Effects of Chemical Co-exposures at Doses Relevant for Human Safety Assessments

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SUMMARY

Chemical regulation is mainly based on single substances, but exposure is to complex mixtures, which raises the question of whether the regulatory framework is adequate and protective. There has long been an interest in the toxicology of mixtures, but most studies have been conducted at effect levels for the components of a mixture. This review focused specifically on studies where a mixture has been tested at doses which are at or below the NO(A)EL for every component of the mixture. This is the only dose region in which mixture toxicity could threaten regulatory safety margins. A thorough literature review was conducted using this selection criterion and others, for example only studies using models of mammalian systems were considered. Most studies that were examined failed to meet the selection criteria, but a total of 91 relevant papers were identified. An additional literature review identified 52 papers in which studies of real or artificial mixtures representing environmental exposures were reported, e.g. tap water. These studies would not meet the criteria used for the main review, but to the extent that they represent the testing of real environmental mixtures they can also shed light on the matter in hand.

For studies conducted at or close to the NO(A)EL of each component, toxicity was quite often seen. This is to be expected on the basis of dose additivity and also considering that small effects for a single chemical can occur at the NO(A)EL. The studies reviewed did not show evidence of a different pattern of combination effects according to the type of toxicity examined, for example acute toxicity, organ toxicity, developmental toxicity, endocrine toxicity or carcinogenicity. Deviations from additivity did not seem to be any more prevalent in any one of these domains. Endocrine toxicity is sometimes said to represent a special case with regard to synergy at low doses, but we found no evidence of this. From all the papers reviewed, mixtures producing more toxicity than expected on the basis of independent action were the exception rather than the rule and the deviation from expectation was in several cases small in magnitude. Small deviations from independent action probably represent natural variability rather than a reproducible deviation. The only robust cases of synergistic interaction were:

- The effect of pyrethrin and piperonyl butoxide on total ATPase in an *in vitro* system (Kakko et al, 2000).
- Two optical isomers of ethoxymethoxymorpholinophosphine oxide showed potentiation in an *in vitro* system (Ashby and Styles, 1980).
- The nephrotoxicity of melamine and cyanuric acid (Choi et al, 2010; Jacob et al, 2011).
- The effect of TCDD and PCB 153 on hepatic porphyrin accumulation (van Birgelen et al, 1996).
- Two mixtures of certain UV-filters in the yeast oestrogen assay (Kunz and Fent, 2006).
- The effect of TCDD and dibutyl phthalate on the developing male reproductive tract and liver pathology, though the authors described these findings as preliminary (Rider et al, 2010).

Seven distinct mixtures out of 139 tested showed convincing evidence of synergy, i.e. about 5%. Amongst these, several findings of synergy were from *in vitro* studies. It would be

extremely valuable to examine these specific effects *in vivo*, so that their practical relevance can be fully evaluated.

The review of studies of environmental exposures identified a number of higher-tier studies of complex mixtures representing relevant drinking water or food exposures (or multiples thereof). There was no convincing evidence of toxicity for combined exposures to substances present at concentrations that are acceptable for single chemicals. Only when single chemicals were at unacceptable concentrations did mixtures sometimes result in toxicity. The results of these studies are consistent with the findings of the other studies reviewed in this report.

Independent action of chemicals was found to be the predominant principle of mixture toxicity, so a default assumption of dose additivity for similarly acting chemicals and response addition for dissimilarly acting chemicals is reasonable. In reality, exposure will be to complex mixtures of similar and dissimilar chemicals, many without mode of action data that could inform chemical grouping considerations. For this reason, dose addition is a conservative default assumption that could be made in the absence of better information, as part of a tiered assessment process.

