture.

PODI **Point of Departure Index**

The Point of Departure Index (PODI) is an approach to component-based mixture risk assessment which is similar to the HI. In contrast to the HI, however, exposure levels (EL) of chemicals in a mixture are not expressed as fractions of individually acceptable levels (AL) but as fractions of their respective points of departure (PODs) such as NOAELs or Benchmark concentrations or doses (BMD). Thereby, different uncertainty factors that may be included in AL values are removed from the calculation (Wilkinson et al 2000):

$$PODI = \sum_{i=1}^{n} \frac{EL_i}{POD_i}$$

RPF **Relative Potency Factor**

The Relative Potency Factor (RPF) approach is a practical regulatory application of the CA concept for mixtures of chemical substances that are assumed to be toxicologically similar (EPA 2000). The concentrations (or doses) of mixture components are scaled relatively to the concentration (or dose) of an index compound, and then summed up. The scaling factor is called *RPF*. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of the index compound. In general, the mixture concentration C_m expressed in terms of the index compound for *n* compounds is

$$C_m = \sum_{i=1}^{n} (c_i * RPF_i)$$

where c_i is the concentration of the i^{th} mixture component, and $RPF_1 = 1$, as $i = 1$ indicates the index chemical.

TEF **Toxic Equivalence Factor**

The Toxic Equivalence Factor (TEF) is a specific type of RPF formed through a scientific consensus procedure (EPA 2000). Based on the assumptions of a similar mechanism of action of structurally related chemicals and parallel concentration (or dose) response curves, they were

first developed for dioxins. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of an index compound. The total equivalent quantity *TEQ* is estimated by summation of the concentrations (or doses) of mixture components c_i multiplied by the respective TEF_i:

$$TEQ = \sum_{i=1}^{n} (c_i * TEF_i)$$

TUS **Toxic Unit Summation**

The method of Toxic Unit Summation (TUS) (Sprague 1970) is a direct application of the CA concept and defined by the formula

$$TUS = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i}$$

where c_i are the actual concentrations (or doses) of the individual substances in a mixture and ECx_i denote equi-effective concentrations (or doses) of these substances if present singly (e.g. $EC50_i$). The quotients c_i / ECx_i are termed Toxic Units (TU). Toxic Units rescale absolute concentrations (or doses) of substances to their different individual toxic potencies. They express the concentrations (or doses) of mixture components as fractions of equi-effective individual concentrations (or doses) ECx_i. Typically, x = 50 % ($EC50_i$) is chosen as the reference level, but TUS can also be calculated for any other effect level x. If TUS = 1, the mixture is expected to elicit the total effect x. If the sum of Toxic Units is smaller or larger than 1, the mixture is expected to elicit effects smaller or larger than x, respectively.

Note

Mathematical definitions of terms and concepts are given in this glossary by using a uniform and consistent set of signs and symbols, and in the most general way of formulation. Numerous different notations are used in the literature and transformed, extended or specialized versions of the formula may be found.

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State of the Art Report on Mixture Toxicity

Final Report

Part 4: Overview of approaches to hazard and risk assessment of chemical mixtures in the USA, Japan, and international bodies

22 December 2009

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Responsible Scientists	**Prof. Dr. Andreas Kortenkamp (ULSOP)** **Assoc.-Prof. Dr. Thomas Backhaus (UGOT)** **Dr. Michael Faust (FBEC)**

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1. Summary

Of all the major competing economies of the EU, the United States of America employ the most advanced approaches to mixtures risk assessment and regulation, whereas Japan appears to be engaged in limited activities in this area. The EU currently takes a middle position.

A major driver for mixtures risk assessment in the USA has been the authorization under the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) which covers incidents with hazardous materials and mandates the US Environmental Protection Agency (US EPA) Superfund programme to clean up the highest priority sites contaminated with chemicals. The exposure scenarios normally encountered at such sites require consideration of scores of chemicals that reach exposed subjects by a variety of uptake routes, potentially resulting in more than one adverse health outcome.

To adequately respond to the challenges posed by such complex exposure scenarios, it is the declared intention of cumulative risk assessment in the USA to develop approaches that allow evaluations of the effects of multiple chemicals

- Via multiple routes,
- Multiple time frames,
- Giving rise to multiple adverse health outcomes.

This contrasts with the EU, where the term “cumulative risk assessment” is usually applied to multiple exposure routes of single chemcicals, but not to mixtures of chemicals. IPCS has pointed out that term “aggregate risk assessment” is often used for single chemicals via multiple routes and pathways. In the interest of avoiding confusion, the term “aggregate risk assessment” should be reserved for single chemicals.

To comply with legislative demands, the competent authorities in the USA have developed extensive policy frameworks and guidance documents to deal with the ambitious task of cumulative risk assessment. Given US EPA’s priority concerns for pesticides, industrial chemicals and environmental media, it is by far the most important governmental organization responsible for cumulative risk assessment. Its Risk Assessment Guidance for Superfund (RAGS) has informed US EPA’s cumulative risk assessment approaches in other areas, and has had influence even beyond EPA, on other regulatory authorities in the USA. Other important organizations are the Agency for Toxic Substances and Disease Registry (ATSDR) which evaluates data on releases of hazardous substances and creates and maintains registers of exposed people. The Food and Drug Administration (FDA) is concerned with the establishment of tolerance levels for hazardous substances in food and consumer items.

In its many policy documents, US EPA has over the years evolved and refined approaches to cumulative risk assessment. By its own admission, there is a lack of approaches that can deal effectively with more than additive combination effects. Due to

a lack of knowledge in key areas, and to an absence of appropriate data, the aim of taking account of multiple chemicals via multiple routes cannot always be realized. For the estimation of risks from chemical mixtures, US EPA, ATSDR and other relevant bodies employ a variety of approaches to assessing mixtures. Depending on the risk assessment context, these range from whole mixture approaches to component-based approaches. Dose addition and independent action are applied, and extensive guidance exists advising when to use either concept. For specific groups of chemicals, including dioxins, organophosphates and polycyclic aromatic hydrocarbons, toxic equivalency factors are employed.

A second major stimulus for the practice of cumulative risk assessment in the USA has been the passing of the Food Quality Protection Act in 1996. The act mandates the assessment of risks from mixtures of pesticides with common modes of action, from any source. In response, US EPA has developed sophisticated guidelines to help decide which pesticides should qualify for inclusion in common mechanism groups. The agency has acknowledged the weaknesses of this approach which it identifies in omitting other chemicals that might also induce the effect of interest, although by different mechanisms.

