Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

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Combined toxic effects of multiple chemical exposures

Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety

Adopted 7 April 2008

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The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed an *ad hoc* group consisting of both VKM members and external experts to answer the request from the Norwegian Food Safety Authority. The members of the *ad hoc* group are acknowledged for their valuable contribution to this opinion.

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CONTENTS

SUMMARY

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has on the request of the Norwegian Food Safety Authority (Mattilsynet) examined the principles of risk assessments of combined toxic effects of multiple chemical exposures. Risk assessment of exposure to chemicals in food, feed and cosmetics are mostly based on data from studies on individual substances. However, humans are most often simultaneously exposed to a large number of chemicals from different sources, and it has been questioned whether combined exposures to low doses of substances that individually do not produce any adverse health effects, could still induce toxic effects when they co-occur or appear in mixtures.

A background document to be used in the work with the opinion was prepared by the Norwegian Institute of Public Health. Thereafter, VKM appointed an *ad hoc* group consisting of both VKM members from the relevant Scientific Panels and external experts to perform this task. The VKM Scientific Steering Committee has discussed and approved the final opinion.

VKM has on the request of the Norwegian Food Safety Authority described the most relevant theoretical principles for various types of combined toxic effects from multiple chemical exposures, even though such information can be found in already published reports. This strategy has been chosen as the most appropriate way to answer the question from the Norwegian Food Safety Authority, whether combined effects have adequately been addressed in the risk assessments of VKM. The intention has been to gather the most relevant information about this topic together with comments and views from VKM into one report.

The main basis for the general part of this opinion from VKM has been three recent reports on combined actions of chemicals, one from the Danish Veterinary and Food Administration, one combined report from the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration, and one report published by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). A review of the available scientific literature published later than 2003 has also been performed. Furthermore, applicable methods and approaches regarding such problems have been described.

The ways that the Scientific Panels of VKM have dealt with the issue of possible combined toxicological effects following exposures to multiple chemicals in their assessments, are summarised in Chapter 5 of this opinion. The primary focus has been on human health, but issues related to animal health and substances in animal feed have also been briefly addressed.

Different algorithms or schemes that have been developed to decide whether combined effects are likely to occur or when the possibility of such effects should be taken into consideration, have been presented and discussed in this opinion. These schemes are often complex and require additional data. However, VKM finds that the previously described schemes in most cases are not suitable for practical use in the Scientific Panels of VKM. In the present report VKM has therefore proposed a tool (flow chart) for use in risk assessments of chemical mixtures or concurrent exposures.

The main conclusions of the opinion are summarised below:

The likelihood of combined toxic effects of multiple exposures at dose levels below the thresholds for effect is low. The objectives of current food, feed and cosmetic regulations are that exposures should not be associated with adverse health effects, which also include the potential for combined effects.

For substances exhibiting similar modes of action (simple similar action), adverse effects from multiple exposures may be experienced due to dose addition, even if the exposures to the individual components of the mixture are below their respective acceptable or tolerable daily intakes (ADIs/TDIs).

For substances exhibiting dissimilar modes of action (simple dissimilar action), adverse effects from multiple exposures are not expected when the exposures to the individual components of the mixture are below their respective ADIs/TDIs.

In situations where there is exposure to multiple chemicals significantly above their respective ADIs/TDIs, enhanced combined effects due to interaction may occur. Such interactions could be due both to toxicokinetic and toxicodynamic mechanisms, and are difficult to predict. Assessments should be performed on a case-by-case basis and ideally be based on data from testing of the relevant mixtures/concurrent exposures.

In the derivation of ADIs/TDIs from animal data, provided data on inter- and intraspecies variation are not available, rather large default uncertainty factors are used in the extrapolations to humans, reflecting potential differences in species sensitivity (default factor of 10) and taking into account variability among humans (default factor of 10). Hence, the levels of exposure corresponding to an ADI/TDI may be more than one order of magnitude below the real dose thresholds of effect if humans are not more sensitive than the test species.

Although the Scientific Panels of VKM so far only to a limited degree have formally taken possible combined effects from multiple chemical exposures into account, VKM does not consider this as a matter of serious concern. However, a flow chart has been developed and will be tested out as a tool in the Scientific Panels, in order to formally address the possibility for combined effects of multiple exposures in the future.

Many plant protection products (pesticides) belong to groups with similar mechanisms of action. When there is combined exposure to pesticides within the same mechanism group, the principle of dose addition for such compounds exhibiting simple similar action would apply. When the sum of the exposure doses of the individual compounds in the mixture does not exceed the ADI for the most potent compound, there should be no apparent concerns. In situations where this sum of exposures exceeds the ADI of the most potent compound, dose additive effects may be expected. Risk assessments could for such situations be based on knowledge of the relative potencies of the pesticides in the mixture. Also, synergistic effects from mixtures could occur when exposures are above dose thresholds. However, with respect to the probability of experiencing interactive effects from combined exposures to pesticides, it should be kept in mind that based on national and Europe-wide monitoring programmes of residues of plant protection products in fruits, vegetables and cereals, levels are infrequently above maximum residue limits and thus considerably below ADIs.

From international studies of pesticide operators, combined effects from multiple exposures have been documented. Although no such studies have been performed in Norway, the premise is that professional use of plant protection products should not exceed acceptable operator exposure levels when applied correctly and any advice on use of personal protection equipment has been followed.

This opinion has not addressed other risk areas equally detailed. Generally, areas of risk are those where multiple exposures act by common modes of action and where there is a risk of exceeding dose thresholds. The Scientific Panels of VKM have in addition to the issue of plant protection products addressed the most important areas, such as dioxins and PCBs and algal toxins. A potential risk area in a Norwegian context is the combined effects of consumption of marine organisms from localised areas where there has been point source release of halogenated organic compounds and heavy metals. Since both types of contaminants are associated with developmental effects (reproductive-, immune- and central nervous system) and the fact that the young child is especially sensitive towards such effects, due consideration should be given to the potential for interactions.

This opinion has not in detail dealt with possible combined effects from multiple exposures in relation to ecotoxicology. However, the toxicological principles for combined effects described in this opinion, are expected to apply also for the environment.

SAMMENDRAG

På oppdrag fra Mattilsynet har Vitenskapskomiteen for mattrygghet (VKM) belyst prinsippene for hvordan man kan foreta risikovurderinger av kombinerte effekter som følge av eksponeringer for flere kjemiske forbindelser samtidig. Risikovurderinger av eksponering for kjemiske stoffer via mat, fôr og kosmetiske produkter er hovedsakelig basert på data om virkninger av enkeltstoffer. Mennesker utsettes imidlertid som oftest for en lang rekke kjemiske forbindelser fra ulike kilder samtidig. Det er blitt stilt spørsmål om hvorvidt samtidige eksponeringer for lave doser av kjemiske stoffer, som hver for seg ikke medfører noen uønsket helseeffekt, likevel kan forårsake toksiske effekter når de opptrer samtidig eller i blanding.

Et bakgrunnsdokument, som er benyttet i arbeidet med uttalelsen ble utarbeidet av Nasjonalt folkehelseinstitutt på oppdrag fra VKM. Deretter oppnevnte VKM en *ad hoc*-gruppe, bestående av både VKM-medlemmer fra relevante faggrupper og ekstern ekspertise, for å utføre oppdraget. VKMs Hovedkomité har diskutert og godkjent den endelige uttalelsen.

VKM har i denne uttalelsen, etter ønske fra Mattilsynet, beskrevet de mest relevante teoretiske prinsippene for ulike typer samvirkende toksiske effekter etter eksponering for flere kjemiske forbindelser samtidig, selv om dette allerede er omtalt i publiserte rapporter. Dette ble ansett som mest hensiktsmessig for å kunne besvare spørsmålet fra Mattilsynet om hvorvidt slike hensyn blir ivaretatt på en tilfredstillende måte i risikovurderinger fra VKM. Intensjonen har vært å samle den mest relevante informasjonen om kombinasjonseffekter, sammen med kommentarer og synspunkter fra VKMs faggrupper i en og samme rapport.

Grunnlaget for den generelle delen av uttalelsen fra VKM har vært tre rapporter som omhandler kombinasjonseffekter av kjemikalier, henholdsvis to danske rapporter publisert av Fødevaredirektoratet og Miljøstyrelsen/Fødevaredirektoratet i samarbeid, og en britisk rapport fra UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). I tillegg er det gjort en gjennomgang av ny tilgjengelig litteratur, publisert etter 2003. Videre omtales hvilke metodikker og tilnærminger som kan anvendes ved risikovurdering av denne type problemstillinger.

Rapportens kapittel 5 beskriver hvordan, og i hvilken grad, problemstillingen med mulig samvirkende toksiske effekter ved eksponering for flere kjemiske forbindelser samtidig blir ivaretatt i risikovurderinger fra VKMs faggrupper. I rapporten omtales i hovedsak human helse, men aktuelle problemstillinger relatert til dyrehelse og kjemiske stoffer i dyrefôr blir også kort diskutert.

Det finnes ulike metoder og modeller for å avgjøre om det er sannsynlig at kombinerte effekter kan opptre, eller som sier noe om når muligheten for kombinerte effekter må tas hensyn til. De mest sentrale av disse modellene er presentert og diskutert i denne uttalelsen. Disse modellene er ofte komplekse og krevende med hensyn til mengden data som er nødvendig. VKM er imidlertid av den oppfatning at i de fleste tilfeller er ikke disse modellene egnet til praktisk bruk i VKMs faggrupper. VKM har derfor utarbeidet et eget verktøy i form av et flytdiagram til bruk i risikovurderinger av kjemiske blandinger eller sammensatte eksponeringer.

De viktigste konklusjonene i uttalelsen fra VKM er oppsummert nedenfor:

Sannsynligheten for at det kan oppstå samvirkende toksiske effekter ved samtidig eksponering for flere kjemiske forbindelser i doser som ligger under en terskeleffekt er liten. Formålet med dagens regelverk på mat-, fôr- og kosmetikkområdet er at eksponeringer for kjemiske stoffer ikke skal medføre uønskede helseeffekter, herunder også mulige kombinasjonseffekter.

For stoffer med samme virkningsmåte (simple similar action) kan helseskadelige effekter forårsaket av eksponering for flere kjemiske forbindelser forekomme som følge av doseaddisjon, selv om eksponeringen for hvert enkelt stoff i den blandete eksponeringen ligger under deres respektive akseptable eller tolerable daglige inntaksverdier (ADI/TDI-verdier).

For stoffer med ulik virkningsmåte (simple dissimilar action) er det ikke forventet helseskadelige effekter ved eksponering for flere kjemiske forbindelser når eksponeringen for hvert enkelt stoff i blandingen ligger under deres respektive ADI/TDI-verdier.

I situasjoner hvor samtidig eksponering for flere kjemiske stoffer ligger betydelig over de respektive ADI-verdiene, kan det forekomme en forsterket samvirkende effekt som følge av interaksjon mellom stoffene. Slike interaksjoner kan være forårsaket av både toksikokinetiske og toksikodynamiske mekanismer og er vanskelige å forutsi. Risikovurderinger bør utføres fra sak til sak (case-by-case) og ideelt sett være basert på data fra undersøkelser av de relevante blandingene/eksponeringene.

Når ADI/TDI-verdier utledes basert på data fra forsøksdyr, blir det, når ikke spesielle data foreligger, benyttet relativt store usikkerhetsfaktorer ved ekstrapoleringen fra dyr til mennesker. Usikkerhetsfaktorene skal ta høyde for ulikheter i artsfølsomhet mellom dyr og mennesker (faktor 10) og variasjoner mellom mennesker (faktor 10). Følgelig, kan eksponeringsnivåer som tilsvarer ADI/TDI være mer enn en størrelsesorden lavere enn den faktiske doseterskelen for effekt, hvis mennesker ikke er mer følsomme enn dyrearten som er testet.

Selv om Faggruppene i VKM, så langt kun i begrenset grad formelt har tatt høyde for mulige samvirkende effekter ved samtidig eksponering for flere kjemiske forbindelser i sine risikovurderinger, anser ikke VKM dette som bekymringsfullt. Det er imidlertid utviklet et flytskjema som vil bli testet ut som et verktøy av de vitenskaplige faggruppene, slik at det i fremtiden kan tas mer systematisk hensyn til potensielle samvirkende effekter av sammensatte eksponeringer i VKMs risikovurderinger.

Mange plantevernmidler (pesticider) tilhører grupper av kjemiske forbindelser som har lik virkningsmekanisme. Ved sammensatte eksponeringer for flere plantevernmidler innenfor samme mekanismegruppe, gjelder prinsippene for doseaddisjon for forbindelser med lik virkningsmekanisme (simple similar action). Når summen av eksponeringsdosene for de enkelte stoffene i blandingen ikke overskrider ADI-verdien for den mest potente forbindelsen, er det ikke grunn til bekymring. I tilfeller hvor summen av eksponeringene overskrider ADI for den mest potente forbindelsen, må det imidlertid kunne forventes additive effekter. Risikovurderingene bør da baseres på kunnskap om den relative potensen til plantevernmidlene i blandingen. Synergistiske effekter fra blandinger kan også forekomme når eksponeringene ligger over dosetersklene. Når det gjelder sannsynligheten for å oppleve

samvirkende effekter ved en sammensatt eksponering for flere plantevernmidler, skal det bemerkes at nasjonale og europeiske overvåkningsprogrammer for rester av plantevernmidler i frukt, grønnsaker og kornprodukter viser at restnivåene sjelden er over maksimale grenseverdier for plantevernmiddelrester, og derfor bare vil føre til inntak betydelig under ADI-verdiene.

Internasjonale studier av personer som utsettes for yrkesmessig eksponering av plantevernmidler har dokumentert at det forekommer samvirkende toksiske effekter ved eksponering for flere stoffer i blanding. Selv om ingen slike studier er utført i Norge, er forusetningen slik at yrkesmessig bruk av plantevernmidler ikke skal overskride akseptable eksponeringsnivå for brukeren (acceptable operator exposure levels, AOEL) når plantevernmidlene brukes riktig og alle råd om bruk av beskyttelsesutstyr er blitt fulgt.

I denne rapporten omtales ikke andre risikoområder i like stor detalj. Generelt vil risikoområdene kunne være knyttet til situasjoner hvor eksponeringen for flere forbindelser har lik vikningsmåte, og hvor det er en risiko for å overskride dosetersklene. VKMs faggrupper har i tillegg til å vurdere plantevernmidler også omtalt de viktigste problemområdene, slik som dioksiner, PCB og algetoksiner. Et potensielt risikoområde i norsk sammenheng er den samvirkende effekten etter inntak av marine organismer fra lokale områder hvor det har vært punktutslipp av halogenerte organiske forbindelser og tungmetaller. Siden begge disse gruppene av kjemiske stoffer er forbundet med effekter på vekst og utvikling (reproduksjon-, immun-, og sentralnervesystemet) og barn er spesielt følsomme for slike effekter, bør man være oppmerksom på muligheten for samvirkende toksiske effekter.

Denne vurderingen har ikke i detalj tatt for seg mulige samvirkende effekter i miljøet, men det er forventet at de samme toksikologiske prinsippene for kombinasjonseffekter som er beskrevet i denne uttalelsen også vil gjelde for dette området.

BACKGROUND

Risk management of substances in food, feed and cosmetics is currently, among other relevant factors, based on health risk assessments which generally take into account data from studies on individual substances. However, humans are simultaneously exposed to a large number of chemicals through consumption of food and drinking water, uptake through the skin and inhalation. Moreover, consumers and non-governmental organisations frequently challenge the Norwegian Food Safety Authority (Mattilsynet) to consider whether combined exposures to low doses of substances that individually do not produce any unforeseen health effects, could induce toxic effects when they appear in mixtures. The Norwegian Food Safety Authority therefore wants more information to evaluate whether such "chemical cocktails" are adequately covered in risk assessments related to human and animal health.

The combined toxic effects of multiple chemical exposures are to a certain degree taken into account in the regulations on food, feed and cosmetics. There have for instance been established group restrictions (group R) for some migrants from food contact materials, such as primary aromatic amines. Analogous regulatory limits, which consider that different substances could act by a similar toxicological mode of action, are established for some food additives.

However, in general regulatory limits for the use of a chemical in food, feed and cosmetics are based on separate toxicological tests of the individual substance in question and not on the basis of combined toxic effects from testing chemical mixtures. Neither do the current regulations take into account that a potential adverse effect of an ingredient could be counteracted effectively by other ingredients in the same or other product.

Several research groups and international risk assessment bodies have addressed some general principles related to how substances with different toxicological properties could act through either a synergistic, additive or antagonistic mode of action. In June 2006, the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) received a request from the Norwegian Food Safety Authority for an evaluation of how combined toxic effects of multiple chemical exposures are included in VKM's risk assessments. VKM was asked to consider the following reports when answering the request:

"Combined Actions and Interactions of Chemicals in Mixtures – The Toxicological Effects of Exposure to Mixtures of Industrial and Environmental Chemicals, FødevareRapport 2003:12" combined report from the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration in 2003 (Danish Environmental Protection Agency & Danish Veterinary and Food Administration, 2003).

"Combined actions of pesticides, FødevareRapport 2002:19" published by the Danish Veterinary and Food Administration in 2002 (Danish Veterinary and Food Administration, 2002).

"Risk Assessment of Mixtures of Pesticides and Similar Substances, FSA/0691/0902" published by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) in 2002 (COT, 2002).

Commissioned by VKM, the Norwegian Institute of Public Health was asked to prepare a background document to be used in the work with the opinion. VKM appointed an *ad hoc* group consisting of both VKM members from the relevant Scientific Panels and external experts to perform this task. Based on the background document from the Norwegian Institute of Public Health, the *ad hoc* group was requested to prepare a draft opinion where they primarily focused on the discussion and conclusions of the report, and described issues related to the different Scientific Panels which had not already been covered in the background document. The VKM Scientific Steering Committee has discussed and approved the final opinion.

TERMS OF REFERENCE

VKM is asked to examine the principles of risk assessments of combined toxic effects of multiple chemical exposures. The request from the Norwegian Food Safety Authority is divided into three parts;

1) The opinion should be based on the three abovementioned reports and recent information published after these reports. The most important conclusions from the reports and the recently published literature should be included and commented as they relate to risk assessments from VKM.
2) Do current risk assessments related to regulatory limits and approval of substances take into account combined toxic effects of multiple chemical exposures when considering if exposure to food/cosmetics does not produce any unforeseen health effects for the consumer? The directions and principles for such risk assessments should be elaborated.
3) Possible areas of risk where combined effects are not adequately covered, within the remit of the Norwegian Food Safety Authority, should be listed.

The opinion should also consider combined toxic effects of multiple chemical exposures related to animal health and substances in animal feed.

Since the new EU legislation for residues of plant protection products (Regulation (EC) No. 396/2005) has specific requirements for assessing combined toxic effects when relevant methods exist, the Norwegian Food Safety Authority has asked for a broader discussion regarding residues of plant protection products.

ASSESSMENT

1 Introduction

When the toxicity of a chemical is evaluated, it is important to consider if there is a dose threshold for the effect. A dose threshold is defined as the dose level above which toxicity or adverse health effect occurs. Below the dose threshold, toxicity or adverse health effects are unlikely to occur. Physiological responses in the body are well regulated by homeostatic mechanisms. Up to a certain point, the body can handle chemicals without adverse effects to which it is exposed, and repair any damage. If exposure becomes too high, detoxication and repair mechanisms are overwhelmed, and toxic effects are induced. The prevailing view has been that chemicals which cause cancer and are genotoxic have been assumed to have no thresholds for their effects, whereas non-genotoxic and non-carcinogenic chemicals have been assumed to have dose thresholds. However, the non-threshold concept for genotoxic compounds has been challenged (Hengstler *et al.*, 2003; Bolt & Degen, 2004; Rietjens & Alink, 2006).

In practice, humans and animals are exposed to complex and variable combinations of chemical compounds. In most cases, however, exposures to each compound are below those causing toxicity. In a situation of multiple chemical exposure, the single chemicals may act independently as in a single exposure, or a number of the chemicals may interact to modulate the effects of the total multiple exposure (Koppe *et al.,* 2006). For instance, cumulative low-dose insult can in some circumstances be more toxic than a single high-dose exposure, e.g. endocrine disruptive effects of a combination of polychlorinated biphenyls and dioxins which disrupt the thyroid status. These cumulative insults may further combine with heavy metals and can disrupt heme synthesis. Similar aspects may be relevant for other groups of potential harmful chemicals, such as in cosmetics and cleaning products. In addition, risk assessment with focus on interactions means that not only chemicals but also concurrent diseases should be taken into account, for instance such as increased risk of liver cancer caused by aflatoxin with concurrent hepatitis B infection.

In order to predict the toxicological properties of chemical mixtures, detailed information on the composition of the mixture, the mechanism of action and potency of each compound, as well as proper exposure data is required. Mostly, such detailed information is not available. *In vivo* data are often scarce since animal experiments are demanding. There is also a general policy to reduce the number of such studies due to animal welfare considerations. Exposure to potentially toxic chemicals in mixtures may be especially relevant for residues of pesticides in food taken into consideration new regulations on plant protection products (Regulation (EC) No. 396/2005). Much focus is also on endocrine disruptors, especially oestrogen receptor-binding compounds and their potential to act as reproductive toxicants in combination.

The present opinion of combined toxic effects of multiple chemical exposures is based on the reports mentioned in the background where this topic has been discussed and evaluated (COT, 2002; Danish Veterinary and Food Administration, 2002; Danish Environmental Protection Agency & Danish Veterinary and Food Administration, 2003). During the work with this opinion, the European Food Safety authority (EFSA) published a report from a scientific

colloquium on cumulative risk assessment of pesticides (EFSA, 2007c), which also have been taken into account.

VKM has chosen to include the most relevant theoretical descriptions of aspects related to combined toxic effects of multiple chemical exposures, even though the same information can be found in the abovementioned reports. This strategy has been chosen as the most appropriate way to answer the request from the Norwegian Food Safety Authority. The intention is to gather the most relevant information about this topic together with comments and views from the Scientific Steering Committee of VKM into one report. Unless otherwise stated, the text in chapter 2, 3 and 4 refers to the two Danish reports on combined actions of chemicals and the report from the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT, 2002; Danish Veterinary and Food Administration, 2002; Danish Environmental Protection Agency & Danish Veterinary and Food Administration, 2003). In addition, a literature search was performed to include papers and reports published after 2003.

In this assessment, VKM discussed ways to address combined toxicological effects following exposure to multiple chemical compounds. Chemical risk assessments performed by VKM aim to protect the general, healthy part of the population. Individuals under medical treatment with drugs are therefore not taken into consideration, and necessary additional advice has to be formulated in this context. Also, the influence of a nutritional status strongly deviating from that of the general, healthy population, as well as the possible effect of non-toxic compounds present in foods (e.g. compounds in grapefruit that inhibit the xenobiotic metabolising enzyme CYP3A4) has not been considered here, nor have any effects of use of tobacco and alcohol been taken into account. Combined effects from exposures of mixtures in relation to ecotoxicology have not been dealt with in detail. The widespread phenomenon of hormesis (Calabrese & Baldwin, 2003), which is generally characterized by low-dose stimulation and high-dose inhibition is not considered further due to very limited studies related to combined effects.

Ultimately, risk-benefit analyses may be performed for multiple chemical exposures, since a certain food may have significant health benefits, irrespective of contaminant content. For instance, many fruits and vegetables have potent chemopreventive activities of far greater importance for health aspects such as cancer development, than any low-level pesticide residues present. Risk-benefit analyses have not been given any attention in this opinion.

In this opinion the following terms are used:

- Combined exposure: Exposures to two or more substances occuring concurrently.
- Cumulative exposure: All exposures to a specified substance from all routes.
- Mode of action: Knowledge of toxicological action at the tissue level.
- Mechanisms of action: Knowledge of toxicological action at the cellular level.

2 Basic concepts and terminology used to describe the combined actions of chemicals in mixtures

The major objective in the risk assessment of chemicals in mixtures is to establish or predict how the toxicological effects of the mixture might turn out, often in comparison with exposure to individual compounds. A risk assessment should be performed where co-exposure is likely to occur, with special attention when combinations are intentionally made. One of the main points to consider is whether chemicals in a mixture interact and produce an increased or decreased overall response compared to the expected sum of the effects if each chemical acts independently of each other. The descriptions of these basic concepts used in the evaluation of toxicology of chemical mixtures were first outlined by Loewe and Muischnek (Loewe & Muischnek, 1926), Bliss (Bliss, 1939) and Placket & Hewlett (Placket & Hewlett, 1952) (Table 1). The terms simple similar action and simple dissimilar action, describe situations where no interaction occurs and addition is the outcome of a combined action. The terms complex similar action and complex dissimilar action are used when interaction occurs and the outcomes differ from addition (synonymous terms are written in parenthesis in Table 1).

Table 1. Classification of combined (joint) toxic action of two compounds in a mixture (when both agents are effective individually). From the combined report from the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration, FødevareRapport 2003:12, modified after Placket and Hewlett (1952).

	Similar mechanism	**Dissimilar mechanisms**
No interaction	Simple similar action (Loewe additivity, Dose addition)	Simple dissimilar action (Bliss independence, Response (effect) addition)
Interaction	Complex similar action (Loewe synergism or antagonism)	Complex dissimilar action (Bliss synergism or antagonism)

2.1 No interaction

According to the classification shown in Table 1, there are two models for combined action without interaction: simple similar action and simple dissimilar action.

Simple similar action: The model for simple similar action assumes that the compounds act on the same biological site (e.g. receptor or target organ), by the same mechanism and that they differ only in their potencies. Each chemical contributes to the toxicity of the mixture in proportion to its dose, and their relative toxicities are assumed to be constant at all dose levels. The effect would be a result of the sum of the contributing dose of each chemical (Figure 1A).

Simple dissimilar action: In the model for simple dissimilar action, the chemicals contribute to a common result, but the mechanisms by which the chemicals act are always different. Also, the nature and site of action may possibly, but not necessarily, differ among the

chemicals in the mixture. Therefore, the presence of one chemical will not affect the toxicity of another chemical (Figure 1B).

There is a major difference between simple similar action and simple dissimilar action when the human situation of low exposure levels is assessed (Figure 1). Simple similar action implies that the combined doses of a mixture may lead to a toxic response even if the compounds individually are at levels below the effect threshold (no-effect level). In contrast, simple dissimilar action implies that when doses of chemicals are below the effect threshold (no-effect levels) of the individual compounds, the combined action of all compounds together will be zero.

