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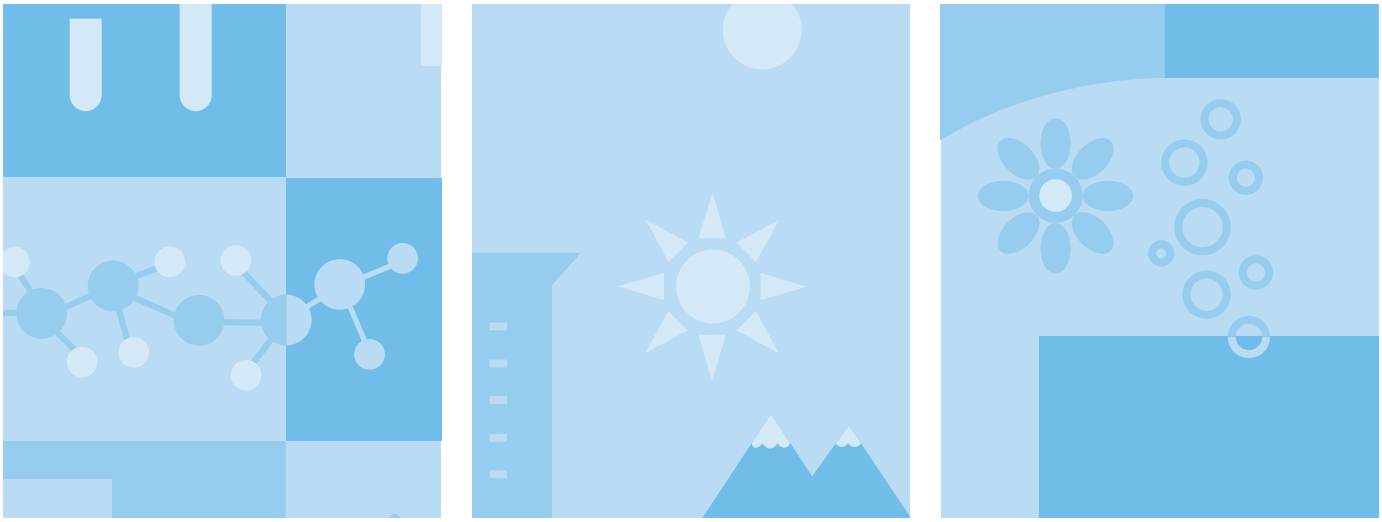
Hazard and Risk Assessment of Chemical Mixtures under REACH

State of the Art, Gaps and Options for Improvement



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Hazard and Risk Assessment of Chemical Mixtures under REACH

State of the Art, Gaps and Options for Improvement

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Order No. 510 968
Sundbyberg, June 2010
Publisher: Swedish Chemicals Agency©
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The report is available as a downloadable pdf on www.kemi.se

Contents

Sammanfattning

Människor och miljön exponeras kontinuerligt för blandningar av flera olika kemikalier vilket visas med flera exempel i denna rapport. Empirisk kunskap om toxicitet och ekotoxicitet av kemikalieblandningar (s.k. cocktails) visar ett samstämmigt mönster, oberoende av kemikalieblandning, testorganism eller vad som mäts i testet. Den kombinerade toxiciteten av en kemikalieblandning (cocktail-effekten) är alltid större än de individuella komponenternas toxicitet, även om man ser till det mest potenta ämnet. Låga halter av enskilda kemikalier kan, även när de inte enskilt ger signifikanta effekter, ge en betydande toxicitet då de förekommer tillsammans i blandningar. Med den gällande exponeringssituationen måste dagens standarder för miljö- och hälsoriskbedömningar ses som bara ett första steg. Nästa steg innebär att även ta hänsyn till kombinationseffekter i riskbedömningen av kemikalier.

Trots detta, och trots att Reach uttryckligen syftar till att "säkerställa en hög skyddsnivå för människors hälsa och miljön" (artikel 1) så ger inte Reach mandat att beakta kombinationseffekter av så kallade "coincidental mixtures" av industrikemikalier. Detta är flerkomponent-blandningar som förekommer i miljön och i våra kroppar som ett resultat av den pågående samtidiga användningen av kemikalier ett visst område. Det är dock möjligt att i existerande lagstiftning införa mandat att göra riskbedömningar även för blandningar, åtminstone för vissa grupper av kemikalier såsom sker i den nya lagstiftningen för växtskyddsmedel (Gemensam ståndpunkt (EG) nr 25/2008 antagen av Europaparlamentet den 13 januari 2009) där det krävs att växtskyddsmedel inte får ge "skadliga hälsoeffekter för människor, inklusive sårbara grupper, eller för djur, med beaktande av kända kumulativa och synergistiska effekter".

Bedömningen av hur människor och miljö påverkas av den uppsjö av olika kemikalier, som de exponeras för samtidigt, kräver mycket mer övergripande och långtgående ansträngningar som skär tvärs över alla existerande delar av dagens kemikalielagstiftning och inte begränsas av typ av substans och produkt vilket sker i dagens lagstiftning, t.ex. i Reach och i förordningen om växtskyddsmedel. Även i mer process- och miljö-inriktade former av lagstiftning måste kombinationseffekterna införas, som till exempel i direktivet Samordnade åtgärder för att förebygga och begränsa föroreningar (IPPC-direktivet) och Vattendirektivet. Strategier som direkt behandlar kumulativa exponeringsscenarior lyfts fram till exempel i Vattendirektivet. Detta kan ge ett förbättrat skydd mot risker för människa och miljö förknippade med kemikalieblandningar.

Flera sätt att utvärdera blandningars toxicitet har beskrivits i den vetenskapliga litteraturen, alla med sina för- och nackdelar. För framåtblickande riskvärderingar (t.ex. inom Reach) är de klassiska toxikologiska koncepten Koncentrations-Addition (Concentration Addition) eller Oberoende Verkan (Independent Action) de mest användbara metoderna. Särskilt Koncentrations-Addition har visat sig kunna bidra med goda approximationer av de förväntade kombinationseffekterna för ett brett register av olika kemiska blandningar, i olika organismer och för olika typer av mätbara effekter. Dessutom, synergistiska (högre toxicitet än koncentrationsaddition) kombinationer är sällsynta. Med Koncentrations-Addition är det möjligt att göra vissa förutsägelser av kombinationseffekter med hjälp av de toxikologiska och ekotoxikologiska datamängder som kommer att vara tillgänglig då ett ämne registrerats enligt Reach.

Två möjligheter för riskvärderingen av "coincidental mixtures" inom Reach diskuteras utifrån detta; (a) en standardiserad säkerhetsmarginal för blandningar "mixture assessment factor" (MAF) eller (b) scenariospecifik kumulativ riskvärdering. Det faktiska genomförandet av dessa

möjligheter begränsas av betydande kunskapsluckor som dock främst är av empirisk karaktär. Särskilt kunskap om "typiska" exponeringsscenarior innefattande Reach-kemikalier saknas.

Dock, om man ser bortom Reach så har giftiga kemikalier och dess blandningar en gränsöverskridande natur och förekommer tillfälligt eller kontinuerligt samtidigt i olika miljömedier och i levande organismer, i mat och i oss människor och utgör en betydande utmaning för dagens system av utvärdering och hantering av kemiska risker.

Summary

As demonstrated by several examples, humans and all other organisms in the environment are exposed to multi-component chemical mixtures. Empirical evidence on the toxicity and ecotoxicity of such chemical cocktails shows one common pattern, independent on the specific chemical composition of a particular mixture, the exposed organism or biological endpoint under observation: the joint toxicity of a chemical mixture is always higher than the individual toxic effect of even the most potent compound present. In particular, even low, individually non-toxic concentrations might result in a significant toxicity, if they co-occur as a chemical mixture. Given the actual exposure situation it has hence to be concluded that ecological or human-health standards for individual chemicals are only a first step. In addition, the toxicity of chemical mixtures has to be adequately considered in the regulatory risk assessment of chemicals.

However, despite these facts and despite its professed aim to "ensure a high level of protection of human health and the environment" (article 1), REACH does not provide a mandate for considering the toxicity of so-called "coincidental" mixtures of industrial chemicals – multi-component cocktails that are found in the environment or the human body as a result from the concurrent use of different chemicals in a given area. That the inclusion of such a mandate for mixture risk assessments provides a feasible option, at least for a certain regulatory groups of chemicals, is demonstrated by the forthcoming new legislation on plant protection products (PPP) (Common Position (EC) No 25/2008 adopted by the EP on 13 January 2009), which requests that PPP's "*shall not have any harmful effects on human health, including vulnerable groups, or animal health, taking into account known cumulative and synergistic effects*". However, comprehensive assessments of the (eco)toxicological impact from the plethora of all the chemicals that humans and the environment are exposed to would require more far-reaching regulatory efforts. There is the need to cut across the existing pieces of chemicals legislation, and not limited the assessment by substance- and product- oriented regulations such as REACH and PPP. Process- and media-oriented forms of legislation, such as for instance the Integrated Pollution and Prevention Control Directive (IPPC) and the Water Framework Directive (WFD) need to be included. Approaches that directly address cumulative exposure scenarios, as put forward for example in the WFD, might provide particularly valuable options for improved protection of humans and the environment from risks from mixtures of chemicals.

Several approaches for the assessment of mixture (eco)toxicities have been described in the scientific literature, each with its own specific pros and cons. For prospective assessments in regulatory contexts (e.g. within REACH), the classic toxicological concepts of Concentration Addition and Independent Action seem to be the most promising methods. Especially Concentrations Addition has proven to provide generally good approximations of expectable mixture toxicities for a wide range of mixtures, exposed organisms and biological endpoints. In particular, synergistic (considerably more than concentration-additive) mixture toxicities seem to be rare. Concentration Addition also allows to predict the expected mixture toxicity (EC50 values) using the toxicological and ecotoxicological data that may be available by the registration of a compound under REACH.

Two options for the risk assessment of "coincidental mixtures" within the context of REACH are discussed on this basis: (a) a default mixture assessment factor (MAF) and (b) scenario specific cumulative risk assessments. The actual implementation of these options are currently hampered

by substantial knowledge gaps, which, however, seem to be mainly empirical in nature. Especially knowledge on "typical" exposure scenarios involving REACH-chemicals is missing.

Nevertheless, from a perspective beyond REACH, the trans-sectorial nature of mixtures of toxic compounds that coincidentally co-occur in an environmental compartment, the organisms living there, food and the human body poses a substantial challenge for the current system of chemical risk assessment and management.

Introduction

As outlined in a recent paper of the Swedish Chemicals Agency (KemI, 2010, chemical risk management aims at four main goals:

1. *"To obtain knowledge of the intrinsic hazardous properties of chemicals.*
2. *To disseminate information on hazardous properties of chemicals placed on the market and on safe use.*
3. *To make informed choices of chemicals in order to avoid hazards.*
4. *To organise safe use of chemicals."*

In view of the agency, the "*primary, and therefore in a sense most important regulations on chemicals control, risk management in the first steps of the supply chain before or when chemicals are supplied, encompass:*

1. *Hazard assessment in the form of classification and hazard communication including requirements for labeling and safety data sheets. These measures guide the flow of information in the supply chain to ensure that enterprises and other users are provided with adequate information on hazards and contents of chemicals and their safe transport, storage, use and disposal.*
2. *Hazard management in the form of bans and restrictions to ensure that highly hazardous chemicals are not marketed or used"* (KemI, 2010.

UN organizations have developed an international standard for classification, labeling and safety data sheets called GHS (Globally Harmonized System). The GHS was implemented in European Community law in 2009 through a Regulation (EC) 1272/2008 on Classification, Labelling and Packaging of substances and mixtures (CLP).

For substances produced or imported in quantities totaling over one ton per producer or importer per year, the new European system for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) laid down in Regulation (EC) 1907/2006 of the European Parliament and of the Council provides complementary approaches. For substances in quantities of 10 tons or more these involve chemical risk assessments which are concerned with the determination and compilation of hazard and exposure information of chemicals and, building on this, the analysis of their risks for human health and the environment.

The scientifically sound risk assessment and management of chemicals is the basis for any chemical control and risk reduction measures and ultimately provides a basis for the sustainable use of substances. One of the professed aims of REACH and CLP is to "ensure a high level of protection of human health and the environment" (REACH and CLP, article 1). Detailed guidance on how chemical substances are to be registered or notified at the European Chemicals Agency (ECHA) and how their risks for human health and the environment are to be assessed are provided in a set of documents on the implementation of REACH and CLP, published by the ECHA. The new Directive (EC) 1272/2008 provides approaches for the classification and labeling of individual chemicals and chemical products, which are actually chemical mixtures.

Substance and product oriented pieces of European chemicals legislation such as REACH and CLP are focused on the assessment and the management of hazards and risks of single substances and individual chemical products. They basically treat these as if they were present in

isolation. Humans and the environment, however, may be exposed to a huge number of different chemicals and chemical products from various sources and via different routes. This has raised concern about the possibility of so-called "cocktail effects" which may be inappropriately addressed by the current regulatory framework. Recently, this debate culminated in the adoption of *Conclusions on Combination Effects of Chemicals* by the Council of the European Union (EU Council, 2009). These conclusions, which were taken under Swedish presidency, *inter alia* call on the European Commission "*to assess how and whether relevant existing Community legislation adequately addresses risks from exposure to multiple chemicals from different sources and pathways, and on this basis to consider appropriate modifications, guidelines and assessment methods, and report back to the Council by early 2012 at the latest"*. In addition, the Council encouraged EU Member States "*to step up research efforts in this area, including a review of the existing research database"*.

This report analyses the hazard and risk assessment of chemical mixtures mainly from the perspective of a substance-oriented regulation (the "chemicals control perspective"). It was commissioned by the Swedish Chemicals Agency (KemI) with the aim to provide a platform for further discussions and initiatives on the issue. The focus is on REACH and CLP, but the report also refers to the forthcoming new legislation on Plant Protection Products (Common Position (EC) No 25/2008 adopted by the EP on 13 January 2009), in the following simply termed "the PPP regulation") and the Water Framework Directive 2000/60/EC (WFD). The report starts with providing some examples on chemical mixtures in the environment and their potential risks. Methods for the hazard and risk assessment of chemical mixtures and an outline on their performance follow. Then the principal mixture types that are considered in environmental regulation are introduced and fundamental gaps are identified. Finally, two options for bridging these gaps are outlined. These options are (a) a default mixture assessment factor (MAF), and (b) scenario specific cumulative risk assessments (CRA).

Annex I provides a glossary of key terms. Annex II of the report provides more detailed explanations and scientific background information on the mixture toxicity concepts of Concentration Addition and Independent Action. This Annex also discusses the intuitively appealing, but toxicologically ill justified approach to predict the joint action of chemicals by simply adding up their individual effects ("Effect Summation"). Annex III lists key guidelines and reports on the risk assessment of chemical mixtures that have been put forward by industrial and academic organizations and national as well as trans-national committees.

Real world exposure is to multi-component chemical mixtures

Between the 1st of June and the 1st of December 2008 roughly 150 000 chemicals were pre-registered for a later full registration within REACH (ECHA, 2008). All of them might potentially end up in air, water and soils and later in food and feed, either as a result of accidental spills or during the normal life cycle of the substance. This results in the typical complex exposure situation where several tens of compounds can be found simultaneously during the chemical analysis of human tissues or an environmental compartment. In the following this is illustrated by some few selected examples.

In December 2003, the World Wildlife Fund (WWF) waged a campaign to raise awareness of the continuous long-term exposure of European citizens to a complex cocktail of persistent, bioac-

cumulative and toxic man-made chemicals. To attract public attention, members of the European Parliament volunteered for this campaign. Blood samples from 47 volunteers from 17 different European countries were analyzed for 101 different chemicals including: 12 organochlorine pesticides, 45 polychlorinated biphenyls (PCBs), 23 polybrominated diphenyl ether (PBDE) and other brominated flame retardants, 8 phthalates and 13 perfluorinated chemicals. The body of every volunteer tested was contaminated by a cocktail of hazardous chemicals from each of the five chemical groups tested. The highest number of chemicals found in a person was 54, the median number of chemicals detected was 41. Thirteen chemicals were found in every single person tested. Such findings support the view that potential cumulative long-term effects of chemical mixtures give reasons for concern as expressed in the European environment and health strategy (Commission of European Communities, 2003).

During 2008 and 2009, Tønning and co-workers investigated the cumulative exposure of 2-year old children in Denmark to a range of chemicals which have shown to cause endocrine disrupting effects in animal studies (Tønning et al., 2009). Based on chemical analysis of various consumer products, experimental migration studies, literature data on contaminant levels in food, indoor air and dust, and exposure scenarios, they performed cumulative health risk assessments for two different sub-sets of endocrine disrupting chemicals: substances with anti-androgenic effects and substances with estrogen like effects. Anti-androgenic substances included 4 phthalates, 5 fungicides and herbicides, PCBs, Dioxins, and DDTs/DDDs. Estrogen-like compounds included 3 parabenes and bisphenol A. Investigated exposure routes included uptake from food, non-food consumer products, indoor air and dust via inhalation, ingestion, and dermal exposure. As a result, the authors came to conclude that the total amounts of endocrine disrupters absorbed by 2 years-old children constitute a risk for both anti-androgenic disruptions and estrogen-like disruptions of their sexual development. They stated a need for exposure reduction measures and as an immediate consequence, the Danish Environment Minister launched an information campaign directed towards parents and grandparents with simple advices related to children s exposure to chemicals (http://www.netdoktor.dk/65000.htm).

Other examples of human exposures to chemical mixtures include the recent report from Reuters[1] (http://www.reuters.com/article/idUSTRE5AI3M820091119) according to which a survey amongst British females revealed that the average UK woman wears 515 chemicals a day. And in 2005 a biomonitoring study of umbilical cord blood of newborns found in average 200 anthropogenic chemicals, including pesticides, dioxins, industrial chemicals, perfluoro-compounds and brominated flame retardants (Houlihan et al., 2005).

Not only humans, but also organisms in the environment are exposed to multi-component mixtures of various chemicals. Swedish monitoring studies conducted by the Swedish University of Agricultural Sciences (SLU) found 57 different pesticides in Swedish streams, many of them co-occurring (Adielsson et al., 2006). Streams in Skåne for example contained between 10 and 22 compounds simultaneously. Also the US geological survey found similarly complex exposure situations: One third of the 139 streams that were surveyed contained 10 or more different chemicals from a broad range of chemical classes and use groups, such as synthetic hormones, other pharmaceuticals, industrial chemicals, pesticides, biocides and flame retardants (Kolpin et al., 2002).

[1] Unfortunately, we were not able to locate the primary source of this press release.

The continuous exposure of humans and environmental organisms to multi-component mixtures has three main reasons. Firstly, they result from the plethora of different compounds that are used concurrently in a given area and enter the environment from different sources and via different routes. Also degradation products of the original compounds add to the complexity of the exposure. Secondly, more than a few chemicals are directly used in the form of chemical combinations. Most chemical products are combinations of various agents (such as active ingredients, surfactants, stabilizers, preservatives, etc.). And finally, complex chemical cocktails are directly emitted into the environment by industrial plants, sewage treatment plants, waste sites as well as agricultural and urban areas.

In summary, there is an unequivocal pattern of chemical contamination: Humans and all other organisms in the environment are regularly exposed to multi-component chemical mixtures, composed of chemicals from a broad range of different groups. Consequently, chemical risk assessments need to be implemented with the awareness of these complex exposure situations.

Assessment of exposure to chemical mixtures

The cumulative exposure assessment and the identification of causal links between an exposure to mixtures of chemicals and observed or anticipated (eco)toxicological effects might pose one of the greatest challenges for the risk assessment of chemical mixtures, in particular because of the high dynamics of chemicals' concentrations in the environment and the possibility of delayed effects. Ideally, an exposure assessment would consider all sources, pathways and routes of exposure as well as the exposure dynamics (acute as well as chronic exposure) and all abiotic and biotic transformation products. However, even for comparatively well investigated compounds, such as pesticides, substantial data gaps have been identified in this respect (Panel on Plant Protection Products and their Residues, 2008).

Exposure assessments can be grouped into so-called macro-assessments on the basis of biomonitoring data, e.g. blood samples or samples from fish that were caught in an exposed river system (IPCS, 2009). The different sources and uptake routes are then of only secondary importance, e.g. for subsequent risk management measures. Closely related are assessments that are based not on direct biomonotoring data in the organism(s) of interest, but from chemical-analytical monitoring data of surrogate entities. An example would be the pesticide exposure assessment for humans, conducted on the basis of the WHO/GEMS cluster diets, i.e. on the basis of the average food consumption of European citizens.