Similarly, the 1996 amendments to the Safe Drinking Water Act required consideration of chemical mixtures in drinking water. US EPA was mandated to develop new approaches to the study of complex mixtures, and this has focused particularly on disinfection byproducts.

Finally, cumulative risk assessment has been applied to attempts to estimate the health effects of air pollutants. In its most recent assessment, US EPA considered 177 chemicals relevant to air pollution.

In contrast, the Japanese Government is considerably less active in the area of mixtures risk assessment. No guidance documents relevant to the issue could be located. However, various governmental organizations acknowledge the need for developing test assays that allow the assessment of risks from complex environmental mixtures, in a whole mixtures approach.

The World Health Organisation (WHO) and its allied International Programme on Chemical Safety (IPCS) are the main drivers behind the development and refinement of the toxic equivalency factor approach for the assessment of mixtures of dioxin-like chemicals. The equivalency factors which are constantly updated have a major impact on the practice of national governments when it comes to the risk assessment of dioxins and related chemicals. Very recently, IPCS has published a workshop report that discusses and reviews methods for assessing the combined risk from exposure to multiple chemicals and initiates the development of a framework for risk assessment for multiple exposures.

The United Nations have developed the Global Harmonised System of Classification and Labelling of Chemicals (GHS) which provides detailed guidance on the classification of

mixtures for human health and the environment. The GHS is currently adopted for application within the EU and is hence discussed within the context of Task 2.

Dose (or concentration) addition has found widespread acceptance as an assessment concept for chemical mixtures. It is extensively used in a variety of settings, including site-specific, media- and product-oriented risk assessments.

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

2. Terms of reference, approach and analyses, definition of terms

The task was to provide a systematic overview on approaches to the hazard and risk assessment of chemical mixtures used by

– competent authorities in the USA,
– competent authorities in Japan,
– the World Health Organisation,
– the International Programm on Chemical Safety,
– the International Agency for Research on Cancer,
– the OECD, and
– the UN Globally Harmonised System for Classification and Labelling of Chemicals

2.1 Approach and steps

Websites of relevant agencies were checked for the availability of relevant documents. Lists of available documents on relevant approaches have been compiled for each of the relevant agencies.

Relevant experts at each of the relevant agencies have been identified and were approached to see whether they would be willing to check the lists of documents for completeness and to provide up-to-date information on recent developments or relevant plans for future action, if applicable. Feed-back from experts has not been received.

All documents were compiled and references stored in a database at *http://www.fb-envico.com/mixture.*

2.2 Analysis

Relevant documents were analyzed with respect to the following aspects:

- Were component based approaches and whole mixture approaches used?
- What are the basic concepts (e.g dose addition) and specific methodologies (e.g. toxic equivalency factors) that are applied in case of component based approaches?
- What is the protection aim (human health risk assessment or environmental risk assessment) of relevant approaches?
- Which specific endpoints are considered?
- What is the type of the risk assessment used in terms of (i) risk assessments for substances or groups of substances, (ii) risk assessments for processes or (iii) products, (iv) site-specific risk assessments for specific environmental media (water, soil, air, food) or (v) population-specific risk assessments (e.g. children in a specific aerea)?

2.3 Definition of terms

Cumulative risk assessment (CRA) is a technical term most frequently used in the context of US American approaches to dealing with combined exposures to chemicals and other stressors. In its most inclusive form, "cumulative risk" is defined as the combination of risks associated with exposure to multiple chemicals and non-chemical stressors by all routes and pathways, and from all sources of each chemical or stressor. This includes multiple time frames and multiple health outcomes.

It is important to realize that the term "cumulative risks" is used in a different way in Europe, where it usually means exposure from multiple routes and pathways, but for only one chemical. Sometimes, the term "aggregate risks" is used to describe risks that stem from exposure to the same substance by multiple pathways and routes (IPCS 2009).

3. United States of America

3.1 Competent authorities

Competent US authorities engaged in chemicals management, risk assessment and regulation include the US Environmental Protection Agency (US EPA), the Food and Drug Administration (FDA), the Consumer Product Safety Commission (CPSC), the Agency for Toxic Substances and Disease Registry (ATSDR), the Occupational Safety and Health Administration (OSHA), the National Institute of Occupational Safety and Health (NIOSH) and the American Conference of Governmental and Industrial Hygienists (ACGIH). There is a host of other specialist institutions, including the National Institute of Health and their institutes.

Of these, US EPA is by far the most important authority for mixtures risk assessment and regulation. Until recently, the most common application of mixtures risk assessment in the USA was to Superfund waste sites. The Comprehensive Environmental Response Compensation and Liability Act (CERCLA) which came into force in 1980 specifically calls for mixture risk assessment during the evaluation of risks that stem from hazardous waste sites and chemical accidents. US EPA is authorized to carry out these assessments, termed ***cumulative risk assessment (CRA)***. With the Risk Assessment Guidance for Superfund (RAGS), the agency has developed extensive and detailed guidance (US EPA 1989). RAGS has informed cumulative risk assessment practice not only within other US EPA programmes, but beyond US EPA, in other US authorities. For these reasons, the section on the USA will focus predominantly on US EPA practices, and will make mention of other governmental authorities only insofar as they developed practices that deviate from those used by US EPA.

3.2 The evolution of cumulative risk assessment within US EPA

To fulfill the mandate of the CERCLA legislation, US EPA developed the first guidelines for assessing Superfund waste sites (US EPA 1987). This basic site assessment called for considering multiple chemicals, exposure routes, and effects (therefore the term: cumulative risks), but few specific suggestions were developed that could guide risk assessors to extend their evaluations much beyond a basic application of additivity concepts (dose addition or independent action). No approaches were described for helping to address the possibility of toxicological interactions, primarily because of limitations in the understanding of such interactions.

In 1989, US EPA published guidance for assessing the health risks specific to Superfund waste sites (US EPA 1989). This site-specific guidance calls for consideration of multiple chemicals, sources, exposure routes, effects and exposed subjects, and represents a major step towards CRA. However, as in the 1986 document, there is little in the 1989 guidance about how to deal with the possibility of toxicological interactions. Instead, a default approach was defined, stipulating application of dose addition or independent action, where appropriate. For the first time, this guidance implemented component-based

approaches for the assessment of the effects of multiple chemicals. It made a distinction between carcinogens, and non-carcinogens.