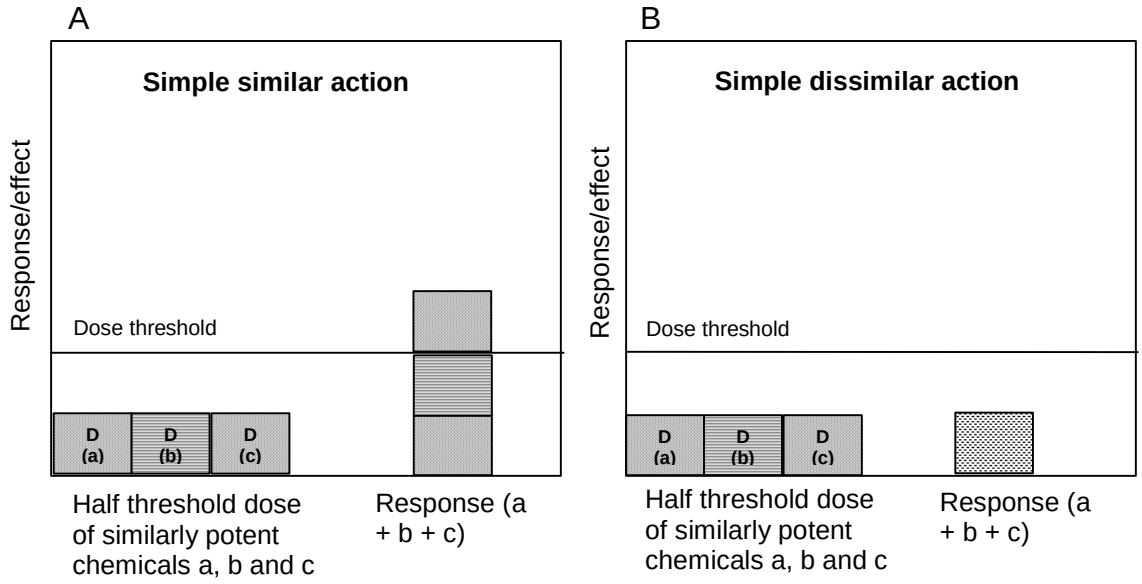

Figure 1. Illustration of the difference in response/effect when chemicals act by simple similar action (A) or simple dissimilar action (B). The hatched parts illustrates exposure to the combination of half threshold doses of three similarly potent components (a, b and c). In the case of simple similar action (A), the total response is above the threshold since the effects of the individual chemicals are additive. In the case of simple dissimilar action (B), there is no observed response since the individual chemicals do not affect each other and the dose of each chemical is below threshold (modified from Borgert et al. (2005)).

2.2 Interactions

The interactions between different chemical components in a mixture may result in either a weaker (antagonistic) or a stronger (synergistic, potentiated) combined effect than the additive effect that would be expected from knowledge about the toxicity and mode of action of each individual compound. Interactions may take place in the toxicokinetic phase (i.e. processes of uptake, distribution, metabolism and excretion) or in the toxicodynamic phase (i.e. effects of chemicals on the receptor, cellular target or organ) (see section 2.2.1).

Addition: An additive effect occurs when the combined effect of two chemicals corresponds to the sum of the effects of each chemical given alone.

Antagonism: An antagonistic effect occurs when the combined effect of two chemicals is less than the sum of the effects of each chemical given alone (this phenomenon is well known for substances competing for the same hormonal or enzymatic receptor sites).

Synergism: A synergistic effect occurs when the combined effect of two chemicals is greater than the sum of the effects of each chemical given alone (e.g. the result of increased induction of metabolising enzymes when the effect is due to a metabolite).

Potentiation: Potentiation occurs when the toxicity of a chemical on a certain tissue or organ system is enhanced when given together with another chemical that alone does not have toxic effects on the same tissue or organ system (e.g. carbontetrachloride (CCl_4) toxicity to the liver is enhanced with isopropanol).

2.2.1 Mechanisms and causes of interactions

Interactions may take place in the chemical/chemical, toxicokinetic phase and/or in the toxicodynamic phase (Figure 2).

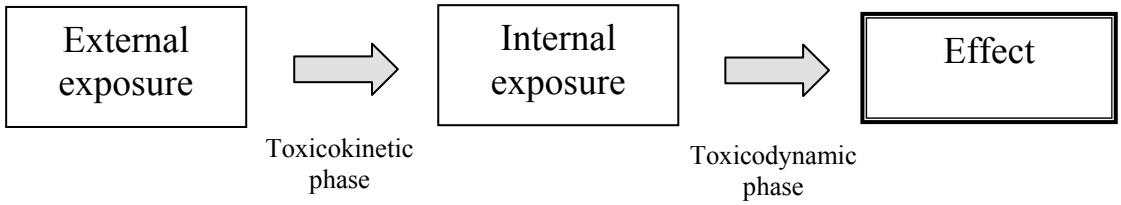

Figure 2. Relationships between external exposure and effect.

Alteration in the absorption, distribution, metabolism or excretion of a toxic compound related to exposure to another toxic compound is called toxicokinetic interaction.

Interactions with absorption can occur when an active transport process is involved, such as absorption of iron and cadmium. For instance, in iron-depleted subjects, an increased uptake of cadmium is seen because the expression of the transport protein is upregulated. However, most toxic compounds are absorbed via passive diffusion.

After absorption, chemicals are distributed throughout the body via the blood circulation or the lymphatic system. E.g. lipophilic compounds (PCB, PCDD/F) are protein-bound, and a more lipophilic compound can displace a less lipophilic one from the binding proteins in plasma. The free concentration of the less lipophilic compound is then increased, and there is a possibility for a more severe toxic effect. This type of interaction is often seen with pharmaceuticals.

For compounds, which are active as the parent compound, enzyme inhibition may reduce detoxication and thus enhance toxicity, whereas enzyme induction could enhance detoxication and thereby reduce toxicity. However, a majority of the chemicals which enter the body are metabolised (biotransformed). Metabolism can either increase or decrease the toxicity of a compound, and there are a number of possible interactions that can influence the outcomes. Different chemicals may compete for a given enzyme or co-factor and thus result in inhibition. Another scenario is interactions with the drug metabolising enzymes, resulting in

induction of the enzymes (increased amounts and activities). Inducers of the microsomal CYP enzymes are well known to result in either increased production of active toxic metabolites or reduced toxicity caused by increased detoxication, depending on which enzymes and pathways are affected and the biological activity of the parent compound and its metabolites.

The tissue doses of chemicals in a mixture can be predicted using physiologically based toxicokinetic (PBTK) models. This requires information about the interaction of all the components in the mixture, and this information has to be determined by experimentation. With complex mixtures, this would require an unrealistic high number of experiments.

Interference (inhibition) with excretion of toxic compounds are mostly seen when active transport processes are involved and can enhance toxicity. Simultaneous exposure to a compound that either alters the pH of the urine, or acts as an osmotic diuretic, can affect the excretion of chemicals and their metabolites.

Toxicodynamic interactions occur at the cellular receptor/functional target level. Generally, the effect of combined action of two components at the same target is unlikely to result in synergism/potentiation. Competition for a receptor will usually result in addition of effects or antagonism (effect inhibition). An antagonist regulates negatively the activity of an agonist. Partial agonists, on the other hand, will act as agonist in the absence or at low concentrations of other ligands. Weak agonists may, however, function as antagonists by occupying the receptor preventing the binding of a more potent ligand. Dynamic interactions may also occur when two or more components act at different receptors/target sites or induce an increased or reduced anti-oxidant capacity.

2.2.2 Complex similar action

Complex similar action occurs when two compounds act by the same mechanism (e.g. on the same target receptor), but do not produce an additive effect as would be expected if it was simple similar action. Both lower effects than additive (antagonism) and higher effects (synergism) may be observed. In cases where compounds are competing for the same hormonal or receptor sites, lower than additive effects are often observed. If the intrinsic activities and affinities for the receptor of two competing substances (similar action) are identical, an additive effect may be the result. This may be considered a special case of simple similar action.

2.2.3 Complex dissimilar action

Complex dissimilar action occurs when there is an interaction in the toxicokinetic or toxicodynamic phase, but the two compounds act by dissimilar mechanisms. For example diethyl ether and styrene have dissimilar action; diethyl ether is hepatotoxic and styrene is carcinogenic when metabolised to the active metabolite styrene oxide. Diethyl ether increases the concentration of CYP2E1, the enzyme that converts styrene into its carcinogenic metabolite, resulting in an increase in the carcinogenic styrene oxide (interaction in the toxicokinetic phase).

2.2.4 Dose-dependent variations in toxic effects of multiple chemical exposures

When studying the combined toxic effects of multiple chemical exposures, the main goal is to determine if additivity is the outcome of a combined action, or if interactions may occur. Interactions may remain constant over the total dose-span, or there may be dose-dependent variations. Critical, limiting steps in toxicokinetic and/or toxicodynamic pathways may become saturated or overwhelmed, and responses may be altered in a non-linear manner with increasing dose. This may affect metabolic processes, endocrine regulation as well as cellular defence and repair mechanisms. An increase in the exposure dose may e.g. shift additivity to synergism, toxic effects not seen without saturation of receptor or enzyme systems may appear or the metabolism of various chemical compounds may be modulated. For risk assessment of combined toxic effects of multiple chemical exposures, it is therefore of importance to know if dose-dependent variations in toxic effects occur, and if the variations take place at doses relevant to human exposure. Dose-dependent additivity and synergism have been demonstrated in female rats exposed to a mixture of thyroid-disrupting chemicals (TDCs) (Crofton *et al.*, 2005). In this study, additivity was seen at the lower doses of the mixture, whereas a greater-than-additive (synergistic) effect was seen at the three highest doses. In a follow-up investigation built on this study, a method for estimation of an interaction threshold limit is presented (Gennings *et al.,* 2007). In two reports by Moser and co-workers, cholinesterase inhibition and behavioral changes were determined in adult and 17-day-old Long Evans male rats following acute exposure to mixtures of organophosphorus pesticides (OPs). At the lower end of the dose-response curves, synergism was observed (Moser *et al.*, 2005). Also, in preweanling rats, the OP mixtures resulted in greater than additive responses, and the effects could only partially be attributed to the presence of malathion in the mixture (Moser *et al.*, 2006).

2.3 Test strategies to assess combined actions and interactions of chemicals in mixtures

Ideally, the chemical identity and the toxicity profile for all chemicals in a complex mixture should be identified, and the potential for combined actions and/or interactions should be determined for a wide range of exposure levels. However, the use of this approach is not realistic in assessing combined actions of most mixtures from a resource and logistical perspective. A number of different test strategies have therefore been presented to obtain toxicological information on mixtures with a limited number of test groups (Cassee *et al.*, 1998; Danish Environmental Protection Agency & Danish Veterinary and Food Administration, 2003).

2.3.1 Testing of whole mixtures

Testing of the whole mixture as such may seem to be the proper way to evaluate the hazard of a mixture. A simple method of carrying out such a study is to evaluate the effects of the mixture and of all individual constituents at one dose level. However, testing of whole mixtures will not provide data on combined actions and/or interactions between the individual components. This can only be achieved when information on dose-response for each single component is available. Therefore, this approach might be applied for assessing the combined toxicity of simple, defined chemical mixtures where the toxicological properties of each component are known or will be investigated. It may also be used for primary screening for potential adverse health effects (hazards) of mixtures that are not well characterised.

2.3.2 Physiologically based toxicokinetic (PBTK) modelling

For many chemicals the outcome of their metabolism is the major determinant of their hazard. In order to elucidate possible toxicokinetic interactions of chemicals in mixtures, PBTK-modelling may be used. PBTK models describe the disposition, metabolism and transfer of chemicals and their metabolites in various tissues of the body. Considerable knowledge about the role of metabolism of toxic substances has become available. Information on reactive intermediates, as well as detoxication pathways, is provided from a variety of *in vitro* and animal studies. In principle, the PBTK modelling may predict human metabolism and the relative contribution of metabolic pathways. When PBTK modelling is used to assess the metabolism of mixtures, it has been suggested that one of the components in the mixture should be regarded as the prime toxicant being modified by the other components (Cassee *et al.*, 1998; Danish Environmental Protection Agency & Danish Veterinary and Food Administration, 2003).

2.3.3 Isobole methods

The isobole method is widely used in the analysis of experimental data to demonstrate simple or complex similar action of binary mixtures in which both components act on the same target and by the same mechanism. An isobole is a line representing exposure doses or concentrations of two components or their mixture that lead to the same level of effect, for instance ED50. The combination index, I_c for the two compounds in the mixture is defined as:

$$I_c = d_1/D_1 + d_2/D_2,$$

where d_1 and d_2 are the dose (or concentration) levels of each chemical in the mixture, and D_1 and D_2 are the dose (or concentration) levels of the single compounds that produce the same level of response as produced by the mixture.

When $I_c = 1$, the combined action is addition (simple similar action)
When $I_c > 1$, the combined action is antagonism (complex similar action)
When $I_c < 1$, the combined action is synergism (complex similar action)

Isoboles representing synergism and antagonism between two compounds (A and B) are shown in Figure 3. In an isobole diagram, additive effect is illustrated by a straight line connecting the iso-effective doses of the single compounds. Dose combinations resulting in an isobole below the straight line correspond to synergism and dose combinations resulting in an isobole above the straight line is usually called antagonism (Sühnel, 1990; Kortenkamp & Altenburger, 1998; Sørensen *et al.*, 2007). However, a variant of the isobole method as described by Parrott and Sprague distinguishes between independent, less-than-additive and antagonistic effects (Parrott & Sprague, 1993).

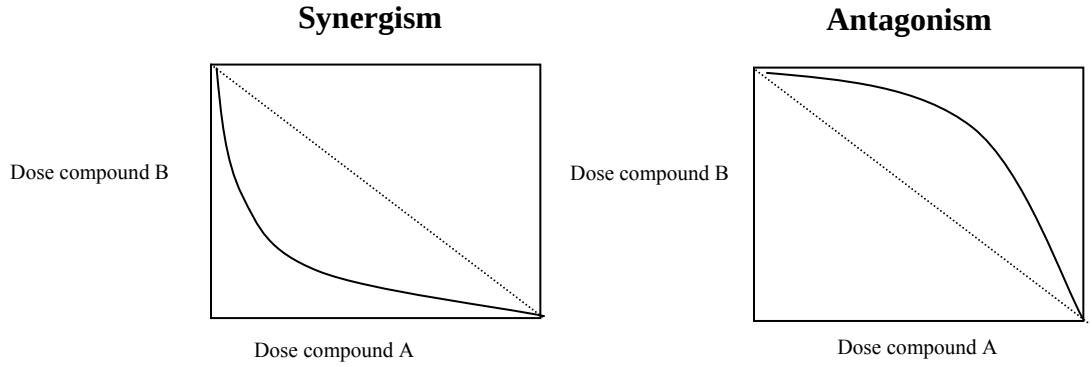

Figure 3. Isoboles for two compounds A and B (solid lines). The isoboles are based on different combinations of A and B that results in the same biological effect (e.g. ED50). The straight (dashed) lines show additive effect of A and B. Synergism (left diagram) is when A and B cause the same response at smaller doses than expected when compared to dose addition. Antagonism (right diagram) is when higher doses of A and B than expected is needed to cause the same biological effect when compared to dose addition (figure modified from the combined report from the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration in 2003).

The isobole method is very illustrative and can be used to analyse combined effects of compounds with different dose-response curves. However, the method requires a large amount of data both on the single components and the mixture. Furthermore, large standard deviations on the estimated isoboles may limit their interpretation.

In a recent study on inhibition of neurite outgrowth in mouse neuroblastoma cells NB2a, the isobole method was used to analyse combined effects of commonly used food additives in an *in vitro* developmental neurotoxicity test (Lau *et al.,* 2006). Significant synergy was observed between combinations of Brilliant Blue (E 133) and L-glutamic acid (E 620), and between Quinoline Yellow (E 104) and aspartame (E 951). The isobole method was also used to illustrate in vitro effects of combinations of the Penicillium mycotoxins citrinin, cyclopiazonic acid, ochratoxin, patulin, penicillic acid and roquefortine on mitogen induced lymphocyte proliferation. The results showed that the majority of toxin pairs tested produced combined effects lower than additive and indicated that the sum effect of all toxins was less than that expected from summation of potency-adjusted concentrations (Bernhoft *et al.,* 2004).

2.3.4 Comparison of individual dose-response curves

Comparison of the dose-response curves for one chemical (A) in the presence or absence of a second chemical (B) has been proposed for prediction of whether the combined action of a mixture is similar or dissimilar. In the case of simple similar action, the dose-response curve should shift to the left, and reach the same maximum response as for A alone, when a fixed dose of chemical B is added to various doses of chemical A. In the case of simple dissimilar action, the dose-response curve will shift upward when a fixed dose of chemical B is added to various doses of chemical A. In the case of simple dissimilar action, individual components in the mixture are not assumed to contribute to the overall mixture if they are present at

subthreshold levels (e.g. No observed adverse effect levels, NOAELs). For more details, see (Cassee *et al.*, 1998).

2.3.5 Other methods

Response surface analysis (RSA) uses multiple linear regressions to produce a statistically based mathematical relationship between the doses of each of the chemicals in a mixture and the effect parameter. Factorial designs, in which *n* chemicals are tested at *x* dose levels (x^n treatment groups) are statistical approaches that have been used to describe interactions between components in chemical mixtures for risk assessment. (For discussions of these matters, see (Groten *et al.*, 1996; Cassee *et al.*, 1998).

Compounds in a chemical mixture which contribute to toxicity can also be studied using advanced statistical multivariate models such as principal component analysis (PCA) (Principi *et al.,* 2006), supervised principal component analysis (SPCA) (Roberts & Martin, 2006) and partial least square projection to latent structures (PLS) (Wold *et al.,* 2001). These methods can also be applied in predictions-based methods such as Quantitative Structure-Activity Relationships (QSARs) (Papa *et al.,* 2005) can thus assist in identifying combined actions and interactions of chemicals in mixtures.

3 Combined actions with different toxicological endpoints

3.1 Local irritation

The main biological barriers protecting the body to xenobiotics are the intact epithelia of the eyes, airways and lungs, gastrointestinal tract together with the stratum corneum of the skin. These barriers may be damaged from combined actions or interactions of chemicals. Cells in the eyes, skin, respiratory tract and gastrointestinal tract are active in metabolising xenobiotics, and induction, depletion or inhibition of the responsible enzymes by the combined action of chemicals can result in local irritative effects. The blinking reflex of the eye and the mucosciliary escalator of the airways constitute additional defence mechanisms.

3.1.1 Skin irritation

The skin consists of two layers: the epidermis, and an underlying layer, the dermis. The epidermis constitutes the major barrier to foreign compounds, mainly those being hydrophilic substances. In contrast, many lipophilic compounds may be absorbed more or less easily through the skin. Several transport processes are involved in absorption of chemicals through the skin, before they reach the circulation. The rate-limiting step in undamaged skin is passive diffusion through stratum corneum of the epidermis. This layer is not impermeable to water, however, it is practically impermeable to large molecules (MW>500) regardless solubility.

Skin irritation is most often studied in animal experiments or human volunteers. However, experiments with skin organ cultures and reconstructed human epidermal tissue cultures are promising and will probably be more used in the future. Compounds that modulate the barrier function of the skin may dramatically change the effects of chemical irritants. Especially dehydration, but also delipidisation of the skin, is known to decrease the permeability function.

Among examples of combined action is tandem application of retinoic acid and sodium lauryl sulphate, which has been shown to cause synergistic, non-specific effects as skin irritants. Shortly after application of sodium lauryl sulphate, increased water loss of the epidermis is measured, but this is delayed after application of retinoic acid. Various chemicals have been used in dermatological preparations in order to enhance the absorption of drugs with both additive and synergistic effects as a result. Dimethyl sulfoxide (DMSO) used as solvent vehicle for radiolabelled parathion resulted in greater absorption of radioactivity than using acetone as solvent. Moreover, sodium lauryl sulphate enhanced parathion absorption with both vehicles. In this experiment, the effect of methyl nicotinate and $SnCl_2$ on absorption of radioactive parathion was also studied and several complex interactions were noted (Qiao *et al.,* 1996).

Addition of lipids to the skin as a component of creams protects workers against damage caused by organic solvents. Other skin protective agents, such as mineral wax or beeswax, protects the skin against sodium lauryl sulphate and combined ammonium hydroxide/urea treatment, and moreover protects against induction of allergic contact dermatitis (Zhai *et al.,* 1998).

3.1.2 Eye irritation

The conjunctiva of the eye consists of a non-keratinized epithelium. This layer contains blood vessels, nerves, inflammatory cells and glands. The conjunctival glands secrete the precorneal tear film, which is very important for proper ocular function. Upon irritation of the eye the flow of tears is increased and the irritant is diluted or rinsed away. The epithelial cell layer constitutes a barrier against entrance of xenobiotics and excess water into the stroma. Several tissues of the eye contain various CYP enzymes, and the enzyme activity is relatively speaking, especially high in the vascularised tissues. Contamination of the eye with surfactants (such as ordinary soap) and detergents causes immediate stinging and burning, without causing serious injury. Threshold responses of nasal and eye irritation of various single solvents and of solvents in combination have been assessed (Cometto-Muniz *et al.*, 1997). Additive effects were observed to varying degree, and as the number of components and the lipophilicity of the compounds increased, so did also the degree of agonism (Cometto-Muniz *et al.*, 1997). The most complex and lipophilic mixtures had synergistic effects especially on eye irritation.

The *in vitro* Hen's Egg Test at the Chorion Allantois Membrane (HET-CAM) has been used to examine combined action of compounds occurring as disinfection by-products in swimming pools. The compounds tested included halogenated carboxyl compounds (HCCs) which act as precursors during the formation of chloroform. These compounds are irritating individually and some of the mixtures were even more active than single compounds at lower concentrations. Moreover, when these mixtures were combined with aqueous chlorine, a number of HCCs exhibited significantly the previously seen enhanced effects (Erdinger *et al.,* 1998).

3.1.3 Irritation to the respiratory tract

Several cell types of the respiratory tract are extremely vulnerable to various types of injury. The Clara cells are the major site of injury (e.g. necrosis) from xenobiotics that are metabolized by the CYP enzymes. Compounds irritating the airways often result in bronchoconstriction. Other types of acute cell toxicity caused by irritation may result in necrosis, increased permeability and oedema. The cytotoxic effect in the respiratory tract is often general and non-specific, and is related to water solubility of the compound. If the xenobiotic is an aerosol, the particle size will determine the site of action in the respiratory tract.

The interaction between ozone and nitrogen dioxide has been studied in a number of human clinical studies and in animal and *in vitro* studies. Based on the results from the human studies only, there is not enough evidence to conclude that there is an interactive effect on lung function after simultaneous exposure to ozone and NO_2. However, both animal and *in vitro* studies suggest at least an additive irritation effect after combined exposure to these two compounds.

3.2 Genotoxicity and carcinogenicity

Genotoxic compounds cause damage to the genetic material (DNA) of cells. The damage can on one hand be repaired or cell death can be induced. However, if DNA repair fails, the DNA change is propagated through subsequent cell divisions and may result in mutations. Mutations caused by genotoxic compounds can cause irreversible, adverse health effects even at low exposure levels.

Humans may be exposed to a number of complex mixtures containing both genotoxic compounds and/or other compounds that can have modifying effects on the genotoxicants. There is much knowledge about the genotoxic effects of individual compounds. However, less is known about possible interactions or combined actions between genotoxic chemicals. Interactions that affect the bioavailability, metabolism (metabolic activation or detoxication), and DNA binding or repair of DNA damage may influence the genotoxicity of a complex mixture. There are a number of different mechanisms by which genotoxic compounds can damage DNA, which make it very difficult to predict the outcome of exposure to chemical mixtures. Examples of types of primary DNA lesions are: DNA adducts, DNA strand breaks, DNA base modifications, loss of DNA bases and DNA cross-links. When repair of these primary changes fails, different types of mutations may arise, such as point mutations, chromosomal or structural mutations, aneuploidy (numerical aberrations) and recombinations.

3.2.1 Interactions between genotoxic substances

There exists quite a lot of knowledge about the overall genotoxic potentital of complex environmental mixtures, but relatively few studies have been performed on mixtures with known chemical composition. Several studies have been performed on the interactions between a mutagen and a co-mutagen or anti-mutagen, and on mixtures of genotoxicants showing no interaction.

In complex mixtures consisting of chemicals of a similar class such as polycyclic aromatic hydrocarbons (PAH), dose or effect addition might be assumed. In binary mixtures of PAH, both synergistic and antagonistic effects have been demonstrated. In a complex mixture of PAH (PAH in urban air), a linear correlation between *Salmonella* mutagenicity and PAH concentrations was demonstrated at lower PAH content, while at higher PAH concentrations the mutagenicity increased much more than the PAH content, indicating a synergistic or potentiating effect.

Toxicodynamic interactions have been demonstrated in *in vivo* studies with heterocyclic aromatic amines (cooked food mutagens), which showed that the combination effect of these mutagens was synergistic at specified dose levels (Hasegawa *et al.,* 1994; Hasegawa *et al.,* 1996).

3.2.2 Carcinogenicity

The understanding of carcinogenesis was in the 1940s, based on experimentation with skin carcinogenesis, operationally divided into two distinct processes, initiation and promotion. Various compounds were shown to affect either process. The first combinational effect in carcinogenesis, demonstrated in 1924, was scarification of the skin prior to application of mineral oil, which enhanced carcinogenesis.

The development of cancer is a complicated multi-stage process in which a large number of factors interact to disrupt normal cell growth and division. Today, there is a general agreement to distinguish between genotoxic and non-genotoxic carcinogens in the risk assessment related to human cancer (O'Brien *et al.*, 2006; Barlow *et al.*, 2006). Genotoxic carcinogens are compounds that affect DNA. Characteristic for these carcinogens is that the compounds itself or its active metabolites react covalently with DNA in the target cells and cause mutations, which thereafter may lead to neoplastic development. Thus, genotoxic

carcinogens are able to act as initiators in the carcinogenic process. Theoretically, exposure to one single molecule could produce DNA damage and as a consequence there is no exposure without a certain level of risk. The traditional approach using a NOAEL is therefore not appropriate for genotoxic compounds. However, when carcinogenicity data from animal bioassays are available, a point of departure (POD) on the dose-response curve (usually the BMDL10) may be defined. Margins of exposures (MOEs) may then be calculated by dividing the POD with human exposure data in order to give an advice to risk managers.

Non-genotoxic carcinogens do not directly affect DNA, but causes cancer via mechanisms that indirectly lead to neoplastic transformation or promote neoplastic development. For the non-genotoxic carcinogens it is generally considered that there is a dose threshold below which no significant effect will be induced (Dybing *et al.,* 2002). Homeostatic mechanisms are able to counteract harmful effects at low intake levels, and adverse effects are only expected to occur at higher intake levels. For non-genotoxic carcinogens, health-based guideline values have therefore been derived from the traditional NOAEL approach.