On the other hand, so-called micro-assessments are based on modeling the different steps from emission to the actual exposure (IPCS, 2009) and here the identification, quantification and assessment of the different sources plays a critical role. Micro-assessments can also serve as conceptual models for estimating exposures before they actually take place, i.e. for prospective risk assessments for the purpose of setting quality standards for human health or the environment. They allow the ranking of different pollutions sources and the prospective analysis of the impact that either additional sources might have, or the benefits that can be expected from specific risk mitigation measures. Examples of such models for micro-assessments with respect to human health and ecotoxicological impacts of chemical mixtures are given e.g. by Menzie et al (2007). They were also the basis for the risk assessment of estrogen mixtures that was conducted by Sumpter and colleagues for the Aire-Calder river catchment and for the assessment of the ecotoxicological risk of pesticide mixtures that was conducted by Verro and coworkers (2008).

For both types of exposure assessment a sound definition of the framework in which the assessment will be implemented (the exposure scenario) is pivotal, which in particular concerns the selection of the compounds to be included.

In contrast to media-oriented regulations such as the Water Framework Directive, REACH is primarily a single-substance oriented regulation at the moment. With respect to chemical mixtures, it might therefore be of main interest to characterize the background exposure to which the particular chemical of interest is added. For this purpose a scenario-specific joint exposure modeling needs to be performed. The feasibility and depth of such a modeling strongly depends on the scenario of interest and on the data that are at hand for the identified substances. A series of exposure modeling approaches and tools has been developed for the cumulative human health risk assessment of pesticides. Such instruments include for example the LifeLine Aggregate and Cumulative Exposure / Risk Assessment Software (http://www.thelifelinegroup.org/lifeline/index.htm), MCRA, the program for Monte Carlo Risk Assessment of chemical intake from food (http://www.biometris.wur.nl/UK/Software/MCRA+Monte+Carlo+Risk+Assessment/) CARES, the Cumulative and Aggregate Risk Evaluation System (http://www.epa.gov/scipoly/sap/meetings/2002/april/cares.htm) or ConsExpo for the modeling of consumer exposures (http://www.rivm.nl/consexpo). These tools have been successfully applied in a range of studies on the cumulative exposure of humans to various pesticides, see for example Caldas et al. (2006). It is, however, completely unclear whether these or similar tools can be applied for industrial chemicals, as usually far less input data are at hand for these compounds. The above mentioned tools for example make heavy use of database information from the national monitoring programs of pesticide residues in marketed food items and similar data not available for a large number of industrial chemicals.

It should finally be pointed out that the necessary exposure knowledge might be substantially different for the various compounds in a scenario. While for major compounds with a potentially high PEC/PNEC or Exposure/DNEL ratio a detailed exposure assessment might be crucial, a semi-quantiative assessment might suffice for minor compounds (e.g. estimating only the order of magnitude of expected exposures). Hence exposure and hazard assessment are closely interconnected during the whole process of chemical mixture risk assessment.

Alternatively to basing the risk assessment on a detailed, scenario specific exposure assessment a more general mixture assessment factor (MAF) might be applied. Both options are discussed in detail further below.

Mixtures are more toxic than their individual components

The scientific evidence on the toxicology and ecotoxicology of chemical mixtures shows one strong unanimous pattern, independent of the chemical composition of the mixture, the exposed organism or the considered biological endpoint: the joint toxic effect of a chemical mixture is always higher than the individual effect of each of its components, see the review by (Kortenkamp et al., 2009). It has even been repeatedly observed that a mixture might have a significant toxicity, even if all individual components are present at low concentrations that indivi-

dually do not provoke significant toxic effects (i.e. at or below individual NOECs[2] or NOEALs[3]) (Altenburger and Greco, 2009; Backhaus et al., 2008; Faust et al., 2001; Kortenkamp et al., 2007; Rajapakse et al., 2002; Silva et al., 2002; Hass et al., 2007). Two examples are depicted in detail in Figures 1 and 2a. Figure 1 provides an example of an ecotoxicological study and Figure 2A the corresponding example of a human health oriented toxicological study.

Hence, if the possibility of a substantial and systematic underestimation of risks from typical chemical exposures is to be avoided – then mixture toxicities need to be specifically considered in chemical regulation.

Approaches for the assessment of mixture toxicities

Several methods for the hazard and risk assessment of chemical mixtures are used in scientific studies of chemical mixtures, for the development of new chemical products and partly in chemical regulation. The most straight forward approach is to simply test the mixture of interest in its totality, in order to provide an experimental estimate of its hazard and risk. A major application of the whole mixture approach is whole effluent testing of wastewater streams, as described e.g. by (OSPAR Commission, 2005) and the testing of chemical products for the purpose of classification and labeling (EU Parliament and the Council, 2008b). Such an approach closely follows standard single substance assessments, does not require special methodologies and is hence appealing.

However, whole mixture testing also has several limitations. Most importantly, the mixture itself has to be available for a direct experimentation, which makes this approach largely unusable for prospective approaches such as the setting of environmental or human health standards. The different compounds that make up a mixture also have vastly differing fates and distribution kinetics in the various tissues, organisms and environmental compartments. Hence, any long-term (chronic) toxicological or ecotoxicological test of a biological mixture, with its very specific experimental conditions, provides only very limited knowledge on the actual effects that might result from a chronic exposure of humans or the environment to chemical mixtures. In the context of the classification and labeling of chemical products, whole mixture testing is hence restricted to the assessment of acute (short-term) toxicities (EU Parliament and the Council, 2008b). Finally, due to large number of compounds that are of proven or potential environmental relevance and the even larger number of mixtures that they might form, it is simply impossible (for pragmatic, economic and ethical reasons) to actually test more than a selected, very limited, subset of all conceivable combinations.

In view of these limitations of whole mixture approaches, several methods have been developed that are based on predicting mixture toxicities from a known or assumed chemical composition and knowledge on the toxicities of the mixture components. These approaches are often termed "component-based " and most of them are based on the classical mixture toxicity concepts of Concentration Addition (CA) and Independent Action (IA).

CA assumes that the mixture components only differ in the concentrations needed to elicit a toxic effect (their relative potency). That is, when corrected for their relative potency, each component can be replaced by equivalent concentrations of another chemical without changing the overall toxicity of the mixture. Due to this conceptual idea, CA is thought to describe the joint

[2] NOEC: No Observed Effect Concentration
[3] NOAEL: No Observed Adverse Effect Level

action of components that have a similar mode or mechanism of action. The concept is the basis of various approaches that have been suggested for the regulatory assessment of mixture toxicities, such as the TEF[4] approach that is used for the hazard and risk assessment of dioxins and related substance (US EPA, 2008; van den Berg et al., 1998) or the sum of PEC/PNEC ratios (Vighi et al., 2003; Vighi and Calamari, 1996), which has been suggested for the establishment of water quality criteria for chemical mixtures.

In contrast, according to the concept of Independent Action (IA), dissimilarly acting chemicals can be thought to contribute to a common biological endpoint, completely independent of the other, simultaneously present, agents. Under this premise, the combined effect that results from an exposure to a combination of dissimilarly acting substances is assumed to be calculable from the effects caused by the individual mixture components by applying the statistical concept of independent random events (Bliss, 1939). IA has often been applied for the risk assessment of carcinogens (U.S. EPA, 2000).

Neither CA nor IA make any assumption about the targeted biological system nor do they consider any specific properties of mixture components beyond the similarity or dissimilarity of their toxic action. This is both the strength and weakness of the concepts. On the one hand, this simplicity allows establishing general rules for mixture toxicity assessment, which are essential for considering the joint action of chemicals in regulatory guidelines. On the other hand, it cannot be assumed that these concepts describe biological reality to its fullest extent. Both concepts provide a frame of reference with IA describing the extreme situation of completely independently acting chemicals and CA describing the opposite extreme of completely interchangeable, i.e. similarly acting, chemicals. Hence with an appropriate experimental resolution, differences between CA- or IA-expectations and the actually observed mixture toxicity might become apparent. The crucial question is therefore not whether deviations between simple concepts and complex biological realities can be observed, but whether the accuracy of a prediction is sufficient for a certain aim.

The predictive power of Concentration Addition and Independent Action

A survey of the scientific literature reveals a surprisingly high power of CA to provide reliable estimates of the toxicity of a broad range of mixtures composed of substances from different chemical classes (Kortenkamp et al., 2009). For example, Belden and his co-workers re-evaluated the results from 207 studies on the aquatic toxicity of different types of pesticides in which the predictive power of CA was investigated and 37 experiments in which IA was used for predicting the toxicity of the mixtures. The studies were conducted mainly with selected species of green algae (*Scenedesmus*), duckweed (*Lemna*), insects (*Chironomus*) and crustaceens (*Hyalella*). If the results from mixtures composed of compounds with a similar mode of action were pooled with the results from mixtures composed of compounds with a dissimilar mode of action, it turned out that the median ratio between CA-predicted and experimentally determined EC50's is just about 1, indicating a very good predictive power of CA in general (Figure 3A). Only about 10% of the data indicated a deviation between CA-prediction and observation by more than a factor of 2, with over- and underestimations distributed roughly equally (5% each). For mixtures that were composed of compounds with dissimilar modes of action, CA was

[4] Toxicity Equivalency Factor

slightly overprotective on average (Figure 3b) and, despite the theoretical foundation of CA, the results indicate that the mixture toxicity of dissimilarly acting compounds is also predictable by CA with an error not exceeding a factor of 2 (see Figure 3b and discussion below). In summary, 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).

It should be emphasized that in total only 5% of the 207 mixtures re-analysed by Belden and his coworkers had a mixture toxicity that exceeded that CA-predictions by more than a factor of 2 (MDR > 2, "synergistic mixture toxicity"). Two examples of these uncommon "more than concentration-additive effects" of 2-compound mixtures that have recently been published are given in Figure 4 (Laetz et al., 2008), and Figure 5 (Lau et al., 2006).

Such more than concentration-additive effects not only seem to be a rare event, they also seem to be restricted largely to 2-compound combinations. In fact, to our knowledge not a single mixture study with more than 5 components has been published in which the experimental EC50 or NOEC was lower by more than a factor 3 than predicted by CA for the most sensitive biological endpoint. It, however, should be pointed out, that current empirical evidence is strongest in the field of aquatic toxicology and specifically designed mixtures of either unspecifically acting industrial chemicals or specifically acting compounds, such as pesticides or biocides. In recent years it has also been repeatedly demonstrated that the effects of multi-component mixtures of environmental estrogens are very precisely predicted by CA (Kortenkamp, 2007) and current studies on other groups of endocrine disrupters point to the same direction.

Although a few studies have shown that IA can be successfully used for predicting the joint effects of multi-component mixtures of dissimilarly acting chemicals, the available empirical evidence is limited, which might be mainly due to the substantially higher demands in terms of input data of this concept (see Appendix 2, Figure 2 and Kortenkamp et al., 2009)

Both, CA and IA, are only applicable to mixtures in which all components affect the endpoint of interest also if applied singly. However, there are situations in which compounds that are inert if present singly still act as a confounder in a mixture. For example, Frische and coworkers recently demonstrated that the common surfactant LAS – which is not endocrine-active alone – increases the joint estrogenicity of a 3-compound mixture of estrogens (Frische et al., 2009), an effect that has also been observed in studies with fish (Harris et al., 2009). Such situations are generally unpredictable using either concept. They can only be considered qualitatively, using pre-existing empirical knowledge or information on so-called "structural alerts", i.e. chemical moieties that are known as signs for a certain characteristic of the whole molecule (e.g. surface activity in the example above).

The interrelation between Concentration Addition and Independent Action

The conceptual idea of CA assumes that the components in a mixture are interchangeable. This is usually interpreted that the compounds compete for the same receptor site and that hence any compound in a mixture can be exchanged by another one with the same mechanism of action, without changing the overall toxicity of the mixture (see detailed discussion in Appendix II). IA is the theoretical counterpart and assumes that the components in a mixture contribute to a common endpoint by completely different and independent mechanisms of action.

However, empirical evidence shows that even the toxicity mixtures of dissimilarly acting substances can often be predicted by CA with a reasonable accuracy (see figure 3b and e.g. Altenburger et al., 1996;Cedergreen et al., 2008) – in apparent contrast to the mutually exclusive foundations of CA and IA. In fact, empirical evidence suggests that the average error that results from the application of CA to mixtures of dissimilarly acting chemicals is comparatively small, the ratio between the CA-predicted and the observed EC50 of a mixture of dissimilarly acting substances has always been smaller than a factor of 5 in published studies, with a tendency of CA to slightly overestimate the mixture toxicity of dissimilarly acting compounds.

The apparent conflict between the mutually exclusive theoretical assumptions and empirical evidence can be resolved by analyzing the quantitative relationship between the two types of predictions. The factors determining this quantitative difference are the number of mixture components, their mixture ratio, the slope of the individual concentration–response curves, and the effect level under consideration (Boedeker et al., 1993; Drescher and Boedeker, 1995). There is mathematical proof (assuming infinitely steep concentration-response curves) that the theoretical maximum ratio between the EC50 values predicted by IA and CA is *n*, the number of components in a mixture (Faust, 1999). Because concentration-response curves are not infinitely steep in reality, the actual ratio between the predictions by IA and CA is considerably smaller than *n* in any real-world mixture. Given an appropriate steepness, CA and IA may even predict the same mixture toxicity. This is not just a theoretical case, but experimental evidence has demonstrated that there are in fact situations were CA and IA provide virtually identical and both accurate predictions of mixture toxicities (Backhaus et al., 2002).

Especially for binary mixtures – where even the theoretically possible maximum ratio between the two alternative predictions of a mixture EC50 is only two – the actual differences are extremely small most of the time and it is hence often undecidable whether experimental data fit better with the assumption of IA or CA. Such small differences can be safely ignored for any practical purpose. For multi-component mixtures, the theoretically possible ratio between the two predictions can in principle become substantial. However, as stated above, the actually documented ratios between CA- and IA-predicted EC50-values are well below a factor of 5, even for mixtures of up to 20 compounds.

Mixture toxicity from low-effect concentrations of single substances

Both concepts are fundamentally different with respect to the question on whether also low, individually non-toxic, concentrations of individual chemicals contribute to the toxicity of a mixture. For mixtures that behave according to CA it is of no importance whether the individual components are present below their individual thresholds, i.e. it does not make a difference whether only one compound is present at a concentration *c* – or whether 100 similarly acting compounds are present, each at a below-threshold concentration of (*c/100)*. In contrast, IA assumes that only those compounds contribute to the toxicity of a mixture that are present in concentrations at which they are toxic if applied singly, i.e. that are present above their individual thresholds.

As pointed out above, both CA and IA describe two opposite extreme situations and any real-world mixture can hence be expected to show an intermediate behavior. The more independent the chemicals in a mixture behave, the better the observed mixture toxicity might be approx-

imated by IA. The more interchangeable (similar) the compounds are, the closer the mixture toxicity might follow CA expectations. Hence the actual behavior of a mixture is always dependent on two factors: the compounds in the mixture and the biological endpoint under observation.

Although widespread agreement seems to have been reached on this conceptual frame, there is an ongoing debate on the degree of dissimilarity needed before the mixture starts behaving significantly different from CA expectations and *vice versa.* It has been argued that in the case of not strictly similarly acting components individual concentrations below individual NOECs do not contribute to a joint toxicity (Feron and Groten, 2002). However, this seems to be in contrast to other experimental studies that have shown that even for mixtures of non-similarly acting chemicals mixture effects from concentrations below individual NOECs/NOAELs of the individual compounds cannot be ruled out, see the in-depth discussion in (Kortenkamp et al., 2007). The experimental exploration of the low-dose issue requires substantial experimental efforts due to the need to record precise (eco)toxicological information over the whole effect range for each involved substance. Nevertheless, 3 studies with uni-cellular organisms are published that tested various mixtures of 14-16 dissimilarly acting compounds each (Altenburger and Greco, 2009; Backhaus et al., 2000; Backhaus et al., 2008; Faust et al., 2003). In all cases concentrations clearly below individual NOECs contributed to a joint toxicity of the mixture.

More details on this issue are given in Appendix II and the discussion on the implications for the application of CA and IA in regulatory contexts below.

Consideration of chemical mixtures in European legislation

Within the context of the REACH regulation and the new regulation on Classification and Labeling (CLP) two types of mixtures are considered:

(I) individual substances in a legal sense, that are in fact mixtures from a chemical perspective (MCS[5], UVCB[6]).

(II) manufactured mixtures of different substances, formerly called "preparations", that are mainly considered within the CLP regulation (EU Parliament and the Council 2008b). Preparations are also specifically considered in other substance-oriented regulations, such as the Biocide Directive 98/8/EC (European Parliament and Council, 1998) and the PPP[7] Directive 91/414/EEC (EU Council, 1991).

However, there are two important additional types of mixtures to be considered in the wider context of chemical risk assessment and management:

(III) mixtures that result from the joint emission of substances from a common source, such as e.g. combustion products from waste incineration plants. Such mixtures are considered under the IPPC Directive 2008/1/EC on industrial emissions (EU Parliament and Council, 2008a) and were hence considered outside the scope of this report.

(IV) mixtures of compounds from different origins that coincidentally co-occur in water, air, soil or sediments, in environmental organisms or in humans.

No specific guidance on the hazard, exposure and risk assessment is provided in the REACH guidance documents for mixtures of type I. They are, however, specifically discussed in Annex I of the ECHA guidance document on the application of the CLP criteria (ECHA, 2009). Chapter I.4 provides guidance on substances which are difficult to test for their aquatic toxicity and type I mixtures are specifically considered in the subchapter I.4.5., where they are termed "complex substances". The aquatic toxicity of such mixtures is based on their water-soluble fraction (WSF) or water accommodated fraction (WAF) and expressed as LL50 (lethal loading level provoking 50% effect).

Mixtures of type I are also discussed in the PBT assessment in chapter R.11 of the REACH guidance documents. As MCS are chemically fully characterized, the PBT assessment is based on the assessment of their individual constituents, down to those that make up 0.1% (w/w) of the MCS. Also the chemical composition of UVCBs is in principle supposed to be disentangled down to the same threshold (0.1 % (w/w)). Fractions that contain substances that are unidentifiable (with a reasonable effort) are supposed to be characterized by "representative structures". An example on the PBT assessment of petroleum products (UVCBs) is given in the guidance document in appendix R. 11-3. It should be mentioned here that suggestions for the environmental hazard characterization of petroleum products have been made by CONCAWE, the oil companies European association for environment, health and safety (www.concawe.be), which is based on the

[5] Multi-constituent substances. These are chemically defined, with the main constituents making up between 10% and 80% of the total mass.
[6] Substances of unknown or variable composition, complex reaction products or biological materials
[7] Plant protection products

classical mixture toxicity concept of Concentration Addition and assumes a narcotic mode of the individual ingredients of petroleum products. The approaches have been implemented in the PETROTOX software package.

The consideration of mixture toxicities within the CLP Regulation 1272/2008

The new CLP Regulation 1272/2008 of the European Parliament and the Council provides a detailed outline on how mixtures of chemicals that are put on the market as chemical products, such as e.g. detergents, shall be assessed for the purpose of classification, labeling and packaging (EU Parliament and Council, 2008b). The principal aim for the classification of a mixture is to achieve a similar classification as for individual compounds. It is important to note in this context, that the supplier (producer, importer or downstream user) of a chemical "*should not be obliged*" to produce any new toxicological or ecotoxicological data, the classification is based on the available data only.

According to the primarily toxicological and ecotoxicological scope of the report, we will restrict the following analysis on the evaluation of toxicological and ecotoxicological properties (classes concerning acute toxicity, skin corrosion, eye damage, respiratory or skin sensitisation, germ cell mutagenicity, carcinogenicity, reprotox, specific target organ tox, aspiration hazard and hazard to the aquatic environment (with the two subclasses acute and chronic hazard)). Table 1 provides an overview of the different classification approaches for the different categories and their order of application.

If data on the mixture itself are available, these are generally used for the classification. If no 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 tested mixture. Mixtures that contain 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) are primarily evaluated using information on the individual ingredients. These component-based approaches can also be applied with respect to other endpoints, if data on the mixture itself are not at hand.

Two principal component-based approaches are outlined: (a) simple concentration limits that are also the basis of the so-called “summation method” or “additivity approach” and (b) Concentration Addition, which in the context of the CLP regulation is termed “the additivity formula”[8] and is applied only for the acute hazard class, and the aquatic hazard class It is highly interesting to note in this context, that the competing concept of Independent Action is not used for any classification purposes, not even for mixtures of dissimilarly acting substances.

Concentration Addition is used for the classification of a mixture for “acute toxicity” and “aquatic toxicity”. For the classification within the class “acute toxicity”, Concentration Addition is the primary component based approach, while it is only applied in the “aquatic toxicity” class when the components are not classified themselves, but (eco)toxicity data are available for them. If the compounds of a mixture are already classified, specific concentration limits are provided for each category If a mixture contains more of an already classified compound than this limit, the whole mixture is classified into the corresponding category.