For carcinogenic substances, component risks are added, following the principles of independent action. For non-cancer endpoints, the doses of mixture components are scaled and added, in an application of the dose addition concept, termed the hazard index (see below). The Superfund guidance also pioneered the quantitative evaluation of exposures via multiple pathways by using the hazard quotient concept (see below). All these combination effect assessments utilize data about the toxic profiles of individual chemicals, readily available in US EPA's IRIS data base. Apart from disregarding toxicological interactions, other weaknesses in the 1989 guidance were in a lack of detail regarding procedures for conducting multi-pathway analyses.

Some of these gaps were filled in US EPA's 2000 Supplementary Guidance for Health Risk Assessments for Mixtures (US EPA 2000). For the first time, a process for the quantitative evaluation of toxic interactions was described. However, there was still a lack of workable procedures for multi-chemical, multi-pathway exposure assessments, as well as for multiple effects produced by mixtures.

Another milestone in the evolution of CRA was the development of procedures for pesticide mixtures. This required approaches that were much more focused than those useful for waste site assessments. The main stimulus for these developments was the passage of the Food Quality Protection Act (FQPA) in 1996 which called for the estimation of health risks from combinations of pesticides with a common mode of action, from any exposure source. Detailed procedures were worked out to assist decision making as to which pesticides should qualify for inclusion into common mode of action groups (US EPA 1999). Further guidance documents concerned the application of the hazard index principle to pesticides, with an aggregate risk formula equivalent to the total hazard quotient in the Superfund guidance (US EPA 2002a). These new principles found entry into an extensive risk assessment exercise for mixtures of organophosphates (US EPA 2002b). They have recently been extended to considerations of mixtures of carbamates (US EPA 2007a), triazines (US EPA 2006a) and chloroacetanilides (US EPA 2006b). The major weakness of the pesticide guidance is that only the toxic effect for the common mode of action is considered, and chemicals not sharing the common mode of action are excluded, although they may also induce the effect under consideration. As in the earlier Superfund guidance, toxic interactions are not considered, but mixture effects are by default assumed to be additive.

Furthermore, the 1996 amendments to the Safe Drinking Water Act required consideration of chemical mixtures in drinking water. US EPA was mandated to develop new approaches to the study of complex mixtures, and this has focused particularly on disinfection byproducts. Relative potency factors were used to aggregate across different chemical components (Teuschler et al. 2004). Again, default assumptions about joint additive effects were adopted, and considerations of synergistic or antagonistic effects remained minimal.

Finally, CRA has been applied to attempts to estimate the health effects of air pollutants across the entire USA, focusing on cancer and non-cancer health effects. In its most recent assessment, US EPA (2006c) considered 177 chemicals relevant to air pollution. Dispersion models were used to estimate concentrations in ambient air, and these were used as input values fort he estimation of both cancer and non-cancer risks. CRA followed the precedents in the Superfund site assessments and summed hazard quotients of individual chemicals that had similar adverse health outcomes, but not necessarily similar modes of action. Like the previous applications of CRA, synergistic or antagonistic effects were not considered, nor were non-chemical stressors taken into account.

In 2003, US EPA produced a Framework for Cumulative Risk Assessment (US EPA 2003), which was further developed with the publication in 2006 of the Considerations for Developing Alternative Health Risk Assessment Approaches for Addressing Multiple Chemicals, Exposures and Effects (US EPA 2006d). The latter document is not intended as guidance, but presents concepts that could assist the development of detailed guidance in the future. It makes key steps towards identifying specific approaches for implementing CRA. These documents are significant because they adopt CRA in its most evolved form to date. CRA is defined explicitly as considering the health risks that stem from multiple chemicals, via multiple routes and exposure pathways, within multiple time frames. Multiple health effects are taken into account.

In the following section basic principles of CRA for Superfund sites, as practiced by US EPA, are described. Subsequent sections illustrate principles for pesticides CRA and outline the perspectives developed in the 2003 Framework document and the 2006 Developing Alternative Health Risk Assessment Approaches document.

3.2.3 Cumulative risk assessment for waste sites in US EPA Risk Assessment Guidance for Superfund (RAGS) (US EPA 1989)

3.2.3.1 Exposure assessment

CRA for waste sites begin with an exposure assessment. This involves an evaluation of exposures to relevant subjects (“receptors”), by all relevant chemicals, through all relevant pathways, by all relevant routes of exposure, and for relevant time periods. The outcome of exposure assessments for hazardous waste sites are estimates of exposure or dose for each chemical for defined people disaggregated by time periods and exposure pathways.

People whose exposures are assessed are those that are “reasonably maximally exposed”. Decision making is based on individuals who experience the highest exposure reasonably likely to occur.

Next, decisions are made about which chemicals should be included in an exposure assessment. To this end, an initial screening exercise is conducted, with the aim of eliminating chemicals that are clearly of no concern.

An exposure assessment is conducted for many chemicals, but often the nature of the expected major contamination is known to some degree, and has been defined in terms of lists of chemicals to be considered. The initial list of chemicals to be evaluated in a typical site risk assessment is the EPA's Contract Laboratory Program (CLP) Target Compound and Target Analyte List (TCP/TALs). The TCP/TALs (as of May 2008) include 52 volatile chemicals, 30 pesticides and Aroclors, 23 metals, cyanide, and 67 semivolatile chemicals.

For the chemicals of relevance, an analysis of exposure pathways (how does a chemical move from the waste site to a human subject?) and exposure routes (ingestion, inhalation, dermal absorption etc.) is carried out for relevant time periods, classed according to likely exposure duration into acute, subchronic and chronic. The outcome of this assessment is a total dose estimate for each individual, disaggregated by chemical, route of exposure, and time period.

3.2.3.2 Toxicity assessment

The exposure dose estimates are mapped against information about the toxicity of each chemical to be considered. Key to these toxicity assessments are so-called reference doses (RfD) or reference concentrations (RfC) for single chemicals that denote doses not associated with discernible risks. Quantitative data about those estimates are found in the IRIS data base. Together with exposure assessment data, these estimates from the input for risk characterization of mixtures.