This review indicates that prioritisation of mixtures for risk assessment should focus on the presence of multiple similar chemicals each close to their acceptable limit and on situations were synergy can be anticipated. Exposure to 10 similar chemicals each at half of their maximum acceptable concentration would results in a safety margin of 20x rather than the 200x that would usually be expected for each chemical singly. The worst-case scenario for this situation is when considering products which are mixtures of similar chemicals or which co-occur due to a shared propensity to accumulate in fat. This situation is already addressed in regulation in the case of dioxin-like chemicals by the use of toxicological equivalence factors and dose addition in their risk assessment. For chemicals that do not bioaccumulate and which are not found together in the same product, recent evidence indicates that the toxicity of real environmental mixtures is often dominated by one and rarely more than three chemicals. Assuming that each chemical in such a mixture is at an acceptable concentration based on its own toxicity, the safety of the mixture would likely also be acceptable. The review of studies of environmental mixtures supports this inference. Equipotent complex chemical mixtures are found only in the laboratory.

The possibility of synergistic interaction has currently to be considered on a case-by-case basis. It would be valuable to formulate evidence-based guidance on when synergy might be anticipated, based in part on the findings in this report. There are a number of widely accepted cases of chemical synergy for which no evidence was found at doses relevant to this review. This might be because the appropriate studies have not been done or because in these cases synergy is restricted to the region of effect levels. Based on our evaluation, there is no evidence that exposure to complex mixtures of components, each well regulated according to established risk assessment approaches, would pose a health risk to humans.

1. INTRODUCTION

Chemical risk assessment is predominantly carried out on individual substances, and this is also reflected in most chemical-related legislation. In reality though, humans, fauna and flora are exposed to a variety of substances concurrently. The toxicology of chemical mixtures is usually addressed through concepts of concentration or dose addition and independent action; synergism is considered to occur only rarely.

More recently, the question arose whether such risk assessment procedures are adequate for assessing combined exposure to multiple chemicals. Much attention is being given to the so-called 'cocktail effect' which is hypothesised to occur due to simultaneous exposure to low levels of environmental chemicals. According to this theory, unexpected effects can occur due to interaction in the body between these chemicals even though the levels could be below the threshold of toxicity for the individual chemicals or their breakdown products. It is claimed that these interactions at low-dose levels may be greater than additive.

The ECETOC Scientific Committee, therefore, recommended forming a Task Force with the following terms of reference:

1. Review the scientific literature on interactions between chemicals at low doses, i.e. particularly those occurring below a toxicological point of departure of the individual chemicals.
2. Evaluate whether the evidence on interactions at low doses demonstrates toxicologically relevant effects, and determine whether there are any associations with specific modes of action.
3. Look at the evidence of chemical interactions and comment on the likelihood of those being important in the context of environmental exposures.
4. Evaluate the adequacy of current human safety risk assessment practice in light of the conclusions of the above.

This report records the extensive literature search, which the Task Force undertook. Papers were evaluated for their relevance with a view to the Task Force's remits. The findings for studies at environmental concentrations and multiples thereof, for mixtures tested at or near the NO(A)EL of their single components, and for mixtures tested well below the NO(A)EL of their single components are given in Chapters 3, 4 and 5, respectively. An overall discussion is in Chapter 6. References identified in three recent major publications are listed in Appendix A, including their evaluation of relevance for this Task Force. Summarised evaluations of those papers considered relevant are attached to the report in Appendix B. Appendix C lists those papers not considered relevant for this Task Force.

An interim report and first conclusions were presented at a workshop on 'Combined Exposure to Chemicals' that addressed the topic in a wider context and with a wider audience of academics, regulators and industry (ECETOC, 2011a).

2. APPROACH OF THE TASK FORCE

In this report the term 'mixtures' is used to represent co-exposures to multiple chemicals and chemical mixtures (e.g. formulations). It should be recognised that for many test systems, *in vitro* and *in vivo*, the doses / concentrations required to induce a measurable and statistically significant effect will often be many orders of magnitude higher than likely to be routinely encountered in the environment. The term 'low dose', when used in the context of discussing a particular test result within this Task Force approach, is applied on the basis of considering the dose-response relationship in that test system and when the dose is very close to, or below, the NO(A)EL of the individual compounds in the mixtures. It is not meant to indicate that this dose approximates real-life human exposure levels or those encountered in the environment, although this may be the case in some instances. For most purposes in this report where one of the terms NOEL, NO(A)EL or NOEC is used, it can be assumed that this refers to all three terms, thus avoiding the use of clumsy terms such as NOEL/NO(A)EL/NOEC. Where a particular study is being referred to then the appropriate term for that study will be used.