3.2.2.1 Combination effects in initiation

Compounds causing mutations or gene rearrangements in a target cell are potential tumour initiators. The initiated cell has an altered response to external stimuli resulting in abnormal division or apoptosis. The initiation-promotion protocols for mouse skin papilloma and rat liver preneoplastic foci formation are the most widely used methods for studying such combined effects. More recently, the mouse and rat newborn systems and transgenic models have also been used. Because the suspected cancer initiating compound is administered to the animal in the newborn systems at day one, and one or two weeks after birth, these methods may be very sensitive.

An effect which is stronger or weaker than the expected additive effect is seen for several combinations of initiators. A synergistic effect was observed with mixtures of heterocyclic aromatic amines in rat liver tumourigenesis, while with mixtures of PAH both synergistic and antagonistic responses have been observed.

In a recent study an experimental 2-year rodent cancer bioassay with either tetrachlorodibenzo-p-dioxin (TCDD), PCB-126 and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or a mixture of these three compounds was performed (Walker *et al.*, 2005). The doses in the mixture study were based on the toxic equivalent factor (TEF) values for the compounds, so that each compound would provide a third of the total dioxin TEFs to the mixture. Dose-response modelling based on statistical analysis indicated that the shape of the dose-response curves for hepatic, lung and oral mucosal neoplasms was the same in studies of the three individual chemicals and the mixture. The main conclusion from the study was that the hypothesis of potency-adjusted dose addition for induction of rodent neoplasms for a defined mixture of dioxin-like compounds cannot be rejected (Walker *et al.*, 2005).

3.2.2.2 Co-carcinogenesis

Co-carcinogenesis occurs when a non-carcinogen increases the action of a carcinogen. Co-carcinogens act mainly by either of three different mechanisms; firstly, by compounds increasing the penetration of carcinogens through epithelial barriers. Examples of this are organic solvents which increase the penetration of PAH through the skin, and a fatty diet which increases the absorption of dietary lipophilic carcinogens. Secondly, there are compounds that increase the activation or the impact of the genetic damage of the initiator. An example is benzo[e]pyrene which increases the potency of benzo[a]pyrene by increasing

its activation and DNA-binding. When a combined exposure of tars and mineral oil residues are applied on skin together with benzo[a]pyrene, the fraction bound to DNA is reduced, resulting in lower carcinogenic potency. In female mice exposed orally to benzo[a]pyrene as a part of a coal tar mixture, the formation of forestomach tumours seemed to be in accordance with the benzo[a]pyrene content of the mixtures (SCF, 2002b). However, in addition, the coal tar mixtures also produced increased incidences of adenomas and carcinomas at several different sites. The third mechanism is when cell turnover is increased by co-carcinogens. This increases the carcinogenic process in at least two ways: dividing cells are more vulnerable to mutations than resting cells, and rapid cell turnover leaves less time for the cell to repair damaged DNA. Two classical examples of this mechanism are scarification of the skin in skin cancer and partial hepatectomy in liver cancer. Chemicals and physical agents that act by this mechanism are phorbol esters, catechol and asbestos. As mentioned above, several combination effects are possible in carcinogenesis, the effects can be specific and a co-carcinogen can increase, decrease or have no impact on the effect of different other carcinogens.

Anticarcinogens are compounds that decrease the response of carcinogens and this effect can occur at any stage in the carcinogenic process.

3.2.2.3 Promotion

Promotion is a process that gives an initiated cell a growth advantage over normal cells, and it is quite similar to co-carcinogenesis. It is demonstrated that promoters can act synergistically when they act by different mechanisms. Due to the fact that tumour promoters in many cases are both organ- and initiator-specific, their identification is difficult.

3.2.2.4 Combination effect at later stages

Subsequent treatment after initiation with a direct-acting initiating agent on mouse skin can be shown to increase the tumour response, an effect that has been called conversion.

3.2.2.5 Conclusion

Carcinogenesis is affected by different chemical, physical or biological agents and it is clear that in most cases tumour development is caused by combined toxic effects. Initiators, promoters, converters and co-carcinogens all may act in concert to potentiate the final tumour outcome. In some cases anticarcinogens can potentiate each others' effect. Potentiation can be caused by compounds affecting the same step by different mechanisms. The possibilities for combination effects in carcinogenesis are therefore many, and they are difficult and often impossible to predict. Combination effects at low and realistic human exposures, which are not possible to explore experimentally in animal studies, may be different from those obtained by experimentation at high doses.

3.3 Reproductive toxicity

Exposure to chemical mixtures has been reported to give impaired male and female reproductive functions which interfere with the capacity to fertilise, with the fertilisation itself or with the development and implantation of the fertilised ovum. Moreover, harmful effects have been documented on the progeny induced by chemical mixtures and can occur both before and after birth and up to puberty.

3.3.1 Testing interaction of teratogenic compounds by using *in vitro* studies

More than 20 *in vitro* tests have been used for testing of teratogenic effects. These can be divided in two different categories; morphogenetic tests, based on the use of isolated embryos, organs or regenerating tissues, and non-morphogenetic tests, based on cell cultures. However, the results from *in vitro* testing in the field of reproduction and development are difficult to interpret and use in risk assessment, because of the complex nature of the *in vivo* reproductive processes involving mother, father and foetus.

3.3.2 Examples of interaction of reproductive toxicants *in vivo*

Three compounds found at hazardous waste sites (trichloroethylene (TCE), di-(2-ethylhexyl)phthalate (DEHP) and heptachlor (HEPT)) were combined using 5 dosages of each agent in a 5x5x5 full-factorial design. Four dose levels (1-4) were selected on the basis of a preliminary dose-ranging study performed for each chemical individually. Dose level 0 indicated the absence of an agent. Combination of TCE and DEHP caused synergism, appearing as a negative effect on maternal weight gain, and induction of prenatal loss, and full litter resorption. Combination of DEHP and HEPT had synergistic effect on maternal death and antagonistic effects on the parameters maternal weight gain, full litter loss and pup weights on day one and on day six. HEPT potentiated the effect of TCE and DEHP on prenatal loss and full litter resorption. The TCE-HEPT interaction was antagonistic concerning adverse effects on full litter loss. Several mechanisms of interaction were found to be involved in these results. The authors concluded that the assumption of additive toxicity may be inadequate in some situations. When these compounds are administered simultaneously at the tested doses, several mechanisms of interaction are involved. Because the mechanism of action was not elucidated, it was, however, not possible to classify whether the interaction was of complex similar or complex dissimilar nature (Narotsky *et al.,* 1995).

In a recent study with C57BL/6 mice, a significant increase in the incidence and severity of forelimb ectrodactyly in foetuses exposed to cadmium and all-trans retionic acid (RA) was reported, when compared to the results with corresponding doses of cadmium or RA given alone (Lee *et al.,* 2006). When mice were exposed to subthreshold doses of both cadmium (0.5 mg/kg) and RA (1 mg/kg), the combined treatment exceeded the threshold, resulting in forelimb ectrodactyly in 19% of the foetuses. Moreover, co-administration of cadmium and RA at doses exceeding the respective thresholds showed a synergistic effect, resulting in 92% of foetuses with the forelimb defect, as opposed to 10% if the response were additive.

In conclusion, data from several *in vivo* experiments studying reproductive toxicity suggest that the prevailing outcome of exposure to mixtures often was dependent of the dose. Low doses of combinations in most cases produced no effects or additive effects, whereas higher doses more typically produced antagonistic or synergistic effects.

3.4 Endocrine disrupting chemicals

During the last years a growing list of chemicals reported to show endocrine activity has appeared. The increased occurrence of abnormal sexual development in wild-life, the world-wide increased incidence of testicular cancer, developmental disorders of the male reproductive tract and female breast cancer have by some scientists been suggested to be related to exposure to these so-called endocrine disrupting environmental chemicals. Most of them are weak agonists or antagonists of the steroid receptors, such as the oestrogen receptor, however their potencies are several thousand fold lower than those of the natural steroid

hormones. It is therefore difficult to explain the possible health effects caused by such compounds only based on the toxicity from exposure to the single compounds. However, humans are exposed to mixtures of xenoestrogens, and it is therefore important to consider whether the interaction effects of these compounds with respect to endocrine disruption might occur. The systematic analysis of combined effects of xenoestrogens has only recently begun and a few examples will be given here.

3.4.1 *In vitro* studies

In a report by Wang and Kurzer, genistein and coumestrol at low concentrations significantly enhanced oestradiol-induced DNA-synthesis in MCF-7 cells, while at high concentrations inhibition was observed (Wang & Kurzer, 1998). Thus, the type of interaction was concentration-dependent. This study was performed very thoroughly, including dose-response curves of the single compounds. In another report, the dose-dependent combination effect of the phytoestrogens genistein, daidzein and coumestrol, and 17β-oestradiol on cell proliferation and apoptosis induction in human MCF-7 breast cancer cells was studied (Schmidt *et al.*, 2005). In the presence of phyto-oestrogens and low levels of 17β-oestradiol, no additive or antagonistic effects on proliferation were seen. However, the observed increase in cell number was explained by inhibition of apoptosis (Schmidt *et al.*, 2005). Mixtures of eight oestrogenic compounds, including hydroxylated PCBs, benzophenones, parabens, bisphenol A and genistein, were tested in a recombinant yeast oestrogen screen. The mixture was prepared at a mixture ratio proportional to the potency of each individual component. Four approaches for calculation of different additive combination effects were used. The authors concluded that dose addition and the use of the toxicity equivalent factor approach, was a valid method for the calculation of additive mixture effects, showing excellent agreement between prediction and observation. Substantial mixture effects were reported, even though each chemical was present at levels well below its no observed effect concentration (NOEC) or EC_{01} (Silva *et al.*, 2002).

In another report, the combined dose additive effect of 11 xenoestrogens on 17β-oestradiol action *in vitro* led to a dramatic enhancement of the natural hormone's action, even when each single agent was present below its NOEC (Rajapakse *et al.*, 2002).

3.4.2 *In vivo* studies

Oral administration for three days of the phytoestrogen genistein (Gen, 100 mg/kg bw) in female Wistar rats resulted in significant increase in uterine weight comparable to the effect of ethinyloestradiol (EE, 30 µg/kg bw), and co-administration of EE and Gen resulted in an additive effect on the uterine wet weight (Schmidt *et al.*, 2006). In the same study, bisphenol A (BPA, 200 mg/kg bw) alone did not stimulate the uterine wet weight significantly. However, BPA significantly antagonised the effect of EE on the uterine epithelium. In combination with Gen, BPA was also able to antagonise the stimulatory effect of Gen on the uterine epithelium (Schmidt *et al.*, 2006). In a different study with female Wistar rats, Gen (10 mg/kg bw for three days) caused a faint stimulation of the uterine wet weight in ovariectomized (OVX) rats (Diel *et al.*, 2006). In both intact and OVX animals co-treated with oestradiol (1 or 4 µg/kg bw for three days), Gen had no effect on uterine wet weight. However, in OVX animals co-treated with oestradiol, Gen antagonised the oestradiol-stimulated increase of the uterine epithelial height and epithelial PCNA (proliferating cell nuclear antigen) mRNA (Diel *et al.*, 2006).

In a study with female neonates of Sprague-Dawley rats exposed to mixtures of non-ortho PCBs, PCDDs and PCDFs, indications of tissue-specific additive and non-additive/ antagonistic effects, but no synergism, were observed with increasing doses of organochlorines, or in combination with ethynyloestradiol (Desaulniers *et al.,* 2003). In female Long Evans rats, dose-dependent addition and synergism of 18 different thyroid-disrupting chemicals (TDCs) were demonstrated (Crofton *et al.*, 2005). In this study, TDCs' effect on total thyroxine concentration in serum was measured. At the lowest doses of the mixtures there was no deviation from dose addition, but at the three highest mixture concentrations there was a greater than additive effect (Crofton *et al.*, 2005).

Recently the developmental effect of three antiandrogens: vinclozolin, procymidone and flutamide, alone or in combination, were studied in male Wistar rats. With anogenital distance as endpoint, the combined effects of the three anti-androgens were dose additive. Nipple retention was slightly higher than expected from prediction of dose addition (Hass *et al.,* 2007). In another report the ability of a mixture of vinclozolin, procymiodone and flutamide to induce disruption of male sexual differentiation after perinatal exposure was investigated in rats. Changes in weight of reproductive organs and of androgen-regulated gene expression in prostates from male pups were chosen as endpoints. The combined effects of the three antiandrogens were dose additive with all endpoints. Single administration of vinclozolin, procymidone and flutamide at low doses did not produce significant effects in the weight of seminal vesicles and on prostate-binding protein subunit C3. These data support the idea that antiandrogens act together to produce marked combined effects after simultaneous exposure at doses that individually produce, small statistically insignificant responses (Metzdorff *et al.,* 2007).

In conclusion, studies have reported both dose addition and synergism of endocrine disrupting chemical mixtures. However, several of the more recent well-designed studies clearly show that the effects of oestrogenic compounds do not deviate from dose addition. At present more data are needed to address whether synergism is a possibility to be taken into account in the risk assessment of weakly oestrogenic chemical mixtures.

3.4.3 Fish and wildlife studies

Several chemicals have been shown to have endocrine disruptive effects in fish and wildlife species. Among chemicals of concern are diethylstilbestrol, coumestrol, bisphenol A, dichlorodiphenyldichloroethylene (DDE), nonylphenol, endosulfan, and dieldrin (Watson *et al.,* 2007), as well as natural oestrogen and ethinylestradiol. The most worrying effects of endocrine disrupters in fish and wildlife have been linked to effects on the reproductive system and the thyroid system, resulting in decreases of fitness and fecundity. With respect to exposure to mixtures of contaminants, most focus has been put on oestrogenic compounds, since they all have a similar mode of action via the estradiol hormone receptor (ER) proteins (Matthiessen & Johnson, 2007). Due to release of oestrogenic compounds to aquatic environments, especially from sewage, there has been particular focus on exposure to mixtures which contain different oestrogenic compounds. Currently available information suggests that mixtures of estrogenic compounds act no more than additively (Brian *et al.*, 2007; Matthiessen & Johnson, 2007). There is however, also concern about antiestrogenic properties of some chemicals, especially of drugs (Kawahara & Yamashita, 2000). Thus, in the environment animals can be exposed to mixtures of both oestrigenic and antioestrogenic compounds (Houtman *et al.,* 2004).

There is also concern about androgenic and anti-androgenic effects of chemicals in fish and wildlife. These effects are thought to be mediated through the androgen receptor (AR) (Goksoyr, 2006). The ability of a range of chemicals to bind to the AR has been tested (Fang *et al.*, 2003). There are also serious concern about effects of thyroid disruptive chemicals in fish and wildlife (Rolland, 2000; Fang *et al.*, 2003; Mastorakos *et al.*, 2007; Tan & Zoeller, 2007). Studies on free-ranging fish, birds, seals and polar bears have shown associations between pollutant levels, in particular PCBs and levels of circulating hormones (Van den Berg *et al.*, 1994; Brown *et al.*, 2004; Jørgensen *et al.*, 2004; Braathen *et al.*, 2004; Sørmo *et al.*, 2005). The modes/mechanisms of action of thyroid disrupters are multiple, and little is known about the combined effects of several chemicals that interfere with the thyroid homeostasis.

In Norway, there has been particular focus on ecological risk assessment of persistent organic pollutants (POPs) in the Arctic (Skaare *et al.*, 2002; Jenssen, 2006), and several studies have focused on endocrine effects related to POPs in Arctic wildlife and fish. Since these generally are field studies, the approach has been to study associations between organ or blood concentrations of various POPs and levels of various endocrine variables, such as plasma hormone levels. It is important to be aware that reports of associations between POP levels and endocrine variables not are direct evidence of cause-effect relationships.

In polar bears (*Ursus maritimus*) from Svalbard and the Barents Sea, levels of various POPs were negatively correlated to plasma levels of thyroid hormones (Skaare *et al.*, 2001; Braathen *et al.*, 2004) and to vitamin A (retinol) (Skaare *et al.*, 2001). Furthermore, pesticides have been shown to contribute negatively, and PCBs to contribute positively, to the variation in the plasma cortisol, even though the overall contribution of the organochlorinated compounds (OCs) to the plasma cortisol variation was negative (Oskam *et al.,* 2004). In male polar bears, plasma testosterone levels correlated negatively with levels of pesticides and PCBs (Oskam *et al.*, 2003; Ropstad *et al.*, 2006), whereas in females a positive correlation between PCBs and progesterone levels was found (Haave *et al.*, 2003; Ropstad *et al.*, 2006). Several studies have also addressed the effects of POPs on health and immune function related aspects (Bernhoft *et al.*, 2000; Skaare *et al.*, 2002; Lie *et al.*, 2004; Lie *et al.*, 2005). These studies show associations between POPs and immune function variables, which strongly indicate that high levels of POPs may impair resistance to infections in polar bears. Furthermore, there are strong indications that POPs may interfere with the size of the reproductive organ in male and female polar bears (Sonne *et al.,* 2006) and with bone density (Sonne *et al.,* 2004), possibly via endocrine disruptive mechanisms.

In glaucous gulls from Bjørnøya, negative associations between plasma levels of thyroxin (T4) and blood levels of several POPs, and positive associations between plasma levels of progesterone and several POPs have been reported in males, but not in females (Verreault *et al.*, 2004; Verreault *et al.*, 2006). Testosteron levels did not correlate with any of the POPs in glaucous gulls (Verreault *et al.*, 2006). Effects of POPs on behavioural and morphological traits in glaucous gulls at Bjørnøya have been suggested to be linked to these endocrine disruptive effects of POPs (Bustnes *et al.,* 2001; Bustnes *et al.,* 2002). Negative associations have also been reported between various POPs and vitamins (A and E) in seabirds, such as European shag (*Phalacrocorax aristotelis*), kittwakes (Rissa *tridactyla*) and Brünnchs guillemot (*Uria lomvia*) (Murvoll *et al.,* 1999; Murvoll *et al.,* 2006a; Murvoll *et al.,* 2006b; Murvoll *et al.,* 2007).

In Arctic charr, PCB appear to affect plasma cortisol regulation (Jørgensen *et al.,* 2002), and the effects were reported to be linked to brain glucocorticoid receptor downregulation (Aluru *et al.,* 2004).

In seals, several studies have reported associations between body concentrations of various POPs and plasma levels of thyroid hormones and between POPs and vitamins (Jenssen *et al.*, 1994; Jenssen *et al.*, 2003; Nyman *et al.*, 2003; Routti *et al.*, 2008). There are also indications that POPs affect bone metabolism in seals via intereference with thyroid hormone and vitamin D homeostasis (Routti *et al.*, 2008). Even though PCB associated alterations of hepatic steroid metabolism in harbour seals have been reported (Troisi & Mason, 2000), associations between POPs and steroid hormones do not seem to be reported in seals.

In conclusion, there are strong indications that POPs have endocrine disruptive effects in Arctic wildlife and fish. These effects may be of ecological significance, and may affect population dynamics of the species.

Since animals in the wild are exposed to complex mixtures of different compounds, these suspected endocrine effects may be caused by single compounds or by mixtures of compounds. Currently, there is little knowledge on the extent to which the effects are caused by single compounds or combinations of different compounds.

3.5 Neurotoxicity

Exposure to some chemicals, including drugs, has been linked to induction of persistent changes in the nervous system. Neurotoxicity is any adverse effect on the chemical signaling, structure and/or function of the nervous system during development or at maturity induced by chemical or physical influences. The nervous system is particularly vulnerable to toxic compounds during the period of development. During this period there is extensive interaction between the brain and other organs, especially the sex organs and the thyroid gland. An example of toxic interaction in developmental toxicity is the interaction between dimethoate (a pesticide) and lead. Pretreating rats with dimethoate accentuates the developmental neurotoxicity of lead. Pretreating with lead has a similar effect on dimethoate toxicity, but not as pronounced (Nagymajtenyi *et al.,* 1998).

3.5.1 Consequences of adverse effects on the nervous system

Mature neurons have limited capability of regeneration, and are therefore at greater risk for permanent damage after a toxic injury than many other cells in the body. Proper function of the nervous system depends on an electrochemical balance and large energy supply. Chemicals affecting neuronal targets such as membranes, intracellular transport or energy supply may cause neurotoxicity.

The molecular mechanism of action is unknown for most of the toxicants affecting the central nervous system. In the peripheral nervous system, a well known mode of action is inhibition of the anterograde transport of cellular components causing peripheral neuropathy. Interaction is possible between chemicals that affect this transport system, regardless of which target organs are involved.

3.5.2 Examples of mechanisms of interactions

Repeated seizure activity in the brain is accompanied by increased synaptic strength, a phenomenon called kindling. Experimentally induced kindling is used to study changes in nervous system excitability. The pesticide chlorpyrifos caused a more rapid occurrence of kindling, an effect that was additive with xylene (Wurpel *et al.,* 1993).

When a chemical without neurotoxic effect interacts with a neurotoxicant and increases its response or effect, this is an example of potentiation. Hexane or hexanedione is more neurotoxic at the same dose level when a ketone is co-administered. The mechanism has not been elucidated; the interaction is probably related to changes in metabolism, distribution or excretion of the neurotoxic agent. Non-neurotoxic compunds can make the blood-brain-barrier (BBB) more penetrable to ionic compounds, mannitol is an example, although not relevant for food. If the cytostatic doxorubicin (adriamycin) is given together with mannitol (1.4 M) enhanced neurotoxicity is observed (Neuwelt *et al.,* 1981; Kondo *et al.,* 1987).

3.5.3 Examples of interactions between agents

Insecticides: The toxic effects of combined exposure to organophosphorus insecticides are generally considered to be a result of dose addition. These structurally related compounds share certain characteristic toxicological actions, specifically the inhibition of acetylcholine esterase leading to accumulation of acetylcholine in the nervous system. However, in addition to inhibition of acetylcholine esterase, a variety of agent-specific clinical signs are also induced in experimental animals after exposure to organophosphorus compounds.

An acute neurotoxicity study in male rats examined the effect of parathion and permethrin in a formulated product and in a commercially formulated mixture of the two compounds. Methyl parathion was found to increase the LD_{50} of permethrin because of an inhibition of carboxylesterase involved in the main metabolic pathway of permethrin. The decrease in carboxylesterase activity presumably caused an increased permethrin concentration with increased toxicity as a result. Permethrin also decreased the methyl parathion-induced inhibition of brain cholinesterase activity (Ortiz *et al.,* 1995).

In two reports, cholinesterase inhibition and behavioral changes were determined in adult and 17-day-old Long Evans male rats following acute exposure to mixtures of organophosphorus pesticides (OPs) (Moser *et al.*, 2005; Moser *et al.*, 2006). Two different mixtures were tested, one containing the following five pesticides: chlorpyrifos, diazinon, dimethoate, acephate and malathion, and another containing the same pesticides except malathion. Malathion has been shown to produce synergistic interactions with certain OPs. In the study with adult rats, significant deviation from dose addition for several neurochemical and behavioural endpoints, except tail-pinch response, was observed. At the lower end of the dose-response curves, synergism was observed (Moser *et al.*, 2005). Also, in preweanling rats the OP mixtures resulted in greater than additive responses, and the effects could only partially be attributed to the presence of malathion in the mixture (Moser *et al.*, 2006).

Organic solvents: Several studies have shown that exposure to mixtures of solvents may be more harmful than single exposures, and in several cases exposure to combinations gave greater effects than mere addition (WHO & Nordic Council of Ministers, 1985).

Metals: Lead, mercury and manganese exhibit interaction with other neurotoxicants. Absorption of lead from the gastrointestinal tract is increased by ethanol. Increased absorption

of neurotoxic divalent metal ions such as lead and manganese is seen in humans or experimental animals with low iron status (Berglund *et al.*, 1994).

In a recent report, antagonistic effects *in vitro* of some concentrations of the neurotoxicants methylmercury and PCB153 in PC12 cells were observed (Vettori *et al.*, 2006). Cell viability and intra-cellular dopamine were used as endpoints.

In conclusion, potential interaction of neurotoxic chemicals by additive and synergistic effects/potentiation should always be considered in the risk assessments, especially when exposures are above effect thresholds.

3.6 Immunotoxicity

3.6.1 Direct toxic effects on the immune system

Toxic responses to the immune system can be determined experimentally *in vivo* using a number of immunological methods. A variety of mechanisms are involved in immunotoxicity, an example being the thymic toxicity of TCDD that is due to an effect on the thymic epithelial cells mediated by the Ah-receptor. Another example is the immuntoxic effect of aflatoxin that presumably is linked to inhibition of protein synthesis.

3.6.1.1 Combined actions and interactions in immunotoxicity

Few genuine immunotoxicology studies have been performed on chemical mixtures. One study in mice with a mixture of 25 groundwater contaminants was found to be myelotoxic (Germolec *et al.*, 1989). Other effects observed were decreased cellularity in the bone marrow, decreased antibody formation to sheep red blood cells and an increased number of parasitised red blood cells in an infection model. None of the individual contaminants were present at sufficient concentration in the chemical mixture to be solely responsible for the observed immunological effects. The total dose, however, was relatively high and the results of this study indicated some kind of additive immunotoxic effect. Based on few experimental data, it seems that chemicals sharing a common immunotoxic mechanism may have additive effects, whereas competition for metabolising enzymes may antagonise the immuntoxic effects.

3.6.2 Allergy

Skin contact with certain allergenic chemical compounds (allergens or haptens) may induce contact sensitisation, which is a cell-mediated process. Most contact allergens are small molecules (MW < 600 Da). Contact sensitisation, once established, is a life-long process, but the effect may become weaker if exposure is avoided. Allergic contact dermatitis, which is a cell-mediated process (type IV immune reaction), can develop in contact-sensitised persons as a result of re-exposure to the specific chemical. Patch testing in humans is used to document contact-sensitisation to environmental chemicals. In this test, contact allergy is assayed by re-exposing a 0.5 cm^2 large skin area on the upper back to a specific chemical. A positive test is seen with clinical signs showing redness, infiltration and possibly vesicles. The degree of symptoms in a test may be quantified by comparisons with standard series using the most frequently sensitising chemicals such as certain metals, preservatives, fragrances and others. Animal models of skin allergy are also available.

Environmental chemical compounds may also induce IgE sensitisation. Sensitisation resulting in IgE production and contact sensitisation may occur concurrently, i.e. develop with the same compound in the same individual. IgE mediated sensitisation is demonstrated with a skin prick test and/or the detection of IgE antibodies in serum. IgE sensitisation may, but not necessarily result in respiratory, gastrointestinal or skin symptoms and even anaphylaxis (type I immune reaction).