[8] For the sake of keeping a consistent nomenclature, we the term Concentration Addition (CA) is used throughout the whole text.

For example, compounds are classified into "acute aquatic hazard, category 1" if they have an EC50 equal or below 1 mg/L. If a particular mixture contains 25% or more of such components, the whole mixture itself is classified as "acute aquatic hazard, category 1"[9]. An obvious advantage of this approach is its ease of use, which will allow the rapid classification of any mixture that is composed by a down-stream user from ingredients with different individual classifications. However, if it is assumed that a CA-based classification more accurately reflects the mixture toxicity (see scientific state of the art above), it has to be regarded a disadvantage of the summation method that its application might result in a lower classification with respect to the "aquatic hazard" class .

For example: according the summation method a mixture is classified into "acute aquatic hazard, category 1" if the following relationship holds (section 4.1.3.5.5.3.2 of Annex I of the Regulation):

$$\sum_{i=1'}^{n} c_{\text{acute cat 1'}} \quad M \quad 25\%$$

M is a weighting factor for highly toxic substances (see footnote), which is (for the sake of simplifying the examples) assumed to be 1 in the following. In this situation $c_{acute\ cat\ 1}$ gives the relative concentration of compounds with 1 <= EC50 > 0.1 mg/L. A mixture that contains 24% of such compounds is hence not classified into "category 1 for acute aquatic hazard". Assuming that the classification for all the 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 predicts an overall toxicity of the mixture of:

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{24}{0.11}} = 0.46\,\text{mg/L}$$

As the EC50 is below the threshold of 1 mg/L, the mixture would now need to be classified into "category 1 for acute aquatic hazard". 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 76% water.

The situation becomes even more aggravated if one would assume that those 76% are made of compounds that just "escaped" a classification as "acute aquatic hazard, category 1", i.e. compounds with an EC50 of for example 1.1 mg/L. For such a mixture – which would not be classified as "acute aquatic hazard category 1" according to the summation rule – CA calculates a mixture toxicity of

[9] The regulation further differentiates highly toxic substances by using so-called "M-Factors", which are multiplication factors of 10, 100, 1000 etc. that give highly toxic compounds an increased weight. M-Factors account for EC50 values below 0.1 mg/L (with limiting values for the EC50 values of 0.01, 0.001, … mg/L). For example, the concentration of a compound with an EC50 of 0.09 mg/L is multiplied with a M-Factor of 10 and is hence treated similar to a compound with an EC50 of 0.9 mg/L which is present at a 10-fold higher concentration. For the sake of simplifying the following examples it is assumed that all compounds have an M-factor of 1 unless otherwise mentioned. For further explanation see CLP regulation, section 4.1.3.5.5.5.

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{24}{0.11} + \frac{76}{1.1}} = 0.35 \text{ mg/L}$$

that is, the application of CA would lead to a clear classification into "category 1, acute aquatic hazard", as the estimated EC50 is clearly below the threshold of 1 mg/L.

It should be kept in mind that these examples are worst case scenarios, as they are based on the maximum allowable amounts of ingredients that are just at the border of a classification band (EC50's of 0.11 respectively 1.1 mg/L). However, under the assumption that CA is a scientifically sound and empirically validated mixture toxicity concept (see discussion above and in Appendix 2), it demonstrates a potential limitation (toxicity underestimation) by applying the 25%-threshold that is used in the summation method. Whether this is of any practical relevance will depend on the specific product(s) under investigation.

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 still come the same conclusion of "no classification for acute aquatic hazard", i.e. a predicted EC50 > 1mg/L. This occurs if no more than 10% of the mixture consist of compounds with an EC50 of 0.11 mg/L (i.e. compounds classified as "acute aquatic hazard"). Under these circumstances CA predicts

$$EC50_{Mix} = \frac{100}{\sum_{i=1}^{n} \frac{c_i}{EC50_i}} = \frac{100}{\frac{10}{0.11}} = 1.1 \text{ mg/L}$$

In summary, according the summation rule 24% of compounds with a classification "acute toxicitiy (environment) category 1"and an EC50 between 1 and 0.1 mg/L are allowed in a mixture, without the mixture itself being subject to classification. If these compounds are more toxic (EC50=0.11 mg/L) CA only allows 10% of such compounds before the mixture becomes subject to classification. That the application of the summation method might result in a lower aquatic hazard classification than the application of Concentration Addition could be an argument for a change of the generic concentration limit from 25% to 10%.

The regulation provides a "catch all you can" phrase in section 4.1.3.5.4 of Annex 1, which states that "*If a mixture is classified in more than one way, the method yielding the more conservative result shall be used"*. However, this would imply that CA and the summation method are always comparatively assessed, which is not demanded by the Regulation.

The consideration of coincidental mixtures (type IV)

Although the adequate consideration of mixture type IV (also simply termed "coincidental mixtures") might easily be considered to be of major importance for the wellbeing of humans and the environment, neither REACH nor other major pieces of EU legislation make specific provisions for their prospective hazard and risk assessment. The only exception, although still referring to one chemical group only, might be the recently adopted new regulation on PPPs where multi-chemical residues in food and feed are considered: It is explicitly stated that residues of PPPs *"shall not have any harmful effects on human health, including vulnerable groups, or animal health, taking into account known cumulative and synergistic effects where the methods to assess such effects are agreed"* (from the forthcoming new legislation on Plant Protection Products (Common Position (EC) No 25/2008, adopted by the EP on 13 January 2009)). However, the

regulation is limited to one particular use group of chemicals, namely PPPs, and the explicit mandate for mixture toxicity assessments covers only the protection of human health, not the effects on the environment.

In addition to substance-oriented regulations, media-oriented pieces of EU legislation such as the Water Framework Directive (WFD) could provide appropriate starting points for controlling potential hazards and risks arising from mixture toxicity. The WFD provides a framework for the assessment of European water bodies and aims to reach "good ecological status" in all natural European surface water bodies by 2015. Within this context all factors that might potentially have an impact are to be considered, namely the hydro-morphological status, physico-chemical status and the biological quality elements. The physico-chemical status is supposed to ensure ecosystem function and focuses on Europe-wide Ecological Quality Standards (EQSs) for all individual substances of a set of 33 priority pollutants and additional regional sets of pollutants. So far, EQSs have been put forward only for individual pollutants, but not for their potential mixture(s) – although procedures for the setting of mixture EQSs have been suggested in the scientific literature quite a while ago (Calamari and Vighi, 1992; Vighi et al., 2003; Vighi and Calamari 1996) and several recent publications estimated the ecotoxicological impact of analytically determined or expected (modeled) pollutant mixtures on freshwater ecosystems using the CA concept (Chevre et al., 2006; Chevre et al., 2008; Junghans et al., 2006; Verro et al., 2009). Although the WFD might in principle provide a basis for regulatory considerations of chemical mixtures in the environment, no mixture specific guidelines have been suggested yet. Additionally, the WFD takes a largely retrospective perspective, as it works its way backward from an impaired or deteriorating ecological status towards identifying causal factors.

In summary it has to be concluded that there are currently no provisions, regulations or even guidelines for the prospective, pro-active consideration of hazards and risks of mixtures of chemicals that are to be expected to co-occur in the environment or the human body as a result of their normal use.

Consideration of chemical mixtures in the US regulatory system

The US Environmental Protection Agency (EPA) has published an extensive series of dedicated guidelines on the assessment of the impact of various types of chemical mixtures from the environment on human health (US EPA, 2000; US EPA, 2007; US EPA, 2008; US EPA, 2003, see also compilation in Appendix III). These are anchored in three major legislative acts: (a) the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) which covers incidents with hazardous materials and mandates the so-called "Superfund programme" of the EPA to assess the toxicological impact at priority contaminated sites and to develop clean-up programs, (b) the Food Quality Protection Act, and (c) the Safe Drinking Water Act. The latter two acts were amended in 1996 to include the risk assessment of cumulative exposures.

Also the Agency for Toxic Substances and Disease Registry (ATSDR) of the Department of Health and Human Services has published a similar guidance document (US Department of Health and Human Services, 2004). Of particular interest might be the so-called "binary weight of evidence" approach (BINWOE) that the ATSDR has been developed to account for interactions within the compounds of a mixture (Agency for Toxic Substances and Disease Registry

(ATSDR) 2006; Pohl et al., 2009; US Department of Health and Human Services, 2001). A list of all US guidelines is provided in Annex III.

Selection between CA and IA for regulatory assessments of chemical mixtures

All published guidelines and suggestions on mixture toxicity assessment in regulatory settings focus on IA and CA as the central mixture toxicity concepts. As environmentally realistic mixtures cannot be expected to be composed of either only similarly or of only dissimilarly acting compounds, two basic options exist for the predictive assessment of pollutant mixtures in a regulatory context: (a) a case by case selection of the most appropriate modeling approach or (b) the *a priori* choice of one of the concepts as a pragmatic default approach. For implementing mixture toxicity assessments into regulation, it is of paramount importance, to analyze whether and how these options are applicable. As both concepts come to fundamentally opposite conclusions with respect to the contribution of low, individually non-toxic concentrations, this issue requires special attention.

Case by case selection of the most suitable modeling approach

All existing experimental evidence clearly shows that the similarity or dissimilarity of the mechanisms of toxic action of the involved compounds is a valid criterion for selecting the appropriate concept for a given mixture (see above). Hence, from a scientific perspective it might be tempting to call for a case-by-case decision on the most appropriate modeling approach for each mixture.

However, there are major practical problems with such an approach: For many, if not most, environmentally relevant mixtures, knowledge about the (dis)similarity of the modes of toxic action of most components is scarce, or even absent. Filling in these gaps for all potentially relevant exposure scenarios requires a substantial effort, especially because the modes of toxic action might be specific for each potentially exposed species and considered biological endpoint. Although some detailed knowledge exists for certain groups of environmental chemicals, such as pesticides, this information is usually restricted to a very few species (target organisms) and/or biological endpoints. Hence, a mechanism-driven case-by-case selection might need to be limited to a few, specific situations, such as for example the human health assessment of mixtures of plant protection products. With the (eco)toxicological data that are generated or compiled during the registration of industrial chemicals with REACH, such a case-by-case approach is certainly not possible.

Using one concept as a default approach

The *a priori* choice of one concept as a pragmatic default approach is only justifiable, if on average only minor errors occur by choosing the "wrong" concept (i.e. estimating the toxicity of a mixture of dissimilarly acting substances with CA instead of IA or *vice versa*). Also, when having the precautionary principle in mind ("better safe than sorry"), that concept should be *a priori* selected, which in the case of an error does not lead to an *under*estimation of the mixture toxicity. Empirical evidence strongly argues for CA from this perspective (see above).

Mathematical analyses showed, that considerable errors (> one order of magnitude) may occur only with large number of individual mixture components (>10) and extremely steep concentration-response relationships (Faust, 1999). The BEAM EU-Project ran simulated mixture experi-

ments with ecotoxicological data for a set of single substances that were collected from Research Facilities and Regulatory Authorities. Results clearly show that CA predicts slightly lower effect concentrations than IA for the vast majority of mixtures that can be composed from the given pool of single substances (Faust and Scholze, 2004). In other words, typical situations from standard ecotoxicological assays with algae, daphnids and fish almost always led to CA predicting slightly higher toxicity values. The simulations furthermore reveal that the differences between CA- and IA-predicted EC50-values are always below an order of magnitude, in the vast majority of cases below a factor of 5 – which corresponds to the empirical evidence that is available from the peer reviewed literature. Unfortunately, similar simulations studies have as yet not been conducted with data from assays for the assessment of human toxicity.

If a concept is selected as a default approach, its demands in terms of input data should be compatible with the typical data situation generated during the routine hazard assessment of single toxicants. CA can be applied for predicting the EC50, the NOEC, or the PNEC of a mixture, using EC50, NOEC, or PNEC values, respectively, for the individual components as input data (for a detailed discussion see Appendix II). That is, the concept can be applied using typical base-set data for providing a (limited) assessment of expectable mixture toxicities. IA, in contrast, can only be applied if full concentration-response curves, that need to be especially reliable in the low-effect range, are available for each compound in the mixture – which is rarely the case.

Thus, in summary it may be concluded, that for the *a priori* selection of a concept empirical evidence and mathematical considerations as well as the precautionary principle point to CA as a pragmatic and defendable default approach.

The relevance of low-effect concentrations

It might be argued that the suggestion to use CA for any type of mixture is overprotective in many situations. After all, IA predicts that mixtures of dissimilarly acting chemicals do not show any mixture toxicity at all, as long as all components are present in concentrations below their individual toxicity thresholds. An answer to that seemingly obvious objection hinges on two separate questions:

(a) When can mixtures be expected to behave perfectly in accordance with Independent Action?

(b) What does the term “below their thresholds” mean?

A) IA is a purely theoretical construct, assuming that all the components in a mixture act completely independent of each other via different modes and mechanisms of toxic action on the endpoint of interest. It is highly questionable, whether such a situation actually can occur in any organism with its tightly interwoven, mutually dependent physiological pathways. Furthermore, as IA assumes that the mechanisms of action are completely dissimilar of each other, mixtures in which any of the involved components has multiple or unspecific mechanisms cannot be expected to completely follow IA. The concept of an unspecific, “narcotic” mode of action, which describes the “baseline toxicity” that all organic components share (Escher and Hermens, 2002) hence immediately leads to an IA-incompatible situation. And especially the (eco)toxicity of many industrial chemicals that are registered within REACH can be expected to be explained to a good deal by such a narcotic mode of action.

B) Threshold values are typically based on experimental No Observed Effect Concentrations, NOECs, which denote the highest test concentration at which the observed response of the

test organisms was not significantly different (in a statistical sense) from the untreated controls. NOECs have been heavily criticized for various reasons (Allen et al,. 1994; Chapman, 1997; Chapman et al., 1996; Dhaliwal et al., 1997; Hoekstra and Ewijk, 1993; van der Hoeven, 1997), and in the context of mixture studies, the most important shortcoming of NOEC-based approaches might be that those values are based on the failure to detect a statistically significant effect in a given biotest with a given experimental design – which of course does not prove that there is no effect in reality. Therefore, NOECs do not describe a "safe" concentration and differ fundamentally from true No Effect Concentrations, although they are frequently equated as such.

Because NOECs might correspond to effects as high as 5%-20% (Allen et al., 1994) or even 30% (Moore and Caux, 1997) IA-compliant mixture effects cannot be ruled out, even if all components are present only at their individual NOECs. If only certain fractions of the individual NOECs are present (e.g. if NOECs are divided by an assessment factor), it depends on the number of mixture components, the precision of the experimental data and the steepnesses of the individual concentration-response curves whether a mixture effect might occur. In fact it has been demonstrated that concentrations of strictly dissimilarly acting substances contribute to the toxicity of a mixture that behaves according to IA even if present at concentrations below their statistically estimated individual EC1 values (Backhaus et al., 2008; Faust et al., 2001). A further, in-depth discussion of the available empirical evidence on low-dose effects of chemical mixtures and their implications for ecological and human health assessment has been provided by Kortenkamp and coworkers (Kortenkamp et al., 2007; Kortenkamp et al., 2009).

In summary, possible mixture effects can only be excluded *a priori*, if all components in the mixture are completely independently acting (which might never be the case in reality) and if all of them are present at concentrations that definitely produce absolutely no effect (which is impossible to prove experimentally). Mixture effects have to be taken into consideration in all other situations (assuming that the compounds actually co-occur and affect the same endpoint) and the default use of CA seems to be a suitable first tier approach for this purpose.

It should be pointed out that the use of CA as a first approach for estimating the toxicity of a mixture does not imply that any combination of chemicals is hazardous or risky *per se*. It depends on the number of components, their concentration in the mixture and their individual toxicity whether this is the case.

Options for including the assessment of “ coincidental” mixtures into REACH

Using the current scientific knowledge on the toxicology and ecotoxicology of chemical mixtures, two options for their consideration may be suggested within the context of prospective hazard and risk assessments, especially REACH, may be suggested. Option A, the application of a default “mixture assessment factor” (MAF) could be a pragmatic amendment to the current assessment procedures as they are conducted for the registration of single substances under REACH. Option B, a scenario-specific cumulative risk assessment (CRA) requires a broader overview of expectable cumulative pollution situations. Hence, CRA is an approach that might mainly be applicable by regulatory authorities, such as the ECHA or the competent national bodies.

Option A: a mixture assessment factor (MAF)

Cumulative risk assessments by means of component-based approaches such as CA first of all require a definition of the mixture of concern in terms of the number and nature of components and their concentrations and concentration ratios. Secondly some quantitative knowledge about their individual toxic potencies with respect to a common endpoint is required as input data. Where such knowledge is unavailable, a default exposure scenario could be defined for regulatory purposes, and such a scenario could then be reflected in single substance assessments by means of a default mixture assessment factor (MAF).

Such a MAF may be proposed as a very pragmatic way to account for the fact that not only the particular substance that is subject to registration under REACH might be harmful to exposed humans and organisms in the environment – but that in fact it may become part of a multi-component mixture, whose cumulative impact can be expected to be higher than that of each individual chemical present.

Before discussing the issue further, it needs to be clearly stated that, contrary to popular belief, there is no indication that current assessment factors in the REACH guidance documents are meant to actually account for mixture effects. While it was stated in the old Technical Guidance Documents[10] that for the assessment factors "additive, synergistic and antagonistic effects from the presence of other substances may also play a role" (p 99), this statement has been omitted in the justification of assessment factors in the current guideline (ECHA, 2008, "Guidance on information requirements and chemical safety assessment", Chapter R10, "Characterisation of dose [concentration]-response for environment", p 17).

In addition to the definition of a standard scenario, the derivation of a MAF requires to adopt a default assumption about the type of joint action of mixture components. For reasons that have been explained in detail above, the assumption of a concentration-additive action of mixture components appears to be justifiable as a precautious but not inadequately overprotective pragmatic approach to the problem. The mathematical formulation of this concept (see Appendix II) implies that the expectable overall effect of a mixture will never exceed a certain critical effect level x, if the concentrations of all components are smaller than $1/n$ -th[11] of the effect concentration (ECx_i) of each individual toxicant that would cause the same effect x if applied singly. For instance, the total expected effect of a mixture of ten compounds (n 10) will always be smaller than 5%, if the concentrations of all components are smaller than 1/10 –th of their individual EC5 values.

For regulatory purposes, pragmatic generalizations of the CA concept have been introduced such as the so-called Point of Departure Index (PODI) and the so-called Hazard Index (HI) (see Appendix I). The PODI means that the use of individual effect concentrations (ECx_i) in the calculation is replaced by so-called points of departures (PODs) such as LOELs, NOAELs or NOECs. The HI means that instead of effect concentrations (ECx_i) regulatory acceptable levels (ALs) such as ADIs[12], DNELs[13] or PNECs are used in the calculation, which in addition to PODs include an assessment factor that accounts for intra- and interspecies variability and other assess-

[10] In support of the old 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

[11] n = number of components in the mixture

[12] ADI = Acceptable Daily Intake

[13] DNEL = Derived No Effect Level

ment uncertainties (but not for mixture effects, as explained above). These approaches are consistent with the CA concept under the simplifying assumptions that (*i*) PODs reflect the same effect level (e.g. 5%) and (*ii*) refer to the same end point in the same species under comparable conditions and that (*iii*) assessment factors that are included in the acceptable levels established for individual toxicants have the same size (e.g. 100). Otherwise, PODI and HI will usually result in more cautious assessments of mixture hazards and risks than the original CA concept. This is why they may be considered to provide first tier worst case estimates.

By definition, PODI and HI do not give an indication for any statistically significant or regulatory unacceptable mixture effects, respectively, if the indices do not exceed a value of one (see the formulas in Appendix I). This value of one will never be reached if the concentrations or doses of all mixture components are not larger than 1/n of their respective individual PODs or ADIs, DNELs or PNECs. If the individual concentrations are between 1/n and 1/1 of the individual PODs or ALs, it will depend on (i) the number of mixture components, (ii) their actual concentrations and (iii) their toxic potencies in terms of individual PODs or ALs, whether PODI or HI indicate a potential for statistically significant or regulatory unacceptable hazards of a mixture.

As a consequence of these considerations, a default MAF of *n* may be considered as an appropriate precautious measure for safeguarding against unwanted mixture effects, whereby *n* is the number of chemicals that are assumed (i) to be simultaneously present in a default scenario and (ii) to contribute to a common (eco)toxicological endpoint. The problem then is to establish a consensually acceptable default value for that *n*.