2.1 Criteria for relevance

The first step was to define a set of criteria which would define papers which were relevant for the purpose of the Task Force's remit. The criteria evolved slightly in the light of experience, and the final sets used were:

- Relevant doses of mixtures are ones where each individual component is present at doses which, if tested singly, would produce no effect. This should be determined by testing each component individually under the same experimental conditions as for the mixture, preferably in the same study. This criterion was strictly applied, and resulted in the rejection of a large percentage of the papers reviewed. Most of the rejected papers only studied mixtures at doses where one or more of the components were present at an effect level. The attention of this report is exclusively focused on whether mixtures can produce effects when all components are present at a no effect level. For some studies a range of mixture doses were tested, in which case only the results for those doses satisfying this acceptability criterion were considered in this review.
- The single class of acceptable papers which do not fulfil the above criterion are studies with mixtures which represent real environmental exposures. For example, if real tap water is dosed to animals and compared to ultrapure water, then this is a study of interest in the context of mixtures. These studies are considered in Chapter 3 as a special case.
- Studies that are in scope are *in vivo* mammalian studies, and *in vitro* models of mammalian systems. If a particular combination effect (e.g. synergy) is seen *in vivo* then this is of definite relevance. If a similar effect is seen *in vitro* then it is certainly of interest and is

potentially relevant, but the effect should really be examined *in vivo* before its relevance can be fully evaluated.

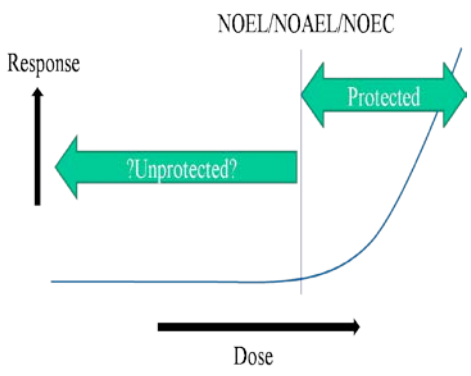
- Effects studied should be systemic, rather than local (e.g. dermal irritation).
- Studies are potentially relevant whether they do or do not demonstrate the occurrence of toxicity.
- The following special cases are excluded: radioactivity, particles (e.g. asbestos, smoke), dietary deficiency (e.g. studies of the effects of chemicals under conditions of dietary zinc deficiency), surgical treatments, other treatments not involving the use of chemicals (e.g. noise, viral infections).
- Studies only reporting the kinetics of the test substance or its metabolites, in the absence of toxicity endpoints, are excluded.
- Primary sources are used. Review papers are only used to identify primary sources.

Within the criteria described above, all types of mixtures were considered. For example, preparations or products may contain a number of different chemical substances. A study testing such a preparation would be relevant, provided data were presented where each component was also tested singly in the same system. Mixtures occurring in the environment, resulting from different sources of contamination or natural occurrence are also potentially relevant. Studies of products that are themselves complex mixtures of chemicals, would in general not be relevant, as each component will not have been tested singly (e.g. mineral oil).

2.2 Dose-level considerations

The most critical component of the literature searching strategy was that the Task Force was only interested in mixtures where all individual components were present at doses which, if tested singly, would produce no effect. This criterion was strictly applied, and resulted in the rejection of a large percentage of the papers reviewed. Therefore, it is worth expanding on why the Task Force felt this was the right approach for its specific purpose.

Figure 1: When considering mixture toxicity, only doses at or below the NO(A)EL of each component are relevant from a public health perspective



Chemical risk assessment is predominantly carried out on individual substances, and this is also reflected in most chemical-related legislation. Exposure to a single chemical at an effect level would represent a failure of existing regulation. Regulation protects against this situation (and adds a safety factor to ensure a margin of safety). Studies which include single chemicals at effect levels have no relevance for this review because they represent an unacceptable situation which existing regulations are designed to negate. However, humans are exposed to a variety of substances concurrently, and it is important to consider whether the mixtures might result in toxic effects at doses where each component is present at the individual no effect level. The target of this review has been to seek evidence on this point.