Food additives, as well as other food substances of non-protein nature, have been demonstrated to elicit allergic and non-allergic food reactions comparable to the above mentioned reactions to chemical compounds. The modes of action involved are IgE mediated, cell mediated or non-immunologic mechanisms.

3.6.2.1 Patch test results with mixtures of chemicals

Clinical experience with patch testing of mixtures of substances, such as a cosmetic product, often causes a positive result, while testing of the individual ingredients show no response. For example, only half of the individuals reacting to a fragrance mixture from the European Standard patch test series also gave a positive response to at least one of the eight individual fragrance mixture constituents when these were tested individually. The same phenomenon is known from testing of other mixtures such as rubber allergens. These test results have often been interpreted as a false positive patch test. Much effort has been taken to eliminate the causes of such false results, but despite of this the frequency of such responses has remained stable over the years (Johansen & Menne, 1995).

In a different study, two groups of individuals with contact dermatitis to perfume ingredients were investigated. Eighteen of the subjects had contact allergy to two fragrance substances and 15 were allergic to only one of these two fragrances. The test and matched control subjects were patch tested with the two allergens applied in serial dilutions in separate chambers on one side, and combined in one chamber on the other side of the upper back. The reactions were assessed on day three by clinical grading and by measurement of blood flow by laser Doppler flow measurement. The 1:1 mixture of allergens elicited responses as if the doses were three to four times higher than the dose actually used. The authors of the study concluded that the compounds acted synergistically (Johansen *et al.,* 1998).

In conclusion, an individual with a negative result in a diagnostic patch test with single chemicals may in some instances have a positive reaction after being tested with a mixture of chemicals.

4 Approaches used in the assessment and regulation of chemical mixtures

4.1 Procedures to assess combined effects of chemicals that work by similar mechanisms of action

The exact molecular mechanism of effect is only known for a few groups of chemicals, therefore the term "mode of action" is often used to describe toxicities that appear to be similar, but the detailed underlying mechanisms are not known. An ILSI Working Group (Mileson *et al.,* 1998) concluded that a common mechanism might exist if two compounds:

- Cause the same critical effect,
- Act on the same molecular target at the same target tissue, and
- Act by the same biochemical mechanism and may share a common toxic intermediate.

If a combined effect is likely to occur, the duration and timing of exposure and the biological half-life of the chemicals in the body are of importance. This is because the internal exposure is more important for the hazard assessment than the external exposure, and the intensity and duration of the response depend on the toxicokinetic properties of the chemicals in question. A major problem associated with methods of combined hazard assessment and derivation of regulatory guidance values, is that different uncertainty factors may be applied in safety assessment of various chemicals, since the size of the uncertainty factor will dominate the derived values of the hazard assessment, such as acceptable daily intakes (ADIs) or acute reference doses (ARfDs).

4.1.1 Group acceptable/tolerable intake (group ADI/TDI)

Some compounds with similar structure and effects have been allocated a group ADI/TDI. EFSA considers that a group ADI/TDI should be employed if: 1) exposure to several members of a structurally related series of chemicals is likely to occur frequently, and 2) several members of the series have been demonstrated to have (a) common target organ(s), cellular target(s) and the same mode of action (EFSA, 2005b). For instance, the members of a group may be metabolised to a common metabolite which determines the toxic effect. Even in cases where there are only limited toxicological data on one or more of the members, it is assumed that these compounds contribute to the same effect on the target organ. The group ADI/TDI is set for the sum of these compounds on the assumption that they have the same potency. If the above-mentioned criteria are met, individual members of the series should be assumed to have an additive effect. This concept has been used for e.g. parabens and food additives (EFSA, 2004d; EFSA, 2004e), but obviously represents an overestimation when some of the individual compounds are less potent than others.

4.1.2 Hazard index (HI)

This method is based on the assumptions that the compounds in the mixture act on the same biological site, by the same mechanism of action, and differ only in their potencies (simple similar action). The HI method has also been used for compounds with similar effect when knowledge about the mechanism of action is insufficient or not available. The HI is the sum

of the hazard quotients (HQ) of the compounds in a mixture. The HQ is the ratio of exposure to a defined limit exposure, such as the ADI. The HI for a mixture of four compounds can be calculated by the following equation:

$HI = HQ_1 + HQ_2 + HQ_3 + HQ_4$
or
$HI = E_1/ADI_1 + E_2/ADI_2 + E_3/ADI_3 + E_4/ADI_4$

where E is the level of exposure and ADI is the acceptable daily intake for each compound.
If HI > 1, the mixture has exceeded the maximum acceptable level for daily intake and there may be a risk. This method is based on an assumption of dose addition and risk is overestimated if the basic assumption of dose addition is wrong. Incorrect estimations will be the consequence if interactions (synergism or antagonism) occur.

4.1.3 Point of departure index (PODI)

The point of departure index is the sum of the exposures of each compound expressed as a ratio between exposure and their respective point of departures (PODs), e.g. NOAEL or Benchmark dose (BMD), instead of comparing them with the ADI/ARfD or TDI. BMD-modelling gives a better basis than NOAEL if there are sufficient data for modelling.

$PODI_i = \Sigma_i Exp/(POD_i \text{ or } NOAEL_i \text{ or } BMD_i)$

There is not a defined magnitude for an acceptable PODI. However, the sum should be less than an agreed uncertainty factor for the group. This uncertainty factor does not have to be the default 10 x 10 usually chosen for setting ADIs.

The PODI is a valuable approach if interactions (synergism/potentiation) are suspected. In such cases an extra uncertainty factor should be incorporated.

4.1.4 Margin of exposure (MOE)/ Margin of safety (MOS)

An international conference organised by EFSA and the World Health Organisation (WHO), with the support of ILSI Europe, concluded that the MOE is the preferred approach for risk assessment of substances that are both genotoxic and carcinogenic (Barlow *et al.*, 2006). According to this, the term Margin of safety (MOS) should be used in the assessment of compounds other than those being both genotoxic and carcinogenic. The calculated value of the MOE (or the MOS) is obtained by dividing the POD from the dose-response curve by the level of exposure.

MOE = POD/Exposure

The POD may be a dose corresponding to a given observed effect level, e.g. ED_{10}, or the NOAEL. When MOE is calculated for single chemicals, values > 100 or > 10 are usually considered as acceptable when toxicological data are from animal or humans, respectively. The MOE approach is the reciprocal of the PODI.The combined MOE for compounds in mixtures is calculated by the following equation:

$MOE_T = 1/(1/MOE_1 + 1/MOE_2 + ---1/MOE_n)$

There is no defined magnitude of an acceptable MOE_T for exposure to a mixture of chemicals. However, when the number of compounds in the mixture increases, the MOE_T decreases. As a consequence, combinations of several compounds with MOEs of 100 will obtain a MOE_T < 100. Therefore, a MOE_T > 100 approach seems inappropriate and a reduction in the level of acceptable MOE_T may be considered as the number of components in a mixture increases. Similar considerations are valid for MOS_T.

4.1.5 Toxic Equivalent Factors (TEF)

The toxic equivalent factor concept is based on the assumption that structurally related chemicals exert their toxic effects by a similar mechanism of action (simple similar action), but they differ in potency. It is also based on the assumption that the dose-response curves for all compounds are parallel. One chemical in the group of related chemicals is chosen as an "index compound" and given a certain TEF, and the toxicities of the other chemicals are calculated as the toxicity of each compound relative to the index compound. The total combined exposure (total equivalent quantity, TEQ) is then estimated by summation of the exposure to each chemical (C_i) multiplied to its respective TEF (TEF_i):

$$TEQ = \Sigma\, C_i \times TEF_i$$

The estimated TEQ expresses the total hazard as the hazard of an equivalent exposure of the index compound, and may therefore be compared to the ADI/TDI of the index compound. The toxic equivalent factor approach is used for the risk assessment of mixtures of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like biphenyls (PCBs). A re-evaluation of human and mammalian toxic equivalent factors for dioxins and dioxin-like compounds has recently been performed by WHO. New TEF values have been proposed by WHO for human risk assessment of these compounds (Van den Berg *et al.*, 2006). A similar approach has also been chosen for the ongoing risk assessment of combined hazard from exposure to acetyl cholinesterase inhibitors by the UK Pesticide Safety Directorate (Advisory Committee on Hazardous Substances, ACHS 07/18A).

4.1.6 Relative potency factor

In the relative potency factor (RPF) method the dose of all compounds in a mixture is also scaled relatively to the dose of an index compound, and then added. This method is used when exposure to the different components in a mixture leads to a common toxic effect, but there may be differences in the mechanism of action. RPF is the same as TEF when the mechanism of action is similar (simple similar action), but may be used in a broader context. This method is used for risk assessment of organophosphorus pesticides (cholinesterase inhibitions) by the US EPA (U.S.EPA, 2006b), and RPF is the preferred method of this agency. The concept of so-called cumulative RPF is used by EPA when oral, dermal and inhalation exposure are taken into consideration.

4.1.7 Interaction-based modification of the hazard index (HI)

A weight-of-evidence (WOE) modification method proposed by (Mumtaz & Durkin, 1992), takes into account both synergistic and antagonistic interactions in the derivation of the HI. The method evaluates the data relevant to combined actions for each possible pair of chemicals in the mixture (binary weight-of-evidence, BINWOE) in order to make weight-of-evidence classification for the effect of each chemical on the toxicity of all the other

chemicals in the mixture. Physiologically based toxicokinetic (PBTK) models may provide data useful for the calculations. A problem with the interaction-based HI method is that due to lack of experimental or epidemiologic data a number of assumptions about the mechanisms are used (Haddad *et al.*, 2001; Teuschler, 2007).

4.1.8. Indicator substance

The Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have introduced a surrogate approach for evaluation of chemicals in mixtures, where one substance has been chosen as an indicator for a group of compounds, e.g. benzo[a]pyrene, as the most potent carcinogen, is used as a marker of exposure and of the effects of the mixture of PAHs (SCF, 2002b; JECFA, 2005). This method uses a single component as the measure of concentration in relation to the response of the whole mixture (13 genotoxic and carcinogenic PAHs).

Table 2 gives an overview of different procedures/methods used to assess combined effects of chemicals.

Table 2. Methods for risk assessment of mixtures, all based on information on individual compounds. Based on Danish Veterinary and Food Administration (2002) and EFSA Scientific Colloquium 7 (EFSA, 2007c).

Risk assessment strategy	Data needed	Applicability	Assumptions	Advantages	Disadvantages
Hazard Index (HI)	ADI, ARfD, TDI	Exposure data at low levels. HI is also used for compounds with similar target organ	Simple similar action (similar mode of action/dose addition)	Understandable and transparent. Relates to well known and widely used measures of acceptable risk (e.g. ADI, ARfD)	ADI, TDI, ARfD are based on NOAELs, which in most cases involve default uncertainty factors in the extrapolations
Point of Departure Index (PODI)	NOAEL or BMD		Simple similar action (similar mode of action/dose addition)	Relates directly to real exposure and toxicity data	No criteria for defining the magnitude of an acceptable PODI
Margin of exposure/margin of safety for mixtures (MOE_T/MOS_T)	Point of departure		Simple similar action (similar mode of action/dose addition)	Relates directly to real exposure and toxicity data	No criteria for defining the magnitude of an acceptable MOE_T (MOS_T)
Toxic Equivalent Factor (TEF)	Toxicity data for each compound and dose-response data for the index compound	Restricted by strong similarity in mechanism of action, few chemical groups will qualify	Simple similar action (similar mechanism of action/dose addition)	Relates directly to real exposure and toxicity data. Understandable and transparent	Relies very much on the toxicity data for the index compound
Relative Potency Factor (RPF)	Toxicity data for each compound and dose-response data for the index compound	Restricted by similarity and to specific conditions	Simple similar action (similar mode of action) is supposed to account for mixtures with different mode of action	Relates directly to real exposure and toxicity data. Understandable and transparent	Relies very much on the toxicity data for the index compound
Response Addition	The fraction of total effect for each component	Data usually not available	Simple dissimilar action (different mode of action), common effect		
Interaction-based Hazard Index	Maximum acceptable level for each compound and weighing factors based on BINWOEs		Binary interactions are the most important		Complex to determine the BINWOE. Weighing factors not supported by experimental data

4.2 Procedures to assess combined effects of chemicals that act by dissimilar mechanisms of action

4.2.1 Simple dissimilar action

If fractional responses of the components of a chemical mixture are known, response addition can be applied. It is assumed that the modes of action and possibly the nature and site of action differ among the chemicals in the mixture. The chemicals exert their individual effect and contribute to a common result without modulating the effect of each other. The term response addition is used to describe proportions of responders in a population and represents the sum of probabilistic risks, whereas effect addition describes the sum of graded biological responses in individuals (Teuschler, 2007). In order to use this method, it is necessary to know the fraction of the total possible effect of each of the compounds in the mixture, as well as the total possible effect of the mixture. Even if the method is easy to use mathematically, the required data are usually not available. Furthermore, the concentrations of the individual components in a mixture are very often below exposures corresponding to the NOAEL. In such situations, the dominating understanding is that effect/response addition is not relevant for mixtures where the exposure is below the NOAEL for each individual compound. This is if there are no interactions between the compounds resulting in synergism/potentiation or antagonism. Exposure below the NOAEL may, however, be relevant if the compounds interact and produce synergism/potentiation (EFSA, 2007c).

4.3 Chemical mixtures and the Threshold of Toxicological Concern (TTC) principle

The TTC is a principle, where level of exposure is used to set priorities for need of toxicological testing, since for most toxic effects there is an exposure dose, or threshold value below which no adverse effects can be expected (Barlow, 2005; VKM, 2006b). The concept should not be confused with thresholds for toxicological effects. The application of the TTC principle is recommended for substances to which humans are exposed at low levels. The approach originated in relation to food packaging migrants, was refined for application to flavouring substances, and will be developed further to allow for a wider application to low-molecular-weight compounds that are present in the human environment in trace amounts, either naturally or as result of human activities.

The assessment of chemical mixtures is a complex issue, and more work is needed to develop methods to deal with this issue, in regular risk assessment as well as when using TTC. In principle, the TTC approach could be used to assess mixtures of substances which have similar toxic modes of action (Barlow, 2005). It would be possible to sum their exposures/intakes and compare the combined exposure/intake with the relevant TTC, provided they were of similar potency or were corrected to a similar potency. If the combined intakes were below the TTC, this would indicate that the substances would not be expected to be of concern. If the mechanisms of action of substances in the mixture were known to be dissimilar, then the TTC approach could be used in assessment of each individual substance, one at a time.

When dealing with complex mixtures of diverse chemicals, assessment using the TTC approach should focus on the exposure to a "marker" compound or a major compound which represents a high proportion of the mixture and is of the highest Cramer class (III), i.e. the chemical with the highest potential for toxicity based on its chemical structure (VKM, 2006b), of the known constituents in the mixture.

As an added complexity, relevant exposures from sources other than the one under evaluation, e.g. from a certain food, need to be taken into consideration. However, data for exposure from other sources are often not available to consider total intake.

4.4 Approaches to assess toxic effects of multiple chemical exposures - suggestions from other European and US regulatory bodies

Several advisory reports and suggested strategies on how to assess toxic effects of multiple chemical exposures have been published by regulatory bodies in several European countries and in the USA. Suggested strategies may be outlined in simple or more complex flow charts that are meant to serve as guidelines for choosing among the methods and models presented earlier in this report. The applicability of the various strategies and flow charts is highly dependent on the amount of available data on the different compounds in a mixture. The following paragraphs contain summaries of some of the suggested approaches on how to assess combined toxic effects of multiple chemical exposures.

4.4.1 Approach to assess simple and complex mixtures – advisory report published by the Health Council of The Netherlands (2002)

In July 2002, the Health Council of The Netherlands published an advisory report "Exposure to Combinations of Substances: a System for Assessing Health Risks" (Health Council of The Netherlands, 2002). Major topics in this report are: a) a distinction between a *specified combination* of substances and a *mixture* of substances, b) a framework for the safety evaluation of combined exposures, including the use of so-called "top *n*" and "pseudo top *n*" approaches, and c) a recommendation to use the Mumtaz-Durkin weight of evidence (WOE) method for prioritisation of combined exposures according to their potential health risks (Feron *et al.*, 2004).

A *specified combination* of chemicals is characterised by known chemicals with the same or different target organ, various exposure routes and independent exposures which may or may not overlap in time. *Mixtures* may contain both known and unknown components, depending on whether they are intentionally or unintentionally formed. However, exposure to a mixture is characterised by exposure to all components at the same time and by the similar route since they occur together. Different approaches are often considered for the two types of risk assessment. The framework for risk assessment of combined exposures is shown in Figure 4 A and B. Combination of chemicals may be treated as a single entity, as divided into fractions according to physical and chemical properties, or as individual components. To reduce the safety evaluation of complex exposures to manageable proportions, the "top *n*" and "pseudo top *n*" approaches were introduced, *n* representing the most hazardous chemicals or groups of chemicals, respectively. The Mumtaz-Durkin WOE method results in qualitative and quantitative refinement of hazard indices, based among other things on classification of binary interactions (BINWOES). For more details, see Feron and co-workers (2004) and the report of the Health Council of The Netherlands (2002).

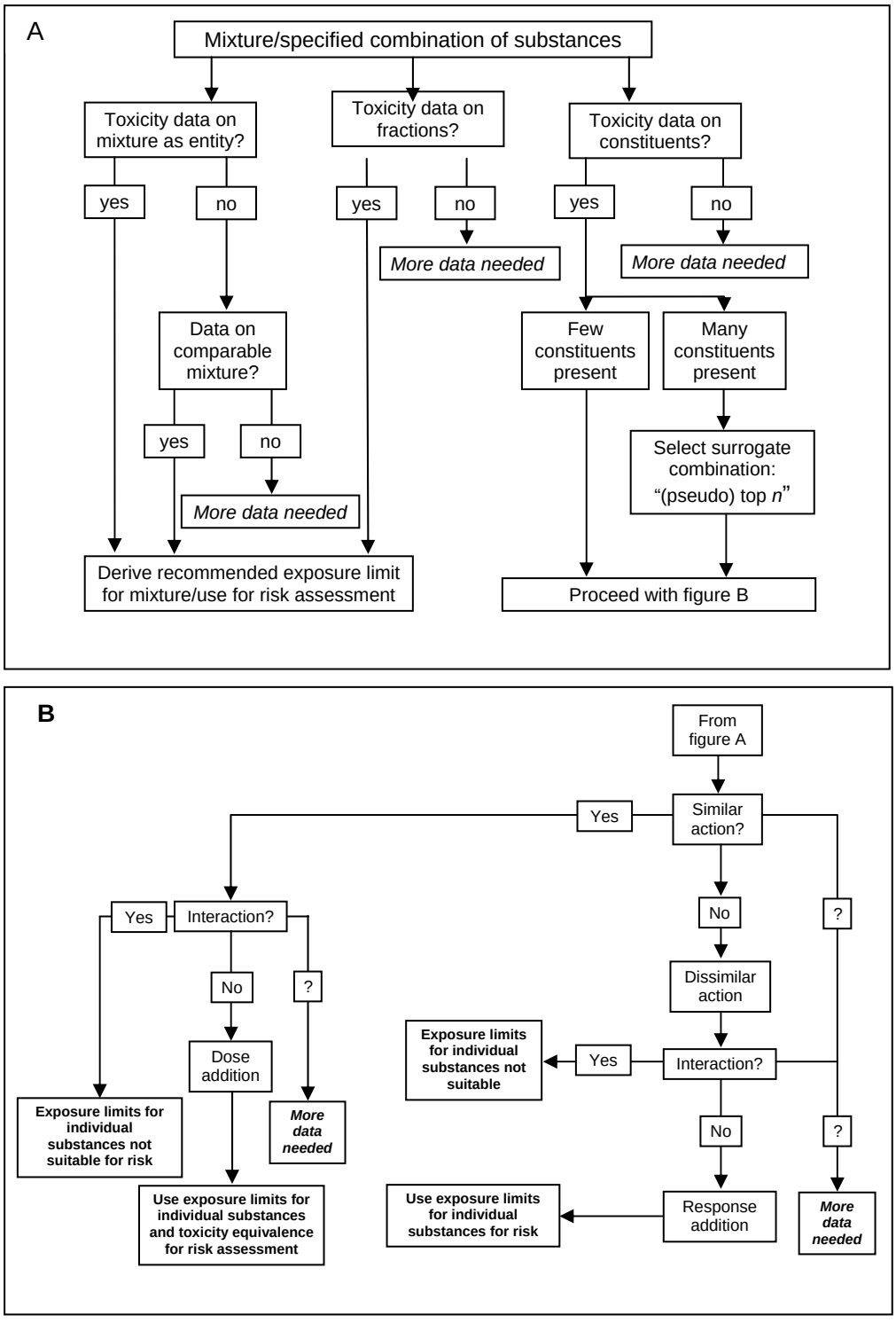

Figure 4. Proposed framework for the risk assessment of combined exposures, modified from Feron et al. (2004).

4.4.2 Approach for assessment of combined toxic action of chemical mixtures – proposal from the American Agency for Toxic Substances and Disease Registry (ATSDR)

The ATSDR "Guidance Manual for the Assessment of Joint Toxic Action of Chemical Mixtures" describes a methodology for how to conduct health risk assessment for chemical mixtures (ATSDR, 2004a). In this process, step-by-step procedures for assessing non-

carcinogenic and carcinogenic effects are outlined in flow charts. The hazard index (HI; the sum of HQ) approach uses the assumption of dose addition to assess non-cancer health effects. The strategy for cancer is similar to the non-cancer effects, except that the cancer risk estimates are used in place of HQ and HI in the analysis. Dose addition usually requires that all components act by the same mechanism, but HI may also be used as a tool after exposure to components affecting the same critical target. The target organ toxicity dose (TTD) modification, a refinement of the HI method, is used to accommodate the assessment of mixtures whose components do not all have the same critical effect, but have overlapping targets. The HI method does not take into account interactions among components of the mixture. This may lead to overestimation or underestimation of the health hazard if the interactions are greater or less, than additive, respectively. To evaluate the potential impact of interactions, a WOE method is used both in the non-cancer and cancer strategies to assess for interactions. For discussion of the strategies, see (Wilbur *et al.,* 2004). ATSDR has also developed six toxicity interaction profiles for chemical mixtures, two on persistent environmental chemicals, two on metals and two profiles on volatile organic chemicals (ATSDR, 2004b; ATSDR, 2004c; ATSDR, 2004d; ATSDR, 2004e; ATSDR, 2004f; ATSDR, 2004g). The profiles are also described in a paper (Pohl *et al.,* 2003).

4.4.3 Combined risk assessment of pesticide chemicals that have a common mechanism of toxicity – US Environmental Protection Agency (US EPA)

The US EPA Office of Pesticide Programs has published the document "Guidance on Cumulative Risk Assessment of Pesticide Chemicals That Have a Common Mechanism of Toxicity" (U.S.EPA, 2002). This provides guidance for the evaluation and estimation of potential human risks associated with multichemical and multipathway exposures, referred to as cumulative risk assessment. The organising principle is the identification of a group of chemicals that induces a common toxic effect by a common mechanism of toxicity (common mechanism group, CMG). In short, a CMG group of pesticides is identified, and the Candidate Cumulative Assessment Group (CAG) is determined. The dose addition and relative potency factor (RPF) approach are used to express the total exposure. Based on dose response analysis, the RPF of each chemical is expressed as the ratio between the index chemical potency and the CMG chemical (e.g BMD or NOAEL). The POD for the index compound is then used to calculate a combined MOS. Route specific MOSs can be combined to generate a total MOS.

The organophosphorus pesticide (OP) class of pesticides was established as the first common mechanism group by EPA in 1999. OPs share the ability to inhibit acetylcholinesterase by binding to and phosphorylate the enzyme in both the central and peripheral nervous system. This results in accumulation of acetylcholine and continuous stimulation of cholinergic receptors. Thirtythree chemicals were included, and methamidophos was selected as index chemical (Dr. Vicki Dellaco, EPA, at EFSA Scientific Colloquium 7, 2006). An updated version of "Organophosphorus Cumulative Risk Assessment" was published in 2006 (U.S.EPA, 2006b). In contrast to risk assessment of acetylcholinesterase inhibitors in other countries, such as UK and The Netherlands, EPA has chosen to distinguish between OPs and carbamates because of toxicokinetic and also toxicodynamic differences.

The "Estimation of Cumulative Risk from *N*-Methyl Carbamate Pesticides: Preliminary Assessment", was published in 2005 (U.S.EPA, 2005). Other pesticide CMG identified and assessed by EPA are "Triazine Cumulative Risk Assessment" (U.S.EPA, 2006c) and "Cumulative Risk from Chloroacetanilide Pesticides"(U.S.EPA, 2006a). EPA has also started

to develop a cumulative risk assessment for the pyrethroids, some of the most common pesticides used in indoor environments. Pyrethroids are a family of pesticides that induce toxicity through a common mode of action, by interacting with sodium channels in the nervous system. They are commonly found as a mixture, and routes of exposure include oral (diet, hand-to-mouth), dermal and inhalation (Soderlund *et al.*, 2002).

In a recent publication existing risk assessment methods are referred to and discussed (Teuschler, 2007). In this paper, a guide for selection among available risk assessment methods for chemicals in food is presented (Figure 5). Cumulative RPF is suggested used when various exposure routes are taken into consideration (see section 4.1.6).