Several knowledge gaps and open issues currently hamper the establishment of such a default MAF. First of all, the actual number of toxic compounds present in realistic exposure situations is largely unknown and might vary considerably for different scenarios. Secondly, at least in the field of human toxicology, there is still a considerable dispute on whether and to what extent CA might be a justifiable default assumption also for mixtures of substances that affect a common endpoint via different mechanisms of action (but see discussion above). In fact, the available regulatory guidelines on the human health assessment of chemical mixtures as suggested by the US EPA or the EFSA devote a considerable effort to a case-by-case selection between CA and IA (Panel on Plant Protection Products and their Residues(PPR), 2008; US EPA, 2000; US EPA, 2003). And thirdly, the assumption of a concentration additive joint action of mixture components does not necessarily mean that all mixture components contribute equally to the overall effect. On the contrary, there are empirical examples where a few components were shown to dominate the overall mixture toxicity in a realistic exposure scenario while all the others substances did not make a significant contribution (see for e.g. Junghans et al., 2006, Kortenkamp and Faust, *in press*). If such episodic findings could be demonstrated to have a more general validity, this could potentially argue for a MAF between 10 and 100. In fact, the Netherlands apply an assessment factor of 100 for the derivation of so-called "negligible concentrations"[14] for the setting of environmental risk limits that are supposed to safeguard against mixture effects (Vlaardingen et al, 2007).

[14] The negligible concentration (NC) is defined as: *"The target value (ed.: the EQS) is, in principle, set at the level of negligible concentration (NC) and is the guideline for the long-term environmental quality to be achieved. The NC has been set to a factor of 100 below the MPC, which defines a safety margin allowing for combination toxicity",* where the MPC is largely synonymous to the PNEC (Vlaardingen et al, 2007).

However, the consensual establishment of a MAF approach in human as well as ecotoxicological assessments requires further investigations into the appropriate definition of realistic mixture exposure scenarios. They may differ for different groups of chemicals, different receiving media (air, water, soil, biota, food, humans), and different toxicological or ecotoxicological endpoints.

Option B: scenario specific cumulative risk assessment (CRA)

The MAF may provide a pragmatic regulatory tool for taking mixture toxicity into account, but better estimates of the expectable (eco)toxicity of realistic mixtures of substances registered under REACH may be gained by considering specific exposure scenarios, similar to the approaches initiated by the new PPP regulation or those suggested for the advancement of the WFD (see above). Examples of such a scenario specific approach include the study on the joint ecotoxicological effects of realistic exposures to pesticide mixtures by Junghans and her coworkers (2006), the analysis of potential cumulative health risks resulting from exposure to various different antiandrogenic compounds from different sources via different routes by Kortenkamp and Faust (in press) or the study by Tønning et al. on the risks of endocrine disrupters for 2-year old children (Tønning et al., 2008).

As the assessment of the number and the concentrations of compounds that may be present in a certain scenario requires a systematic overview of registered and used chemicals, such a scenario-specific CRA is beyond the horizon of an individual registrant. Carrying out such advanced assessments could hence be regarded as a task for a national or European regulatory authority. Menzie and coworkers recently discussed a conceptual outline of how combined effects of stressors (including non-chemical ones) can be assessed (Menzie et al. 2007). Following their outline, but simplifying it for mixtures of chemical stressors only, a scenario-specific assessment of chemical mixtures could contain the following steps:

(a) Problem formulation and development of a conceptual model of the scenario.
(b) Analysis of the joint exposure: which compounds are known to occur simultaneously (evidence from chemical-analytical monitoring) or are expected to be present simultaneously (joint exposure modeling, see above)?
(c) Analysis of the joint action (see below).
(d) Mixture risk assessment.
(e) Risk management, risk mitigation, if necessary.

The analysis of the joint action of a mixture (step c) might require an iterative (tiered) approach, starting with a semi-quantitative analysis and a stepwise refined assessment if a potential risk is indicated. The following major steps can be identified:

(i) Compilation of toxicological and/or ecotoxicological data
(ii) Decision on whether a particular substance needs to be considered for the joint toxicity of the mixture (does it impact the biological endpoint of interest? Other reasons for waiving?)
(iii) Prediction / modeling of mixture toxicity, using CA, IA, TSP[15], mixture-msPAF[16] or advanced modeling (PBPK/PD[17]), depending on the amount of available (eco)toxicological

[15] Two-stage prediction, a combination of CA and IA in order to predict the toxicity of a mixture of similarly as well as dissimilarly acting substances

[16] msPAF = multi-substance potentially affected fraction of species, the relative number of species expected to be affected by exposure to a mixture

information. Only CA might be applicable when only the limited amount of data from the registration of a chemical under REACH is at hand.

(iv) Analysis of interactions, i.e. incorporation of existing knowledge on synergistic or antagonistic interactions that might lead to more or less than expected mixture effects. The BINWOE[18] approach that has been developed by the US Dep. of Human Health and Services might provide a suitable methodology here.

The frame of reference of a given study guides the selection and definition of the biological endpoint of interest. It is selected *a priori* if a certain biological phenomenon or effect type defines the scope of the study. The study by Tønning et al. (2008) for example focused from the very beginning exclusively on the risks of endocrine disruption in children. This tremendously focuses the analysis (the available resources). In particular the selection of compounds to be included in the scenario evaluation might be substantially simplified and hence the consideration of different pollutant sources and pathways is facilitated. On the other hand, such an *a priori* focus might risk overlooking effects on other biological endpoints, perhaps being equally or even more sensitive and important than the one that was selected in the first place. The aforementioned study does for example not provide any information on the risks of allergy developments.

A study might also focus on a pre-defined set of substances that are expected and/or detected in a scenario. The studies by Verro and colleagues (2009) and by Junghans et al (2006) for example focused on mixtures of pesticides only. Under these circumstances, the selection of the biological endpoint of interest is usually guided by its expected sensitivity towards the mixture. Formulating an informed hypothesis on the potentially affected endpoints might be one of the critical steps in the whole assessment process, depending on the pre-existing knowledge on the modes and mechanisms of action of the involved compounds. Ecotoxicological studies have the opportunity – much more so than human health related studies – to employ highly integrating endpoints such as mortality, growth or reproduction. This provides an overall picture of the consequences of impairing different physiological processes. Complementary investigations on a genetic or molecular level might then help to pinpoint the physiological pathways most severely affected by the mixture(s). Such studies on the environmental impacts of mixtures additionally have to face the challenge that the biological endpoint of interest is not only related to physiological or population-level processes, but also to the different sensitivities of the exposed species and ecological processes (e.g. nutrient cycles, ecological competition and food webs).

Suggestions for further case studies in the context of REACH

In order to gain further experience with the feasibility of such scenario specific cumulative assessment within the context of REACH it might be suggested to implement a series of dedicated case studies, using the available data from the REACH registration of the involved chemicals. The existing categorization of chemicals within REACH might provide different starting points for the development of such case studies. They could be set up along the lines of the sectors of use (e.g. a cumulative assessment of chemicals in a typical private household (private household use, REACH category of use SU2), product categories (e.g. typical personal care product mixtures, REACH product category PC39) or article categories (e.g. typical chemicals that are found in paper products, REACH product category AC8).

[17] Physiologically based pharmacokinetic/pharmakodynamic modeling

[18] Binary Weight of Evidence

Should the final mixture risk assessment for a given scenario indicate reasons for concern, the assessment data would also allow to pinpoint the major contributing chemicals. Regulatory instruments for a risk management and mitigation could then focus on targeted exposure reduction measures.

However, it should be pointed out that REACH currently does not provide any provisions for the consideration of any type of "coincidental" mixture (type IV). As scientific evidence strongly suggests that such chemical mixtures require attention, it may be required to amend the REACH regulation in a way analogous to the new PPP regulation, this time considering both environmental safety and human health. Otherwise the professed aim of REACH to "ensure a high level of protection of human health and the environment" might be severely compromised.

Summary of recommendations

1) As the example of the new PPP regulation shows, protection goals with respect to hazards and risk arising from chemical mixtures can be incorporated into existing substance-oriented pieces of legislation. In order to ensure that the professed aim of REACH to "ensure a high level of protection of human health and the environment" can be fulfilled, an analogous amendment should be incorporated into the current REACH regulation.

2) The data that will be compiled for the registration of a compound within REACH would allow two principal options for considering chemical mixtures: (a) a default mixture assessment factor (MAF) and (b) a scenario specific cumulative risk assessment (CRA).

3) Several guidelines and outlines for conducting chemical risk assessments for mixtures have been suggested in the scientific literature, put forward by national and trans-national committees from EU member states and the US, as well as academic and industrial groups (see Appendix III). Although all these documents center around IA and CA as central mixture toxicity concepts, they differ in scope and hence suggest approaches with varying levels of complexity. These guidelines provide a good starting point for the development of analogous guidelines within the context of REACH. A critical review and analysis of existing guidelines and scientific suggestions would also prepare the ground for more general, trans-sectorial strategies for mixture risk assessment.

4) All guidelines that have been brought forward by regulatory authorities so far focus on human health oriented assessments, in particular the recent work by the EFSA on the development of a methodology for the risk assessment of multiple pesticide residues in food and the cumulative risk assessment guidelines developed by the US EPA. No comprehensive guideline for the ecotoxicological assessment of chemical mixtures has been developed yet, although approaches have been developed and discussed in the scientific literature. The Water Framework Directive with its ecological protection goals might provide a good regulatory frame for the initial development of such a dedicated guideline for the ecotoxicological assessment of chemical mixtures.

5) The new classification, labeling and packaging Regulation suggests two component-based approaches for the classification of chemical mixtures (chemical products, preparations): the summation rule and Concentration Addition. However, both approaches might come to different conclusions for the classification for environmental (aquatic) hazards, with the Summation Rule providing the less conservative classification in certain situa-

tions (see examples above). Although the Regulation requests to base the final classification on the more conservative method, it does not request to comparatively apply both approaches, if the available data allow doing so. However, the Regulation could easily be amended accordingly.

6) A major knowledge gap that hampers the actual implementation of options such as a mixture assessment factor is the currently limited knowledge on expectable or typical exposure situations with respect to industrial chemicals. REACH already provides a series of classifications that may provide a basis for the analysis of exposure scenarios from different perspectives: sectors of use, product categories and article categories.

Challenges beyond REACH

The compounds that make up chemical mixtures in the real-world do not belong to a single regulatory group (i.e. not all of them are pesticides, not all of them are industrial chemicals, etc.). This trans-sectorial nature of chemical mixtures in the environment poses a major challenge for the current regulatory system. However, media-oriented pieces of legislation, such as the Water Framework Directive, may potentially provide a useful platform for attacking this problem effectively, and substance-oriented pieces of regulation seem to provide options for the complementary assessment of particular scenarios as can be seen from the recent revision of the legislation on plant protection products.

Figure 1: Mixture toxicity of 11 priority pollutants from different chemical classes, selected from EEC list 1, Council Directive 76/464.

Each individual compound is present at its individual No Observed Effect Concentration (NOEC). Figures above the bar of each individual compound denote the average inhibition that was observed at the NOEC, which is not significantly different (α=0.05) from the untreated controls. However, a clear mixture toxicity of 63.9%, far higher than each individual effect, can be observed. This mixture toxicity falls into the span between the predictions by CA and IA, but are in this study better approximated by IA. From (Walter et al., 2002), reprinted with permission.

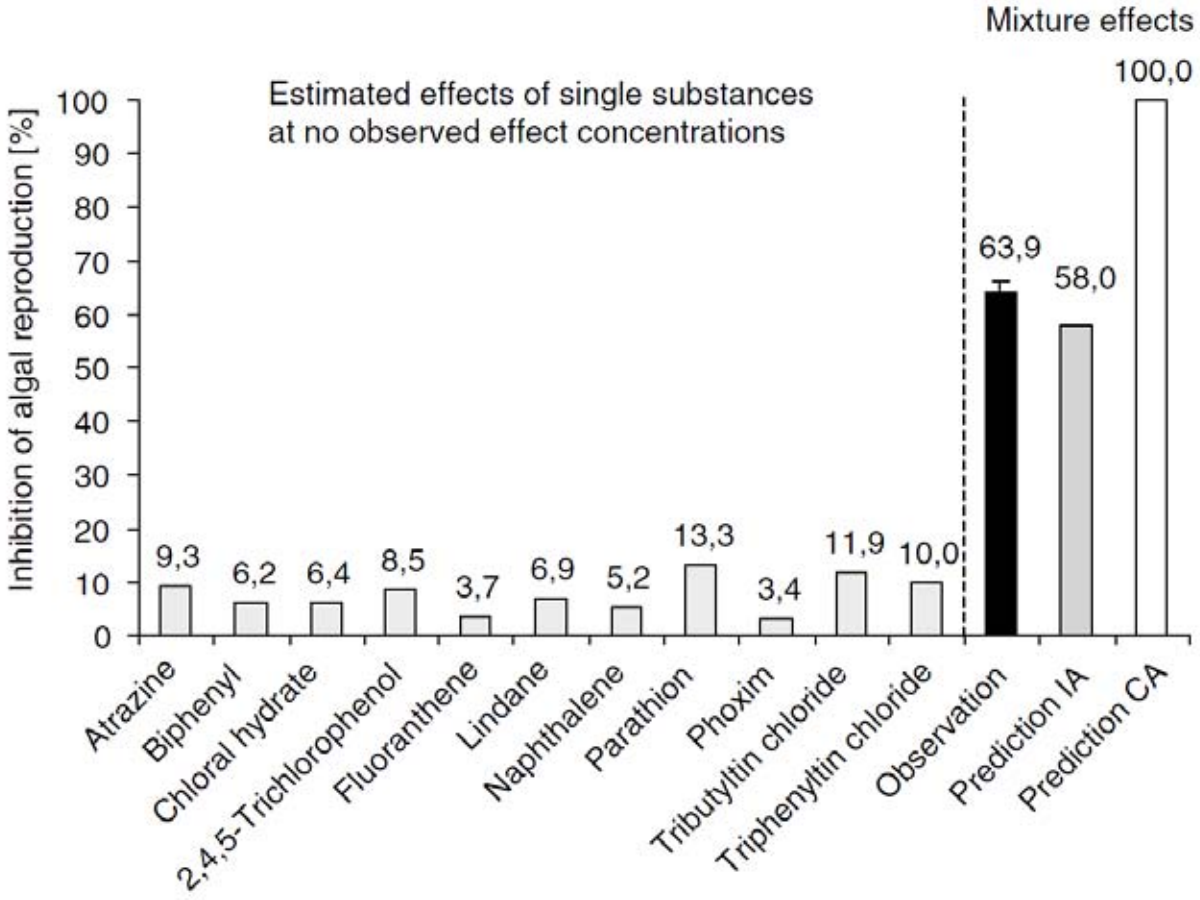


Figure 2: Effects of a low-dose mixture of the three anti-androgens Vinclozolin (VZ), Flutamide (FLUT) and Procymidone (PRO) on markers of sexual differentiation in rat

A: Anogenital distance, B: Nipple-retention. * and ** indicate significant differences (α< .0.05). Gray bars indicate response of untreated male and females, respectively. Blue bars indicate the observed response to the exposure to the single substances and the corresponding mixture, respectively (24.5 mg/kg vinclozolin (VZ), 0.77 mg/kg flutamide (FLUT), and 14.1 mg/kg prycymidone (PRO) (blue), and the mixture dose of 39.37 mg/kg (blue bar)). The white bar indicates the prediction according to Concentration Addition. From (Hass et al., 2007), reprinted with permission.

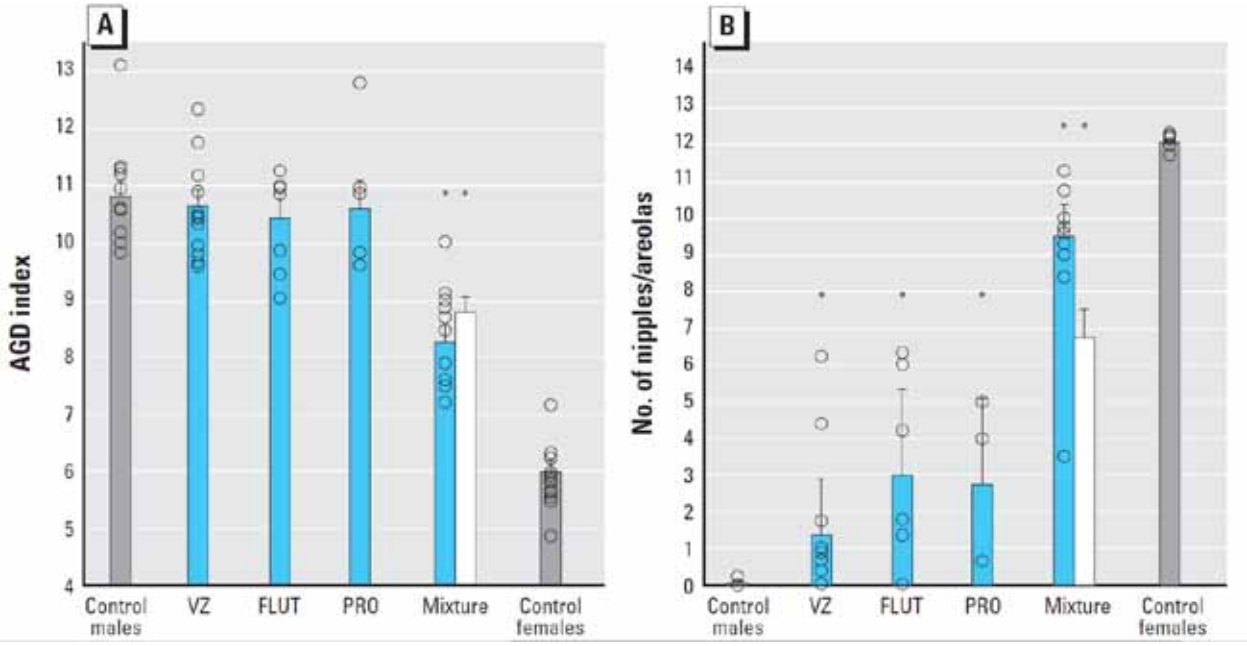


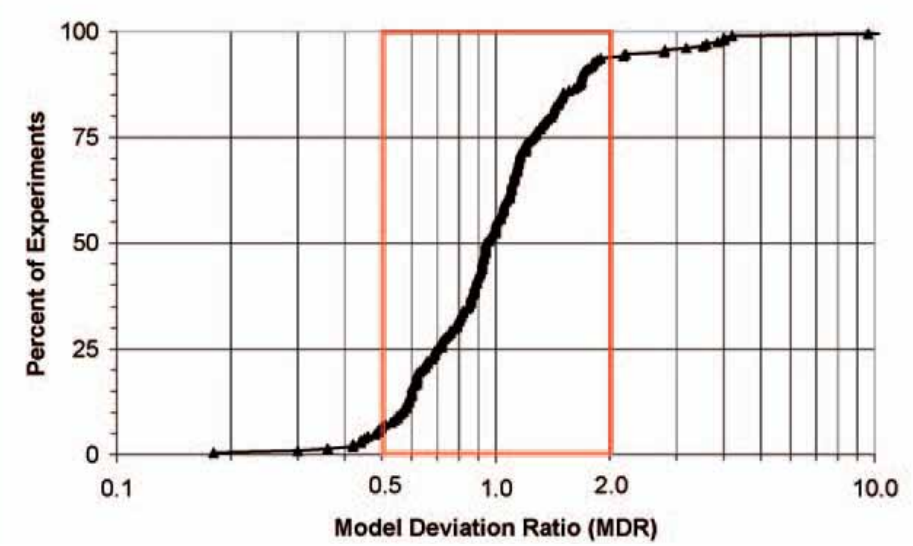
Figure 3: Analysis of the mixture toxicity of pesticide mixtures.

A. Cumulative Model-Deviation Ratio (MDR) for Concentration Addition for all 207 re-analysed mixture experiments.

B. MDR 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, i.e. a MDR of 1 indicates a perfect prediction of the experimentally observed mixture toxicity. From (Belden et al. 2007), reprinted with permission.

A.



B.

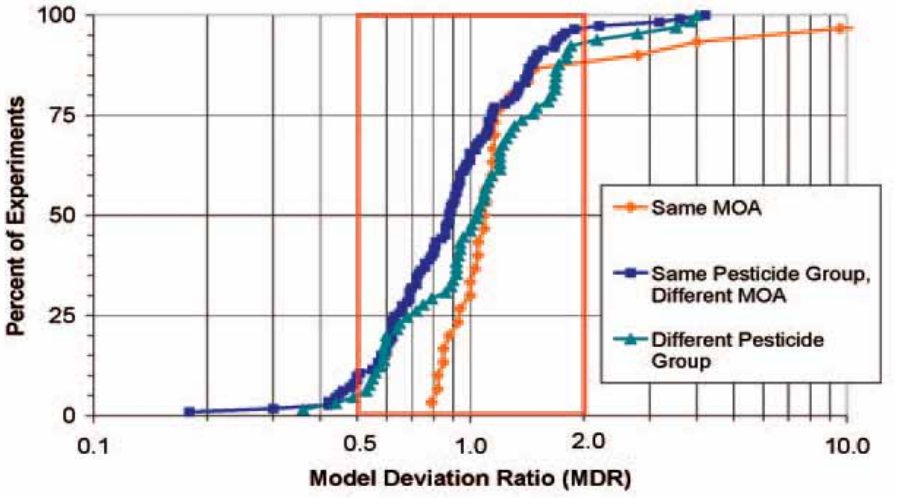


Figure 4: More than concentration-additive mixture toxicity (inhibition of acetylcholine esterase activity) of binary mixtures of carbamates (CB) and organophosphate (OP) pesticides.