One of the reasons for carrying out this review was to examine whether existing regulation is adequate with respect to mixtures. Thus, the standard regulatory paradigm of the NOEL (or NO(A)EL) was used, i.e. there is a dose above which there are treatment-related (adverse) effects and below which no statistical difference in (adverse) effect can be found between a treated group and the control. The Task Force has not adopted the approach of benchmark doses nor has it considered dose-response modelling. This is not because they lack scientific merit, but because in almost all cases the NOEL (or NO(A)EL) forms the basis for human health protection with respect to chemicals, and the questions to be addressed for mixtures are ones related to human health protection. The consequence of using the standard regulatory paradigm could be conservative in this case, i.e. it could make it more likely to find toxicity for mixtures where each component is at a NOEL. This is because effects can already occur at the NOEL, but not reach the size needed to be statistically different from the control. Depending on the variability of the endpoint considered and the group size there can sometimes be as much as a 20% difference from the control at the NOEL (Kortenkamp et al, 2009). If several chemicals, each having such an effect, were combined at their NOEL they might result in a statistically significant effect, and the study would then qualify as of value for this review. Broadly spaced doses can result in uncertain estimates of the NOEL, so in some cases the NOEL can represent a much lower dose than one that would still not result in a statistically significant effect.

As far as dose level selection is concerned, the Task Force has found that the majority of mixture studies have been conducted at doses where one or more of the components, often all of them, have an effect. Despite this, many of the authors of these studies claim to have studied low doses or doses around the NOEL. This is perhaps to be expected, as the authors may have designed the studies in order to ‘look for effects’, i.e. a study with no effects is less likely to be satisfying for those conducting or funding it. There is also a possible effect of publication bias, in that negative studies can be hard to publish.

2.3 Quality of the papers reviewed

A quality standard was not applied at the stage of determining which studies are relevant. As a result, the intrinsic quality of the studies reviewed varied greatly, as did the standard of reporting of what was done in these studies. A study and its reporting should be of sufficient quality to be able to establish whether effects seen are likely to be real and due to treatment. In some cases, this standard was not reached.

Another aspect of quality, for the Task Force's purposes, is the availability of toxicity data for the individual components of a mixture, preferably in the same test system as for the mixture or mixtures, and ideally run concurrently with the testing of the mixture. The availability of such toxicity data for individual components varied. In a few cases, the Task Force was able to access data from elsewhere for the individual components, for example in cases where the toxicity test used a standardised regulatory test protocol. In other cases, toxicity data for individual components were present, but did not cover the full range of doses / concentrations to enable a full interpretation of the nature of any mixture toxicity.

In a number of cases, interesting mixture data are presented, but dose responses for each component are lacking in the paper. If the authors cite earlier papers where they used the same test system on each component, then the Task Force sought to use these data. If the test system is a standard regulatory one, e.g. a rat carcinogenicity study, then it has sometimes been possible to obtain broadly comparable data for each component from other sources; however with non-standard test systems this is more problematic. To compare NOELs from a narrowly-focused mixture study with NOELs from more wide-ranging studies of each component would introduce considerable uncertainty. For example, a 90-day rat study focused only on thyroid effects of a mixture is hard to compare with regulatory NOELs from standard 90-day rat studies on the single components; there may be many differences between the studies, even when considering only thyroid effects (e.g. group size, thyroid endpoints measured, which is deemed to be adverse).

An additional problem occurs with narrowly focused studies. Using the example of the 90-day study focused only on the thyroid; at the thyroid NOEL there might have been substantial toxicity in other organs, e.g. liver, which went unobserved. From a regulatory point of view the dose of predominant interest is the one that is a NO(A)EL for all endpoints examined in a wide-ranging regulatory study, with dozens or hundreds of endpoints. In a narrowly focused study the effects seen may not be the most sensitive endpoints, but it is often not possible to determine if this is the case. However, in some cases the reason for conducting a narrowly focused study may be that it is already established that the few endpoints examined represent the most sensitive ones on the basis of earlier work.