3.2.3.3 Risk characterization for mixtures

The risk characterization for mixtures distinguishes carcinogens from non-carcinogens. A total cancer risk estimate is obtained by summing individual chemical risk estimates across pathways and routes. In dose ranges corresponding to low cancer probabilities, it is assumed that there is no dose threshold, and that the dose-response function is essentially linear. Under such conditions the use of simple effect summation for the estimation of joint risks produces results similar to independent action, because the predicted cancer probabilities are very much smaller than 0.001.

For each non-carcinogenic chemical, each pathway, and each averaging period, a hazard quotient (HQ) is calculated as:

$$HQ = \sum_{routes} \frac{\text{Average dose rate}}{\text{RfD}} \quad \text{or} \quad \frac{\text{Average concentration}}{\text{RfC}}$$

where the summation is over all routes of exposure and time frames.

An overall summary hazard index (HI) is then calculated as the sum of HQs for each pathway and each chemical, so

$$HI = \sum_{pathways} \sum_{chemicals} HQ$$

The concept of HI is an application of dose addition. If the HI is less than or equal to unity, it is assumed that there is unlikely to be an appreciable risk of deleterious effects and the analysis is complete. If the summary HI is larger than unity, further analysis may be performed with the aim of determining whether application of dose additivity to all the chemicals simultaneously is justifiable.

3.2.4 US EPA pesticides cumulative risk assessment (US EPA 2002)

US EPA's pesticide CRA follows the broad principles developed for Superfund sites, with important modifications and extensions. A first challenge was to extend the procedures devised for site-specific exposure assessments to dealing with simultaneous exposures from food, drinking water and residential (non-occupational) use of pesticides for the general population. A second modification of the Superfund CRA was in the selection of chemicals which should be considered.

3.2.4.1 Identification of chemicals

CRA for pesticides begin with the identification of a group of chemicals that are considered to induce a common toxic effect by a common mechanism, a so-called common mechanism group (CMG). Detailed guidance exists about how to identify CMGs.

Once a CMG is established, registered and proposed uses for each chemical in the CMG are evaluated in order to identify potential exposure pathways (food, drinking water, residential pesticide application) and exposure routes (oral, dermal, inhalative).

3.2.4.2 Hazard identification and dose response analysis

In a hazard characterization phase, the various endpoints associated with a common mechanism of toxicity are identified. An important aspect of this assessment step is to determine if the common effect is expressed across all exposure routes for each chemical in the CMG.

The assessment then proceeds to a quantitation of cumulative risks. Not all chemicals in the CMG need to be subjected to quantitative dose response analyses; pesticides that contribute to exposures by minor pathways can be excluded. Thus, a subset of the CMG is defined, termed cumulative assessment group (CAG) which is subject to quantitative analyses. For each CAG member, dose response analyses are performed with the aim of determining its toxic potency for the common effect. For the estimation of the combined

risks in the CAG, the concept of dose addition is normally used, but departures from this basic principle are permitted if there are data that indicate that this is appropriate.

In a next step, the relative potencies of the CAG members are established by selecting one chemical from the CAG that can serve as an index chemical. This is then used as a point of reference for the standardization of the common toxicity of the other CAG members in terms of relative potency factors (RPF). RPF are used to convert exposures of all chemicals in the CAG into exposure equivalents of the index chemical, rather like the procedure used with TEFs for dioxin-like chemicals.

3.2.4.3 Detailed exposure assessments

Detailed exposure scenarios for all CAG members are developed. This includes determination of potential human exposures by all relevant pathways, durations and routes where simultaneous exposure may occur. Sequential exposures are also considered. The output of this analysis is an aggregation of exposures via all routes and pathways, for each chemical, which is then expressed in terms of equivalent exposures of the index chemical, by using RPFs.

3.2.4.4 Risk characterisation

The exposure assessment yields a dose measure for the mixture that is expressed as equivalent exposures to the index chemical. The index chemical should be well evaluated for its toxicity, because such data are then used to characterize risks as margins of exposure. The risk characterization step also includes descriptions of variability and major areas of uncertainty.

3.2.5 US EPA National Air Toxics Assessment

The National-scale Air Toxics Assessment (NATA) estimates concurrent exposures to the selected chemicals at the census tract, county or state level at a point in time (US EPA 2006c). The cumulative methods applied for NATA are dose addition and independent action. The common non-cancer health effect of concern is respiratory irritation (irritation of the lining of the respiratory system) and single-chemical hazard quotients for respiratory irritants are added to yield a “respiratory hazard index” (dose addition). For the carcinogens, lifetime cancer risk estimates for inhalation exposures are added (independent action) (US EPA 2007b)

3.2.6 Further guidance in US EPA framework documents

US EPA has produced a Framework for Cumulative Risk Assessment (US EPA 2003), which was further developed with the publication in 2006 of the Considerations for Developing Alternative Health Risk Assessment Approaches for Addressing Multiple Chemicals, Exposures and Effects (US EPA 2006d). These documents are not intended as

guidance, but rather elaborate concepts that could assist the development of detailed guidance in the future.

These documents identify specific approaches for implementing CRA, and respond to some weaknesses in the earlier Superfund and pesticides guidance, most notably with respect to dealing with toxic interactions and further aspects of exposure assessment. These documents are significant because they adopt CRA in its most evolved form to date. CRA is defined explicitly as considering the health risks that stem from multiple chemicals, via multiple routes and exposure pathways, within multiple time frames. Multiple health effects are taken into account.

3.3 Agency for Toxic Substances and Disease Registry (ATSDR)

The ATSDR is also engaged in Superfund sites, but does not carry out site specific assessments (this is the domain of US EPA). Rather, ATSDR assesses whether adequate information on health effects is available for the priority hazardous substances, a mandate under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). Where such information is not available or under development, ATSDR initiates, in cooperation with the National Toxicology Program, a program of research to determine these health effects. The Act further directs that where feasible, ATSDR shall develop methods to determine the health effects of substances in combination with other substances with which they are commonly found.

Similarly, the Food Quality Protection Act (FQPA) requires that factors be considered in establishing, modifying or revoking tolerances for pesticide chemical residues in food, and that these should include available information about cumulative effects of substances with common mode of action.