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

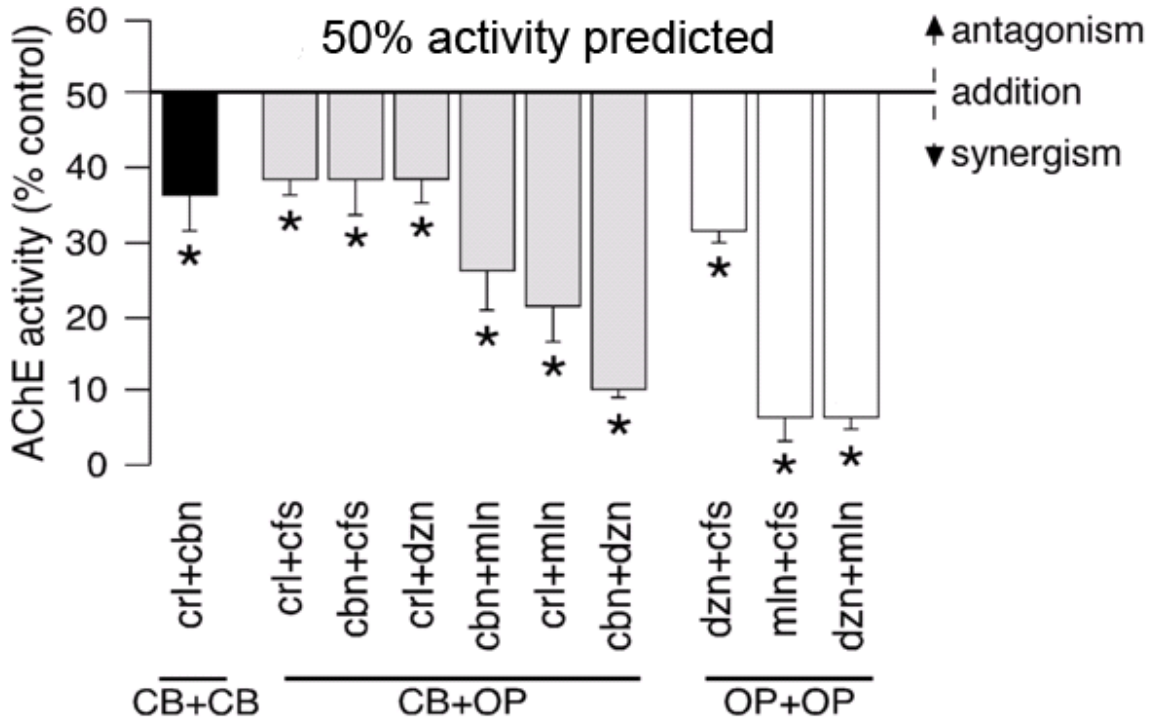


Figure 5: More than additive mixture effects of (a) Brilliant Blue (BB) and L-Glutamic Acid and (b) Quinoline Yellow (QY) and Aspartame.

Brilliant Blue and Quinoline Yellow are common food additives. Experiments show the observed and predicted neurotoxicity (neurite growth) in mouse neuroblastoma cells. Given are the isoboles for CA (straight line) and for the experimentally observed EC50 values. It can be seen that the EC50 of the experiments is clearly lower than expected by CA.
Taken from (Lau et al., 2006), reprinted with permission.

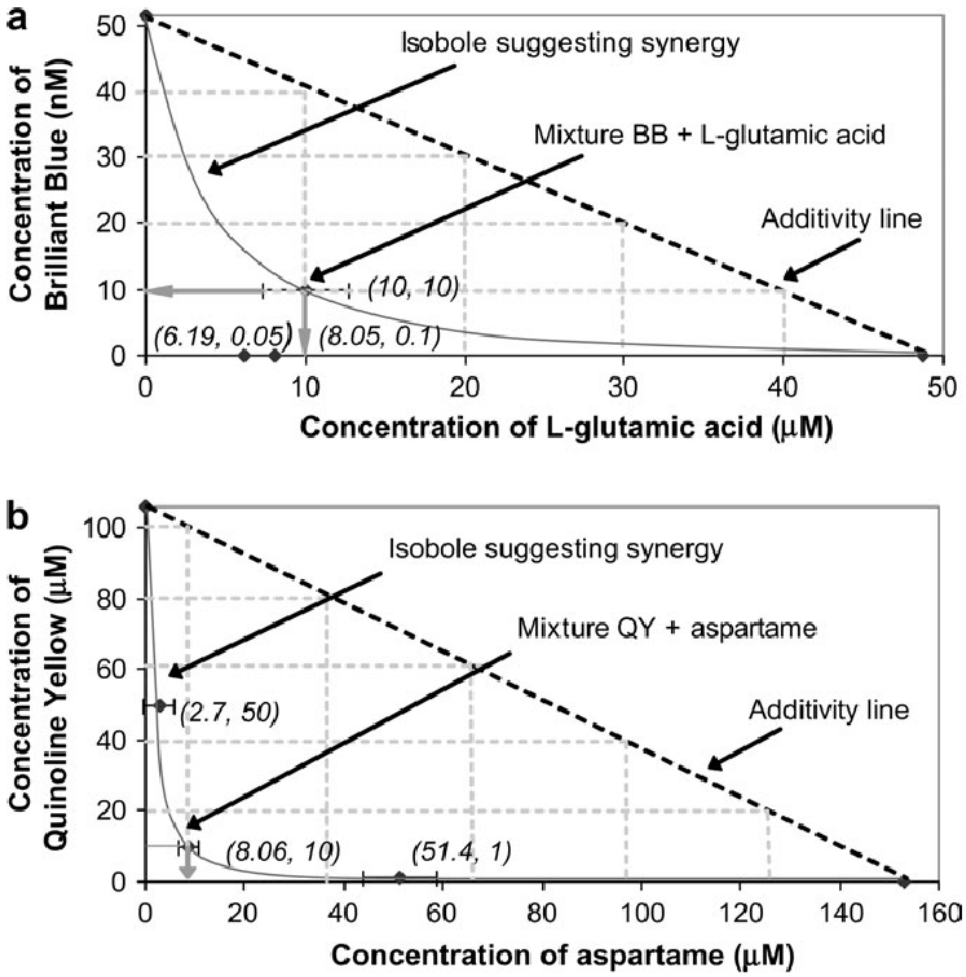


Table 1: Mixture classification approaches as suggested in the CLP regulation 1272/2008

Category	Whole mixture tests	Bridging principles	Concentration Addition	Summation Method / Concentration Limits	Application Order	Comments
acute toxicity	.	.	.	.	WM-BP-SM-CA	
skin corrosion,	.	.	.	.	WM-BP-CA-SM	- CA is not provided as a formula, the "theory of additivity" is put forward in section 3.2.3.3.2
eye damage	.	.	.	.	WM-BP-CA-SM	- CA is not provided as a formula, the "theory of additivity" is put forward in section 3.2.3.3.2
respiratory or skin sensitisation	.	.		.	WM-BP-SM	- CA not considered
germ cell mutagenicity	.	.		.	SM-(WM-BP)	- CA not considered - WM only considered on a case-by-case basis
carcinogenicity	.	.		.	SM-(WM-BP)	- CA not considered - WM only considered on a case-by-case basis
reprotox	.	.		.	SM-(WM-BP)	- CA not considered - WM only considered on a case-by-case basis
specific target organ tox	.	.	.		WM-BP-SM	- CA not considered, - separate evaluation for single-dose and repeated-dose toxicity
aspiration hazard	.	.		.	WM-BP-SM	- CA not considered
aquatic hazard (acute)	.	.	.	.	WM-BP-SM-CA	
aquatic hazard (chronic)				.	SM	- CA not considered - WM not considered

CA: Concentration Addition, i.e. the "Additivity Formula"; WM: Data from whole mixture tests; BP: Application of bridging principles; SM: Summation Method or Concentration Limits for already classified ingredients.

References

Adielsson S, Törnquist M, and Kreuger J. (2006) Bekämpningsmedel i vatten och sediment från typområden och åar samt i nederbörd under 2005. ISRN SLU-VV-EKOHYD-94-SE. Swedish University of Agricultural Sciences, Uppsala.

Agency for Toxic Substances and Disease Registry (ATSDR). 2006. Interaction Profiles for Toxic Substances.

Allen, B.C., Kavlock, R.J., Kimmel, C.A., and Faustman, E.M. (1994) Dose-response assessment for developmental toxicity II. Comparison of generic benchmark dose estimates with no observed adverse effect levels. *Fundamental and Applied Toxicology*. 23:487-495.

Altenburger, R., Boedeker, W., Faust, M., and Grimme, L.H. (1996) Regulations for combined effects of pollutants: consequences from risk assessment in aquatic toxicology. *Food and Chemical Toxicology*. 34:1155-1157.

Altenburger, R. and Greco, W. (2009) Extrapolation concepts for eadling with multiple contamination in environmental risk assessment *Integrated Environmental Assessment and Management*. 5:62-68.

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M. and Grimme, L.H. (2000) Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. *Environmental Toxicology and Chemistry*. 19:2348-2356.

Backhaus,T., Faust, M., Scholze, M., Gramatica, P., Vighi, M. and Grimme, L.H. (2002) The joint action of phenylurea herbicides is equally predictable by Concentration Addition and Independent Action." *Environmental Toxicology and Chemistry*. 23:258-264.

Backhaus, T., Sumpter, J.P. and Blanck, H. (2008) On the ecotoxicology of pharmaceutical mixtures. In *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Kümmerer, K. (ed), Springer, 3rd edition.

Belden, J.B., Gilliom, R.J., and Lydy, M.J. (2007) How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integrated Environmental Assessment and Management.* 3:364-372.

Bliss, C.I. (1939) The toxicity of poisons applied jointly. *Annual Journal of Applied Biology*. 26:585-615.

Boedeker,W., Drescher, K., Altenburger, R., Faust, M. and Grimme, L.H. (1993) Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. *The Science of the Total Environment*. Supplement:931-938.

Calamari, D. and Vighi, M. (1992) A proposal to define quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere*. 25:531-542.

Caldelas, E.D., Boon, P.E. and Tressou J. (2006) Probabilistic assessment of the cumulative acute exposure to organophosphorus and carbamate insecticides in the Brazilian diet. *Toxicology*, 222:132-142.

Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Mathiassen, S. K., Streibig J. C., and Sörensen, H. (2008) A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environmental Toxicology and Chemistry*. 27:1621-1632.

Chapman, P.F. and Chapman, P.M. (1997) Reply To: "Warning : Replacing NOECs with point estimates may not solve regulatory contradictions" by Dhaliwal et al." *Environmental Toxicology and Chemistry*. 16:125-126.

Chapman, P.M., Caldwell, R.S. and Chapman, P.F. (1996) A warning: NOECs are inappropriate for regulatory use. *Environmental Toxicology and Chemistry*. 15:77-79.

Chevre, N., Loepfe, C., Singer, H., Stamm, C., Fenner, K. and Escher, B.I. (2006) Including mixtures in the determination of water quality criteria for herbicides in surface water. *Environmental Science and Technology* 40:426-435.

Chevre, N., Maillard, E., Loepfe, C., and Becker-van Slooten, K. (2008) Determination of water quality standards for chemical mixtures: Extension of a methodology developed for herbicides to a group of insecticides and a group of pharmaceuticals. *Ecotoxicology and Environmental Safety*. 71:740-748.

Commission of European Communities. (2003) *A European environment and health strategy.* Communication from the Commission to the Council, the European Parliament and the European Economic and Social Committee. Brussels, COM (2003) 338 final.

Dhaliwal, B.S., Dolan, R.J., Batts, C.W. and Kelly. J.M. (1997) Warning: replacing NOECs with point estimates may not solve regulatory contradictions. *Environmental Toxicology and Chemistry*. 16:124-126.

Drescher,K. and Boedeker, W. (1995) Assessment of the combined effects of substances: The relationship between concentration addition and independent action. *Biometrics*. 51:716-730.

European Chemicals Agency (ECHA). (2008) Press release of the European Chemicals Agency, ECHA/PR/08/59, 19.12.2008

Escher, B.I. and Hermens, J.L.M. (2002) Modes of action in ecotoxicology: Their role in body burdens, species sensitivity, QSARs, and mixture effects. *Environmental Science and Technology* 36 (20):4201-4217.

Houlihan, J., Kropp, T., Wiles, R., Gray, S., and Campbell, C. (2005) Body Burden, the pollution in newborn, Report, The Environmental Working Group, Washington, USA

EU Council (Council of the European Union). (1991) Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market, *Official Journal of the European Communities*, L230 , 1-32.

EU Council (Council of the European Union). (2009) Council conclusions on combination effects of chemicals. 2988th Environment Council meeting, Brussels, 22 December

EU Parliament and the Council. (2008a) Directive 2008/1/EC of the European Parliament and the Council of 15 January 2008 concerning integrated pollution prevention and control. *Offical Journal of the European Communities*, L 24, 8-29.

EU Parliament and the Council. (2008b) 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. *Official Journal of the European Communities*. L353.

EU Parliament and the Council. (1998) Directive 98/8/EC of the European Parliament and of the council of of 16 February 1998 concerning the placing of biocidal products on the market, *Official Journal of the European Communities*, L 123/1.

European Chemicals Agency (ECHA) (2009) Guidance on the Application of the CLP Criteria - Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures *ISBN not yet available at the time of this report.*

Faust, M. (1999) Kombinationseffekte von Schadstoffen auf aquatische Organismen: Prüfung der Vorhersagbarkeit am Beispiel einzelliger Grünalgen. ISBN 3934389287, GCA-Publishers, Herdecke, Germany.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V. Scholze, M., Vighi, M., and Grimme, L. H. (2003) Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquatic Toxicology*. 63:43-63.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V. Scholze, M., Vighi, M., and Grimme, L. H. (2001) Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquatic Toxicology*. 56:13-32.

Faust, M., and Scholze, M. (2004) Competing Concepts for the Prediction of Mixture Toxicity: Do the Differences Matter for Regulatory Purposes? Final Report of the European R D Project BEAM (EVK1-CT1999-00012).

Feron V.J., and Groten, J. P. (2002) Toxicological evaluation of chemical mixtures. *Food and Chemical Toxicology* 40 (6):825-839, 2002.

Frische,T., Faust, M., Meyer, W. and Backhaus, T. (2009). Toxic masking and synergistic modulation of the estrogenic activity of chemical mixtures in a yeast estrogen screen (YES). *Environmental Science and Pollution Research*. 16:593-603.

Harris,C.A., Brian, J.V., Pojana, G., Lamoree, M., Booy, P., Marcomini, A., and Sumpter J.P. (2009) The influence of a surfactant, linear alkylbenzene sulfonate, on the estrogenic response to a mixture of (xeno)estrogens in vitro and in vivo. *Aquatic Toxicology*. 91:95-98.

Hoekstra,J.A. and van Ewijk, P.H. (1993) Alternatives for the no-observed-effect level. *Environmental Toxicology and Chemistry*. 12:187-194.

International Programme on Chemical Safety (IPCS). (2009) Assessment of combined exposures to multiple chemicals. Report of a WHO/IPCS International Workshop.

Junghans, M., Backhaus, T., Faust, M., Scholze, M., and Grimme, L.H. (2006) Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquatic Toxicology.* 76:93-110.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton H. T. (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology.* 36 (6):1202-1211.

KEMI (Swedish Chemicals Agency). (2010 Capacity Building for sound management of Chemicals. Organisation, responsibilities and tasks of governmental institutions and enterprises. Report No. 510 961, PM 1/10.

Kortenkamp, A. (2007) Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environmental Health Perspectives.* 115 Suppl 1:98-105.

Kortenkamp, A., Backhaus, T. and Faust, M. (2009) State of the Art Report on Mixture Toxicity. Report for Directorate General for the Environment of the European Commission.

Kortenkamp, A., and Faust, M. (In press) Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. *International Journal of Andrology.*

Kortenkamp, A., Faust, M., Scholze, M., and Backhaus, T. (2007) Low-level exposure to multiple chemicals: reason for human health concerns? *Environmental Health Perspectives.* 115 Suppl 1:106-114.

Laetz, C.A., Baldwin, D.H., Collier, T.K., Hebert, V., Stark, J.D. and Scholz, N.L. (2008) The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon. *Environmental Health Perspectives*. 117(3):348-353.

Lau, K., Mclean, W.G., Williams, D.P. and Howard, C.V. (2006) Synergistic interactions between commonly used food additives in a developmental neurotoxicity test. *Toxicological Sciences*. 90:178-187.

Menzie, C.A., MacDonell, M.M. and Mumtaz, M. (2007) A phased approach for assessing combined effects from multiple stressors. *Environmental Health Perspectives*. 115:807-816.

Moore, D.R.J. and Caux, P.Y. (1997) Estimating low toxic effects *Environmental Toxicology and Chemistry*. 16:794-801.

OSPAR Commission (2005) Whole Effluent Assessment. Report.

Panel on Plant Protection Products and their Residues(PPR). (2008) 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) 396/2005 *The EFSA Journal*. 704:1-85.

Pohl, H.R., Mumtaz, M.M., Scinicariello, F. and Hansen, H. (2009) Binary weight-of-evidence evaluations of chemical interactions-15 years of experience *Regulatory Toxicology and Pharmacology*. 54:264-271.

Rajapakse, N., Silva, E., and Kortenkamp, A. (2002) Combining xenoestrogens at levels below individual no- observed- effect concentration dramatically enhances steroid hormone action *Environmental Health Perspectives*. 110:917-921.

Silva, E., Rajapakse, N., and Kortenkamp, A. (2002) Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environmental Science and Technology.* 36:1751-1756.

Sumpter, J.P, Johnson, A.C., Williams, R.J., Kortenkamp, A and Scholze, M. Modeling effects of mixtures of endocrine disrupting chemicals at the river catchment scale. *Environmental Science and Technology.* 40 (17):5478-5489, 2006.

Tønning K., Jacobsen E., Pedersen E., Strange M., Poulsen P.B., Møller L., and Boyd H.B. (2009) Survey and Health Assessment of the exposure of 2 year-olds to chemical substances in Consumer Products. Survey of Chemical Substances in Consumer Products, No. 102, Environmental Protection Agency, Danish Ministry of the Environment.

US Department of Health and Human Services. (2001) Guidance for the preparation of an interaction profile.

US Department of Health and Human Services. (2004) Guidance manual for the assessment of joint toxic action of chemical mixtures.

US EPA. (2002) Guidance on cumulative risk assessment of pesticide chemicals that have a common mechanism of toxicity.

US EPA. (2000) Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures.

US EPA. (2000) Guidance for Conducting Health Risk Assessment of Chemical Mixtures..

US EPA. (2007) Concepts, methods and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects: a resource document.

US EPA. (2008) Framework for application of the toxicity equivalence methodology for polychlorinated dioxins, furans, and biphenyls in ecological risk assessment.

US EPA (2003) Framework for cumulative risk assessment.

Van den Berg, M., Birnbaum, L., Bosveld, A.T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P. Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X. Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environmental Health Perspectives.* 106:775-792.

van der Hoeven,N. (1997). How to measure no effect.Part III:Statistical aspects of NOEC,EC_x and NEC estimates. *Environmetrics*. 8:255-261.

Verro, R., Finizio, A., Otto, S., and Vighi, M. (2009) Predicting Pesticide Environmental Risk in Intensive Agricultural Areas. II: Screening Level Risk Assessment of Complex Mixtures in Surface Waters. *Environmental Science and Technology*. 43:530-537.

Vighi, M., Altenburger, R., Arrhenius, Å., Backhaus, T., Boedeker, W., Blanck, H., Consolaro, F., Faust, M., Finizio, A., Froehner, K., Gramatica, P., Grimme, L. H. Grönvall, F., Hamer, V., Scholze, M., and Walter, H. (2003) Water quality objectives for mixtures of toxic chemicals: problems and perspectives. *Ecotoxicology and Environmental Safety*. 54:139-150.

Vighi, M. and Calamari, D. (1996) Quality objectives for aquatic life: the problem of mixtures of ' chemical substances. *Human and Ecological Risk Assessment*. 2:412-418.