Quality and relevance criteria for mixture studies in general are for example discussed by Borgert et al (2001) and Teuschler et al (2002).

2.4 Definitions

Definitions used

As this ECETOC report addresses 'low-dose interactions' the Task Force thought it would be useful to provide some underlying scientific principles and definitions used during the evaluation and interpretation of the obtained results. The most contradicting debates would probably be expected by trying to define the term 'low dose'. In a number of reports mixtures of substances are tested in combinations of individual substances at so called 'low doses' without providing a definition of what constitutes a low dose. One possible definition of a low dose which comes more from the hazard side could be that a 'low dose' is every dose below a NO(A)EL of a substance. Or more specifically a 'low dose' might be defined as the virtually safe dose as defined by regulatory authorities (e.g. reference dose [RfD], acceptable daily intake [ADI] or predicted no effect concentration [PNEC]) which is based on the lowest no observed effect level from a package of toxicity studies divided by the uncertainty factors used in the respective context. Coming from the exposure side, a 'low dose' would probably be best defined as a human-relevant or environmentally relevant dose. Concerning the selection criteria used for the evaluation of references by this Task Force, all studies have been considered relevant, where

- Two or more substances were tested at or below their individual NO(A)ELs for the studied effects in the mixture study, although this might not necessarily have to be regarded as a low dose, because it is sometimes a much higher dose than an actual human- or environmentally relevant dose.

  Or:
- Mixtures representing real environmental exposures were tested.

When entering the scientific and regulatory debate on combinations or mixtures, differentiation of mixture approaches is often related to common or uncommon mode or mechanism of toxicological action of the substances in a mixture. Definitions exist for both terms, mode and mechanism of action, but there is a lack of consistency in their use (Borgert et al, 2004).

The distinction between 'mode' and 'mechanism' is critical when conducting a mixture risk assessment. This is because the choice of a model to predict the effects of chemical mixtures (i.e. a dose addition model versus a response addition model) can depend on whether mechanistic data for the chemical components of the mixture are described in terms of the mode or mechanism of action

(Borgert et al, 2004). However, in the majority of cases the exact modes or mechanisms of actions leading to adverse effects after administration of test substances are not known.

Table 1: Definitions of 'mode of action' and 'mechanism of action'

Term	Definition	Reference
Mode of action	Mode of action analysis is based on physical, chemical, and biological information that helps to explain key events in an agent's influence on development of tumours. … An agent may work by more than one mode of action, both at different sites and at the same tumour site. Thus the mode of action and human relevance cannot necessarily be generalised to other toxic endpoints or tissues or cell types without additional analyses	Meek et al, 2003 US EPA, 2005 : http://www.epa.gov/raf/publications/pdfs/CANCER_GUIDELINES_FINAL_3-25-05.PDF
Mechanism of action	Mechanism of action is detailed, stepwise information at various levels of biological organisation = the molecular sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response in the target organ. Requires that an understanding of a chemical's mechanism necessarily entails understanding of the causal and temporal relationships between the steps leading to a particular effect, as well as the steps that lead to an effective dose of the chemical at the relevant biological target(s) of action	Borgert et al, 2004

For the purposes of risk assessment, compounds could be grouped even in the absence of such detailed data, on the basis of a less refined evaluation of the mode of action (e.g. based only on target organ toxicity) (EFSA, 2008).

The definition of the term 'interaction' can more easily be based on cited references and accepted terminologies and might best be described by the following table (Meek et al, 2011; Borgert et al, 2004).