To fulfill these legislative mandates, ATSDR's Division of Toxicology has developed a chemical mixtures program. It includes a trend analysis to identify mixtures most often found in environmental media. As part of the mixtures program, ATSDR devised a guidance manual that outlines the latest methods for mixtures assessment (ATSDR 2004). In addition, a series of documents called interaction profiles are developed for certain priority mixtures that are of special concern to ATSDR. The purpose of the interaction profile is to evaluate data on the toxicology of the 'whole' priority mixture (if available) and on the joint toxic action of the chemicals in the mixture in order to recommend approaches for the exposure-based assessment of the potential hazard to public health.

3.3.1 The ATSDR mixtures guidance manual (ATSDR 2004)

ATSDR devised a mixtures guidance manual with the intention of assisting their Division of Toxicology in determining whether exposure to chemical mixtures at hazardous waste sites may impact public health. The manual is also intended to serve as a basis for the development of interaction profiles (see below).

The approaches developed in the mixtures guidance manual are consistent with US EPA guidance articulated since 1989. The preferred basis for assessments of the health risks stemming from mixtures at waste sites, is information about the mixture of concern, both with respect to exposure, and to toxicological profile. For this purpose, whole mixture data are used, if they are available. Examples of such whole mixture assessments include coke oven emissions and a cocktail of groundwater contaminants. If data on a whole mixture are not available, recourse is made to a "similar" mixture. That is meant to be a combination of the same chemicals as in the mixture of concern, but at different mixture ratios. If analyses of "similar mixtures" are not possible, ATSDR adopts component-based approaches.

Component-based mixture assessments make use of the hazard index and are fully compatible with US EPA approaches. There are two departures from the guidance articulated by US EPA: the target organ toxicity dose modification of the hazard index, and the weight-of-evidence approach to dealing with possible deviations from additivity.

3.3.1.1 Target organ toxicity dose (TTD)

In deriving hazard quotients, US EPA recommends the use of RfD also for effect that occur at higher doses, not only the critical effects. It is acknowledged that this may lead to overestimations of risks. To deal with this potential complication, TTDs were developed for chemicals that affect an endpoint at a dose higher than for the critical effect.

3.3.1.2 Weight of evidence (WOE) modification of hazard index

As discussed above, the hazard index concept assumes dose additivity, and does not take account of the possibility of toxic interactions. The WOE modification to the hazard index is intended to fill this gap. It builds on a suggestion by the National Research Council of the US National Academies to use additional uncertainty factors to accommodate the possibility of deviations from expected additivity (NRC 1989). It evaluates binary mixtures and introduces a classification that indicates the expected direction of interaction (synergistic or antagonistic) by using an alphanumerical scoring system. The scores are then combined with the hazard index.

3.3.2 Interaction profiles

The purpose of interaction profiles is to evaluate data on the toxicology of "whole" priority mixtures, and on the joint toxic action of the chemicals in the mixture, with the aim of recommending approaches for exposure-based assessments of hazards to the public. The topic of interaction profiles are mixtures of concern found in environmental media, in food, or in site specific exposure settings. Interaction profiles for the following mixtures of concern have been published:

- Arsenic, Cadmium, Chromium, Lead
- Benzene, Toluene, Ethylbenzene, Xylenes

- Lead, Manganese, Zinc, Copper
- Persistent chemicals found in breast milk
- Persistent chemicals found in fish
- 1,1,1-TCE, 1,1-DCE, TCE, PERC
- Cesium, Cobalt, Polychlorinated Biphenyls, Strontium, and Trichloroethylene
- Arsenic, Hydrazines, Jet Fuels, Strontium-90, and Trichloroethylene
- Cyanide, Fluoride, Nitrate, and Uranium
- Atrazine, Deethylatrazine, Diazinon, Nitrate, and Simazine
- Chlorpyrifos, Lead, Mercury, and Methylmercury

Draft interaction profiles are available for

- Carbon Monoxide, Formaldehyde, Methylene Chloride, Nitrogen Dioxide, Tetrachloroethylene
- Chloroform, 1,1-Dichloroethylene, Trichloroethylene, and Vinyl Chloride

3.4 Methods used by other agencies

3.4.1 American Conference of Governmental Industrial Hygienists (ACGIH)

The ACGIH (2000) recommends additivity approaches similar to the hazard index. For mixtures that act on the same organ system, the ratio of exposure concentration to threshold limit values is summed. If the sum of this index exceeds unity, the threshold limit value for the mixture is deemed exceeded.

If the mixture is judged to act according to the principles of independent action, the threshold limit value of the mixture is judged to be exceeded only if the hazard quotient of at least one component is larger than 1.

3.4.2 Occupational Safety and Health Administration (OSHA)

OSHA (1993, 2001) also recommends the hazard index approach for the evaluation of occupationally relevant combined exposures, where the ratio of exposure concentrations to PEL is considered.

3.4.3 National Institute of Occupational Safety and Health (NIOSH)

NIOSH adopted a similar approach in developing recommendations for exposure limits for combined exposures to methylenechloride and carbonmonoxide. This is based on observations of additive effects of the two chemicals with regard to the formation of carboxy hemoglobin. The sum of the ratios of each agent to recommended occupational exposure limits must not exceed unity. The permissible exposure limits for methylenechloride are corrected downwards when carbonmonoxide levels exceed 9 ppm, in order to keep the sum of hazard quotients from exceeding unity (NIOSH 1976, NIOSH 1992).

3.4.4 Food and Drug Administration (FDA)

The Food and Drug Administration has issued a guidance document for industry drug-drug interaction studies (FDA 2006). The focus of this guidance is to advise on studies aimed at establishing whether one drug influences the absorption, distribution, metabolism, excretion, or the effects of another drug. The idea is not to determine additive combination effects between drugs, and consequently no component-based approaches are suggested.

Guidance relevant to pesticide residues or additives in food could not be located.

3.4.5 National Academy of Sciences, National Research Council (NRC)

In 1974, the National Academy of Sciences (NAS 1974) investigated mixtures of toxicants in freshwater aquatic systems, at the request of US EPA. For multiple chemical exposures, the NAS recommended a hazard index approach, where the sum of the ratios of the measured concentrations to the acceptable concentrations for the individual components has to be kept at levels equal to, or lower than, unity.

In 1989, the Safe Drinking Water Committee of the National Research Council (NRC) of the NAS discussed possible modifications to the then current approaches for estimating the toxicity of mixtures in drinking water. The NRC suggested to group mixture components according to toxicity endpoints, such as specific organ toxicity or carcinogenicity, with the aim of assessing their combined hazards and risks (NRC 1989).