Walter, H., Consolaro, F., Gramatica P., Scholze, M., and Altenburger, R. (2002) Mixture toxicity of priority pollutants at No Observed Effect Concentrations (NOECs). *Ecotoxicology* 11:299-310.

Appendix I: Glossary of Terms

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.

Additivity

Combined effect of agents expected from the effects of the individual substances. Expected effects are calculated using models that are based on either the concept of (->) Concentration Addition, (->) Independent Action, or (Effect Summation). Without explicit reference to such a concept the exact meaning of the term *additive* is unclear.

Binary Weight-of-Evidence (BINWOE)

Binary Weight-of-Evidence (BINWOE) determinations are a modification to the (->) Hazard Index (HI) approach. While the HI does not incorporate information on interactions among the components of a mixture, the BINWOE determinations are an attempt to systematically address this point. As detailed in the ATSDR Guidance Manual for the Assessment of Joint Toxic Action of Chemical Mixtures (ATSDR 2004) the method evaluates available data relevant to joint action for each possible pair of chemicals in a multicomponent. The aim is to make qualitative determinations for the effect of each chemical on the toxicity of every other chemical. "*Two BINWOEs are needed for each pair: one for the effect of chemical A on the toxicity of chemical B, and another for the effect of chemical B on the toxicity of chemical A. The BINWOE determination is a classification that indicates the expected direction of an interaction (greater than additive, less than additive, additive, or indeterminate), and scores the data qualitatively, using an alphanumeric scheme that takes into account mechanistic understanding, toxicological significance, and relevance of the exposure duration, sequence, bioassay (in vitro versus in vivo), and route of exposure. The alphanumeric terms in the classification scheme can then be converted to a single numerical score, by multiplying the corresponding direction factor by the data quality*" (ATSDR 2004).

Component Based Approach

Mixture toxicity studies and assessments that apply a *Component Based Approach* use estimates of the expectable total toxicity of a mixture that are calculated from available toxicological information for individual mixture components. To this end either the concept of (->) Concentration Addition, (->) Independent Action, or (Effect Summation) is applied, may be directly or by means of assessment methodologies that are based on one or more of these concepts, such as the (->) Hazard Index, the (->) Point of Departure Index, the (->) Relative Potency Factor, the (->) Toxic Equivalence Factor, or the (->) Toxic Unit Summation.

Concentration Addition (or Dose Addition) **(CA)**

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).

Cumulative Risk Assessment (CRA)

The Framework for Cumulative Risk assessment developed by the US EPA defines *cumulative risk assessment* (CRA) as an analysis, characterization, and possible quantification of the combined risks to human health or the environment from multiple agents or stressors. Unfortunately the term is not consistently used in this way throughout the literature and regulatory documents. In particular some pieces of European legislation use the terms cumulative risks or cumulative toxic effects to denote effects or risks 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. The US EPA in contrast uses the term *aggregate risks* to denote such risks that merely result from exposure to a single specific agent or stressor via different relevant routes, pathways, and sources, while cumulative risks means risks from aggregate exposure to multiple agents or stressors.

Effect Summation (ES)

Effect Summation (ES) is a concept for combination effects that is based on the simple idea that the effect of a mixture should equal the sum of the effects caused by the individual mixture constituents if applied singly. The idea is intuitively appealing but toxicologically ill justified as explained in detail in Appendix II.

Hazard Index (HI)

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

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

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.

Independent Action (also called Response Addition) **(IA)**

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

$$E(c_{mix}) = E(c_1) + E(c_2) \quad E(c_1) \bullet E(c_2) \quad ,$$

which can be extended to any number of mixture components, giving '

$$E(c_{mix}) = 1 \quad \prod_{i=1}^{n} \left(1 \quad E(c_i)\right) \quad ,$$

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.

Interaction / Non-Interaction

Interaction is used as an umbrella term for (->) synergistic (greater than expected) and (->) antagonistic mixture effects (smaller than expected). The case of *Non-interaction* is usually described by the (->) additivity expectation of a suitable prediction concept (->) (Concentration Addition, Independent Action or Effect Summation). Interactions may occur when the compounds in a mixture interfere with each others toxicokinetics, e.g. by facilitating or diminishing each others uptake, transport, metabolism or excretion.

Isoboles

Plotting of quantitative effects of two or *n* substances combined in various proportions against their individual concentrations leads to the presentation of the concentration-response relationships as a concentration-response surface in a three- or (*n*+1)-dimensional space. Lines on such a surface that connect all points where the total concentrations of mixture components combined in different proportions result in the same quantitative response are called *Isoboles.* Examples are given in Fig. 5 of this report.

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 mixtures of unknown or not completely known composition, such as samples from environmental media or biota. Some authors also use the term *complex mixture* to describe any combination of a relatively large number of chemicals (e.g. more than three or more than ten), but for the purposes of this report, the term *multi-component mixture* is preferred.

Mixture Effect / Combination Effect / Joint Effect

These terms are used synonymously to describe the response of a biological system to several chemicals after simultaneous exposure.

Point of Departure Index (PODI)

The Point of Departure Index (PODI) is an approach to component-based mixture risk assessment which is similar to the (->) Hazard Index (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):

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

Relative Potency Factor (RPF)

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 (US 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

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

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

Synergistic / Antagonistic Mixture Toxicity

Terms denoting adverse effects of mixtures that are greater or less than expected on the basis of an appropriate mixture toxicity concept such as (->) Concentration Addition or (->) Independent Action. Without explicit reference to such a concept the exact meaning of the terms is unclear.

Toxic Equivalence Factor (TEF)

The Toxic Equivalence Factor (TEF) is a specific type of (->) Relative Potency Factor (RPF) formed through a scientific consensus procedure (US 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:

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

Toxic Unit Summation (TUS)

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

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

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 res-

cale 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.

Whole Mixture Approach

Mixture toxicity studies that use a *Whole Mixture Approach* are based on the direct toxicity testing of a given chemical mixture, such as a sample from wastewater treatment plant effluent for instance. Whole mixture approaches closely resemble the experimental assessment of individual chemicals.

References

ATSDR (Agency for Toxic Substances and Disease) (2004) Guidance Manual for theAssessment of Joint Toxic Action of Chemical Mixtures. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Division of Toxicology, May 2004.

Berenbaum, M.C. (1985) The expected effect of a combination of agents: the general solution. *J. Theor. Biol.* 114:413-431.

Bliss, C.I. (1939) The toxicity of poisons applied jointly. *Ann. Appl. Biol.* 26:585-615.

Loewe, S. and Muischnek, H. (1926) Über Kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* 114:313-326.

Sprague, J.B. (1970) Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Water Res.* 4:3-32

Teuschler, L.K. and Hertzberg, R.C. (1995) Current and future risk assessment guidelines, policy and methods development for chemical mixtures. *Toxicology* 105:137-144.

US EPA (United States Environmental Protection Agency) (2000) Supplementary guidance for conducting health risk assessment of chemical mixtures. Risk Assessment Forum, U.S. Environmental Protection Agency, Washington, DC 20460, August 2000.

US EPA (United States Environmental Protection Agency) (2003). Framework for Cumulative Risk Assessment. U.S. Environmental Protection, Agency, Office of Research and Development, National Center for Environmental Assessment, Washington Office, Washington, DC, EPA/600/P-02/001F, 01 January 2003.

Wilkinson, C.F., Christoph, G.R., Julien, E., Kelley, J.M., Kronenberg, J., McCarthy, J. and Reiss, R. (2000) Assessing the risks of exposures to multiple chemicals with a common mechanism of toxicity: How to cumulate? *Regul. Toxicol. Pharmacol.* 31:30-43.

Apendix II: Background on component based approaches (Concentration Addition, Independent Action and Effect Summation)

The assessment of joint effects of chemicals has therefore become an increasingly important topic for scientific research as well as chemical regulation and several reports, conceptual outlines and guidance manuals have been published by various committees and working groups, from national authorities, academia, international bodies as well as industry (see Appendix 1). The work on combination effects of chemicals has a long standing in experimental sciences, where three major areas of research can be identified: (i) research on the molecular mechanisms of action and interactions of the mixture components (e.g. (Bernhagen et al. 2007)), (ii) the optimization of products (e.g. (Dutcosky et al. 2006)) and (iii) the hazard and risk assessment of chemicals. A diverse set of approaches has been developed for these different aims, which can be grouped into 2 major classes: whole mixture approaches and component based approaches.

Whole mixture approaches and reasoning from data for similar mixtures

Whole mixture approaches, i.e. the direct toxicological or ecotoxicological assessment of a given chemical mixture closely resembles the assessment of individual chemicals and therefore does not require mixture-specific methodologies. This approach is therefore obviously appealing, but also has several limitations: First of all, the mixture itself has to be available for a direct experimentation, which makes this approach largely unusable for prospective approaches such as the setting of environmental or human health standards. And secondly, the obtained results are strictly speaking only applicable to the actually investigated mixture. As the actual exposure situation is often highly dynamic, frequent re-testing might be required. A major application of this approach is whole effluent testing (WET), e.g. (OSPAR Commission 2005) and the classification and labeling of chemical products (EU Parliament and the Council 2008).

A closely related approach is to draw conclusions from documented analyses of similar mixtures performed previously. For example, the US Environmental Protection Agency uses this methodology for estimating the risk for different combustion processes (Teuschler and Hertzberg 1995). Employing this approach requires reliable data for a mixture 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.

In view of these limitations of whole mixture approaches, several methods have been developed that are based on drawing conclusions on mixture toxicities from a known or assumed chemical composition and knowledge on the toxicities of its individual components. They are often summarized as "component-based approaches" and numerous methods and approaches for this purpose have been described in the literature.

Component-based approaches

Inferences from the effects of the mixture components to their joint action can be drawn in a purely empirical manner, i.e. by simply testing all possible combinations (mixture ratios and concentrations) of the components and then interpolating between the tested concentrations if necessary, see overview in e.g. (Cornell 2002) . 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, while the hyperplane of multi-component mixtures is beyond simple visualization.

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. Additionally, all conclusions are restricted to a given set of components. Both issues seriously hamper the applicability of such purely empirical approaches for environmental issues where organisms are typically exposed to multi-component mixtures with often changing compositions. However, a conceptually sound link between the individual components and the effects of the mixture would allow predictions of mixture toxicities and hence to draw more general conclusions.

The most advanced method for developing a biologically justified link from the toxicology of individual compounds to the toxicology of a chemical mixture uses physiologically based pharmacokinetic/pharmakodynamic modelling (PBPK/PD) modelling. As the name implies, this approach strives to model the uptake and distribution of the mixture components in an organism. PBPK/PD models are hence highly specific for a particular animal and require detailed knowledge on its physiology, such as for example the exposed skin surface or alveolar ventilation rates. Also specific data on the involved mixture components are needed, such as e.g. their blood/air, blood/tissue partition coefficients and metabolic rate constants. Krishnan for example lists some 45 parameters that build up these models (Krishnan et al. 1994). In view of these huge knowledge demands, this approach has been restricted to toxicological studies with a few selected animal test systems and mixtures with only a few compounds, e.g. (Haddad and Krishnan 1998; Krishnan et al. 1994; Verhaar et al. 1997). Teuschler et al. discuss this approach for the assessment of combinations of disinfection byproducts (Teuschler et al. 2004) and they have also been put forward for the cumulative risk assessment of specific groups of pesticides (Conolly et al. 2005; Lowit et al. 2004).

The classical mixture toxicity concepts of Concentration Addition and Independent Action

Three different fundamental concepts, which will be termed Effect Summation (ES), Concentration Addition (CA) and Independent Action (IA) in the following text, are established in the literature. They take an intermediate position between purely empirical approaches and very detailed PBPK/PD models. It should be pointed out that these concepts can also be found under various other names (Altenburger et al. 1993; Berenbaum 1989; Greco et al. 1992; Greco et al. 1995; Hertzberg and MacDonell 2002; Hertzberg and Teuschler 2002; Hewlett and Plackett 1979; Kodell and Pounds 1991; Könemann and Pieters 1996; Pöch 1993) and are implemented in a diverse set of models for predicting or assessing mixture toxicities, see compilations in(Berenbaum 1989; Boedeker et al. 1990; Groten et al. 2001; Kodell and Pounds 1991).

ES is based on the intuitive notion that the effect E of a mixture at a given concentration c_{Mix} simply equals the arithmetic sum of the effects that the components will provoke if present singly at the concentration at which they are present in the mixture, c_i:

$$E(c_{Mix}) = \sum_{i=1}^{n} E(c_i) \qquad \text{(eq 1)}$$

However, as has been repeatedly pointed out, ES lacks a sound pharmacological basis (Berenbaum 1985; Boedeker et al. 1992; Boedeker et al. 1993; Greco et al. 1995). In particular, ES predicts more-than-expected or less-than-expected mixture effects even in the simple case of a so-called "sham combination", a combination of a compound with itself. This shall be illustrated on the concentration-response curve (from (Christiansen et al. 2009) that describes the developmental effects of Finasteride in rats. Finasteride synthetic anti-androgen, used for the treatment of prostrate cancer. As can be seen from the plot in figure 1, 0.046 mg/kg/day Finasteride cause 5% relative effect. According to ES, a 4-component sham combination in which each "component" is present at 0.046 mg/kg/day would cause 5 + 5 + 5 + 5 20% effect, assuming that all components in the sham combination are in fact Finasteride. Or in other words: according to ES 0.046 + 0.046 + 0.046 + 0.046 = 0.183 mg/kg/day Finasteride should cause 20% effect. However, as it can be seen from the concentration-response function in figure 1, in fact 8.27 mg/kg/day Finasteride are needed to give rise to 20% effect. That is, the application of ES to the sham combination leads to a clear overestimation of the actual effect of the "mixture", a phenomenon which is caused by the non-linear form of the concentration-response curve. Another obvious shortcoming of ES is the fact that it would predict 200% relative effect for a 20-compound mixture if each component is present at a concentration provoking 10% single substance effect (EC10).

CA, on the other hand, is thought to adequately describe the joint action of compounds in a sham combination. The concept takes the general mathematical form of:

$$\frac{c_{mix}}{ECx_{mix}} = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \qquad \text{(eq 2)}$$

with c_{mix} being the total concentration of the mixture and c_i denoting the concentrations of each individual compound in the mixture. ECx_{Mix} and ECx_i are the mixture and single substance concentrations, respectively, that provoke an effect of *x%*.

Each concentration c_i in equation 2 can also be expressed as a fraction of the corresponding ECx_i, in which case all fractions in equation 2 take the form of dimensionless so-called "toxic units" (TUs). If it is assumed for the sake of argument that each of the four "components" in the Finasteride sham combination is present at just $1/4^{th}$ of its individual EC50 then each component adds with a TU of 0.25 to the mixture, and the sum would just be 1 TU, i.e. the EC50 of the "mixture".

Alternatively, CA can be formulated as:

$$ECx_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad \text{(eq 3)}$$

From a mathematical perspective CA hence simply represents the weighted harmonic mean of the individual *ECx*-values, with the weights just being the fractions p_i of the mixture components.

According to CA the individual mixture components behave as if they were dilutions of each other – they only differ in the relative potency and amounts in the mixture. Due to this conceptual idea, CA is thought to describe the joint action of components that have a similar mode or

mechanism of action and forms the conceptual basis of the TEF approaches used for the hazard and risk assessment of e.g. dioxins (US EPA 2008; Van den Berg et al. 1998).

By activating different molecular effector chains, each component in a mixture of dissimilarly acting chemicals can be thought to contribute to a common biological endpoint, completely independent of the other simultaneously present agents. The resulting combined effect can then be calculated from the effects caused by the individual mixture components by the statistical concept of independent random events (Bliss 1939) and the expected combination effect can be mathematically expressed as:

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

for the situation that the effect increases with increasing concentrations (e.g. when mortality is the biological endpoint under observation), or

$$E(c_{Mix}) = \prod_{i=1}^{n} [E(c_i)] \quad \text{(eq. 4)}$$

when the effect decreases with increasing concentrations (e.g. when survival rates are studied).

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 strength and weakness of the concepts. On the one hand, this simplicity allows establishing broader 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 biologically very simple systems. Hence, with an 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 when applying either concept is therefore not 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 aim.

Input requirements

The fundamental characteristics and assumptions of both concepts are summarized in figure 2. Both concepts rely on quantitative input data, either in terms of effects or effect concentrations. It follows, that biological variation, technical variability and reproducibility play a crucial role when designing experiments for putting the concepts to the test. Both concepts are also only applicable to mixtures of known chemical composition. However, CA and IA operate on fundamentally different levels: IA uses single substance effects, $E(c_i)$, for predicting a mixture effect, while CA is based on effect concentrations (*ECx*-values) of each compounds and predicts on this basis the concentration of the mixture that would give rise to the same effect. *ECx*-values are the result of a concentration-response analysis and hence concentration-response curves for all mixture components need to have been previously recorded in order to apply CA. Such curves also allow in principle to calculate individual $E(c_i)$-values and therefore also provide the necessary input data for an application of IA. But in contrast to CA, IA is not relying on concentration-response curves. It can also make use of single experimentally observed effect values as input

data (e.g. (Laetz et al. 2008)), although the variability of those individual effect values has to be critically assessed.

As both concepts make use of toxicity data for 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 that is needed per 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 binary or a 50 component mixture is analyzed. 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. IA predicts for example that 30% effect of each individual component leads to 50% combination effect in a 2-compound mixture. In a 10-compound mixture each component needs to be present only at a concentration that would give 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(ci)*-values for each component are needed for actually calculating IA-predictions is a serious drawback of IA, as this tremendously increases experimental demands.

Toxicological and ecotoxicological studies often report their findings in terms of EC50 values or NOECs (No Observed Effect Concentration). NOECs do not provide suitable input data for the application of either concept. This becomes apparent when reviewing the mathematical formulations of the concepts in equations 1 and 3. NOECs neither represent effect concentrations (*ECx* values as required for CA) nor effect levels (*E(ci)* as required by IA). 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 NOECs in order to predict a mixture NOEC, but this would imply that all NOECs provoke the same, insignificant effect, i.e. that all individual values have been determined in an identical experimental setup (in terms of replicates, spacing of test concentrations, variance structure), which is hardly ever the case. Only when supported by additional information, such as data on the underlying concentration-response relationships, NOECs can be used in the context of either concept (Backhaus et al. 1999; Faust et al. 2001; Faust et al. 2003; Rajapakse et al. 2002; Silva et al. 2002; Walter et al. 2002). Nevertheless, a range of methods such as TEFs (Toxicity Equivalency Factors) or the Hazard Index for mixtures make use of a CA-like approach and sum up NOEC-based hazard quotients (US EPA ; US EPA 2000). This introduces an additional source of uncertainty in the assessment, which is fundamentally different from the question on whether CA is an appropriate concept for the mixture of interest.

EC50 values are of only limited use for the application of IA in the context of multi-component mixtures, as they only allow to predict mixture effects of 75% (2-component mixtures) and higher (multi-component mixtures), which usually is of no practical relevance. CA on the other hand can make use of those values for assessing how the total concentration in an exposure scenario relates to its anticipated EC50: is it above or below and how far away is the CA predicted EC50 from the concentration found in the scenario of interest? This allows for the possibility to use assessment factors analogous to standard single substance assessments. Without any further knowledge on the individual concentration-response curves, no statement on the expected mixture effect can be made though.

Due to the probabilistic background of 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 et al. 2004). It also requires suitable controls in the experiments. For endpoints that are naturally confined, such as mortality, negative controls suffice. For other endpoints appropriate positive controls might also be necessary (see e.g. the vitellogenin induction studies in (Brian et al. 2005; Brian et al. 2007)). Although such a re-scaling is not strictly required for the application of CA, it 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 the biometrical data analysis is to handle every new set of experimental data independently, e.g. by following the so-called "best-fit" approach by (Scholze et al. 2001)in which a whole set of different concentration-response models is applied to each data set in order to maximize the quality of the concentration-response fits. 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.