Table 2: Definition of different types of combined effects, adapted from Meek et al, 2011; Borgert et al, 2004

Type of combined effect	Subtypes	Synonyms	Effects observed
Non-interactive	Simple similar action Simple dissimilar action	Additivity Independent action	Dose addition Response addition
Interactive	Synergy and potentiation Antagonism		Greater than dose additivity Less than dose additive effects

Simple similar action is likely to occur when the chemicals in the mixture act in the same way and/or by the same mechanisms (possibly at the same macromolecule). Dose / concentration additivity is subsequently observed when chemicals that act by the same mode of action and/or at the same target cell, tissue or organ act in a potency-corrected 'dose additive' manner (Meek et al, 2011; OECD, 2011; Price and Han, 2011).

Simple dissimilar action results in response or effect addition. It occurs when the modes, nature and/or sites of action differ between the chemicals in the mixture and constituents do not modulate the effect of other constituents of the mixture (Meek et al, 2011; OECD, 2011; Price and Han, 2011).

Positive interaction would be called synergy, which means a greater than additive effect, with the model of addition defined. Potentiation would be a special case of synergy where one agent has no activity at any dose, but increases the potency of another agent. An antagonism (of a toxicological effect) is *vice-versa* a combined effect which is less than that predicted on the basis of additivity. Although this effect might occur in some cases, there is no public health concern. The hypothesis of the Task Force was that for the majority of different combinations or mixtures a non-interactive type of effect is to be assumed with dose / concentration addition in case of a common model of action or response addition in case of different modes of actions. However, the postulated interactive type of combined effects has always been a reason for safety concerns because synergistic effects, if they occurred, would be difficult to predict. The Task Force therefore evaluated all publications carefully for evidence of 'synergistic effects'.

According to Choudhury et al (2000) and ATSDR (2001), dose addition should be used for chemicals that produce the same toxic effect in the same target organ via the same mechanism of action. A more pragmatic approach to be taken, in order to decide on common toxicity in the absence of more detailed mechanistic data might be to assess the target organs of the compounds under investigation or to focus on specific common effects (e.g. reduction of the anogenital distance). This approach of defining appropriate cumulative assessment groups (CAG) based on less restrictive criteria is under investigation by the EU project ACROPOLIS[1] and by EFSA (European Food and Safety Authority).

Other relevant definitions

NO(A)EL (no observed adverse effect level): The highest exposure level at which there is no statistically or biologically significant increase in the frequency or severity of adverse effects

[1] http://www.acropolis-eu.com/

between the exposed population and its appropriate control. Some effects may be produced at this level, but they are not considered to be adverse or precursor events. NO(A)ELs are derived for specific studies in specific species. For the derivation of reference values, the lowest NO(A)EL determined will generally be used.

LOAEL (lowest observed adverse effect level): The lowest exposure level at which there are statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control.

Adverse effect: A biochemical, behavioural, morphological or physiological change (in response to a stimulus) that either singly or in combination adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge. In contrast to adverse effects, non-adverse effects can be defined as those biological effects that do not cause biochemical, behavioural, morphological or physiological changes that affect the general well-being, growth, development or life-span of an animal. Effects are less likely to be adverse if:

- There is no alteration in the general function of the test organism or of the organ / tissue affected.
- It is secondary to other adverse effects.
- It is an adaptive response.
- It is transient.
- Its severity is limited e.g. below thresholds of concern.
- Effect is isolated or independent, i.e. changes in other parameters usually associated with the effects of concern are not observed.
- Effect is not a precursor, i.e. the effect is not part of a continuum of changes known to progress with time to an established adverse effect.
- It is a consequence of the experimental model.

2.5 Ecotoxicology

The Task Force recognised that much important work on combined effects of chemicals had been done in the field of ecotoxicology. However, as previously stated, the focus of this report was on evaluating the evidence related to low-dose interactions in mammalian test systems to gain an insight into the robustness of the current human risk assessment paradigm.

Although studies on ecological systems do not fall within the remit of this report, it is evident that in many cases, the test systems are particularly amenable to studying combination effects of defined mixtures with large numbers of components, and consequently to investigating

interactions when components are present at concentrations which individually produce no, or very small, effects.