In 1994, the NRC was charged by US EPA to review the methods used by the agency for the determination of carcinogenic risks associated with exposure to hazardous air pollutants. NRC (1994) pointed out that people at risk are exposed to a mixture of chemicals, each of which might be associated with an increased probability of one or more health effects. Because data are often available on only one of the adverse effects (e.g., cancer) associated with each chemical, the issue is how best to characterize and estimate the potential aggregate risk posed by exposure to a mixture of toxic chemicals. The method used by US EPA of adding the risks related to each chemical in a mixture for developing a risk estimate was considered appropriate when the only risk characterization needed is a point estimate for use in screening. When a more comprehensive uncertainty characterization is desired, NRC recommended that US EPA adopt appropriate statistical (e.g., Monte Carlo) procedures to aggregate cancer risks from exposure to multiple compounds. In the analysis of animal bioassay data on the occurrence of multiple tumor types, the cancer potencies should be estimated for each relevant tumor type that is related to exposure, and the individual potencies should be summed for those tumors.

Very recently, NRC was asked by US EPA to look into the necessity of conducting cumulative risk assessment for phthalates. In their report (NRC 2008) the Academy advised that risk assessment should consider not only certain phthalates, but other chemicals that could potentially cause the same health effects as phthalates, instead of

focusing solely on chemicals that are structurally or mechanistically similar, which is US EPA's current practice. The NRC committee recommended that phthalates and other chemicals that affect male reproductive development in animals, including antiandrogens, be considered in the cumulative risk assessment. A focus solely on phthalates to the exclusion of other chemicals would be artificial and could seriously underestimate the risk posed by phthalates. This is a departure from current practice where US EPA often considers only chemicals that are structurally related, on the assumption that they exert their effects by similar mechanisms leading to a final health outcome. The NRC committee pointed out that this practice ignores how exposures to different chemicals may result in the same health effects. The conceptual approach taken for phthalates -- to consider chemicals that cause similar health effects -- should also be applied when completing any cumulative risk assessment, the committee recommended. For instance, US EPA could evaluate the risk of combined exposures to lead, methylmercury, and polychlorinated biphenyls because all contribute to cognitive deficits consistent with IQ reduction in children, albeit by very different mechanisms.

3.4.6 US Geological Survey

The US Geological Survey (USGS 2006) provides scientific information to help facilitate effective management of natural resources, including water supply. In 1991 the Survey implemented the National Water Quality Assessment (NAWQA) Program, with the aim of supporting rational management of water resources. As part of NAWQA, the levels of 185 pesticides were measured regularly in US surface waters.

To assess water quality conditions, NAWQA developed a Pesticide Toxicity Index (PTI) which combines measures of pesticide exposures of aquatic biota with acute toxicity data derived from laboratory assays to produce a single index for a sample or a site. Being the sum of “toxicity quotients”, the PTI is in effect an indicator of combined effects from pesticides that are to be expected under the assumption of concentration addition. The toxicity quotient for each pesticide is formed by dividing its concentration in water by its median toxicity. This approach is very similar to the sum of Hazard Quotients used by U.S.EPA (see 3.2.3.3.).

Noteworthy is the way in which the median toxicity measures were formed. A data base was used for this purpose, where several bioassay outcomes per compound were aggregated into a single toxicity value. The bioassays represented three categories of endpoints, i.e. EC50 for cladocerans, LC50 for benthic invertebrates and LC50 for fish. For many taxa, the outcome of only one bioassay was available, and LC50 for fish were somewhat over-represented. Sometimes the median number of bioassays per compound that found entry into deriving a median toxicity measure was relatively low.

In their report, the USGS emphasized that the PTI is not intended to determine whether a specific water sample is toxic to aquatic life. Rather, it is used for ranking purposes, or to compare samples from different sites. The index is also useful for assessing the relative contribution of specific pesticides to an overall assumed effect. USGS listed several shortcomings of PTI:

- A PTI does not indicate actual toxicity of a sample.
- The median toxicity values are based on short-term laboratory assays with high effect levels (50%), which may limit the usefulness of PTI for estimations of long-term effects.
- In using measured water levels of pesticides, factors that may impact on the bioavailability of compounds could not be considered, e.g. dissolved carbon or variations in sediment levels.
- Because PTI are based on pesticide concentrations in the water column, lipophilic compounds with their tendency to adsorb to particles are under-represented.
- The PTI makes allowance only for additive effects and does not take possible synergisms or antagonisms into account.

Despite these limitations, the PTI's make best use of available data and are a valuable tool for comparative assessments of water quality.

4. Japan

4.1 Competent authorities

Many of the Japanese authorities responsible for chemicals management, risk assessment and regulation are concentrated in the Ministry of Economy, Trade and Industry (METI), and in the Ministry of the Environment (MOE).

METI's Office for Chemicals Safety is responsible for law enforcement in the areas of chemical safety evaluation and regulations regarding chemical production. The Office for Chemical Risk Assessment Policy carries out risk assessments for chemical substances.

MOE's Environmental Health Department is organized into a Division for Environmental Health and Safety which is responsible for standard setting for substances that are released into the environment, and for all matters regarding the examination, research and assessment of contamination of the environment with chemicals. It's Office for Environmental Risk Assessment deals with matters related to defining tolerable daily intakes for dioxins and the examination, research and assessment of chemical contaminations. The Office for Chemicals Evaluation of MOE deals with standard setting and regulations for the evaluation, manufacture, import and use of chemical substances in order to protect the environment.

The Ministry of Health, Labour and Welfare (MHLW) has a Bureau of Pharmaceutical and Food Safety whose Office of Chemical Safety is responsible for the control of poisonous and harmful substances, regulations of the use of substances which may damage human health from an environmental view point, household products that contain hazardous substances and matters related to the tolerable daily intake of dioxins.

The National Institute of Health Sciences is an organization affiliated to the MHLW responsible for the testing and evaluation of chemicals and devices, in support of the ministry's mission. There are Divisions of Risk Assessment, Food Additives, Safety Information on Food Drugs and Chemicals, Genetics and Mutagenesis and Environmental Chemistry.

A number of incorporated administrative agencies also deal with chemicals regulations, including the Japanese National Institute of Occupational Safety and Health (JNIOSH), the National Institute of Technology and Evaluation (NITE) with its Chemical Management Centre, and the National Institute for Environmental Studies (NIES). Since 2003, Japan has established a Food Safety Commission which prepares reports on the risks associated with relevant chemicals, as requested by Ministries.