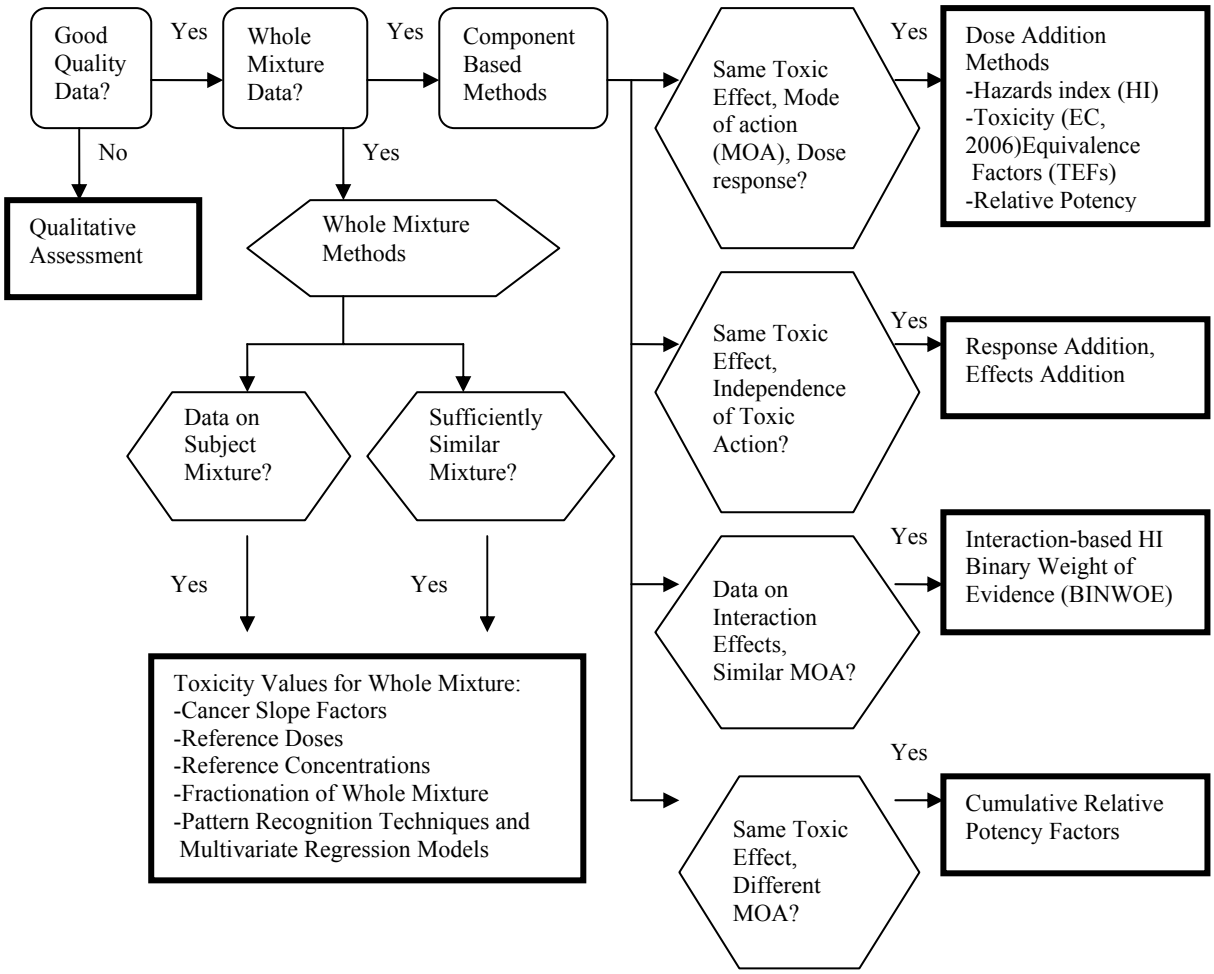

Figure 5. Flow chart for risk assessment of combined exposures, modified from Teuschler (2007).

4.4.4 Examples on risk assessment of pesticide residues in food by the Danish Veterinary and Food Administration

4.4.4.1 Selection of methods for risk assessment of pesticide residues in food

The whole mixture approaches would, as mentioned earlier, be the preferable risk assessment methodology for pesticide residues in foods. However data to be used for such approaches are

usually not available. This leaves the single compound approaches as the more realistic methods to use. These can be divided into three categories depending on whether the compounds act by similar mechanism, independently or whether they interact with each other. At the moment there exists no readily applicable risk assessment method that accounts for interactions.

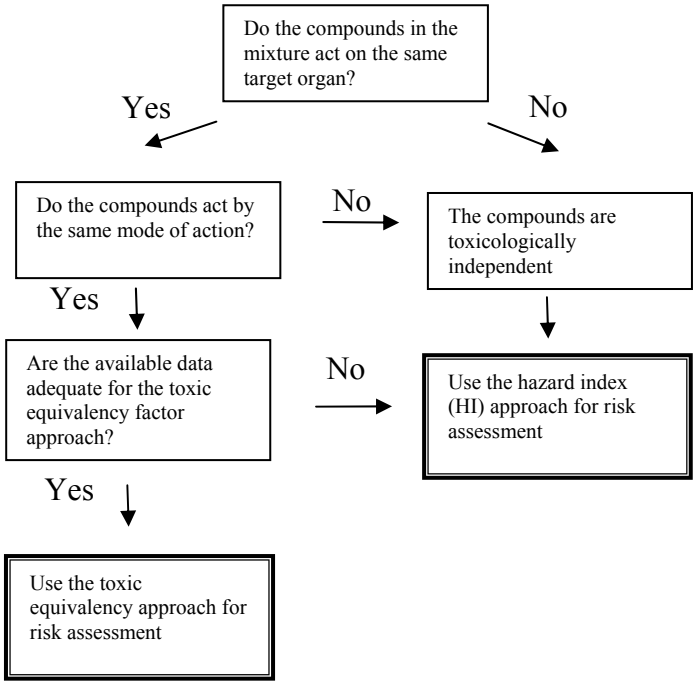

Figure 6. Proposed flow chart for risk assessment approach for mixtures of pesticides in food, adapted from Danish Veterinary and Food Administration (2002).

The Danish Veterinary and Food Administration proposes to use the flow chart shown in Figure 6. The risk assessments of pesticide residues have to be done on a case-by-case basis in which the available chemical and toxicological data are evaluated in a weight-of-evidence process. The hazard index (HI) method is seen as the most appropriate in most cases for toxicologically similarly acting compounds, but the HI method may also be used for compounds with similar effects when knowledge about the mode of action is insufficient or unavailable. The HI method is transparent, understandable and it relates directly to ADI, a well known measure of acceptable risk. The method has also been used for mixtures of compounds that have the same target organ, but different modes of action. In Figure 6, the HI method is also suggested used for mixtures of pesticide residues. VKM is of the opinion that this approach does not comply with the theoretical principle on which the method is based. Food residues of pesticides are generally found at exposure levels well below their respective NOAEL and they are therefore not expected to cause more than an additive effect. In cases where the weight-of- evidence points out that the compounds share a common mechanism (e.g. as for the organophosphorus pesticides, the chloroacetanilides, the dithiocarbamates and the thiocarbamates) the toxic equivalent factor (TEF) should be used.

4.4.4.2 Examples from Denmark

In the Danish report, 10 examples of risk assessments of crops containing pesticide residues are given, of which two are included in the present report (Table 3).

Example 1: Oranges, Spain

Seven pesticides were found in oranges from Spain: chlorpyrifos, dicofol, imazalil, malathion, orthophenylphenol, prothiofos and tetradifon. The compounds do not belong to the same chemical classes, and they are not similar in chemical structure. The compounds have the following similarities with respect to their toxic action and target organ:

- dicofol, imazalil, malathion, orthophenylphenol and tetradifon have an effect on the liver, but they do not have a common mode of action
- dicofol, malathion and orthophenylphenol cause adenomas in the liver and dicofol can also cause liver carcinomas
- chlorpyrifos, malathion and prothiofos act on the nervous system by inhibiting acetylcholinesterase
- dicofol and malathion have an effect on the thyroid gland.

None of the compounds in this mixture do have a common target organ or mode of action, even though some of them show some similarities. The compounds are toxicologically independent, and according to the flow chart proposed by the Danish Veterinary and Food Administration (Figure 6), the mixture is evaluated by the hazard index approach.

The average daily intake of oranges in Denmark is 15 g/person/day for adults. For children under 3 years, the intake is estimated to half of this amount: 7.5 g/person/day. The residue of chlorpyrifos in this type of fruit was 0.04 mg/kg orange, which gives the following exposure in a 60 kg adult or in a 15 kg child:

$$E_{1,\,adult} = \frac{0.04mg/kg\ x\ 0.015kg/person/day}{60kgbw} = 0.00001mg/kgbw/day$$

$$E_{1,\,child} = \frac{0.04mg/kg\ x\ 0.0075kg/person/day}{15kgbw} = 0.00002mg/kgbw/day$$

These calculations are performed for all compounds found in the oranges and the results are shown in Table 3. The next step is to calculate the hazard index, from the equation shown in section 4.1.2.

$$HI_{adults} = \frac{1x10^{-5}}{0.01} + \frac{3.5x10^{-5}}{0.002} + \frac{9.5x10^{-4}}{0.03} + \frac{1x10^{-5}}{0.3} + \frac{3.8x10^{-4}}{0.4} + \frac{4.5x10^{-6}}{0.0001} + \frac{1.5x10^{-5}}{0.03} = 0.0097$$

$$HI_{children} = \frac{2x10^{-5}}{0.01} + \frac{7x10^{-5}}{0.002} + \frac{1.9x10^{-3}}{0.03} + \frac{2x10^{-5}}{0.3} + \frac{7.5x10^{-4}}{0.4} + \frac{9x10^{-6}}{0.0001} + \frac{3x10^{-5}}{0.03} = 0.19$$

The hazard indices for both adults and children are well below 1 and it is concluded that the exposure to the residues is not expected to constitute a risk.

Table 3. Raw data used to calculate exposure of pesticide residues in oranges and kiwi.

Crop	Compounds	Residue (mg/kg)	Exposure adult (mg/kg bw/day)	Exposure child (mg/kg bw/day)	ADI (mg/kg bw)
Oranges 15 g/ person/day for adults, 7.5 g/person/day for children	Chlorpyrifos	0.04	$1x10^{-5}$	$2x10^{-5}$	0.01
	Dicofol	0.14	$3.5x10^{-5}$	$7x10^{-5}$	0.002
	Imazalil	3,8	$9.5x10^{-4}$	$1.9x10^{-3}$	0.03
	Malathion	0.04	$1x10^{-5}$	$2x10^{-5}$	0.3
	Ortho-phenylphenol	1.5	$3.8x10^{-4}$	$7.5x10^{-4}$	0.4
	Prothifos	0.018	$4.5x10^{-6}$	$9x10^{-6}$	0.0001
	Tetradifon	0.06	$1.5x10^{-5}$	$3x10^{-5}$	0.03
Kiwi, Italy 3 g/person/ day for adults, 1.5 g/person/day for children	Iprodione	0.14	$7x10^{-6}$	$1.4x10^{-5}$	0.06
	Vinclozolin	5.7	$2.9x10^{-4}$	$5.7x10^{-4}$	0.01

Example 2: Kiwi, Italy

This is an example of kiwi with high levels of two dicarboximides pesticide residues; iprodione and vinclozolin. Both compounds have an effect on the liver and the adrenal gland. Iprodione has been reported to cause non-neoplastic lesions in the liver and vinclozolin causes hepatocellular carcinomas. Iprodione is reported to cause histopathological changes in the adrenals, and vinclozolin to cause tumours in the adrenals. The toxicological data for the compounds indicate toxicological similarity and the data were for these organs considered to be good enough for using the toxicological equivalent approach (Figure 6). The data for both compounds were considered to be of equal quality and therefore either of them can be chosen as the index compound. ADI for iprodione is 0.06 mg/kg bw and ADI for vinclozolin is 0.01 mg/kg bw. If iprodione is chosen to be the index compound, TEF for vinclozolin is 6 (0.06 mg/kg bw/0.01 mg/kg bw). The toxic equivalent for adults can then be calculated as in section 4.1.5: $TEQ= \Sigma C_i \times TEF_i$.

Using data from Table 3, TEQ for adults is calculated as follows:
TEQ = ($7x10^{-6}$ mg/kg bw/day) x 1+ ($2.9x10^{-4}$ mg/kg bw/day) x 6 = $1.7x10^{-3}$ mg/kg bw/day. This is 35 times lower than the ADI for the index compound (iprodione), and the exposure to the residues is therefore not considered to be of any risk.

TEQ for children is calculated as follows:
TEQ = ($1.4x10^{-5}$ mg/kg bw/day) x 1+ ($5.7x10^{-4}$ mg/kg bw/day) x 6 = $3.4x10^{-3}$ mg/kg bw/day. This is a factor 17.5 below ADI for iprodione, and the exposure to the residues is therefore not considered to constitute a risk.

4.4.5 Approach from the EFSA Scientific Colloquium - Cumulative Risk Assessment of Pesticides to Human Health

Criteria for grouping compounds into CMGs were one of the topics discussed at the EFSA Scientific Colloquium: Cumulative Risk Assessment of Pesticides to Human Health: The Way Forward (EFSA, 2007c).

The following points of view on defining CMGs were presented after discussions:

- The criteria should be science-based. This is easy when there is a well established target (OPs and carbamates), but is much more complicated when there are multiple targets and feedback mechanisms, e.g. as with endocrine disruptors and pyrethroids.
- There are options regarding the strength of evidence for commonality of mode of action. E.g. should grouping be performed only when the scientific basis is sound enough or also when there is no evidence of the contrary? If a minimum is that the compound must have the same end-effect, this approach opens for a lot more compounds to be grouped.
- The problem is the lack of information on mechanism/mode of action for a lot of chemicals:
    - Standard studies that are adequate for use in risk assessment of each compound are the minimum data requirement
    - Information on time-course of effects is essential for acute and long-term exposures
    - The ideal data to have would be: dose-response for benchmarking; time-course of toxic effects, data for defining key events
    - Data on mixture studies, also in order to reveal complex similar mode of action (synergism/potentiation/antagonism)
- Complex similar action has to be considered (synergism/potentiation(/antagonism))

The following points regarding non-dose addition were discussed:

- Simple dissimilar action is not of concern at levels below the ADI of all compounds. However, it may be relevant to consider synergism or potentiation.
- Complex dissimilar action is considered to be rare at levels of regulated residue exposure (below MRL).
- Assessment of combined effects should be performed where co-exposure is likely to occur, with special attention when combinations are intentionally made.
- Discriminate between acute and chronic effects of exposure in the risk assessment. Timing in exposure is essential since it influences any kinetic interactions.
- In cases where interactions are foreseen, an additional UF may be used.

5 Approaches used by VKM to assess combined toxic effects of multiple chemical exposures

In the following, information is given on how combined toxic effects of chemicals in mixtures are handled at present within the areas covered by the Scientific Panels 2, 4, 5, 7 and 8 in VKM. Combined toxic effects of multiple chemical exposures were not considered to be relevant for VKMs Panels on Biological Hazards (Panel 1), Genetically Modified Organisms (Panel 3) and Plant Health (Panel 9).

A discussion and description of relevant approaches within the remit of VKM is given below. Relevant research on combined toxic effects of chemical mixtures within some areas is also included.

5.1 VKM Scientific Panel 2 (Panel on Plant Protection Products)

This Panel is concerned with the safety of chemical and biological plant protection products, and their residues in food. Most of the work in this Panel relates to evaluation of pre-marketing dossiers submitted when importers/producers apply for registration of plant protection products formulations. In Norway, the situation related to plant protection products is different than for other chemicals in foods and consumer products. In other areas, Norway has to comply with EU regulations as laid down in the EEA-agreement between the EU and EFTA. For plant protection products, Norway has its own specific regulation concerning approval for use, laid down in the Act pertaining to Food Production and Food Safety (The Norwegian Food Law). For residues of plant protection products in food, Norway has to comply with the EU regulations. The Panel assesses the risk to operators when exposed during application of plant protection products. This includes an evaluation of the use of any personal protection equipment.

The Panel is also consulted by the Norwegian Food Safety Authority in instances where the national monitoring programme for residues of plant protection products has identified samples with residues levels indicating a case of concern, such as when estimated exposures result in intakes above acute reference doses (ARfD).

5.1.1 Multiple Exposures to Plant Protection Products

Multiple exposures to plant protection products may chiefly occur in one of two situations: 1) Exposures in the occupational setting when plant protection products are loaded, mixed and sprayed, as well as during re-entry into sprayed crops, either in greenhouses or in the field; or 2) from exposures when consuming foods such as fruits and vegetables that contain multiple plant protection products residues.

For risk assessment of plant protection products exposures in the registration of new plant protection products formulations or re-registration of plant protection products already on the market, the applicant must submit dossiers enabling an exposure assessment to be performed. Ideally, such information should be based on real-life measurements. However, often the exposures are estimated by using various models (e.g. UK, Europoem and German models). Measurement of occupational exposure to applicators and workers in the field may be performed by quantifying parent compound and/or metabolites in blood and urinary samples,

but this is seldom done in practice. Biomonitoring of exposures to organophosphate and carbamate pesticides can relatively easily be measured by using levels of plasma and red blood cell cholinesterase activity. Since 1974, the US State of California has required testing of pesticide applicators by measuring these cholinesterase enzyme levels. This has been reported to be a useful and cost-effective means of preventing organophosphate and carbamate overexposure (Lessenger, 2005). This type of biomonitoring would integrate multiple exposures from the cholinesterase-inhibiting pesticides.

The Norwegian monitoring programme for plant protection products residues in fresh fruit and vegetables including potatoes, cereals, baby food and other vegetable products has in the last years included 1700-2000 samples per year. The monitoring programme 2006 covered 244 plant protection products including some isomers and breakdown products. Of the samples of fresh fruit and vegetables including potatoes, 55% were without detectable residues. The Maximum Residue Levels (MRLs[1]) were exceeded in 2.6% of the samples (0.9% in domestic and 3.6% in imported samples). Residues of 102 different plant protection products were found. Eight consignments contained residues in amounts that were considered to represent a health risk (omethoate/dimethoate and EPN). Of 1540 samples in 2006, 891 had no residues (58%), 292 (19%) samples contained one type of plant protection products residue, 165 samples (11%) contained two residues, 98 samples (6%) three residues, 57 samples (4%) four residues, 19 samples (1%) five residues and 18 samples (1%) six or more residues. Samples with multitude of residues could contain plant protection products showing both similar and dissimilar mechanisms of action.

During the period 1997-2004, samples of fruit, vegetables and cereals monitored in the EU contained 15.5% (1997) to 23.4% (2004) multiple plant protection products residues (EC, 2006). For 53-64% of the total samples monitored, no detectable residues could be found. There were 32-42% of the samples which contained residues below or at the level of national or EC-MRLs. In 3.3-5.5% of the samples, national or EC-MRLs were exceeded (EC, 2006).

5.1.2 Risk Assessment of Combined Exposures of Plant Protection Products to Human Health

Regulation (EC) No. 396/2005 on maximum residue levels of plant protection products in or on food and feed of plant and animal origin emphasises the importance of developing a methodology to take into account combined and possible synergistic effects of plant protection products to human health. There is no generally agreed framework/approach yet for combined risk assessment of plant protection products at the European or International level. US EPA has given considerable emphasis and work concerning approaches to combined (cumulative) risk assessment of pesticides the later years (U.S.EPA, 2002). This effort has focused on combined effects of pesticides, called a common mechanism group, that bring about the same toxic effect by a common mechanism of toxicity. So far, the following mechanism groups have been identified: 1) organophosphates, 2) *N*-methyl carbamates, 3) triazines and 4) chloroacetanilides. In light of the interest in this area, EFSA organised a scientific colloquium in November 2006 to evaluate existing methodologies and identifying new approaches (see section 4.4.5).

[1]MRLs are defined as the maximum concentration of pesticide residue (expressed as mg residue/kg of food/animal feeding stuff) likely to occur in or on food and feeding stuffs after the use of pesticides according to Good Agricultural Practice (GAP), i.e. when the pesticide has been applied in line with the product label recommendations and in keeping with local environmental and other conditions.

Risk assessments of combined exposures in relation to registration of plant protection products, can either be done based on already available knowledge from studies on combined effects of compounds or based on existing toxicological assessments for each of the compounds. Today only a few studies on combined effects of plant protection products exist that can be used for evaluation of combined interactions. However, the toxicological documentation on each of the plant protection products is extensive and gives a good knowledge on endpoints/target organs and sometimes on effects at the cellular level and mode of action.

The US EPA points out that not all assessments of combined exposures need to be of the same depth and scope (U.S.EPA, 2002). A screening-level assessment may be conducted that applies more conservative approaches than would a comprehensive and refined combined assessment. For example, a margin of safety may be based on the ADIs for the common toxic effect rather than modelling dose-response curves of each chemical member to derive more refined relative potencies and points of reference. For exposure to food, treatment of 100% of crops and MRLs may be assumed for each chemical belonging to a common mechanism group registered for use on a crop. If a screening-level analysis including such overestimates of exposure indicates that there is no reason for concern, no further detailed assessment may be necessary. But if this conservative approach indicates a potential for unacceptable risk, then a refined assessment should be conducted.

In a refined assessment, a dose-response analysis is performed on each member of a common mechanism group in order to establish its toxic potency. Once the toxic potency of each common mechanism chemical is determined, the relative potencies of the common mechanism group chemicals are established. To determine relative potency, a chemical from the common mechanism group is selected to serve as the index chemical. Relative potency factors (RPFs) are used to convert exposures of all chemicals in the common mechanism group to exposure equivalents of the index chemical. The last step in the dose-response assessment is to calculate a point of reference (NOAEL, BMD etc.) for the index chemical so that the risk of the common mechanism group can be extrapolated to anticipated human exposure (see also section 4.1.6).

5.1.2.1 Criteria for grouping compounds

A number of existing frameworks and guidelines are already available that set out criteria to identify and define a common mechanism group of compounds (e.g. EPA1, ILSI2, IPCS).

Simple similar action

There are a number of plant protection products which share a common mechanism of action or for which a common mechanism cannot be excluded:

- Organophosphorus (OPs): cholinesterase-inhibiting agents such as acephate, azinphos-methyl, chlorphenvinphos, chlorpyrifos, diazinon, dichlorvos, dimethoate, fenthion, malathion, methyl parathion
- *N*-Methyl carbamates: cholinesterase-inhibiting agents such as aldicarb, carbaryl methiocarb, methomyl, primicarb, propoxur, dithiocarb). Only acute exposure may need to be considered, and there have to be reasons for combining the assessment with those for the OPs

- Triazines (atrazine, simazine, propazine): disruption of the hypothalamic-pituitary-gonadal axis with changes of hormone levels and developmental delays, as well as rat-specific mammary gland tumours
- Conazoles: there are many compounds within the group and presently no common endpoint and mechanism has been decided
- Pyrethroids: effect on the nervous system. The possibility of sub-grouping is presently under consideration
- Dicarboximides (vinclozolin, procymidone, chlozolinate and iprodione): alterations in hormonal homoestasis
- Chloroacetanilides (alachlor, acetohclor): nasal olfactory tumours in rats
- Microtubule/spindle inhibitors: benomyl, karbendazim and tiofanatmetyl..
- Phthalimides: captan and folpet
- Dithiocarbamates: mankozeb, maneb etc.

For the latter two groups, a combined assessment is normally performed by default when the analytical method determines a common residue such as CS_2 for dithiocarbamates.

Compounds that show the same toxicological effects can be grouped under a dose addition principle when it is reason to believe that the compounds do not have a different mode/mechanism of action.

For plant protection products with simple similar action and sharing a common mechanism of action, it is assumed that an effect of combined exposures would be the result of the sum of the contributing dose of each chemical (see section 2.1). When the combined exposures are below the ADI of the most potent compound in a mixture, no concern would be raised. If exposures should exceed the ADI, then a detailed, specific risk assessment related to the common mechanism would be necessary.

Simple dissimilar action

When combined exposure to plant protection products with simple dissimilar action (non-dose addition, response addition) are below their respective effect threshold levels (NOAELs, BMDs), it is assumed that combined action of all plant protection products will be zero (see section 2.1). In situations with exposures above the respective effect thresholds, each plant protection product in the mixture and their accompanying risks resulting from exposure would have to be evaluated separately.

Interaction

Available data indicate that interaction does not occur at doses that are at or below the NOAEL of plant protection products in a mixture (Richardson *et al.,* 2001; Gordon *et al.,* 2006). When exposures exceed the respective NOAELs of the plant protection products in the mixture, both toxicodynamic and toxicokinetic interactions resulting in inhibition or synergy can occur (Moser *et al.*, 2005; Moser *et al.*, 2006), see section 3.5.3. For example, organophosphates that require metabolic activation could compete for those activating sites resulting in less-than-additive bioactivation and toxicity. In contrast, inactivation of detoxification pathways by one organophosphate could increase the net toxicity of another. In mixture studies of five organophosphorus pesticides, greater-than-additive interactions occurred at the lower end of the dose-response curve (Moser *et al.*, 2005). Also, if the dose levels of compounds in the mixture were large enough to cause enzyme induction, this could

also lead to synergy for plant protection products requiring metabolic activation. A study of the interactive toxicity of two organophosphates (chlorpyrifos and parathion) reported a marked influence by the sequence of exposure (Karanth *et al.,* 2001). Taken together, it may be quite difficult to predict the toxic outcome from combined exposures to multiple compounds eliciting toxicity through a common mechanism.

5.1.2.2 Methods for assessing combined effects

Four methods are used for dose addition of plant protection products in mixtures having simple similar action. In selecting the method, consideration is given to knowledge of mechanisms and the toxicological profiles of the compounds in question. The four methods being used are: Toxic Equivalency Factors (TEF), Relative Potency Factors (RPF), Hazard Index (HI) and Point of Departure Index (PODI). These methods are described in more detail in section 4.1.

5.1.2.3 Occupational combined exposures to plant protection products

Exposure conditions during plant protection products application may be very complex. The possibility for interactive effects would depend on the number and types of plant protection products being used, their mechanisms of action, the exposure conditions (levels, frequency, duration), greenhouse or field application, climatic conditions, use of protective equipment, life-style factors, etc.

Measuring cholinesterase enzyme levels in red blood cells and plasma has become a practical and useful tool in the early detection of organophosphate and carbamate poisoning. This is a practical example of an integrated measure of a likely additive effect when there has been combined exposures to such compounds, and would also reflect any joint effects of such mixed exposures.

5.1.2.4 Risk assessment of combined exposures to plant protection products conducted by VKM Scientific Panel 2

Panel 2 has evaluated the combined effects of plant protection products in connection with residues levels in vegetable food resulting in exposure doses which could potentially exceed acute reference doses (ARfD). In 2006, this was performed in connection with maximum residue levels of dimethaote and its metabolite omethoate were exceeded in green beans and apples. Both dimethoate and omethoate affect the nervous system in high doses via acetylcholine esterase inhibition, the metabolite omethoate being more potent than dimethoate. The risk assessment took this metabolic conversion into account. The TEF approach was used and the calculation showed that the maximum intake of the residues in apples and green beans by children aged 1-4 years would have been 3-7 times more than recommended as safe for this age group.