Empirical Evidence on the predictive power of CA and IA

CA and IA are both inherently simple concepts. A substantial effort has therefore been devoted in toxicology as well as ecotoxicology to the question as to what extent they are able to predict actual “real world“ mixture effects. Experimental studies in particular analyzed the predictive power of the concepts in relation to the type(s) of chemicals in the mixture and the employed biotest (test organism, exposure conditions and biological endpoint), see reviews in (Backhaus et al. 2008; Belden et al. 2007; Cassee et al. 1998; Committee on Toxicity of Chemicals in Food 2002; Deneer 2000; ECETOC 2001; Kortenkamp 2007b; McCarty and Borgert 2006; Norwegian Scientific Committee for Food Safety 2008; Norwood et al. 2003; Warne 2003). The majority of documented studies has focused on mixtures of only two compounds and evaluated the observed mixture toxicity in relation to the predictions provided by CA. Although in some studies statistically significant differences between CA-predicted and observed mixture toxicities were observed, the overall pattern shows a surprisingly high predictive power of CA with the ratio between predicted and observed mixture EC50 being far below a factor of 5 in the vast majority of studies. This pattern does not seem to be restricted to a particular biological system, biological endpoint, exposure situation or class of chemicals. For example, Belden and his coworkers recently re-analyzed for example the predictive power of CA for 207 published studies on pesticide mixtures (Belden et al. 2007). The average ratio between CA-predicted and observed EC50 values (the MDR) was 1, with only 5% of the analyzed experiments having an MDR>2 and 5% having an MDR<0.5. Several other reviews came to similar conclusions (Backhaus et al. 2008; Cedergreen et al. 2008; Kortenkamp 2007b; Warne 2003). It should be pointed out here, that for binary mixtures, CA often provides a good prediction even for mixtures of dissimilarly acting compounds (e.g. (Cedergreen et al. 2008; Faust et al. 1994)), which might be due to the fundamental quantitative relationship between both predictions (Drescher and Boedeker 1995): the theoretically possible difference between the predicted EC50 values by CA and IA is exactly n,

the number of mixture components (Faust et al. 1996; Junghans et al. 2006). That means that for a binary mixture the maximum theoretically possible difference of the predicted EC50 values is 2 and in practice the difference is even much smaller. Hence for binary mixtures the EC50 values that are predicted by CA and IA are often experimentally indistinguishable.

Studies with multi-component mixtures are rarer than experiments with binary mixtures, but they also reveal a very stable pattern: strong deviations from the CA-predicted mixture toxicity are extremely rare for mixtures of similarly acting compounds, usually the predictive power of CA is extremely good (Altenburger et al. 2000; Backhaus et al. 1999; Faust et al. 2001; Rajapakse et al. 2002; Silva et al. 2002). The recent study by Chistiansen and coworkers on the developmental toxicity of anti-androgens in the rate provides one of the few examples for clearly more than concentration-additive mixture effects (Christiansen et al. 2009), and even in this study the toxicity of the mixture with respect to the most sensitive endpoint was very well predicted by CA.

In comparison, the evidence on the performance of IA is considerably smaller than our knowledge on the predictive power of CA, which holds especially true for multi-component mixtures. In particular, only three studies seems to have been published in which the mixture toxicity of more than 10 structurally dissimilar and/or dissimilarly acting compounds was compared to the predictions by IA (Backhaus et al. 2000; Faust et al. 2003; Walter et al. 2002) and due to the large required experimental effort those studies employed simple unicellular microorganisms. Already in 1985 Hermens et al published a study on the fish toxicity of 33 aquatic pollutants from 3 assumed groups with different modes of action (Hermens et al. 1985). However, the experimental results were only compared to the mixture toxicity expectation according to CA.

The contribution of low-effect concentrations to the toxicity of a chemical mixture

The question, whether and to what extent ecological or human health oriented thresholds for chemical exposure hold even if an exposure towards chemical mixtures is taken into consideration has sparked considerable interest from regulatory authorities as well as the scientific community, especially because CA and IA come to opposite conclusions on whether even concentrations below individual thresholds might contribute to the overall toxicity of a mixture.

As discussed above, CA builds on the idea of a sham combination, i.e. that the mixture components act as if they were actually one and the same compound at different dilutions (potencies). For components that behave according to CA it hence does not matter, whether the individual components are present below their individual thresholds. Or in other words: it does not matter for the overall toxicity whether only one compound is present at a concentration c – or whether 100 compounds are present, each at a concentration of $c/100$. According to CA all components hence contribute to the toxicity of the mixture in proportion to their individual potency and concentration, see also the discussion in (Kortenkamp 2007a). This is also apparent in the mathematical representation of CA (eq. 2): as long as a component is present in the mixture (i.e. $c_i > 0$), it contributes to the total toxicity of the mixture. This theoretically expected contribution of even low, individually not significantly effective concentrations to the toxicity of a mixture has been repeatedly demonstrated in experimental mixture studies from toxicology as well as ecotoxicology (Altenburger and Greco 2009; Arrhenius et al. 2004; Backhaus et al. 1999; Brian et al. 2005; Crofton et al. 2005; Faust et al. 2001; Hass et al. 2007; Jonker et al. 1996; Rajapakse et al. 2002; Silva et al. 2002; Walter et al. 2002), see also discussion in (Kortenkamp et al. 2007).

In contrast, IA assumes that only those compounds contribute to the toxicity of a mixture that are present in concentrations at which they are toxic if applied singly, i.e. that are present above their

individual thresholds ($E(c_i) > 0$, eqns. 3 and 4). It has therefore repeatedly been argued that "as a rule, exposure to mixtures of chemicals at (low) non-toxic doses of the individual constituents is of no health concern" (Cassee et al. 1998), see also (Feron and Groten 2002; Groten 2000) and the rebuttal by (Kortenkamp et al. 2007).

Threshold values are typically based on experimental No Observed Effect Concentrations, NOECs, which denote the highest test concentration in which the observed response of the test organisms was not significantly different from the untreated controls. NOECs have been heavily criticized for various reasons (Allen et al. 1994; Chapman and Chapman 1997; Chapman et al. 1996; Dhaliwal et al. 1997; Hoekstra and Ewijk 1993; van der Hoeven 1997), and in the context of mixture studies, the most important shortcoming of NOEC based approaches might be that those values 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, NOECs do not describe a "safe" concentration and differ fundamentally from true No Effect Concentrations, although they are frequently equated as such.

Because NOECs might correspond to effects as high as 5%-20% (Allen et al. 1994) or even 30% (Moore and Caux 1997) IA-compliant mixture effects cannot be ruled out, even if all components are present only at their individual NOECs. If only certain fractions of the individual NOECs are 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 a mixture effect might occur. In fact it has been demonstrated that concentrations of strictly dissimilarly acting substances contribute even if present at only below their individual EC1 to the toxicity of a mixture that behaves according to IA (Backhaus et al. 2008; Faust et al. 2001). A further, in-depth discussion of the available empirical evidence on low-dose effects of chemical mixtures and their implications for ecological and human health assessment is provided by Kortenkamp and coworkers (Kortenkamp et al. 2007).

The assessment of cumulative chemical exposure in the context of epidemiology and eco-epidemiology

Epidemiology has a long tradition in describing the human health effects of complex mixtures, such as for example tobacco smoke, e.g. (Alberg and Samet 2003; Glantz and Parmley 1991; Law and Hackshaw 1996), other types of air pollution e.g. (Hajat et al. 2007; Peden 2005; Pope III and Burnett 2007; Simkhovich et al. 2008), or mixtures of endocrine disrupting chemicals, e.g. (Fernandez et al. 2007). Those mixtures are most often studied as if they were single stressors and studies often focus on the description of vulnerable populations and effect sizes. However, in concordance with the available evidence from toxicology and ecotoxicology there are indications that the epidemiological effects or risks of individual compounds are adding up to a combined effect that is bigger than each of the individual effects (Damgaard et al. 2006; Hauser et al. 2003; Swan et al. 2005). Hence, in order to inform the risk assessment of complex exposure situations, information is needed on the causative agents, their relative contribution to the total risk and whether and how the different stressors interact. Only such knowledge can provide management options with a view to reduce or prevent harm from a complex exposure situation or to remedy existing problems.

Despite its lack of a sound pharmacological foundation, the dominant null-hypothesis for the joint action of stressors in epidemiological studies still seems to be the simple summation of individual risks, i.e. effect summation (ES, eq. 1), although CA and IA have been repeatedly put

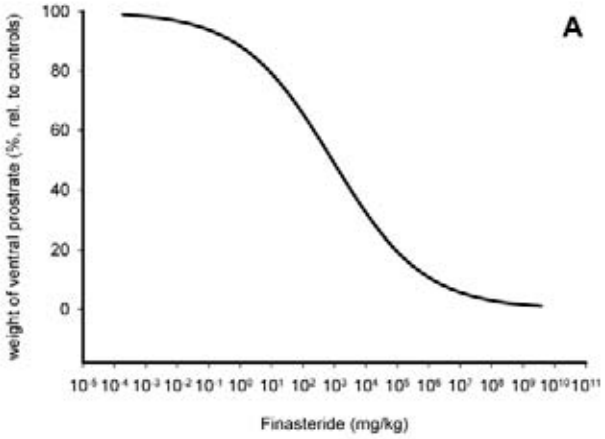
forwards as appropriate tools for human health risk assessment of exposures to chemical mixtures (US EPA ; US EPA 1986; US EPA 2000; US EPA 2007). CA and IA have also been successfully applied in a large-scale eco-epidemiology study in the Ohio river basin where they were instrumental in exploring the factors that are responsible for shaping fish populations at polluted and non-polluted sites (De Zwart et al. 2006; Posthuma and De Zwart 2006). An additional series of ecotoxicological studies was published by Grote and coworkers, in which CA and IA were successfully applied for identifying the roles and contributions of individual toxicants in complex chemical mixtures found in sediments (Grote et al. 2005a; Grote et al. 2005b; Grote et al. 2005c). The CONTAMED R D project, which is funded within the 7th European Framework Programme, uses a combination of epidemiological approaches and exposure assessments in human cohorts with tailored experimental animal studies to investigate whether and to what extent observed developmental effects in human populations can be traced back to a cumulative exposures to endocrine disrupter mixtures. CA and IA are applied for modeling expected mixture effects in the animal model and for then developing future policy options (www.contamed.eu, accessed 19. Nov. 2009).

It should be emphasized that the principal methodologies and concepts for the assessment of the consequences of combined exposures are almost identical for human health and ecological risk assessment, although the protection goals in both fields differ. Ecological assessment can also often rely on a more extensive body of empirical evidence and it has therefore been argued that cumulative human health risk assessment should "draw greater insights from ecological risk assessment" (National Academy of Sciences 2009). Accordingly, Menzie and his coworkers recently published a conceptual outline of a tiered approach for the assessment of combined effects from multiple stressors which covers both, ecological as well as human health related assessments and which even goes beyond the consideration of chemical stressors only (Menzie et al. 2007). A recent compilation of methodologies and data sources for the human health assessment of cumulative exposures has also been published by the US EPA (US EPA 2007).

Figure 1: The sham combination though experiment and the inadequacy of Effect Summation

A: Concentration-response curve of Finasteride in rat (endpoint: prostrate weight). From (Christiansen et al. 2009).

B: Inset of Figure A.Indicated are the concentration of Finasteride resulting in 5% relative effect (0.046 mg/kg), the concentration resulting in 20% effect (8.287 mg/kg) and the concentration that should give 20% in the "sham-combination" thought-experiment according to Effect Summation (0.183 mg/kg). The dotted line gives the concentration-response curve according to Effect Summation. For details see text.



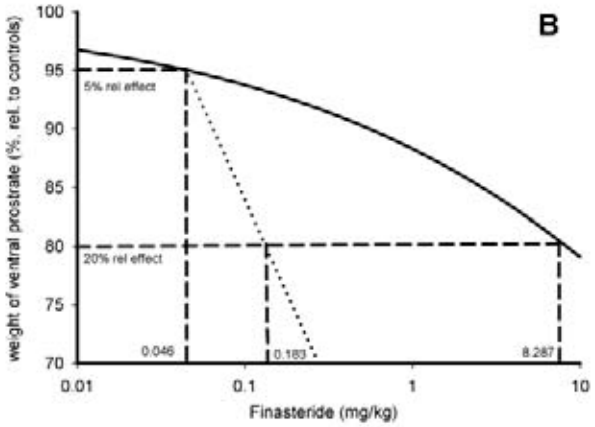


Figure 2: Fundamental properties of Concentration Addition and Independent Action

A. Concentration Addition

$$ECx_{Mix} = \frac{c_{Mix}}{\sum_{i=1}^{n} \frac{c_i}{ECx_i}} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1}$$

Legend

n: number of mixture components

c_i : the concentration (or dose) of the i-th component in a n-compound mixture

ECx_i: concentration or dose of the i-th component that provokes x % effect if applied singly

ECx_{Mix}: concentration of the total mixture that provokes x % effect

Conceptual idea

§ The components only differ with respect to their potency, i.e., they behave as if they were dilutions of one another.

§ Pharmacological interpretation: all components share the same mechanism of action, i.e. they compete for the same receptor site and hence all affect the same biological endpoint

Input requirements

§ concentrations or relative fractions of all mixture components, i.e. 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

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 are considered.

§ All of them contribute to the toxicity of the mixture, i.e. individual threshold are of no importance.

§ The number of components in the mixture does not influence the input requirements. Especially, the considered effect level x is only dependend on the mixture ECx that is to be calculated, not on the number of mixture components.

§ The bias in the CA-calculated mixture toxicity never exceeds the maximum bias that is present in a single substance ECx-value

§ NOECs are unsuited as input data

§ Calculated ECx values of the mixture always fall into the span between the lowest and highest single substance ECx value

§ CA allows the direct calculation of the mixture EC*x*-concentration (i.e. a concentration that is assumed to provoke a predefined effect *x*), but for the calculation of an effect that is expected to occur from a given mixture concentration, the CA-equation has to be solved iteratively.

B. Independent Action (Response Addition)

$$E(c_{Mix}) = 1 \prod_{i=1'}^{n} [1 - E(c_i)]$$

Legend

n: number of mixture components

$E(c_i)$: the effect that the *i*-th component would provoke, if applied singly at a concentration c_i

$E(c_{Mix})$: the effect that the total mixture at a concentration $c_{Mix} = \sum_{i=1}^{n} c_i$ provokes

Conceptual idea

§ All components have a unique mechanism of action, i.e. they contribute independently to a common effect (biological endpoint)

Input requirements

§ Knowledge on the qualitative as well as quantitative mixture composition

§ Relative effects 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 defined composition

§ No specific assumptions on the biotest are needed, nor considered by the concept

§ Prediction is limited to 0-100% relative effect

§ The calculated mixture effect is always higher than the highest single substance effect, i.e. $E(c_{mix}) > \max(E(c_1),E(c_2),\ldots,E(c_n))$

§ The more mixture components, the lower the single substance effects that are required for the calculation of a given mixture effect. The following equation holds for a n-compound mixture: $E(c_{\min}) \quad 1 - \sqrt[n]{1 - E_{Mix}}$ where $E(c_{min}) = \min(E(c_1),E(c_2),\ldots,E(c_n))$, i.e. the minimum of the required input single substance effect values.

§ Only components that are also effective if applied singly have an impact on the toxicity of the mixture.

§ Only those compounds contribute to the mixture toxicity that are present in concentrations that would also provoke an effect if applied singly, i.e. that are present in a concentration above their biological threshold values.

§ NOECs are unsuited as input data

§ Calculation of mixture NOEC is not possible

§ Direct calculation of the effect that is expected to occur from a given mixture concentration is possible. For the calculation of a mixture EC*x* (i.e. a concentration that is assumed to provoke a predefined effect *x*), iterative procedures have to be used.

References

Alberg,A.J. and J.M.Samet. 2003. "Epidemiology of lung cancer." *Chest*. 123:21S-49S.

Allen,B.C., R.J.Kavlock, C.A.Kimmel, and E.M.Faustman. 1994. "Dose-response assessment for developmental toxicity II. Comparison of generic benchmark dose estimates with no observed adverse effect levels." *Fundamental and Applied Toxicology*. 23:487-495.

Altenburger,R., T.Backhaus, W.Boedeker, M.Faust, M.Scholze, and L.H.Grimme. 2000. "Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting compounds." *Environmental Toxicology and Chemistry*. 19:2341-2347.

Altenburger,R., W.Boedeker, M.Faust, and L.H.Grimme. 1993. "Aquatic toxicology, Analysis of Combination effects." In M.Corn, editor, *Handbook of hazardous materials*. Academic Press. San Diego. 15-27.

Altenburger,R. and W.R.Greco. 2009. "Extrapolation Concepts for Dealing with Multiple Contamination in Environmental Risk Assessment." *Integrated Environmental Assessment and Management*. 5:62-68.

Arrhenius,A., F.Grönvall, M.Scholze, T.Backhaus, and H.Blanck. 2004. "Predictability of mixture toxicity of 12 similar-acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities." *Aquatic Toxicology*. **68**:351-367.

Backhaus,T., R.Altenburger, W.Boedeker, M.Faust, M.Scholze, and L.H.Grimme. 2000. "Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri." *Environmental Toxicology and Chemistry*. 19:2348-2356.

Backhaus,T., A.Arrhenius, and H.Blanck. 2004. "Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition." *Environ.Sci.Technol.* 38:6363-6370.

Backhaus,T., M.Scholze, and L.H.Grimme. 1999. "The single substance and mixture toxicity of quinolones to the bioluminescent bacterium Vibrio fischeri." *Aquatic Toxicology*. 49:49-61.

Backhaus,T., J.P.Sumpter, and H.Blanck. 2008. "On the ecotoxicology of pharmaceutical mixtures." In K.Kümmerer, editor, *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Springer.

Belden,J.B., R.J.Gilliom, and M.J.Lydy. 2007. "How well can we predict the toxicity of pesticide mixtures to aquatic life?" *Integr.Environ.Assess.Manag.* 3:364-372.

Berenbaum,M.C. 1985. "The expected effect of a combination of agents: the general solution." *Journal of Theoretical Biology*. 114:413-431.

Berenbaum,M.C. 1989. "What is synergy?" *Pharmacological Reviews*. 1989:93-141.

Bernhagen,J., R.Krohn, H.Lue, J.L.Gregory, A.Zernecke, R.R.Koenen, M.Dewor, I.Georgiev, A.Schober, L.Leng, T.Kooistra, G.Fingerle-Rowson, P.Ghezzi, R.Kleemann, S.R.McColl, R.Bucala, M.J.Hickey, and C.Weber. 2007. "MIF is a noncognate ligand of

CXC chemokine receptors in inflammatory and atherogenic cell recruitment." *Nat.Med.* 13:587-596.

Bliss,C.I. 1939. "The toxicity of poisons applied jointly." *Annual Journal of Applied Biology*. 26:585-615.

Boedeker,W., R.Altenburger, M.Faust, and L.H.Grimme. 1990. "Methods for the assessment of mixtures of plant protection substances (pesticides): Mathematical analysis of combination effects in phytopharmacology and ecotoxicology." *Nachrichtenblatt des deutschen Pflanzenschutzes*. 42:70-78.

Boedeker,W., R.Altenburger, M.Faust, and L.H.Grimme. 1992. "Synopsis of concepts and models for the quantitative analysis of combination effects: from biometrcis to ecotoxicology." *Archives of Complex Environmental Studies*. 4:45-53.

Boedeker,W., K.Drescher, R.Altenburger, M.Faust, and L.H.Grimme. 1993. "Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology." *The Science of the Total Environment*. Supplement:931-938.

Brian,J.V., C.A.Harris, M.Scholze, T.Backhaus, P.Booy, M.Lamoree, G.Pojana, N.Jonkers, T.Runnalls, A.Bonfa, A.Marcomini, and J.P.Sumpter. 2005. "Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals." *Environ.Health Perspect.* 113:721-728.

Brian,J.V., C.A.Harris, M.Scholze, A.Kortenkamp, P.Booy, M.Lamoree, G.Pojana, N.Jonkers, A.Marcomini, and J.P.Sumpter. 2007. "Evidence of estrogenic mixture effects on the reproductive performance of fish." *Environ.Sci.Technol.* 41:337-344.

Cassee,F.R., J.P.Groten, P.J.Bladeren, and V.J.Feron. 1998. "Toxicological evaluation and risk assessment of chemical mixtures." *Critical Reviews in Toxicology*. 28:73-101.

Cedergreen,N., A.M.Christensen, A.Kamper, P.Kudsk, S.K.Mathiassen, J.C.Streibig, and H.Sörensen. 2008. "A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites." *Environmental Toxicology and Chemistry*. 27:1621-1632.

Chapman,P.F. and P.M.Chapman. 1997. "Reply To: "Warning : Replacing NOECs with point estimates may not solve regulatory contradictions" by Dhaliwal et al." *Environmental Toxicology and Chemistry*. 16:125-126.

Chapman,P.M., R.S.Caldwell, and P.F.Chapman. 1996. "A warning: NOECs are inappropriate for regulatory use." *Environmental Toxicology and Chemistry*. 15:77-79.

Christiansen,S., M.Scholze, M.Dalgaard, A.M.Vinggaard, M.Axelstad, A.Kortenkamp, and U.Haas. 2009. "Synergistic disruption of external male sex organ development by a mixture of four anti-androgens." *Environmental Health Perspectives*. in press.

Committee on Toxicity of Chemicals in Food,C.P.a.t.E. 2002. *Risk assessment of mixtures of pesticides and similar substances*.

Conolly,R., J.Wang, and Y.Tan. 2005. "Physiologically based pharmacokinetic (PBPK) modeling as an alternative to relative potency factors (RPFs) in cumulative risk assessment: An example with acetylcholinesterase- (AChE) inhibiting pesticides." *Toxicological Science*. 84:Abstract 34.