4.2 The use of TEF and TEQ in regulations relevant to polychlorinated dioxins and furans

In 1999 Japan passed a Law Concerning Special Measures Against Dioxins (Law No. 105 of 1999) which aims to protect the health of citizens by establishing exposure

reduction measures regarding polychlorinated dioxins, furans and biphenyls. Chapter 2, Article 6 of the Law defines a tolerable daily intake of 4 pg/kg. The implementation of the Law relies on WHO toxicity equivalency factors for dioxins, furans and PCBs. In their Report on tolerable daily intake of dioxins and related compounds the MOE's Environment Agency and the MHLW utilize these TEF to evaluate current Japanese exposures to dioxins and dioxin-like chemicals (MOE 1999).

This is the only example in Japanese chemicals regulation that could be located where mixture effects are taken into consideration.

4.3 Other activities

An extensive search of the websites of the Japanese authorities listed below was conducted with the search terms outlined in the First Interim Report.

Ministry of Health, Labour and Welfare (MHLW)
National Institute of Health Sciences (NIHS)
Ministry of Agriculture, Forestry and Fisheries (MAFF)
Ministry of Economy, Trade and Industry (METI)
Ministry of the Environment (MOE)
National Institute of Occupational Safety and Health, Japan (JNIOSH)
National Institute of Advanced Industrial Science and Technology (AIST)
National Institute of Technology and Evaluation (NITE)
Chemical Management Center (CMC), National Institute of Technology and Evaluation (NITE)
Agricultural Chemicals Inspection Station (ACIS)
Fertilizer and Feed Inspection Station (FFIS)
National Institute for Environmental Studies (NIES)

The search yielded 620 documents, which reduced to 380 in the English language, once documents in Japanese were eliminated. Of these, 8 documents had relevance to chemical mixtures assessments.

Of note is the Occupational Health Research Strategy in the 21st Century (NOHRS 2001) which aims to address, through research, priority occupational health problems in the coming decade. Key research area 2, "Research on the human health effects of hazardous workplace factors", highlights research into the effects of multiple mixtures as a priority.

Annual reports of the National Institute for Environmental Studies (for a recent example see NIES 2006) have regularly contained expressions of the intention to develop bioassays that allow the measurement of mixture effects of endocrine disrupters.

5. International bodies

5.1. World Health Organsiation, International Programme on Chemical Safety (IPCS), Joint FAO/WHO Expert Committee on Food Additives (JECFA)

5.1.1 Dioxin TEFs

During the last 15 years, the International Programme on Chemical Safety (IPCS) of the World Health Organisation has established and regularly re-evaluated toxic equivalency factors (TEFs) for dioxins and related compounds. WHO-TEF values have been established for humans and mammals, birds and fish. These international consensus TEFs have been used for the risk management in various UNO Member States and have been adopted formally by a number of countries and supranational bodies, including, amongst others, Canada, Japan, the United States and the European Union.

During the assessment 1997 at the WHO/IPCS expert consultation in Stockholm, it was agreed to re-evaluate TEF values on a regular basis, preferably at five-year intervals. Such a re-evaluation should be based on new scientific information published in the peer reviewed literature subsequent to the last expert consultation.

WHO considered this re-evaluation of TEF values an important effort and has initiated a project to review the current human and mammalian TEFs. The project has served to update the database summarizing all studies published on the relative potency of dioxins, furans, and dioxin-like PCBs. The results of these activities have been published (van den Berg et al 2006).

5.1.2 Integrated risk assessment

The IPCS has prepared a report on integrated risk assessment for the purposes of human and environmental risk assessment in one coherent framework (IPCS 2001). It details a general framework and contains four case studies, intended to illustrate the benefits of integrated risk assessment. Considerations of the effects of sequential and simultaneous exposure to several chemicals are an integral part of the framework, but specifics of mixtures hazard characterization are not described.

5.1.3 Project to update the principles and methods for the assessment of chemicals in food

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) serve as scientific advisory bodies to the Codex Alimentarius Commission since its inception in the early 1960s. In response to requests by JECFA and JMPR for general guidance for risk assessments, the International Programme on Chemical Safety (IPCS) sponsored in the 1980s the preparation of two Environmental Health Criteria (EHC) monographs, EHC 70 (Principles for the safety assessment of food additives and contaminants in food) and EHC 104 (Principles for the toxicological assessment of pesticide residues in food). JECFA has regularly held

meetings at which veterinary drug residues in foods were assessed. While general principles have been developed in these meetings, they have not been consolidated in a similar document.

In light of the advances in the science of risk assessment and the recognition that the evaluations performed by JECFA and JMPR serve as the scientific foundation for international food standards that are of increasing importance within the Codex Alimentarius Commission and the World Trade Organization, FAO and WHO have initiated a joint Project to Update and Consolidate Principles and Methods for the Risk Assessment of Chemicals in Food. A website has been set up to provide reports and other information on the Project as they become available (http://www.who.int/ipcs/food/principles/en/index.html).

The information available on this website does not indicate that the Project will take account of combination effects of pesticides and/or food additives.

5.1.4 Development of a framework for the consideration of combined exposures in risk assessment

In March 2007, IPCS convened an international workshop to discuss methods for assessing the combined risk from exposure to one or more agents via all relevant routes and pathways. The development of a framework for such assessments was initiated. IPCS plans to publish the report of the workshop, together with a draft framework for aggregate/cumulative risk assessment for peer and public review (see http://www.who.int/ipcs/methods/harmonization/areas/aggregate/en/index.html).

The workshop report appeared in 2009 (IPCS 2009). It summarises the objectives of the 2007 workshop, which were to discuss and review available methods for assessing combined risks from exposures to multiple chemicals, to review knowledge gained from approaches adopted in different sectors, to develop working definitions for the different types of exposures, effects and risks of chemicals and to initiate the development of a framework for assessment of risks to multiple chemicals.

The workshop agreed on working definitions for key terms and concepts, as follows:

- Exposure to the same chemical by multiple pathways and routes should be described as "*Single Chemical, All Routes*" (sometimes also referred to as "aggregate exposure"), although this was not the topic of the workshop.

- Exposure to "*Multiple Chemicals by a Single Route*" should be distinguished from "*Multiple Chemicals by Multiple Routes*", and both these possibilities are the topic of the framework development.