5.1.3 Concluding remarks from VKM Scientific Panel 2

Many plant protection products belong to groups with similar mechanisms of action. When there is combined exposure to compounds within the same mechanism group, the principle of dose addition for such compounds exhibiting simple similar action would apply. When the sum of the exposure doses of the individual compounds in the mixture does not exceed the ADI for the most potent compound, there should be no apparent concerns. In situations where this sum of exposures exceeds the group ADI, dose additive effects may be expected. Risk

assessments could for such situations be based on knowledge of the relative potencies of the plant protection products in the mixture. Also, synergistic effects from mixtures could occur when exposures are above dose thresholds, due to both toxicodynamic and toxicokinetic interactions. However, with respect to the probability of experiencing interactive effects from combined exposures to plant protection products, it should be kept in mind that based on national and Europe-wide monitoring programmes of plant protection products residues in fruits, vegetables and cereals, levels are infrequently above maximal residue limits and thus well below effect levels.

5.2 VKM Scientific Panel 4 (Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics)

This Panel addresses questions on safety of the use of food additives, flavourings, processing aids, materials in contact with food and drinking water, and chemicals used for water treatment. In addition the Panel is responsible for risk assessments of cosmetics.

5.2.1 Food additives

For a number of food additives, group ADI/TDI values have been established (see also below under Food contact materials). Examples are groups of food preservatives such as sorbic acid and sorbates, benzoic acid and benzoates and the parabens (ethyl-, methyl- and propylpara-hydroxybenzoates). They have all been allocated group ADI values, which mean that the intake of the sum of the amounts of each compound in the group, obtained through simple addition, should not exceed the group ADI. For benzoic acid and the benzoates, the group ADI of 0-5 mg/kg bw/day also includes the intake of the flavouring agents benzyl acetate and other benzyl esters, benzyl alcohol and benzaldehyde. The rationale behind this is that these compounds are all rapidly and efficiently metabolised to benzoic acid.

Food additives are authorized in the EU on the basis that they constitute no health risk to the consumer at the proposed level of use. Although additives at their permitted levels of use are considered safe, there are concerns that simultaneous intake of different additives could be of potential health significance. Therefore, the International Life Sciences Institute (ILSI) Europe Acceptable Daily Intake Task Force established an expert group of scientists to analyse the possibility of health implications of joint actions and interactions between the 350 food additives currently approved in the EU (Groten *et al.*, 2000). All approved additives allocated a numerical ADI value were studied. Target organs were identified on the basis of the effects reported at doses above the NOAEL in animal or human studies. Descriptions of the pathological and other changes reported were used to assess whether different additives sharing the same target organ would produce a common toxic effect. In all but a very few cases, the possibility of joint actions or interactions could be excluded on scientific grounds. The exceptions were some additives with effects on the liver (curcumin, thiabendazole, propyl gallate and butylhydroxytoluene), the kidneys (diphenyl, *o*-phenylphenol and ferrocyanide salts), the blood (azorubine and propyl gallate), and the thyroid (erythrosine, thiabendazole, nitrate salts). However, in-depth consideration of both the specific use and the intake levels of these additives led to the conclusion that joint actions or interactions among these additives are a theoretical rather than a practical concern.

The ILSI expert group proposed that when approving future additives that induce target organ toxicity at doses above the NOAEL, risk assessors should consider the possible joint actions

or interactions with previously approved additives on the basis of a common mechanism of toxicity (Groten *et al.*, 2000).

The VKM Scientific Panel 4 has so far not evaluated health risks of exposure to combined effects of mixtures of food additives, either because the terms of reference concerned one substance only, and/or because of lack of data.

5.2.2 Natural flavouring complexes (NFC) and chemically identified flavourings

Natural flavouring complexes are mixtures of constituents obtained by applying physical separation methods to botanical sources, including pulp, bark, peel, leaf and flower of fruit, vegetables, spices and other plants. Many of the approximately 300 NFC have a food origin, e.g. lemon and basil. The method for the safety evaluation of NFC in the US, called the naturals paradigm, is intended only for the safety evaluation of NFC derived from higher plants to be used as flavouring substances for food and beverages. The naturals paradigm is a procedure that begins with a review of available data on the history of dietary use of the NFC, then prioritises constituents according to their relative intake from use as a NFC and their chemical structure (Newberne *et al.,* 1998; Smith *et al.,* 2004). The method further uses the concept of threshold of toxicological concern (TTC) (see section 4.3), and assigns constituents to one of three structural classes (Cramer classes I, II or III) (VKM, 2006b).

Another aspect of the naturals paradigm involves the evaluation of constituents of unknown chemical structure. As a conservative default assumption, the total intake of all unknowns is considered together and placed in the structural class of greatest toxic potential and thus compared with the most conservative exposure threshold. The paradigm also addresses the concept of joint action among structurally related constituents. If a common pathway of toxicity has been identified or can be reasonably predicted on the basis of structure-activity relationships for a group of constituents, the combined intake of those substances will be compared with the appropriate human exposure threshold of concern. Ultimately, the procedure focuses on those constituents or groups of constituents which, because of their intake and structure, may pose significant risk from consumption of the NFC. With the developed strategy, the overall objective of the naturals paradigm can be attained; that no reasonably significant risk associated with the intake of NFC will go unevaluated.

JECFA (Munro *et al.,* 1999) and the EU Flavour Information System (FLAVIS Working Group) (Council Directive 88/388/EEC) also evaluate structurally related chemically defined flavouring substances in groups, by conducting individual assessments using the TTC approach on each compound and then considering the safety of the group as a whole. Simple addition of the intakes would not allow for differences in potency or interactions, and would assume that the risk related to exposure from each substance, based on its structure, is not altered by the presence of the other substances.

The VKM Scientific Panel 4 has so far not evaluated any cases regarding health risks of exposure to NFC and chemically identified flavourings.

5.2.3 Food contact materials

Approximately 3000 substances may potentially migrate into food from food contact materials (Kroes *et al.,* 2000). Many of them are present simultaneously, for instance in polymeric plastics. To ensure the protection of the health of the consumer and to avoid any

contamination of the foodstuff, two types of migration limits have been established for polymeric materials: 1) an Overall Migration Limit (OML) of 60 mg substance/kg foodstuff or food simulant, that applies to all substances that can migrate from food contact materials to foodstuffs, and 2) a Specific Migration Limit (SML), which applies to individual authorised substances or groups of substances, and is fixed on the basis of the toxicological evaluation of the substance(s). For substances with adequate toxicological data, an ADI or a TDI value is set, and this value is used to calculate the SML. For substances where an ADI/TDI is not established, restrictions (R) can be set, frequently derived from limited toxicity dossiers, leading directly to fixed migration limits, e.g. R = 0.05 or R = 5 mg/kg food or food simulant. For these substances, the SML generally corresponds to the restriction which appears in the toxicological assessment by the former EU Scientific Committee on Food (SCF) or the present European Food Safety Authority (EFSA).

Restrictions for a group of substances may be given as group ADI or group TDI, or group R in the Synoptic document, the provisional list of monomers and additives notified to The European Commission as substances which may be used in the manufacture of polymeric plastics intended to come into contact with foodstuffs (EC, 2005). No general criteria for the evaluation of groups of substances or mixtures were established by SCF, who decided to evaluate them on a case-by-case basis (EC, 2003). Often the compounds that were given a group restriction had the same use (e.g. as plasticizers) and were applied as a mixture. However, EFSA has later given more specific criteria for employing a group ADI/TDI (see section 4.1.1).

According to the SCF, the evaluation and listing of groups of substances and mixtures should be replaced, where possible, by treatment of individual substances (EC, 2003). If the petitioner is unable to specify the individual substances in a mixture, the SCF/EFSA requires an explanation and will usually authorise only the mixture for which the petitioner supplied the technical data. Therefore, the petitioner should precisely describe the mixture.

Quantitative structure-activity relationship (QSAR) analysis, where the activity of a substance is deduced from the structural resemblance of its functional chemical groups to those of other substances, is used increasingly in risk assessments, of food contact materials, as well as of other chemicals.

The VKM Scientific Panel 4 has so far evaluated health risks from exposure to single substances in food contact materials, since the basis for the requests for risk assessments were analytical data of single substances found to migrate from food contact materials to foods or food simulants. No processing aids have yet been evaluated by the Panel.

5.2.4 Drinking water

Chemical contaminants in drinking water supplies are present with numerous other inorganic and/or organic constituents. Disinfected drinking water should be regarded as a variable, complex, very diluted chemical mixture with the following main characteristics: large numbers of chemicals occurring at very low levels, a large fraction (about 50%) of unidentified drinking water by-products, a lifetime exposure of the consumer, and huge numbers of consumers. WHO's Guidelines for drinking-water quality sets guideline values for drinking water contaminants (WHO, 2004). For contaminants considered to be genotoxic carcinogens, the guideline values are the concentrations in drinking water associated with an estimated upper bound excess lifetime cancer risk of 10^{-5} when consuming 2 litres per day. In

cases where the concentration associated with this risk level is not practical because of inadequate analytical or treatment technology, a provisional guideline value is set at a practicable level and the estimated associated cancer risk presented. The guideline values take into consideration also contribution to exposure from other sources. The values generally vary from 10% for substances for which exposure from food is probably the major source to 80% for substances for which exposure is primarily through drinking water. These guideline values are calculated separately for individual substances, without specific consideration of the potential for interaction of each substance with other compounds present. However, the large margin between exposure levels and levels which cause adverse effects incorporated in the majority of guideline values is considered to be sufficient to account for such potential interactions. In addition, the majority of contaminants will not be continuously present at concentrations at or near their guideline value. In Norway, these guideline values set by WHO are used in risk assessments of substances not specified in the Norwegian drinking water regulations.

For many chemical contaminants, the underlying mechanisms of toxicity are different; consequently, there is no reason to assume that there are interactions between them. There may, however, be occasions when a number of contaminants with similar toxicological mechanisms are present at levels near their respective guideline values. In such cases, potential combined effects should be taken into consideration. Unless there is evidence to the contrary, it has been regarded as appropriate to assume that the toxic effects of these compounds are additive.

This hypothesis was studied experimentally using the Eker rat, a model of hereditary renal cell carcinoma (McDorman *et al.,* 2003). The effects of mixtures of either low or high doses of carcinogenic drinking water by-products having distinctly different modes of action on nephrotoxicity and/or renal carcinogenicity were compared with similar doses of individual substances. It was found that while some of the mixture responses observed in male rats did fall within the range expected for additive responses especially at the high doses, predominantly antagonistic effects on renal lesions were observed in response to the low dose mixture in male rats and the high dose mixture in female rats. These data suggest that current default risk assessments assuming additivity may at least not underestimate the cancer risk associated with exposure to mixtures of drinking water by-products at low concentrations.

Since disinfected drinking water is characterized by containing high numbers of chemical by-products at low levels simultaneously, large efforts are being put into development of new methodology to evaluate the health effects of such complex mixtures. Many drinking water utilities are at present changing their primary disinfectant from chlorine to alternative disinfectants, e.g. ozone, chlorine dioxide and chloramines, which generally reduce regulated trihalomethane (THM) and haloacetic acid (HAA) levels, but can increase the levels of other potentially toxicologically important by-products (Richardson, 2007). In addition, significant amounts of the material that make up the total organic halide and the total organic carbon portions of the drinking water by-products have not been identified. Epidemiological studies, while not conclusive, are suggestive of possible reproductive/developmental or carcinogenic effects in humans exposed to drinking water by-products. These effects cannot be explained by the effects of the low doses of known individual by-products. Therefore, approximately 50 high-priority drinking water by-products are being measured as part of the US Nationwide Drinking water By-Product (DBP) Occurrence Study (Simmons *et al.,* 2002; Simmons *et al.,* 2004; Krasner *et al.,* 2006). This research also involves the joint chemical and toxicological evaluation of mixtures of drinking water by-products produced by different water-treatment

processes, at first being chlorination and ozonation/chlorination. Especially, the critical data gap of toxicological evaluation in experimental animals of those endpoints identified as of concern in epidemiological studies as mentioned above will be studied. Both a component-based approach and a whole-mixture approach are used by the US Environmental Protection Agency (EPA) to understand the toxicity of these compounds. The advantages of the last type of approach are that they account for the toxicity of the unknown by-products fraction, as well as any interactions (additive, synergistic or antagonistic) that might occur between the known and unknown drinking water by-products. Three quantitative statistical and risk assessment methods will be developed and evaluated; the detection of departure from dose additivity, the interaction-based Hazard Index, and the proportional-reponse addition (a new method).

Others feel that the strategy described above is unrealistic and that prioritisation of the various groups of drinking water by-products on the basis of potential health hazards is badly needed, focusing on an approach that takes into account the fraction of unidentified drinking water by-products, and includes the TTC concept. The "top *n*" and "pseudo top *n*" methodologies (described in section 4.4.1) were suggested to be useful in such an approach (Groten, 2000; Feron & Groten, 2002).

Health Canada considered the limitations and data requirements of the various mixture risk assessments methods (i.e. whole mixture approach, similar mixture approach, components-based approaches, interaction-based assessment) for incorporation into their risk assessment of drinking water contaminants (Krishnan *et al.,* 1997). They concluded that among the existing mixture risk assessment methods, the components-based and interactions-based approaches could be applicable. Specifically, among the components-based approaches, dose addition, response-addition and the toxic equivalent factor approaches were the most applicable ones for drinking water contaminants. Until an interactions-based, mechanistic risk assessment approach, e.g. physiological model-based approach, becomes available for routine use, the components-based approaches remain the default methods for consideration. A suggested working strategy for the development of a physiological modeling approach to the assessment of drinking water by-products would involve: 1) the construction of dose-response curves for each of the principal components of drinking water by-product mixtures based on relevant tissue-dose surrogates (using physiologically based toxicokinetic (PBTK) models), 2) a study of the potential metabolic interactions between trihalomethanes (THM), and between THM and haloacetic acids (HAA) (the most important by-products based on occurrence and health effects), and their effect on the modulation of the tissue-dose surrogates in animals and humans, and 3) use of these data to estimate quantitative risk for human exposure to these chemicals present as a mixture, by computer simulation. This integrated approach would indicate the exposure concentrations of each chemical, in comparison to its drinking water guideline value, at which significant interactions can be anticipated to occur during mixed exposures. Finally, the potential impact of temporal and spatial variations in the concentrations of one or more THM or HAA in the mixture on the predicted risk estimates can be evaluated by probabilistic methods (Monte Carlo simulation).

In Norway, disinfection of drinking water with ozone, chlorine dioxide and chloramines is less wide-spread than in the USA and Canada. In addition to chlorination, more waterworks are using UV disinfection, although most of these waterworks are relatively small. By-product formation is lower when disinfecting drinking water with UV than with these other alternative methods. The levels of chlorine used in Norway are rather low compared with other countries. Therefore, by-product formation in drinking water is a relatively small problem in Norway.

A method has been presented for the risk assessment of mixtures of cyanobacterial toxins with hepatotoxic, neurotoxic and carcinogenic effects in drinking water, based on derivation of toxicity equivalent factors (TEF) (see section 4.1.5) obtained from toxicological data in the literature (Wolf & Frank, 2002). Generally, all toxicological relevant toxins should be included in the assessment. When using the total sum of toxic equivalents, this approach seemed to lead to a more realistic assessment of cyanobacterial toxin mixtures than the worst-case approach. However, the availability of data about acute, and especially chronic toxicity, must be increased significantly to establish toxicologically validated exposure limit values to such cyanobacterial toxins.

The VKM Scientific Panel 4 has so far not evaluated any cases regarding health risks of exposure to contaminants in drinking water, including migrants from materials in contact with drinking water, or chemicals used for water treatment or their by-products.

5.2.5 Cosmetics

The EU Scientific Committee on Consumer Products (SCCP) is responsible for risk assessments of consumer products, i.e. non-food products intended for the consumer, which include cosmetics. A detailed guideline for their risk assessments of cosmetics is available (SCCP, 2006). The safety of a cosmetic product is based on the safety of its ingredients, which are evaluated individually by toxicological testing. The guidelines are extensive, and include a full evaluation of the effects of a chemical on all toxicological endpoints. All relevant ways of exposure are taken into account, being dermal, oral and/or by inhalation, depending on the particular product/ingredient.

Although not all of them presently in use, there are some 8000 cosmetic ingredients listed in the reference book Blue List (2001) and even more in the International Nomenclature of Cosmetic Ingredients (INCI) list, of which only about 5% have been evaluated for their effects on human health (Bridges J., 2003). The Seventh Amendment to the EU Cosmetics Directive 76/768/EEC (Directive 2003/15/EC of the European Parliament and of the Council) will ban the marketing of cosmetic/personal care products that contain ingredients that have been tested in animal models, and this is due to come into force in March 2009. New and improved *in vitro* methods for testing of ingredients in such products are therefore needed. Since cosmetics/personal care products may contain many ingredients simultaneously, possible interactions also need to be taken into account.

Parabens may in varying degree bind to the oestrogen receptors and exert weak oestrogenic activity *in vitro* and *in vivo*, and show anti-androgenic effects *in vivo*. In its risk assessment of parabens in cosmetics (VKM, 2006a), VKM Scientific Panel 4 did not evaluate combined effects because of lack of *in vivo* data. In its other risk assessments of chemicals in cosmetics the terms of reference concerned one substance only.

5.2.6 Concluding remarks from VKM Scientific Panel 4

The chemical contaminants of relevance for VKM's Scientific Panel 4 are normally present in food or drinking water in low concentrations, and in principle do not have any intended effects, such as e.g. pharmaceuticals (although exceptions can be found). Exposures to a combination of compounds most likely do not cause effects stronger than the effects of their most active component. This is provided that the components are present at low concentration levels, such as at the ADI, TDI or reference dose (RfD) levels, i.e. well below their respective

NOAEL levels. The safety of the overall evaluation is also secured by including an uncertainty factor to cover any limitations in the data.

It has been demonstrated in various studies of mixtures of two or a few chemicals together that a combination of compounds with the same target organ or the same or very similar mechanisms of action, may cause additive or synergistic effects. However, the chance of such effects will most likely diminish with decreasing mixture exposure levels. Synergism and antagonism may also both occur at the same time at different organs or targets in the same organism. However, and despite some exceptions, it has been demonstrated for e.g. plant protection products residues in food or drinking water (Carpy *et al.,* 2000) that interaction between components is not a common event at low levels of human exposure.

In cases where interaction between compounds has been demonstrated in cell cultures (i.e. *in vitro*) and the combined effects are of biological significance, it is important to follow up with experimental animal (i.e. *in vivo*) studies. Risk assessment of ingredients of cosmetics for instance, is usually based on toxicological information following long-term systemic exposure of animals. Such testing takes into account the complex interplay between the organs as well as influences caused by developmental stage, age or life cycle. However, this will not be possible within the EU after 2009.

The VKM Scientific Panel 4 has so far not evaluated health risks of exposure to combined effects of mixtures of chemicals in their risk assessments, either because the terms of reference concerned one substance only, and/or because of lack of data, especially *in vivo* data.

5.3 VKM Scientific Panel 5 (Panel on Contaminants)

This Panel addresses questions on environmental contaminants and other pollutants, process-related chemicals, natural toxins, and residues of medicines in food.

5.3.1 Environmental contaminants

A wide variety of potential hazardous contaminants, including both organic and inorganic compounds, may be present in food. These contaminants may have very different sources and fate in the environment. Common features for many of them are that they are persistent and may accumulate in animal tissues, biomagnify in food webs and may exert toxic effects in humans. Many of the contaminants may share the same toxic endpoints via similar or dissimilar mechanism of actions; however, for most of the contaminants the mechanism of action is unknown.

5.3.1.1 Organochlorinated compounds

The industrial waste polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), the industrial chemicals polychlorinated biphenyls (PCBs, which can be divided into two groups according to their toxicological properties; dioxin-like PCBs (dl-PCBs) and non-dioxinlike PCBs (ndl-PCBs)), and various formerly used chlorinated pesticides (e.g. lindane, DDT and its metabolites chlordanes, dieldrin, aldrin, endrin, heptachlor, hexachlorbenzene and toxaphene) are all very persistent chemicals which are ubiquitous in the environment. However, the chlorinated pesticides and PCBs are no longer produced, and the emissions of PCDD/Fs into the environment during the past decade has been significantly reduced (>80%). Thus, the levels of these compounds in the environment and foods are

generally decreasing. Even though most of these compounds tend to occur in parallel and are still generally present in food, in particular fat-containing food including milk, egg, meat and fish, most attention has been given to the PCDD/Fs (dioxins) and PCBs. In Norway occurrence of dioxins and PCBs has resulted in recommendations of restricted consumption of various food products.

Dioxins (PCDD/Fs) and dioxin-like PCBs (dl-PCBs)

Dioxins and PCBs are persistent organochlorine compounds that are globally dispersed environmental contaminants and which accumulate in fatty foods. The term "dioxins" is commonly used to refer to a group of 75 PCDD and 135 PCDF congeners, of which 20 are biologically active. 1,3,7,8-TCDD and 1,2,3,7,8 penta-CDD are the most potent. There are 209 theoretically possible PCB congeners, of which 12 non-ortho and mono-ortho compounds exhibit similar biological activity to PCDD/Fs, and are therefore referred to as "dioxin-like (dl) PCBs". Exposure of the general population to dioxins and dl-PCBs is primarily from food (> 90%), and fatty fish is an important source. Dioxins and dl-PCBs exhibit a broad range of toxic and biological effects, and most, if not all, are mediated through the aryl hydrocarbon receptor (AhR). AhR is a cytosolic receptor protein present in most vertebrate tissues with high affinity for 2,3,7,8-substituted PCDD/Fs and some dl-PCBs. The general acceptance of an additive model has resulted in the toxic equivalent concept and the toxicities of 17 dioxins and 12 dl-PCBs relative to 2,3,7,8-TCDD are expressed as toxic equivalent factors (TEFs) (Van den Berg *et al.*, 1998; Van den Berg *et al.*, 2006). The total toxic equivalency (TEQ) is operationally defined as the sum of the concentration of each compound multiplied by its TEF value and is an estimate of the total 2,3,7,8-TCDD-like toxicity of the mixture. The level of TEQs in a food sample is a measure of the total dioxin effect and simplifies risk assessment of complex mixtures of dioxins and dl-PCBs. Expert groups in SCF (SCF, 2001) and JECFA (JECFA, 2001b) have assessed health risk of intake of dioxins and dl-PCBs from food, and they based their updated assessment on rodent studies providing a NOAEL and LOAELs for the most sensitive effects of 2,3,7,8-TCDD exposure in experimental animals, i.e. developmental effects in rat male offspring. The tolerable weekly intake (TWI) of dioxins and dl-PCBs is 14 pg TEQ/kg bw (SCF, 2001). The VKM Scientific Panel 5 has used this TWI in their risk assessment of exposure to dioxins and dl-PCBs in fish and other food known to be important sources of these compounds.

Non-dioxin-like PCBs

The PCBs referred to as the "non-dioxin-like PCBs" (ndl-PCBs) (197 congeners) are found in relatively high concentrations compared to dl-PCBs in food; in some food matrices they can be several orders of magnitude more concentrated than the dl-PCBs. More than 90% of the ndl-PCB exposure in the general population is via food; foods of animal origin are most important. The ndl-PCBs do not show dioxin-like toxicity, but have other toxicological profiles and about 10 different toxicological endpoints have been identified. The adverse effects reported in laboratory animals following exposure to individual ndl-PCBs were biochemical evidence of effects on the thyroid, liver and brain, as well as immunotoxicity, oestrogenicity, and reproductive and neurodevelopmental effects. The most sensitive effects seen in studies with individual ndl-PCB congeners in experimental animals were liver and thyroid toxicity.

This group of PCB congeners has recently been evaluated by (EFSA, 2005a) who concluded that no health-based guidance value for humans can be established because simultaneous exposure to ndl-PCBs and dl-PCBs hampers the interpretation of the results of the toxicological and epidemiological studies, and the database on effects of individual ndl-PCB

congeners is rather limited. Furthermore, through their evaluation EFSA found indications that subtle developmental effects, being caused by ndl-PCBs, dl-PCBs or dioxins alone or in combination, may occur at maternal body burdens that are only slightly higher than those expected from average daily intakes in Europe. In their risk assessment of exposure to PCBs in food, VKM's Panel 5 has used the health-based guidance value for dl-PCBs and dioxins. According to the available toxicological database the toxicological potencies of the dioxins and the dl-PCBs are orders of magnitude higher than the ndl-PCBs. Thus, assuming that dioxins and dl-PCBs are occurring in parallel with ndl-PCBs in most food items and that the concentration ratio does not differ significantly within and between food groups, the approach to assessing the health risk of exposure to the most potent compounds in a mixture should give an acceptable protection of the population. However, occurrence and levels should be taken into consideration.

5.3.1.2 Organobrominated compounds

Other persistent organic contaminants that have received a lot of attention recently are the brominated flame retardants, polybrominated diphenyl ethers (PBDEs; 209 congeners), hexabromocyclododecane (HBCD; 3 isomers) and tetrabromobisphenol A (TBBPA). In contrast to the abovementioned chlorinated organic contaminants, the levels of these compounds, particularly PBDEs, have until recently been increasing in the environment and in humans. Since 2004, the production of specific technical mixtures of PBDEs has been banned in Europe and a decline in levels for specific biota and humans has been reported. Low levels of brominated flame retardants are generally found in parallel with the dioxins and PCBs in fat-containing food, particularly in fatty fish. Fatty fish is an important source for human exposure to chlorinated and brominated persistent organic pollutants.

PBDEs

The contamination in food reflects the composition of the three commercial mixtures (penta, octa and deca mixtures) that have been used worldwide. The most common PBDE congeners found in food are tetra-BDE 47, penta-BDE-99, penta-BDE-100, hexa-BDE-153, and hexa-BDE-154 in addition to the fully brominated deca-BDE-209.

Both single substances and commercial mixtures have been tested in laboratory animals. Among these, penta-BDEs appear to be most toxic. Effects on thyroid hormone balance, histopathological changes in thyroid and liver, and neurotoxic effects/behavioural changes leading to sustained increase in locomotor activity level after exposure *in utero* have been reported in mice and rats. For several of the reported effects the toxicological relevance for humans is not clear. Also, there are important physiological species differences in thyroid hormone homeostasis. There is very limited knowledge of mechanisms of action, metabolism, half-life and accumulation in both laboratory animals and in humans. In a risk assessment of exposure to PBDEs from 2005, VKM's Panel 5 concluded that there is not sufficient knowledge to set a tolerable daily intake for PBDEs, and chose to present the margin of exposure (should probably have been called margin of safety, MOS.) which is the ratio between the no effect level in laboratory animals and exposure to the sum of BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154 in different parts of the Norwegian population.

The structure of PBDEs resembles that of PCBs. However, the PBDEs do not activate the Ah-receptor, indicating that their mechanism of action is different from that of dioxin-like PCBs. On the other hand, several of the effects reported after PBDE-exposure in rodents, such as neurotoxic effects, have also been reported after PCB-exposure. There is a theoretical

possibility that PBDEs may act additively to non-dioxin-like PCBs. If this turns out to be the case, the combined exposure needs to be accounted for in future risk assessments.