Cornell,J. 2002. *Experiments with Mixtures: Designs, Models, and the Analysis of Mixture Data*.

Crofton,K., E.S.Craft, J.M.Hedge, C.Gennings, J.E.Simmons, R.A.Carchman, W.H.Jr.Carter, and J.M.deVito. 2005. "Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism." *Environmental Health Perspectives*. 113:1549-1554.

Damgaard,I.N., N.E.Skakkebaek, J.Toppari, H.E.Virtanen, H.Q.Shen, K.W.Schramm, J.H.Petersen, T.K.Jensen, and K.M.Main. 2006. "Persistent pesticides in human breast milk and cryptorchidism." *Environmental Health Perspectives*. 114:1133-1138.

De Zwart,D., S.D.Dyer, L.Posthuma, and C.P.Hawkins. 2006. "Predictive models attribute effects on fish assemblages to toxicity and habitat alteration." *Ecological Applications*. 16:1295-1310.

Deneer,J.W. 2000. "Toxicity of mixtures of pesticides in aquatic systems." *Pest Management Science*. 56:516-520.

Dhaliwal,B.S., R.J.Dolan, C.W.Batts, and J.M.Kelly. 1997. "Warning: replacing NOECs with point estimates may not solve regulatory contradictions." *Environmental Toxicology and Chemistry*. 16:124-126.

Drescher,K. and W.Boedeker. 1995. "Assessment of the combined effects of substances: The relationship between concentration addition and independent action." *Biometrics*. 51:716-730.

Dutcosky,S.D., M.V.E.Grossmann, R.S.S.F.Silva, and A.K.Welsch. 2006. "Combined sensory optimization of a prebiotic cereal product using multicomponent mixture experiments." *Food Chemistry*. 98:630-638.

ECETOC. 2001. *ECETOC Technical Report No. 80 - Aquatic Toxicity of Mixtures*.

EU Parliament and the Council. 2008. "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." *Official Journal of the European Communities*. L353.

Faust,M., R.Altenburger, T.Backhaus, H.Blanck, W.Boedeker, P.Gramatica, V.Hamer, M.Scholze, M.Vighi, and L.H.Grimme. 2003. "Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action." *Aquatic Toxicology*. 63:43-63.

Faust,M., R.Altenburger, T.Backhaus, W.Boedeker, P.Gramatica, V.Hamer, M.Scholze, M.Vighi, and L.H.Grimme. 2001. "Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants." *Aquatic Toxicology*. 56:13-32.

Faust,M., R.Altenburger, W.Boedeker, and L.H.Grimme. 1994. "Algal toxicity of binary combinations of pesticide." *Bulletin of Environmental Contamination and Toxicology*. 53:134-141.

Faust,M., R.Altenburger, W.Boedeker, and L.H.Grimme. 1996. "Kombinationswirkungen von Schadstoffen in Gewässern: Vorhersagbarkeit und Beurteilung." *Impulse aus der Forschung*. 21:36-40.

Fernandez,M.F., B.Olmos, A.Granada, M.J.Lopez-Espinosa, J.M.Molina-Molina, J.M.Fernandez, M.Cruz, F.Olea-Serrano, and N.Olea. 2007. "Human Exposure to Endocrine-Disrupting Chemicals and Prenatal Risk Factors for Cryptorchidism and Hypospadias: A Nested Case-Control Study." *Environmental Health Perspectives*. 115:8-14.

Feron,V.J. and J.P.Groten. 2002. "Toxicological evaluation of chemical mixtures." *Food and Chemical Toxicology*. 40:825-839.

Glantz,S.A. and W.W.Parmley. 1991. "Passive smoking and heart disease. Epidemiology, physiology, and biochemistry." *Circulation*. 83:1-12.

Greco,W., H.-D.Unkelbach, G.Pöch, J.Sühnel, M.Kundi, and W.Boedeker. 1992. "Consensus on concepts and terminology for combined-action assessment: The Saariselkä agreement." *Archives of Complex Environmental Studies*. 4:65-69.

Greco,W.R., G.Bravo, and J.C.Parsons. 1995. "The search for synergy: a critical review from a response surface perspective." *Pharmacol.Rev.* 47:331-385.

Grote,M., W.Brack, and R.Altenburger. 2005a. "Identification of toxicants from marine sediment using effect-directed analysis." *Environmental Toxicology*. 20:475-486.

Grote,M., W.Brack, H.A.Walter, and R.Altenburger. 2005b. "Confirmation of cause-effect relationships using effect-directed analysis for complex environmental samples." *Environmental Toxicology and Chemistry*. 24:1420-1427.

Grote,M., W.Brack, H.A.Walter, and R.Altenburger. 2005c. "Confirmation of cause-effect relationships using effect-directed analysis for complex environmental samples." *Environmental Toxicology and Chemistry*. 24:1420-1427.

Groten,J.P. 2000. "Mixtures and interactions." *Food Chem.Toxicol.* 38:S65-S71.

Groten,J.P., V.J.Feron, and J.Sühnel. 2001. "Toxicology of simple and complex mixtures." *Trends in Pharmacological Sciences*. 22:316-322.

Haddad,S. and K.Krishnan. 1998. "Physiological modelling of toxicokinetic interactions: implications for mixture risk assessment." *Environmental Health Perspectives*. 106:1377-1384.

Hajat,S., B.Armstrong, P.Wilkinson, A.Busby, and H.Dolk. 2007. "Outdoor air pollution and infant mortality: analysis of daily time-series data in 10 English cities." *Journal of Epidemiology and Community Health*. 61:719-722.

Hass,U., M.Scholze, S.Christiansen, M.Dalgaard, A.M.Vinggaard, M.Axelstad, S.B.Metzdorff, and A.Kortenkamp. 2007. "Combined Exposure to Anti-Androgens Exacerbates Disruption of Sexual Differentiation in the Rat." *Environmental Health Perspectives*. 115:122-128.

Hauser,R., Z.Y.Chen, L.Pothier, L.Ryan, and L.Altshul. 2003. "The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p '-DDE." *Environmental Health Perspectives*. 111:1505-1511.

Hermens,J., P.Leeuwangh, and A.Musch. 1985. "Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (Poecilia reticulata)." *Ecotoxicology and Environmental Safety*. 9:321-326.

Hertzberg,R.C. and M.M.MacDonell. 2002. "Synergy and other ineffective mixture risk definitions." *The Science of the Total Environment*. 288:31-42.

Hertzberg,R.C. and L.K.Teuschler. 2002. "Evaluating quantitative formulas dor dose- response assessment of chemical mixtures." *Environmental Health Perspectives*. 110:965-970.

Hewlett,P.S. and R.L.Plackett. 1979. *The interpretation of quantal responses in biology*. Edward Arnold.

Hoekstra,J.A. and v.P.H.Ewijk. 1993. "Alternatives for the no-observed-effect level." *Environmental Toxicology and Chemistry*. 12:187-194.

Jonker,D., R.A.Woutersen, and V.J.Feron. 1996. "Toxicity of mixtures of nephrotoxicants with similar or dissimilar mode of action." *Food and Chemical Toxicology*. 34:1075-1082.

Junghans,M., T.Backhaus, M.Faust, M.Scholze, and L.H.Grimme. 2006. "Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures." *Aquatic Toxicology*. accepted for publication:2-93.

Kodell,R.L. and J.G.Pounds. 1991. "Assessing the toxicity of mixtures of chemicals." In D.Krewski and C.Franklin, editors, *Statistics in toxicology*. Gordon and BREACH. New York. 559-591.

Könemann,H. and M.N.Pieters. 1996. "Confusion of concepts in mixture toxicology." *Food and Chemical Toxicology*. 34:1025-1031.

Kortenkamp,A. 2007a. "Low dose mixture effects of endocrine disrupters: implicatoins for risk assessment and epidemiology." *International Journal of Andrology*. 31:233-240.

Kortenkamp,A. 2007b. "Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals." *Environ.Health Perspect.* 115 Suppl 1:98-105.

Kortenkamp,A., M.Faust, M.Scholze, and T.Backhaus. 2007. "Low-level exposure to multiple chemicals: reason for human health concerns?" *Environ.Health Perspect.* 115 Suppl 1:106-114.

Krishnan,K., M.E.Andersen, H.J.Clewell, and R.S.H.Yang. 1994. "Physiologically based pharmacokinetic modeling of chemical mixtures." In R.S.H.Yang, editor, *Toxicology of chemical mixtures: case studies, mechanisms and novel approaches*. Academic Press. 399-437.

Laetz,C.A., D.H.Baldwin, T.K.Collier, V.Hebert, J.D.Stark, and N.L.Scholz. 2008. "The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon." *Environmental Health Perspectives*. in press.

Law,M.R. and A.K.Hackshaw. 1996. "Environmental tobacco smoke." *British Medical Bulletin*. 52:22-34.

Lowit,A., C.Darit, F.Powers, J.Blancato, R.W.Setzer, R.Conolly, and M.Seaton. 2004. "Physiologically-based pharmacokinetic/pharmacodynamic modeling and cumulative risk

assessment: Case study for the N-methyl carbamate pesticides." *EPA Science Forum, http://www.epa.gov/nerl/news/forum2004/lowit_web.pdf*.

McCarty,L.S. and C.J.Borgert. 2006. "Review of the toxicity of chemical mixtures containing at least one organochlorine." *Regulatory Toxicology and Pharmacology*. 45:104-118.

Menzie,C.A., M.M.MacDonell, and M.Mumtaz. 2007. "A phased approach for assessing combined effects from multiple stressors." *Environmental Health Perspectives*. 115:807-816.

Moore,D.R.J. and P.Y.Caux. 1997. "Estimating low toxic effects." *Environmental Toxicology and Chemistry*. 16:794-801.

National Academy of Sciences,B.o.E.S.a.T. 2009. *Advancing Risk Assessment - Report in Brief*.

Norwegian Scientific Committee for Food Safety. Combined toxic effects of multiple chemical exposures. 2008.

Norwood,W.P., U.Borgmann, D.G.Dixon, and A.Wallace. 2003. "Effects of metal mixtures on aquatic biota: A review of observations and methods." *Human and Ecological Risk Assessment*. 9:795-811.

OSPAR Commission. Whole Effluent Assessment. OSPAR Report . 2005.

Peden,D.B. 2005. "The epidemiology and genetics of asthma risk associated with air pollution." *Journal of Allergy and Clinical Immunology*. 115:213-219.

Pöch,G. 1993. *Combined effects of drugs and toxic agents. Modern evaluation in theory and practice*. Springer Verlag. Wien, New York.

Pope III,C.A. and R.T.Burnett. 2007. "Confounding in air pollution epidemiology: The broader context." *Epidemiology*. 18:424-426.

Posthuma,L. and D.De Zwart. 2006. "Predicted effects of toxicant mixtures are confirmed by changes in fish species assemblages in Ohio, USA, rivers." *Environmental Toxicology and Chemistry*. 25:1094-1105.

Rajapakse,N., E.Silva, and A.Kortenkamp. 2002. "Combining xenoestrogens at levels below individual no- observed- effect concentration dramatically enhances steroid hormone action." *Environmental Health Perspectives*. 110:917-921.

Scholze,M., W.Boedeker, M.Faust, T.Backhaus, R.Altenburger, and L.H.Grimme. 2001. "A general best-fit method for concentration-response curves and the estimation of low-effect concentrations." *Environmental Toxicology and Chemistry*. 20:448-457.

Silva,E., N.Rajapakse, and A.Kortenkamp. 2002. "Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects." *Environ.Sci.Technol.* 36:1751-1756.

Simkhovich,B.Z., M.T.Kleinman, and R.A.Kloner. 2008. "Air Pollution and Cardiovascular Injury. Epidemiology, Toxicology, and Mechanisms." *Journal of the American College of Cardiology*. 52:719-726.

Swan,S.H., K.M.Main, F.Liu, S.L.Stewart, R.L.Kruse, A.M.Calafat, C.S.Mao, J.B.Redmon, C.L.Ternand, S.Sullivan, and J.L.Teague. 2005. "Decrease in anogenital distance among

male infants with prenatal phthalate exposure." *Environmental Health Perspectives*. 113:1056-1061.

Teuschler,L.K. and R.C.Hertzberg. 1995. "Current and future risk assessment guidelines, policy, and methods development for chemical mixtures." *Toxicology*. 105:137-144.

Teuschler,L.K., G.E.Rice, C.R.Wilkes, J.C.Lipscomb, and F.W.Power. 2004. "Feasibility study of cumulative risk assessment methods for drinking water disinfection by-product mixtures." *Journal of Toxicology and Environmental Health-Part A-Current Issues*. 67:755-777.

US EPA. Guidance on cumulative risk assessment of pesticide chemicals that have a common mechanism of toxicity.

US EPA. 1986. "Guidelines for the health risk assessment of chemical mixtures." *Federal Register*. 51:34014-34025.

US EPA. Supplementary guidance for conducting health risk assessment of chemical mixtures. EPA/600/8-90/064 . 2000.

US EPA. 2007. *Concepts, methods and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects: a resource document*.

US EPA. 2008. *Framework for application of the toxicity equivalence methodology for polychlorinated dioxins, furans, and biphenyls in ecological risk assessment*.

Van den Berg ,B.M., L.Birnbaum, A.T.Bosveld, B.Brunstrom, P.Cook, M.Feeley, J.P.Giesy, A.Hanberg, R.Hasegawa, S.W.Kennedy, T.Kubiak, J.C.Larsen, F.X.van Leeuwen, A.K.Liem, C.Nolt, R.E.Peterson, L.Poellinger, S.Safe, D.Schrenk, D.Tillitt, M.Tysklind, M.Younes, F.Waern, and T.Zacharewski. 1998. "Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife." *Environ.Health Perspect.* 106:775-792.

van der Hoeven,N. 1997. "How to measure no effect.Part III:Statistical aspects of NOEC,EC_x and NEC estimates." *Environmetrics*. 8:255-261.

Verhaar,H.J.M., J.R.Morroni, K.F.Reardon, S.M.Hays, Jr.D.P.Gaver, R.L.Carpenter, and R.S.H.Yang. 1997. "A proposed approach to study the toxicology of complex mixtures of petroleum products: The integrated used of QSAR, lumping analysis and PBPK/PD modelling." *Environmental Health Perspectives*. 105:179-195.

Walter,H., F.Consolaro, P.Gramatica, M.Scholze, and R.Altenburger. 2002. "Mixture toxicity of priority pollutants at No Observed Effect Concentrations (NOECs)." *Ecotoxicology*. 11:299-310.

Warne,M.St.J. 2003. "A review of the ecotoxicity of mixtures, approaches to, and recommendations for, their management." *Proceedings of the Fifth National Workshop on the Assessment of Site Contamination*.253-276.

Appendix III: Overview of major reports and guidelines on the hazard and risk assessment of chemical mixtures

All Documents are made available as PDF files. The name of each file starts with the listed number for each report.

1. Bengtsson,G. and J.Holmqvist. (2008) Kombinationseffekter av föroreningar. SWECO VIAK Rapport (in Swedish).
2. Bjarnason, S.G. (2004) Toxicology of Chemical Mixtures: a Review of Mixtures Assessment, Defence R D Canada, Technical Memorandum.
3. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, Food Standards Agency, UK (2002) Risk assessment of mixtures of pesticides and similar substances.
4. Danish Veterinary and Food Administration (2003) Combined Actions and Interactions of Chemicals in Mixtures - the toxicological effects of exposure to mixtures of industrial and environmental chemicals. Fødevare Rapport 2003:12. ISSN: 1399-0829.
5. ECETOC - European Centre for Ecotoxicology and Toxicology of Chemicals. (2001) Technical Report No. 80 - Aquatic Toxicity of Mixtures. ISSN -0773-8072-80.
6. IGHRC - The interdepartmental group on health risks from chemicals. (2009) Chemical Mixtures: A framework for assessing risks to human health. Published by the Institute of Environment andHealth, Cranfield University. ISBN 978-1-899110-44-5.
7. Munn, M.D., Gilliom, R.J., Moran, P.W. and Nowell, L.H. (2006). Pesticide Toxicity Index, US Department of the Interior, US Geological Survey, Scientific Investigations Report 2006-5148, 2nd edition.
8. Norwegian Scientific Committee for Food Safety. (2008) Combined toxic effects of multiple chemical exposures - Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety. ISBN 978-82-8082-233-8.
9. EFSA Panel on Plant Protection Products and their Residues(PPR). (2008). 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) 396/2005. The EFSA Journal. 704:1-85.
10. EFSA Panel 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 throughout food from these pesticides on human health on request of EFSA. The EFSA Journal. 7 (9); 1167-1271.
11. Sterner, T.R., Robinson, P.J., Mattie, D.R. and Burton, G.A. (2005) The toxicology of chemical mixtures - risk assessment for human and ecological receptors. Report for the US Air Force Laboratory, Human Effectiveness Directorate, Biosciences and Protection Division.

12. US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Division of Toxicology. (2001). Guidance for the preparation of an interaction profile.
13. US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Division of Toxicology. (2004). Guidance manual for the assessment of joint toxic action of chemical mixtures.
14. US EPA, Office of the Science Advisor, Risk Assessment Forum (2008) Framework for Applicat on the Toxicity Equivalence Methodology for Polychlorinated Dioxins, Furans, and Biphenyls in Ecological Risk Assessment, EPA/100/R-08/004.
15. US EPA, Office of Pesticide Programs. (2002) Guidance on cumulative risk assessment of pesticide chemicals that have a common mechanism of toxicity.
16. US EPA, Risk Assessment Forum Technical Panel. (2000). Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures. EPA/630/R-00/002.
17. US EPA, National Center for Environmental Assessment, Office of Research and Development and U.S. Department of Energy, Argonne National Laboratory, Environmental Assessment Division (2007). Concepts, methods and data sources for cumulative health risk assessment of multiple chemicals, exposures and effects: a resource document. EPA/600/R-06/013F.
18. US EPA Risk Assessment Forum (2003). Framework for cumulative risk assessment. EPA/630/P-02/001F.
19. World Health Organization / International Programme on Chemical Safety (2007) Assessment of Combined Exposures to Multiple Chemicals: Report of a WHO/ICPS International Workshop, ISBN 978 92 4 156383 3.
20. World Health Organization (2009). Risk assessment of combined exposures to multiple chemicals: a WHO/IPCS framework (external review draft).
21. Kortenkamp,A., T.Backhaus, and M.Faust. 2010. State of the Art Report on Mixture Toxicity. Report for Directorate General for the Environment of the European Commission.

Abbreviations

Terms denoted by an asterisk (*) denote key concepts in mixture toxicology and are explained in detail in the Glossary of Terms (Appendix I). Other terms are defined where they appear in the text or in corresponding footnotes.

ADI	acceptable daily intake
BB	brilliant blue
BINWOE	Binary Weight-of-Evidence*
BP	bridging principle
CA	Concentration Addition* (or Dose Addition)
CB	carbamate
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act (US law)
CLP	Classification, Labelling and Packaging of Substances and Mixtures (EU law)
CRA	Cumulative Risk Assessment*
DNEL	derived no-effect-level
DDD	dichlorodiphenyldichloroethane
DDT	dichlorodiphenyltrichloroethane
ECx	effect concentration causing x% effect
EEC	European Economic Community
EFSA	European Food Safety Authority
EQS	ecological quality standards
ES	Effect Summation*
EU	European Union
GEMS	Global Environment Monitoring System
GHS	Globally Harmonised System
HI	Hazard Index*
LOEL	lowest observed effect level
IA	Independent Action* (also called Response Addition)
IPPC	Integrated Pollution and Prevention Control (EU law)
MAF	mixture assessment factor
MCS	multi-constituent substance
MDR	model derivation ratio
MOA	mode of action

MPC	maximum permissible concentration
msPAF	multi-substance potentially affected fraction of species
NC	negligible concentration
NOEC	no-observed-effect concentration
NOEL	no-observed-effect level
NOAEL	no-observed-adverse-effect level
OP	organophopsphate
QY	quinoline blue
PBPK/PD	physiologically based pharmacokinetic/pharmacodynamic
PCB	polychlorinated biphenyl
PEC	predicted environmental concentration
PNEC	predicted no-effect-concentration
PPP	plant protection product
PODI	Point of Departure Index*
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals (EU law)
RPF	Relative Potency Factor*
SM	summation method
TEF	Toxic Equivalence Factor*
TGD	Technical Guidance Document (in support of EU law)
TSP	two-stage prediction
TUS	Toxic Unit Summation*
US EPA	United States Environmental Protection Agency
UVCB	substances of unknown or variable composition, complex reaction products or biological materials
WFD	Water Framework Directive (EU law)
WHO	World Health Organization
WM	whole mixture

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