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

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

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

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

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

The workshop came to propose a preliminary framework for consideration of risk from exposure to multiple chemicals. The application of this framework is intended as an iterative process which involves step-wise consideration of exposures and hazards in several tiers, depending on the data available to support the analysis. The analysis begins with a consideration of the potential for cumulative exposure, before any assessments of hazards take place.

In its earliest tier, the workshop report recommends **adopting dose addition**, if there is no evidence for synergisms or antagonisms. Chemicals to be subjected to this procedure should be grouped according to their chemical structure, similarity of target tissue and/or similarity in the manifestation of toxicity.

Should the combined risks turn out not to be acceptable, the assessment should be refined further by additional consideration of temporal aspects of the common toxic effect, the presence of a common metabolite, analysis of key biological targets and consideration of information about environmentally relevant mixture ratios and exposure levels.

IPCS has published a draft document for public and peer review (see http://www.who.int/ipcs/methods/harmonization/areas/aggregate/en/index.html).

5.2 Organisation for Economic Cooperation and Development (OECD)

OECD Member countries and the OECD Secretariat cooperate to develop and co-ordinate chemical and pesticide related activities on an international basis. The main objectives of the OECD Chemicals Programme are to assist OECD Member countries' efforts to protect human health and the environment through improving chemical safety, to make chemical control policies more transparent and efficient and save resources for government and industry, and to prevent unnecessary distortions in the trade of chemicals and chemical products. An important focus of this work is on the production, processing and use of industrial chemicals, and some aspects include work on pesticides, chemical accidents. A key mission of OECD is to work for mutual acceptance of data on the hazardous effects of chemicals. To achieve this aim, a great deal of activities focuses on the development of guidelines for chemicals testing. Guidance on the conduct of

experimental work to deal with the effects of multiple chemicals has not yet found entry into OECD activities.

In 2004, the UN sub-committee of experts on the GHS mandated OECD to work on classification criteria for toxic gas mixtures. When mixtures of gases containing hazardous gases are classified and labeled according to existing GHS criteria, certain mixtures would not be classified or labeled as posing an acute inhalation hazard, because the GHS cut-off values are too low to provide adequate protection. The OECD has proposed to solve the problem by using an additivity formula equivalent to dose addition to determine the concentration at which a mixture of hazardous gases would be classified in a GHS category (OECD 2005).

OECD (2007) has produced guidance on limiting the number of toxicological tests to be carried out by grouping chemicals into closely related categories. In this so-called category approach, not every chemical has to be tested. Instead, data for chemicals and toxicological endpoints that have been tested are used to estimate the corresponding properties of untested chemicals. In principle, the category approach can also be used to define groups of chemicals to be subjected to mixtures risk assessment.

An example is the TEQ approach that is used for polychlorinated dioxins and furans (PCDD/F). Originally designed to estimate the toxicity of untested congeners, TEQ have matured into a framework for assessing mixtures of PCDD/F. There is merit in considering systematically whether methods for grouping chemicals into categories for purposes of toxicity predictions have value for mixtures risk assessment. However, apart form PCDD/F there is too little experience with exploiting these methods.

5.3 The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC)

In their report “Aquatic Toxicity of Mixtures” ECETOC (2001) emphasized the need for practical methods to deal with the possibility that mixtures of chemicals present in aquatic systems in the environment express additive effects, even when substances are present at concentrations not expected to lead to chronic toxicity on their own. Five approaches were listed, and their advantages and disadvantages discussed:

Toxic unit summation using actual environmental concentrations
Individual substances in aquatic systems should be identified and their concentrations determined analytically. For each chemical, QSAR-based toxicity values (NOECs, LC50 and similar) are derived in order to establish Toxic Units (TU, the ratio of concentration to toxicity value). Finally, these TU are summed up, and if the numerical value of the sum of TU exceeds 1, further evaluations are considered.

The advantages of this procedure lie in the fact that only substances actually present in the environment are included. However, the approach is judged by ECETOX to be only viable in situations where the number of chemicals is comparatively low. In many

situations the method is unlikely to be useful because the identification and quantification of individual chemicals is time consuming, analytical methods may be lacking, and special problems may arise when the limit of detection is larger than the biologically effective concentrations of chemicals (as is the case with some hormonally active chemicals).

PEC/PNEC summation
Instead of using measured values as in the previous method, this approach utilizes predicted values (predicted environmental concentrations, PEC; predicted no-effect-concentrations, PNEC) which are relatively easily available for a large number of chemicals.

The approach is straightforward, uses available data and, at least initially, does not require environmental measurements. However, problems may arise from the fact that predicted values are used. These may not always be reliable and may over-estimate risks where individual PEC/PNEC ratios are overly conservative.

Use of a correction factor to modify individual chemical assessments
In essence, this approach aims to adapt and modify existing risk assessment procedures for individual chemicals by applying a "mixtures correction factor" to each individual substance. It is based on conventional risk assessment for deriving PEC/PNEC ratios, but this time, ratios of PEC to PNEC times X are determined, where X is the number of chemicals also present in a mixture.

A strength of this method is in its ease of use, especially on a case-by-case basis, but distinct disadvantages are that X, the number of chemicals occurring together with the one to be assessed, is largely unknown, and may fluctuate. Furthermore, the method assumes that substances are present at concentrations proportional to their PEC/PNEC ratios. This means that more prevalent substances would be weighted in the same way as all others, leading to a skewed analysis of the situation.

Environmental monitoring
Chemical and/or biological monitoring techniques, e.g. biomimetic approaches using membrane devices, can provide valuable surrogate measures of bioavailable substances. Although relatively ease to use, these methods are as yet poorly validated.

Biological field monitoring
Biological field monitoring is a well established approach to assess whether effects have actually occurred in ecosystems. It provides an integrated biological picture. Chemical measurements are deemed unnecessary if effects are not observed. However, in case effects are noticeable, causes are difficult to establish. Importantly, monitoring techniques are not protective because they can establish effects only after they have occurred.

6. Résumé

Dose addition (or concentration addition) has found widespread acceptance as an assessment concept for chemical mixtures. It is extensively used by US American authorities and regulatory bodies in a variety of settings, including site-specific, media- and product-oriented risk assessments. International bodies also recommend application of dose (or concentration) addition. Of note is the recent recommendation by an IPCS workshop report to adopt dose addition, if there is no evidence for synergisms or antagonisms.

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

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