5.3.1.3 Polycyclic aromatic hydrocarbons (PAHs)

Another group of potential hazardous organic contaminants are the PAHs consisting of a large number of compounds, some of which are fairly persistent. PAH may contaminate food either by emissions from e.g. transport/traffic and industrial processes to agricultural areas and aquatic bodies, or through food processing (e.g. frying and grilling). The most important food sources of PAHs quantitatively are food oils, cereals, and leafy vegetables, but processed (fried and grilled) meat and fish may also contribute to the exposure. Mussels may accumulate PAHs from water. Low levels of PAHs may thus be present in mussels and certain processed food of animal origin in parallel with dioxins, PCBs and brominated flame retardants.

PAHs contain two or more aromatic rings with no side chains. They are formed during incomplete combustion of organic material. Among several hundred PAHs, 14 have been classified as genotoxic and carcinogenic (SCF 2002b, IARC in press). It is believed that the carcinogenic mechanism is primarily by covalent binding to DNA, leading to mutations, but several PAHs also have tumour promoting effects. PAHs always occur as a mixture of several substances. The carcinogenic potency depends on the mixture, since the combination effect of different PAHs is not always additive, but has also in some instances shown to be synergistic and antagonistic. Since different PAHs probably have different mechanisms of action, a TEF principle is not suitable for risk assessment (SCF, 2002b; VKM, 2007b).

Benzo[*a*]pyrene (BaP) is the most thoroughly investigated and is one of the most potent carcinogenic substances among the PAHs. BaP-exposed mice develop tumours in the forestomach, but when mice are exposed to lower BaP doses administered together with other PAHs in a mixture, they develop tumours in the lung and other organs. However, BaP concentration is a good indicator of total carcinogenic potency of a mixture, provided that the composition of PAH in the mixture is relatively constant. SCF (2002b) found that the relative contribution of BaP to total PAH in foods was relatively constant (varied with a factor less than 10), and concluded that BaP could be used as an indicator substance of all carcinogenic PAHs found in food. JECFA used the same principle in their evaluation of PAH (JECFA, 2005). An on-going assessment in EFSA, based on new data on the composition of PAH in food will examine if this assumption is valid.

The VKM Scientific Panel 5 has used BaP as indicator of total carcinogenic PAHs in risk assessment of PAHs in mussels and in barbequed food, and determined the margin of exposure (MOE) between a specified low defined effect level in laboratory animals and human exposure.

5.3.1.4 Metals

The presence of potentially hazardous metals in the environment is a result of both natural geological conditions and anthropogenic factors, such as industrial emissions. Hazardous metals including cadmium, lead, inorganic/organic mercury and organotin may be present in various food items. Methyl mercury, cadmium and organotin are the most important hazardous metals in food of marine origin (VKM, 2007a).

Mercury, cadmium, lead
Despite that metals at a certain dose pose a health threat, about 20 of them are regarded essential. Exposure to some metals such as mercury, cadmium and lead are given special focus as human health and environmental hazards.

Lead (Pb)
Lead accumulates in several tissues and organs of the body, and the intake of lead may result in many different toxic effects, i.e. on the nervous system, blood formation and the kidneys. The half-life for lead in blood and other soft tissues is about 28-36 days, but it is much longer in the various bone compartments. The most important target organ following long-term, low level exposure to lead is the nervous system. Small children, and foetuses in particular, are most vulnerable, and exposure to lead may result in impaired development of cognitive functions (learning ability) and motor skills. The effects of lead exposure have been well-documented and have also been described in epidemiological studies (WHO-IPCS, 1995).

The mechanism underlying the neurotoxicity of lead is that lead passes easily through the blood-brain barrier, causing cell death and interference with the transfer of signals between nerve cells and in supporting cells. Lead is not genotoxic, but it can cause tumours in laboratory animals. Lead has been classified in Group 2A by the International Agency for Research on Cancer (IARC) (IARC, 2004). Due to the effects of lead on children and foetuses, the provisional tolerable weekly intake (PTWI) was set at 25 μg/kg bw by JECFA in 1986. The PTWI was based on studies on lead in children and was set with the aim of avoiding the accumulation of lead in the body. In 1993 and 2000 JECFA confirmed this PTWI value and expanded it to include all age groups (JECFA, 1993; JECFA, 2000a).

Cadmium (Cd)
Cadmium is absorbed in the intestines and accumulates in the kidneys and liver in particular. In individuals with iron deficiency, the intestinal uptake of cadmium may increase considerably. The metal is excreted slowly (the biological half-life is 10-30 years) and is accumulating with age. The largest concentration can be found in the cortex of the kidney. In the cells, cadmium binds to the protein metallothionein, which it also induces. The toxicity depends on the amount of free cadmium in the cells, and is manifested especially when the capacity for detoxification with metallothionein is exceeded (Jin *et al.,* 1998).

The effects of cadmium have been well-documented in a number of experimental and epidemiological studies (WHO-IPCS, 1992). Cadmium has also been classified as a human carcinogen (Group 1) by IARC, but this applies to exposure by inhalation.

The PTWI has been set by JECFA at 7 μg/kg bw on the basis of studies on humans (JECFA, 2001b). JECFA re-evaluated cadmium in 2003 (JECFA, 2003). Recent epidemiological studies indicate that low exposure, at the PTWI level, is associated with an increased incidence of minor kidney changes. Because the long-term significance of these changes is unknown, JEFCA upheld the previously established PTWI at 7 μg/kg bw.

Mercury (Hg)
There are different forms of mercury, both inorganic and organic. In fish and other seafood, methylmercury is most prevalent (75-100% of total Hg in various fish species) and represents the greatest health hazard. Methylmercury is absorbed in the intestine (95%), crosses the placenta and blood-brain barrier and is also excreted in breast milk. The average half-life is 70 days in adults (JECFA, 2003).

Methylmercury is neurotoxic. While the peripheral nervous system is most vulnerable in adults, the central nervous system is most vulnerable in the early stages of human development. The foetal brain is especially sensitive, and increased concentrations of methylmercury may result in impaired cognitive skills as well as motor skills. The human foetus is believed to be most sensitive during the last six months of pregnancy and in the first period following birth due to the rapid development of the nervous system during these periods. Some studies also indicate that methylmercury may affect blood pressure (Grandjean *et al.,* 2004). Also, a number of studies, including studies from Finland (Landmark & Aursnes, 2004; Virtanen *et al.*, 2005) and a large-scale European study (Guallar *et al.,* 2002), show an association between exposure to methylmercury and risk of cardiovascular diseases.

In 2003, JECFA revised its earlier assessment of mercury. The previous PTWI value for methylmercury was reduced from 3.3 to 1.6 μg/kg bw (JECFA, 2003).

In 2004, EFSA assessed the European population's exposure to mercury from fish and gave its support to the PTWI established by JECFA in 2003 (EFSA, 2004a).

Interaction within and between toxic metals

There are several complicating factors regarding possible interactions within and between toxic metals. These include the fact that the metals occur in different chemical forms and bound states in food. Interactions between toxic metals and between other bioactive molecules in food components can occur in several ways:

1. Through different chemical forms and possible interactions in food
2. Through interactions during absorption in the intestine
3. Through metabolic interactions.

It is generally assumed that through the competition between divalent ions at the absorption sites, diets and food items with higher levels of non-toxic divalent ions will reduce the toxicity of toxic elements. The incidence of itai-itai disease in Japan was coupled to a high cadmium intake, but it is also assumed that a low calcium intake enhanced the toxic effect by influencing uptake. Other examples of such interactions may be the positive effect due to uptake interference of selenium on mercury (Ralston, 2005).

Both at the absorption level and for the toxicodynamics of elements it is assumed that metallothionein (MT) plays a crucial role in the homeostasis of elements. MT is a family of low molecular weight proteins with very high content of the sulphur-containing amino acid cystein. It binds readily several divalent ions such as Cd^{++}, Hg^{++}, Cu^{++}, Zn^{++}, Ag^{++} and Pb^{++}.

MT is believed to function as a sequestering agent and possible intracellular storage molecule for essential transition elements. MT may also function as a detoxifier through binding of toxic elements. The protein is also highly inducible, and it is therefore assumed that previous exposure can reduce the toxic effect of a subsequent exposure.

Organotin compounds

The main sources of organotin compounds (OTC) in food are commercial products used as biocides, antifouling paints and as stabilisers of polyvinyl chlorides (PVC). The most toxic OTCs are tributyltin (TBT), dibutyltin (DBT) and triphenyltin (TPT). Known toxicological

endpoints of OTC are reproductive and developmental toxicity, genotoxicity, carcinogenicity, neurotoxicity, immunotoxicity, as well as endocrine disruption.

EFSA was asked to assess the possible risk to human health from the consumption of food contaminated with OTC, based on intake estimates for Europe. The critical toxicological endpoint used in the risk assessment was immunotoxicity (EFSA, 2004b). EFSA focused on the most toxic OTCs: TBT, DBT and TPT which primarily are found in (VKM, 2007d) of the compounds were considered to be dose additive with equal potencies, and a group TDI was established (EFSA, 2004b).

NOAEL for immunotoxicity was set at 0.025 mg/kg bw/day based on chronic feeding studies in (TBTO, *bis*(tri-n-buthyltin)oxide) (Wester *et al.,* 1990; Vos *et al.,* 1990). EFSA used an uncertainty factor of 100 to establish a group TDI of 0.25 µg/kg bw for the sum of TBT, DBT, TFT and DOT. The group TDI is based on the molecular weight of TBTO. However, in the EFSA report it is unclear if they have used TBTO, a *bis* molecule with two tin atoms, in their calculation. EFSA has also given a group TDI based on the molecular mass of tin (0.1 µg/kg bw), as well as one expressed as TBTCl, tributyltin chloride (0.27 µg/kg bw).

The VKM Scientific Panel 5 has used EFSAs group TDI expressed as the mass of tin (0.1 µg/kg bw) in their risk assessment of organotin compounds in fish and fishery products (VKM, 2007d).

5.3.2 Biotoxins

Biotoxins are toxic substances with a biological origin (produced by bacteria, fungi, algae, cyanobacteria, plants or animals). Biotoxins are very diverse chemical compounds with a diversity of biological effects. Biotoxins can be divided either by source of origin, biological effects or chemical structure and toxins with similar chemical structures may have different biological origin.

VKM has conducted risk assessments of algal toxins, which is discussed here. Mycotoxins (toxins produced by fungi) have also caused some concern for public and animal health, and a brief discussion of mycotoxins is also included.

5.3.2.1 Mycotoxins

There are a vast number of described mycotoxins with a diverse chemical structure. However, a limited number have been associated with human diseases and a selection of a few mycotoxins most often considered to pose a risk to human health is discussed.

Fungal species are often co-occurring in food and feed raw materials and products. Contamination of food and feed by mycotoxins may be a result of fungal infection and growth both in the fields and during storage under suboptimal storage conditions where fungal infection and mycotoxin production are known to occur. Furthermore, a large number of the most common toxin-producing fungal species are producers of more than one mycotoxin. During processing of food and feed, a variety of raw materials, each with their set of possible fungi and mycotoxins are mixed. Consequently, mycotoxins are often found as mixtures of different toxins from different chemical classes originating from both field and storage fungi.

A limited number of experimental studies testing different mixtures of mycotoxins *in vivo* have been reported, but the outcomes vary from no interaction to additive or antagonistic effects. However, available data are limited and generally an insufficient number of combinations have been tested to make any firm conclusions. Studies of the effects in different *in vitro* systems have been found to range from antagonistic to synergistic effects, depending on concentrations tested and test system used.

Aflatoxins B_1, B_2, G_1 and G_2 are mainly produced by three *Aspergillus* species, *A. flavus*, *A. parasiticus* and *A. nomius.* The fungi are most prevalent in tropical areas, and in Norway the aflatoxin problem is mainly related to imported food and feedingstuffs. Aflatoxins M_1 and M_2 are hydroxylated metabolites occurring in milk from ruminants exposed to aflatoxins in the feed. Aflatoxin B_1 is found most frequently and in the highest concentrations. Aflatoxins B_2, G_1 and G_2 are generally not found in the absence of B_1. Aflatoxins are potent genotoxic carcinogens causing liver carcinomas. Aflatoxin is also acute hepatotoxic in higher doses causing acute lethal intoxications of animals, and even humans, in tropical areas. Both (EFSA, 2007b) and (JECFA, 1998; JECFA, 2001a) have made recommendations applying to the sum of aflatoxins B_1, B_2, G_1 and G_2, based on the available data base, which to a large extent is either the natural mixture of aflatoxins or aflatoxin B_1. JECFA prepared a separate assessment for M_1, concluding that this toxin is not likely to have any significant contribution to the incidence of liver cancers.

Ochratoxin A is produced by *Penicillium verrucosum* and a number of *Aspergillus* species. The fungi also produce other ochratoxins, including ochratoxin B. The latter toxins are rarely found and in much lower concentrations. Ochratoxin B is also considered to be much less toxic (EFSA, 2006). Ochratoxin A is nephrotoxic inducing cellular damage and a progressive nephropathy. Ochratoxin A has been associated with human kidney diseases and kidney tumours in the Balkan area, but the epidemiological studies are incomplete and do not provide sufficient evidence for classification of ochratoxin as a human carcinogen. Ochratoxin A induces renal tumours in rodents at high doses. EFSA concludes in their evaluation that the renal toxicity, as well as the DNA damage and genotoxic effects, are most likely caused by oxidative damage.

There is no risk assessment where the combined effects of ochratoxin A and other mycotoxins have been included.

Trichothecenes

The EU SCF evaluated a range of toxins produced by *Fusarium* species, the most prevalent toxin-producing genus in the temperate climate zones, including areas such as Europe and North America. Trichothecenes reduce feed intake and growth, and have immunomodulatory effects in low doses. At higher doses, the toxins have a wide range of effects in most animal species, such as diarrhoea, vomiting, pathological changes in the gastrointestinal tract and reduced reproduction. Trichothecenes are generally not considered to be genotoxic or carcinogenic (Rotter *et al.*, 1996; JECFA, 2001a).

SCF made a group evaluation of the trichothecenes T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol, but did not include other important *Fusarium* toxins such as zearalenone and fumonisins. Due to uncertainties about the exact mechanisms of action, very limited database and large non-systematic potency differences between the toxins when different toxic effects are considered, SCF concluded that a group TDI could not be set, with the exception of a group TDI for the sum of T-2 and HT-2 toxins. The effects of the two latter toxins could not

be separated from each other since T-2 toxin is partly and rapidly metabolised to HT-2 toxin *in vivo* (SCF, 2002a). *Fusarium*-infected cereals are to a variable degree more toxic to animals than what should be expected from the known levels of *Fusarium* toxins (Rotter *et al.*, 1996; Eriksen & Pettersson, 2004; EFSA, 2004c). It is not known whether this is due to interactions, the presence of unknown fungal metabolites or some other unknown explanation.

Zearalenone was evaluated by JECFA in 2000. This is a *Fusarium* metabolite with oestrogenic properties and effects in animals. Zearalenone affects reproduction and other physiological processes influenced by the natural hormone. JECFA concluded in the evaluation that for zearalenone the provisional maximum tolerable daily intake (PMTDI) of 0.5 µg/kg bw should apply to the sum of the mycotoxin and all of its metabolites, including zeranol (zearalanol), which is used as a growth promoter in parts of the world (JECFA, 2000b).

5.3.2.2 Algal toxins

Marine algal toxins include many different toxin groups. Many of them accumulate primarily in bivalves, and may present a threat to the consumers. Exposure to marine algal toxins occurs sporadically, and most toxicological data are based on acute or short-term exposure. Consequently, acute reference doses (ARfD) have been established. These are an estimate of the amount of toxin, expressed in terms of mg/kg bw, which can be consumed during a 24-hour period without significant health risk for the consumers.

In 2004, a FAO/IOC/WHO expert group (FAO/IOC/WHO, 2004) established a scientific basis for deriving maximum levels of algal toxins in bivalves based on risk assessments. Their recommendations are now under evaluation by the Codex Alimentarius Committee for Fish and Fishery Products. In addition, the Expert Consultation categorised the marine biotoxins in a new way, based on their chemical structure. In 2006, the EFSA established an expert working group on marine biotoxins, asking them to provide an evaluation of todays EU regulation on maximum levels and methods of analysis.

The following section provides a description of the most important groups.

The saxitoxin group (STX). Paralytic shellfish poisoning (PSP) has been known for several hundred years and is caused by toxins in the STX group. The STX toxins are classic neurotoxins that afflict both the peripheral and central nervous systems with sensory impairment, paralysis and possibly death. The mortality rate in adults is 5-10% without treatment and higher in children. There is great uncertainty regarding the dose/responses. A temporary ARfD was set at 0.7 µg STX-eq/kg bw by the (FAO/IOC/WHO, 2004), based on an estimated LOAEL of 2 µg STX-eq/kg bw in humans, and including an uncertainty factor of 3. The STX equivalents are calculated by adding up each analogue present after multiplication with the assigned TEF-value. The STX group comprises about twenty STX analogues, most of them with established TEFs, based on their intraperitoneal toxicity in a mouse bioassay (Oshima, 1995).

The okadaic acid group (OA). The toxins cause effects in the gastrointestinal tract in the form of diarrhoea and vomiting, and the syndrome is named Diarrhetic Shellfish Poisoning (DSP). A temporary ARfD at 0.33 µg OA-eq/kg bw was suggested by the (FAO/IOC/WHO, 2004), based on a LOAEL of 1 µg OA-eq/kg bw in humans, including an uncertainty factor of 3. The OA group consists of okadaic acid and the closely related dinophysistoxins (DTX-1 and

DTX-2). In addition, fatty acid esters of all three analogues, commonly named DTX-3, contribute in varying degree to the total toxicity. Most recent TEFs recommended by international experts are 1 for OA and DTX-1, and 0.6 for DTX-2. The TEFs for DTX-3 are calculated after hydrolysis to the non-acylated analogues. See also the recent evaluation by EFSA (EFSA, 2007a).

The azaspiracid group (AZA). The major human route of exposure to AZA is ingestion, resulting in toxicity characterised by gastrointestinal disturbances e.g. diarrhoea, vomiting, abdominal pain and cramps. The first case of azaspiracid poisoning (AZP) was reported in the Netherlands in 1995, associated with consumption of blue mussels from Ireland. More intoxication took place in the following years, all from consumption of blue mussels from Ireland. Several international expert groups have evaluated the AZA group recently. AZA-1, AZA-2 and AZA-3 are known to be the most important toxins in the AZA group. Based on the few data available, the TEFs for AZA-2 and AZA-3 are 1.8 and 1.4, compared with AZA-1 at 1. The VKM Scientific Panel 5 has evaluated the risk related to AZA in Norwegian crabs based on the risk assessment from the Food Safety Authority of Ireland in 2006 (FSAI, 2006). The Panel recommends an ARfD of 17 µg AZA-1 equivalents/person (60 kg) based on the lowest estimated LOAEL (50 µg/person) and a safety factor of 3 (VKM, 2007c). The FSAI recommended an ARfD in bivalves at 0.67 µg/kg (40 µg/person), based on the median of the estimated LOAELs from epidemiological studies. Current regulation of AZAs in the EU (maximum level at 160 µg/kg AZA-1 equivalents in shellfish) is to a large extent based on the sensitivity of the mouse bioassay, and not on a comprehensive risk assessment.

The domoic acid group (DA). In 1987, an outbreak of a newly recognized acute illness caused by eating blue mussels and characterized by gastrointestinal and unusual neurological symptoms occurred in Canada. More than one hundred people were affected. The symptoms were vomiting, abdominal cramps, diarrhea within 24 hours and neurological symptoms within 48 hours (severe headache and memory loss). The syndrome was named Amnesic Shellfish Poisoning (ASP). The dominating toxin is an unusual amino acid, domoic acid. A few analogues are known, but they do not contribute much to ASP. No poisoning incidents from ASP have been reported in Norway so far. The FAO/IOC/WHO expert group (FAO/IOC/WHO, 2004) suggested an ARfD at 100 ug/kg bw for DA.

The yessotoxin group (YTX). Since 1987, the toxins in this group have been found together with toxins from the OA group, and until recently they were included in the DSP toxin complex. The main reason for this was that YTXs contribute to the outcome of the mouse bioassay for lipophilic toxins (OA-, AZA- , YTX- and several other groups) The YTX group has not been associated with any known human intoxications. A temporary ARfD has been set at 50 µg/kg bw (FAO/IOC/WHO, 2004), based on studies of oral toxicity of YTX in mice, including a safety factor of 100. An ever increasing number of YTX analogues are discovered (between 50 and 100), but only a few of them exert toxicity even upon intraperitoneal injections in mice.

Until now, possible combined effects of mixtures of toxins from different groups have not been included in regulation, with the exception that pectenotoxins (a non-diarrheagenic group) is included in the EU regulation for the DSP toxin complex. This is due to the EU requirement for using the mouse bioassay, and not a comprehensive risk assessment. In the future, it is important to examine whether exposure to mixtures from different groups may increase the toxicity of shellfish.

5.3.3 Residues of veterinary medicinal products

Within the European Union (EU) veterinary medicinal products (VMP) will not be the subject of a marketing authorisation for the purpose of administering it to one or more food-producing animals, unless this has been granted by the competent authorities of that member state (EU Directive 2004/28/EC or Regulation (EC) No 726/2004). Marketing authorisation requires, amongst others, that both the pharmacologically active substances and their metabolites, which may remain in foodstuffs obtained from treated animals, have been evaluated with regard to human safety (Regulation (EEC) No 2377/90). As a consequence of the European Economic Agreement (EEA), this is also the case for Norway. The tests required are described in various scientific guidelines for the evaluation of residues of VMPs.

For VMP, the approach used by the Committee of Veterinary Medicinal Products (CVMP), European Medical Evaluation Agency (EMEA), for the evaluation of the safety of residues is based on the determination of ADI for the pharmacologically active substance contained in the VPM. The ADI may be set on the basis of toxicological, microbiological (only antimicrobial drugs) or pharmacological data; whichever is the lowest is used.

The ADI values, whether toxicological, microbiological or pharmacological, are set for single substances. This can be justified because, among others, the numbers of combination preparations, i.e. preparations containing two or more active drug substances, approved for use in food producing animals are very limited. This is especially true for Norway. Furthermore, food producing animals are in general rarely treated with several preparations concurrently, because they seldom suffer from several diseases concomitantly. This is a consequence of their short life-span (e.g. broilers: approximately 35 days; dairy cows: approximately 3 years) and because continuing breeding programmes have systematically selected for animals resistant to various diseases or with a low frequency of diseases.

5.3.4 Concluding remarks from VKM Scientific Panel 5

Environmental contaminants, chemicals produced during food processing, biotoxins, and residues of medicines represent a wide variety of potential hazardous substances that may be present in food either as single compounds, mixtures of related compounds, or multiple complex mixtures of related and unrelated chemicals. The individual compound or compound groups may exert a variety of toxicological effects. They may impair the same toxicological endpoints via similar or different modes of action which, however, for most of these hazards are still unknown. Furthermore, the potencies of the hazards may vary by several orders of magnitude. The presence of the individual compounds in a mixture may be below threshold levels for effects. However, the chemicals may interact in the toxicokinetic and/or the toxicodynamic phases resulting in modulation of the effects of the various substances in the mixture. The toxicological data are limited for most of the individual hazardous compound or compound groups, and only for a few groups health-based guidance values for humans have been established (dioxins and dl-PCBs; organotins, selected mycotoxins (group evaluation of T-2 and HT-2 toxins, and a group TDI for zearaleone and all its metabolites) and algal toxins (group ARfDs for the saxitoxin group, the okadaic acid group, the azaspiracid group and the yessotoxin group). For the process-related chemical group the carcinogenic PAHs, one compound, BaP, is used as an indicator of the total carcinogenic PAH-mixture present in food. For other hazardous individual compounds or compound groups, comprehensive risk assessment is not possible at the present time. A database allowing for assessment of multiple exposures is not available. Thus, neither internationally nor nationally a tradition exists for assessing combined toxic effects of exposures to multiple mixtures of environmental

contaminants, process related chemicals, natural toxins, or residues of medicines present via food.

The VKM Scientific Panel 5 has used the toxic equivalent concept and group TWI and ARfDs in their risk assessment of exposure to the environmental contaminants dioxins and dl-PCBs and the natural occurring algal toxins the azaspiracid group, respectively. The Panel has also used EFSA's group TDI expressed as the mass of tin in their risk assessment of organotin compounds in fish and fish products. The Panel has not received any request for risk assessment of mycotoxins and has therefore no experience in evaluation of combined actions of mycotoxins in risk assessment. For the carcinogenic PAHs, The Panel has used BaP as an indicator of the total carcinogenic PAH compound mixture found in food.

A potential risk area in a Norwegian context is the combined effects of consumption of marine organisms from localised areas where there has been point source release of halogenated organic compounds and heavy metals. Since both types of contaminants are associated with developmental effects (reproductive-, immune- and central nervous system) and that the young child is especially sensitive towards such effects, due consideration should be given to the potential for interactions

5.4 VKM Scientific Panel 6 (Panel on Animal Feed)

This Panel is concerned with hygienic and toxicological quality of animal feed, along the whole production chain of feed for terrestrial and aquatic animals, including production methods, raw materials, additives, biological hazards, and GMOs. The Panel assesses the implications of animal feed for animal health and consumers of animal products.

Farmed animals and fish are exposed to multiple chemicals via their feedingstuff. If the feed contains high levels of one or multiple contaminants, the health of the animals may be negatively affected. The concern of the VKM Scientific Panel on Animal Feed (Panel 6) is on feed quality, where undesirable contaminants are one aspect. The rationale for concern about feedingstuff quality is two-fold. Firstly, there is a natural concern about the harmful effects of the contaminants on the health and welfare of farmed animals and fish. However, there is also the concern that hazardous levels of additives or contaminants accumulated in farmed animals and fish can be transferred to humans through the diet.

Most contaminants and toxins are subject to EU legislation and identified in Directive 2002/32/EC on undesirable substances in animal feed and in Commission Regulation (EC) No 466/2001 which set maximum levels for certain contaminants in animal foodstuffs. Maximal levels of these undesired chemicals in farmed animals and fish are often based on the principle that levels for human exposure should not be exceeded. However, for some chemicals certain farmed animals may be especially vulnerable. For information on toxic effects of chemicals and the application of methods for assessing combined toxic effects of multiple chemical exposures in humans, farmed animals and fish, see section 5.2, 5.3 and 5.6.

For most contaminants, except for dioxins, furans and dioxin-like PCBs, maximum levels in animal and fish feed are defined for single substances. For dioxins, furans and dioxin-like PCBs, maximal levels are expressed as TCDD-equivalents (TEQ) based on toxic equivalence factors (TEF), using the WHO-TEFs for PCDDs, PCDFs and dioxin like-PCBs. This is possible because of a similar mechanism of action. These compounds are persistent and are biomagnified in the food chain, thus potentially resulting in high levels in animal and fish

products for human consumption. Thus, for these compounds which are bioaccumulated, the primary concern is not on animal and fish health, but on human exposure.

5.4.1 Naturally occurring contaminants and additives

Examples of naturally occurring contaminants or additives in feedingstuff are trace elements, including toxic metals such as mercury, copper, arsenic and lead. Several trace elements are authorised in feedingstuff to meet the dietary requirements for normal physiological function of farmed animals and fish. If levels in feedingstuff become too high, this can result in toxic effects in the animals. Thus, there are legislations for the use of trace metals (including toxic metals), as well as for other additives (Council Directive 70/524/EEC with updates) in animal and fish feedingstuff. The list of authorised additives, including maximum levels for trace metals are regulated by the EU (Regulation (EC) No 871/2003).

The VKM Scientific Panel 6 has commented on a document to the EU Commission proposing to increase the maximum content for cadmium in fish feed from 0.5 to 1.0 mg/kg (88% dry matter). In the comments, which focused on fish health and consumer threat, interactions with other elements, such as iron, zinc and copper in fish were considered. However, little is known about the combined toxic effects of these elements in fish, and health-related combined effects were not assessed.

Following findings of high levels of cadmium in feedingstuff for ruminants, pigs and farmed fish (11-17 mg/kg) in Norway, Panel 6 gave an opinion on the consequences for fish health and food safety when farmed fish has been fed with feeds containing 11-17 mg of cadmium per kg feed for a limited time period (up to four months). The Panel concluded that feeding Atlantic salmon or rainbow trout with feeds containing 11-17 mg of cadmium per kg is unlikely to cause significant fillet contamination. However, dietary cadmium levels of 11-17 mg/kg have been shown to cause biochemical and physiological responses in Atlantic salmon parr following 4 months exposure. In this opinion, interactions with other elements were also considered. However, little is known about the possible antagonistic interactions of dietary cadmium with elements such as iron, zinc and copper in fish, and health-related combined effects could not be assessed.

Biotoxins in feedingstuff are also of concern for animal health. For a review on effects of biotoxins, and in particular the effects of mycotoxins which may occur in animal feedingstuff, see section 5.3.2. For Aflatoxin B1, maximum content in various products indented for animal feed is given by the EU Commission and vary from 0.005-0.02 mg/kg (ppm) (Commission Directive 2003/100/EC).

5.4.2 Xenobiotic contaminants

Combined maximum levels are given for dioxins (and furans) and dioxin-like PCBs, and the sum of them in feed (EU Commission Directive 2006/13/EC). In addition, there are lower EU action levels for these compounds that are used to highlight those cases where it is appropriate to identify a source of contamination and to take measures for its reduction or elimination (EU Commission Recommendation 2006/88/EC). Maximum levels in animal and fish feed is also given for some xenobiotic compounds, such as camphechlor (toxaphene) (sum of congeners CHB 26, 50 and 62) (EC Directive 2002/32/EC) and for endosulfan (Commission Directive 2003/100/EC).

In products intended for animal feed, maximum levels of dioxins and furans varies from 0.75 to 6.0 ng WHO-PCDD/F-TEQ/kg depending on the products. The maximum limit in feed for fish is for instance 2.25 ng WHO-PCDD/F-TEQ/kg, whereas action thresholds are 1.75 ng WHO-PCDD/F-TEQ/kg. For the sum of dioxins, furans and dioxin-like PCBs, the maximum levels for animal feed vary from 1.25 to 24.0 ng WHO-PCDD/F-PCB-TEQ/kg. In feed for fish, the maximum limit is 7.0 ng WHO-PCDD/F-PCB-TEQ/kg. For dioxin-like PCBs, action threshold for feedingstuffs for fish is 3.5 ng WHO-PCB-TEQ/kg (EU Commission Directive 2006/13/EC). Furthermore, for toxaphenes (sum of the indicator congeners CHB 26, 50 and 62) the maximum content is 0.05 mg/kg in feedingstuff for fish (EC Directive 2005/86/EC), whereas for endosulfan (sum of alpha- and beta-isomers and of endosulfansulphate expressed as endosulfan) is 0.005 mg/kg for complete feedingstuffs for fish (Commission Directive 2003/100/EC).

5.4.3 Concluding remarks from VKM Scientific Panel 6

Hazardous compounds may be present in animal feed either as single compounds, mixtures of related compounds, or as multiple mixtures of related and unrelated chemicals. The individual compounds or compound groups may exert a variety of effects on animal health. They can also be transferred to humans through dietary routes (carry-over), and lead to adverse human health effects. The chemicals may impair the same toxicological endpoints via similar or different modes of action. For animal feedingstuff there are legislations for contents of trace metals and some biotoxins on an individual compound basis. For some groups of organochlorinated compounds, such as dioxins and furans and dioxin-like PCB congeners, camphechlor (toxaphene) and endosulfan, combined maximum levels based on the toxic equivalence concept are given for a range of different animal feedingstuffs. For dioxins and furans and for dioxin-like PCBs action threshold levels in animal feedingstuff are also given.

The VKM Scientific Panel 6 has considered interactive effects of cadmium and other trace metals in feed on fish, but due to lack of relevant studies on fish, no conclusion could be made with respect to animal health effects.

5.5 VKM Scientific Panel 7 (Panel on Nutrition, Dietetic Foods, Novel Food and Allergy)

This Panel addresses questions associated with human nutrition, dietetic products, novel foods, and food allergies, including fortification, food supplements, and health claims.

Human nutrition, which involves a complex interplay between nutritional components, physiology, molecular biology, genetics and metabolism, has been developed and adapted during a long human history. The nutritional sciences constantly reveal how dietary components may play important roles in the protection against disease. The research indicates that the pathology of virtually all age-related or chronic diseases is dependent on multifactorial elements that include diet, exposure to environmental agents, and genetic susceptibility (Hennig *et al.*, 2007). It is evident that nutrition can modulate metal toxicity (Gaetke & Chow, 2003; Ahamed *et al.*, 2007; Rooney, 2007), oxidative stress and cancer (Ferguson *et al.*, 2004; Finley, 2005; Moon *et al.*, 2006; Valko *et al.*, 2006), and biotransformation and detoxification (Moon *et al.*, 2006). Disturbing the complex balance of a traditional diet may therefore represent a health risk. In the group discussions in the VKM Scientific Panel 7 there are frequently raised questions whether the evaluated food may change the balance of the diet in a negative direction. Would a possible dietary change

represent a larger risk than the risk related to consumption of the new product itself? Will the new product disturb protective components of the diet?

When the safety of "energy" drinks was evaluated by the Panel (VKM, 2005), a possible combined effect of caffeine, taurine, and glucuronolactone was taken into consideration. It was also evaluated whether the combination of "energy" drinks and alcohol could represent enhanced toxicological hazards, particularly in combination with physical exercise. In the report, it was commented that the safety assessments were based on information on each of the ingredients, whereas the effect studies were performed with the product. More animal studies with the combination of ingredients or the product itself were recommended.

5.6 VKM Scientific Panel 8 (Panel on Animal Health and Welfare)

This Panel addresses questions on animal health and welfare, with particular focus on food-producing animals, including both farmed and wild fish.

Since the mandate from the Norwegian Food Safety Authority also includes a request for considerations of chemical substances in mixture with respect to effects on animal health and animal feed, the assessment of risk concerning animal health was assigned to Panel 8. Indirectly, adverse effects of mixtures of toxic substances that affect animal health in food producing animals may compromise human health also, whereas exposure of wildlife species or exposure of non-foodproducing animals is a health and welfare issue of individual animals, or may have an impact on the environment. With regard to mammals the risks are similar to human exposure, and of particular concern are the endocrine disrupting chemicals that are further discussed in section 3.4.

Although information on single-chemical effects is valuable, contaminants such as persistent organic pollutants often appear as congeners or as commercial mixtures in the environment. Examples of such mixtures are comercial mixtures of PCBs, hexachlorohexanes (HCHs), hexachlorobenzenes (HCBs), dichlorodiphenyltrichloroethane (DDT), chlordanes and toxaphenes extracted from cod liver oil waste products from Atlantic cod (*Gadus morhua*), freshwater mixtures from Lake Mjøsa and in Losna area in burbot (*Lota lota)* liver oil extracts. Experiments with those naturally occurring mixtures of persistent organic pollutants (POPs) found in marine and freshwater fish harvested from the Atlantic ocean and the freshwater lake systems in Norway have shown effects on sexual steroid production in cell cultures obtained from prepubertal pig ovaries (Gregoraszczuk *et al.*, 2008) and real life POP mixtures displayed negative effect on survival of zebrafish (*Danio rerio*) *in vitro* (Stavik, 2007; Almås, 2007).

In general, the exposure to these substances is below the concentrations that cause overt toxicity. Substances that act synergistic or display antagonistic key effects on sexual steroids may affect not only the development and differentiation of the foetus, but may have long-term effects on animal behaviour and the individual's ability to withstand stress or disease post partum. In the study by (Stavik, 2007), oestrogenic effects and effect on thyroid hormones of real life mixtures of POPs were detected in zebrafish.

Most studies on toxicological evaluations, toxicokinetics, toxicodynamics, and establishment of upper safe levels for humans and farmed animals, however, involve experiments with laboratory rodents, animals that may be as "different" to humans as they are to domestic animals or wildlife. On the basis of this, the VKM Scientific Panel 8 suggested that this

opinion should not include separate chapters on toxicological effects in animals, unless special situations could be identified with particular relevance to one or more animal species, beyond the included considerations of chemicals in drinking water, food additives, food contaminants etc. However, the chapter on toxicological effects of endocrine disrupters (see section 3.4) covers a paragraph with special reference to fish and wildlife. For information on toxic effects in animals resulting from multiple chemical exposures from animal feed, see section 5.3.2.1 and 5.4.

However, considering animal models and animal welfare in toxicological experiments on multiple chemical exposures, there could be concern regarding the extensive use of laboratory rodents and fish in toxicological experiments, and the practise of the three R's (Reduce, Refine, Replace).

Examples of combined actions in different toxicological endpoints obtained from *in vitro* and *in vivo* studies are given elsewhere in the report. To reduce the use of experimental animals, scientifically acceptable strategies of toxicological assessments, including alternative cell culture technologies and molecular tools particularly in the introductory assessment of chemicals should be emphasised. The assessment of effects of several substances in mixtures is necessary to evaluate risks of combined effects that may differ from the effects of each single substance. This often requires a higher number of animals to be statistically significant compared to testing of single substance effects. The use of alternative statistical models is mentioned in the report (see section 2.3.5). Such multivariate statistical models would also be useful for toxicological interaction studies, which could lead to a reduction in the number of animals required.

6 Discussion

6.1 General comments

Current risk assessments of pesticide and other chemical exposures are based on toxicological evaluation of single compounds. The toxicological data come from studies that generally have been done in order to set guidance values, such as TDIs, ADIs or ARfDs. Since humans often are exposed to different chemical components at the same time, the possibility for combined effect of exposure to mixtures needs to be addressed in risk assessments related to food and feed consumption and the use of cosmetics. However, the complexity and variability of multiple chemical substances that may occur in the environment, as well as the general dearth of data from studies using standard toxicological methods on combined actions of chemical mixtures, make the risk assessment of the potential combined toxic effects a considerable challenge. Since experimental testing of all the numerous mixtures is not practically feasible, there is a need for science-based advice on how combined exposures should be addressed in risk assessments of exposures related to food, feed and cosmetics (areas covered by the VKM).

The discussion on possible toxicological effects of multiple chemical exposures has a very long history. In the later years, there has been an extensive scientific development in the understanding of the influence of chemicals on biological systems and organisms. Based on evaluation of the scientific literature and monitoring reports, the exposure of humans to multiple components through food seems in general to occur at low levels, and at such exposures there are no substantiated accounts that demonstrate any frequent occurrence of adverse reactions, except in relation to accidents and misuse.

In this opinion, VKM has reviewed recent reports and scientific literature on combined toxicological effects following multiple chemical exposures. Different algorithms or schemes have been developed to decide whether combined effects are likely to occur or when the possibility of such effects should be taken into consideration.

When data on combined toxicological effects of multiple exposures are available, there are methods to analyse the data in order to determine the nature of the combined effects. An example is the isobole method, which is one way to distinguish between synergism, addition and antagonism. Generation of data from *in vivo* studies is resource-demanding due to the need for studies of multiple combinations of the test compounds. The potential for toxicokinetic interactions (induction/inhibition of biotransformation pathways) should be addressed and the usefulness of physiologically based toxicokinetic (PBTK) modelling should be explored.

For the risk assessments of exposure to chemicals being present as complex mixtures, the actual exposure may be difficult to estimate. Reliable information about the presence and concentrations of mixture components in various foods may be problematic to obtain.

The ways that the Scientific Panels of VKM have dealt with the issue of combined toxicological effects following exposures to multiple chemicals in their assessments of chemicals, are summarised in Chapter 5 of this opinion. Generally, combined effects have been taken into account when groups of structurally similar compounds have been assessed (e.g. dioxins and dl-PCBs, organotin compounds, parabens and some algal toxins). However,

possible effects of other chemical exposures in combination have so far not been formally discussed in assessments by the Panels. On the other hand, the overall objective of the risk assessments is to give advice on when the various exposures can be recognised as safe, i.e. below the dose thresholds of effect such that no appreciable health effect is expected to occur. To this end, uncertainty factors are incorporated in the derivation of ADI/TDIs, hence, additional safety has been included in the assessments.

6.2 Combined effects of multiple exposures below or above the dose thresholds of effect

The nature of the combined effects of multiple chemical exposures may vary significantly with dose, and change upon increasing doses above the thresholds of effect for the various chemicals involved. Thus, it may be difficult to predict the outcome at high exposures above the dose thresholds of effect where both toxicokinetic and toxicodynamic interactions could occur. However, as noted above, the general objective of chemical risk assessment is to give guidance on when an exposure to a single chemical compound is below the threshold of effect. When deriving these thresholds, uncertainty factors to account for intraspecies and interspecies variability are also incorporated. When all the exposures to multiple chemicals are below their respective dose thresholds for effect, the risk of adverse effects from combined effects is presumed to be limited. Crucial information needed in combined low-dose toxicological assessments is insight into the molecular mechanisms underlying the adverse effects. As such detailed insight is often not available, the more broad term ‘mode of action’ is used when common molecular mechanisms are likely or cannot be excluded.

Studies have demonstrated that the toxicity of a mixture of chemicals that affects the same cellular target and act by the same mechanism/mode of action corresponds to the effect expected when the toxicity of the components in the mixture is added together. Thus, in the cases where the individual compounds of a mixture act by the same mechanism and have the same target site (simple similar action), the dose addition model represents the basic method for hazard assessment. This implies that even if the exposure to all the individual components in a multiple exposure are below their respective NOAEL values, the combined dose may result in a toxic effect induced by the whole mixture due to dose addition. The basis for the use of this model is that there is sufficient information available to assign the mixture components into a common mechanism group. For a number of mixtures, the modes/mechanisms of action are not known. When a common target organ is affected, often similar mechanism cannot be excluded for each chemical in the mixture. In these cases it would be prudent to not exclude the possibility of dose addition. Several models are available for taking dose additive effects into consideration. Which model to choose will often depend on the underlying mechanism and data being available.

Generally, when exposure levels of a mixture of individual chemical components exhibiting different modes/mechanisms of action are in the range of their respective NOAELs, no dose addition (simple dissimilar action) or synergistic/potentiating interactions have been observed. Thus, when exposures to multiple chemical substances with dissimilar actions are lower than their respective ADIs (after applying uncertainty factors to the NOAELs), adverse responses to such combined exposures are unlikely.

In situations were concurrent exposures to multiple chemicals significantly exceed their respective ADIs, there is a potential for interactive effects, both for substances having similar

mechanisms/modes of action (complex similar action) as well as for substances with different mechanisms/modes of action (complex dissimilar action). Such interactions can be due to toxicokinetic and/or toxicodynamic mechanisms and may in some instances result in enhanced toxicity (synergism/potentiation) in comparison to the effects caused by the individual component of the mixture. The potential for interaction can be very difficult to predict from a theoretical viewpoint and should be addressed from a case-by-case perspective and ideally be based on data from testing of the mixture. However, it should be kept in mind that situations where such interactions might occur would necessitate risk management measures for the exposures to the individual chemicals exceeding their respective ADIs.

6.3 Proposed procedure for risk assessment of chemicals in mixtures

VKM finds that the previously described schemes in most cases are not suitable for practical use in the Scientific Panels of VKM. Some of the previous proposed models do not even follow the theoretical basis for assessing combined effects (see section 4.4). VKM has therefore proposed a procedure (flow chart) for use in risk assessments of chemical mixtures or concurrent exposures (Figure 7).

Presently, in the view of VKM, the best proposal for doing risk assessment of multiple chemical exposures is to perform a step-wise case-by-case evaluation of the toxicological data on the components and the exposure data. In order to secure that the Scientific Panels in VKM pay appropriate attention to the possibility of combined toxic effects in their assessments of chemical exposures, we propose using the flow chart presented in Figure 7 as a guidance in choosing the most relevant method for a specific risk assessment. This step-by-step approach is based on the presumption that toxicological data on the components and the exposure data are sufficient in order to successively answer “yes” or “no” to the questions presented in the flow chart.

Basis for use of the flow chart:

- The term 'similar mode of action' has a wider definition than the term 'similar mechanism of action'. 'Similar mode of action' includes mechanisms that lead to a common effect. There may be insufficient knowledge about the precise molecular mechanisms, but the principle of dose addition may still be used.

- If exposure to all components in the mixture is below their individual NOAELs and they act by a similar mode of action, no more than additive effect is expected.

- If exposure to component(s) in the mixture is above the individual component NOAELs, combined effects due to interaction may occur.

- If there is simple similar action for all components, a common expression of the hazard can be assigned for the mixture or the concurrent exposures:
  - When ADIs are available, the HI method may be used.
  - When ADIs are not available, the MOS or PODI method may be used.
  - If there is a well-known and strong similarity in 'mechanism of action' for all components in the mixture, the use of an index compound and the TEF model may be considered.

- If the components act by simple dissimilar action:
  - no combined effect is expected if exposure to all components in the mixture are below their individual NOAELs
  - combined effects due to interaction may occur if exposure to component(s) in the mixture are above their individual NOAELs

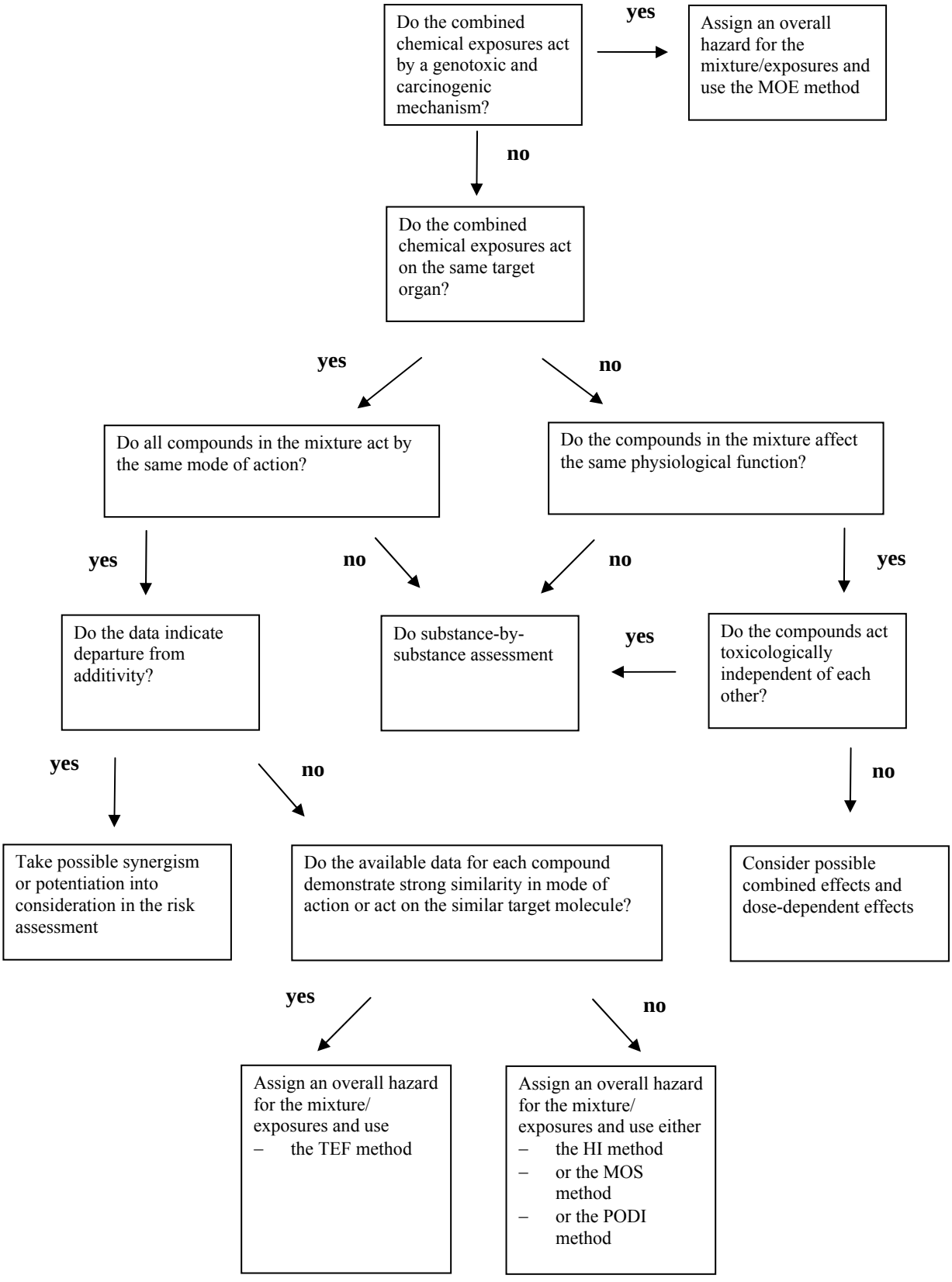

Figure 7. Flow chart for use in risk assessments of multiple chemical exposures proposed by VKM.

CONCLUSIONS

In this opinion, the theoretical principles for the various types of possible combined toxic effects from multiple chemical exposures have been described, and models for handling cases where such combined effects are likely to occur have been presented and discussed. The basis for this opinion has been three reports on combined actions of chemicals, one from the Danish Veterinary and Food Administration, one combined report from the Danish Environmental Protection Agency and the Danish Veterinary and Food Administration, and one report published by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT), as well as a review of the available recent literature. The primary focus of the opinion has been human health, but issues related to animal health and substances in animal feed have also been briefly addressed.

- The likelihood of combined toxic effects of multiple exposures at dose levels below the thresholds for effect is low, since many substances have different modes of action and many exposures exhibit large safety margins. The objectives of current food, feed and cosmetic regulations are that exposures should not be associated with adverse health effects, which also include the potential for combined effects.

- For substances exhibiting similar modes of action (simple similar action), adverse effects from multiple exposures may be experienced due to dose addition, even if the exposures to the individual components of the mixture are below their respective acceptable or tolerable intakes (ADIs/TDIs).

- For substances exhibiting dissimilar modes of action (simple dissimilar action), adverse effects from multiple exposures are not expected when the exposures to the individual components of the mixture are below their respective ADIs/TDIs.

- In situations where there is exposure to multiple chemicals significantly above their respective ADIs/TDIs, enhanced combined effects due to interaction may occur. Such interactions could be due both to toxicokinetic and toxicodynamic mechanisms, and are difficult to predict. Assessments should be performed on a case-by-case basis and ideally be based on data from testing of the relevant mixtures/concurrent exposures.

- In the derivation of ADIs/TDIs from animal data, provided data on inter- and intraspecies variation are not available, rather large default uncertainty factors are used in the extrapolations to humans, reflecting potential differences in species sensitivity (default factor of 10) and taking into account variability among humans (default factor of 10). Hence, the levels of exposure corresponding to an ADI/TDI may be more than one order of magnitude below the real dose thresholds of effect if humans are not more sensitive than the test species.

- Although the Scientific Panels of VKM so far only to a limited degree have formally taken possible combined effects from multiple chemical exposures into account, VKM does not consider this as a matter of serious concern. However, a flow chart has been developed and will be tested out as a tool in the Scientific Panels, in order to formally address the possibility for combined effects of multiple exposures in the future.

- Many plant protection products (pesticides) belong to groups with similar mechanisms of action. When there is combined exposure to pesticides within the same mechanism group, the principle of dose addition for such compounds exhibiting simple similar action would apply. When the sum of the exposure doses of the individual compounds in the mixture does not exceed the ADI for the most potent compound, there should be no apparent concerns. In situations where this sum of exposures exceeds the ADI of the most potent compound, dose additive effects may be expected. Risk assessments could for such situations be based on knowledge of the relative potencies of the pesticides in the mixture. Also, synergistic effects from mixtures could occur when exposures are above dose thresholds. However, with respect to the probability of experiencing interactive effects from combined exposures to pesticides, it should be kept in mind that based on national and Europe-wide monitoring programmes of residues of plant protection products in fruits, vegetables and cereals, levels are infrequently above maximal residue limits and thus considerably below ADIs.

- From international studies of pesticide operators, combined effects from multiple exposures have been documented. Although no such studies have been performed in Norway, the premise is that professional use of plant protection products should not exceed acceptable operator exposure levels when applied correctly and any advice on use of personal protection equipment has been followed.

- This opinion has not addressed other risk areas in detail. Generally, areas of risk are those where multiple exposures act by common modes of action and where there is a risk of exceeding dose thresholds. The Scientific Panels of VKM have in addition to the issue of plant protection products addressed the most important areas, such as dioxins and PCBs and algal toxins. A potential risk area in a Norwegian context is the combined effects of consumption of marine organisms from localised areas where there has been point source release of halogenated organic compounds and heavy metals. Since both types of contaminants are associated with developmental effects (reproductive-, immune- and central nervous system) and that the young child is especially sensitive towards such effects, due consideration should be given to the potential for interactions.

- This opinion has not in detail dealt with possible combined effects from multiple exposures in relation to ecotoxicology. However, the toxicological principles for combined effects described in this opinion, are expected to apply also for the environment.

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