Aquatic toxicity tests, such as algal growth inhibition tests or long-term bioluminescence inhibition assays with marine bacteria, are quick to perform, relatively cost-effective in terms of test material requirements, and importantly have low inter-experimental variability. Thus, robust characterisation of large numbers of both single substance and mixture concentration-response relationships has enabled ecotoxicologists to begin to establish a mechanistic understanding of how substances interact, and to test and refine mathematical models for predicting combination effects. For example, having an understanding of the mechanism of action of their molecules of interest has allowed researchers to test mixtures of specifically similarly or dissimilarly acting substances to demonstrate how response addition and effect addition, respectively, accurately predicted the combination effects (Altenburger et al, 2000; Faust et al, 2001).

These ecotoxicological mixture studies do not form part of the literature reviewed and are not discussed further in this report. However, the principles established in aquatic toxicity tests have formed a key part of the proof of principle in showing how the effects of multi-component mixtures can be systematically tested to gain meaningful information on combination effects and interactions. As a result there was an increased interest in testing defined mixtures in mammalian test systems (*in vivo* and *in vitro*), which is evident in this report.

Moving from the laboratory to the field implies moving from a situation with defined mixtures to one with largely undefined mixtures. A recent ECETOC Task Force has addressed the question of how to evaluate the potential impact of chemicals or chemical mixtures on organisms in specific aquatic environments (ECETOC, 2011b).

2.6 Identification of primary papers for review

The goal of the Task Force was to obtain as complete an overview as possible of the relevant literature concerning chemical mixtures and their combined effects, particularly at doses below effects. The search was restricted to only mammalian studies and *in vitro* mammalian systems. A variety of search strategies were used, including reviews, systematic literature database searching, and selective use of the ‘reference explosion’ technique (i.e. obtaining relevant papers and then looking through their reference lists for further potential relevant papers).

Several review papers and reports (listed in the bibliography) were used to provide an initial list of articles in which the original data were presented and discussed (referred to as primary articles herein). For example, the recent ‘State of the Art Report on Mixture Toxicology’ by Kortenkamp et al (2009) provided a good source of primary articles as well as additional review

articles (also see CoT, 2010). Other major sources of primary articles were provided by the National Toxicology Program (NTP) of the USA, where testing mixtures at environmentally relevant concentrations were reported. WHO International Programme of Chemical Safety (IPCS) publications and reports from previous working groups investigating mixtures were also a useful source of primary articles.

Naturally a major drawback with relying on review papers is that it could not identify more recent articles. Thus, to complement the use of review papers, further primary articles were identified by performing a number of searches using either known scientific search engines (e.g. PubMed; TOXLINE) or Google.

- A literature search, identifying publications up to the end of 2010, was conducted using professional search engines based on the following inclusion criteria:
  - Standard toxicological search profile including ‘acute toxicity’, ‘carcinogenicity’, ‘reproduction toxicity’, ‘endocrine disruption’.
  - Publication year > 2005.
  - Using one of the key-words: ‘cumulative’, ‘combined’, ‘mixture’, ‘synergistic’, ‘joint effect’, ‘co-aggregate’, ‘cumulative exposure’, ‘combination’.
  - Using the term ‘low dose’.
- Publications up to the end of 2011 were identified through the Toxicology Bibliographic Information (TOXLINE), a database of the National Library of Medicine’s TOXNET system (http://toxnet.nlm.nih.gov) using the following query criteria: All of the words: ‘low dose’, ‘interactions’, ‘mixtures’ (all fields). Singular and plural forms.
- A systematic search on Google, covering publications up to the end of 2011, used the following key words: either ‘low dose’, ‘environmentally relevant’, or ‘human relevance’ in combination with one or more of the following terms: ‘interaction’, ‘combinations’, ‘mixtures’, ‘complex mixtures’, ‘co-exposure’, ‘aggregate exposure’, ‘cumulative exposure’, ‘additivity’, ‘synergy’.
- The preceding systematic search was repeated in March 2012 to cover articles published in the years 2010 and 2011 using a larger choice of keywords to include all toxicological papers from the MEDLINE database (updated with the National Library of Medicine´s revised 2011 MeSH terms) and combining this with any one of the terms: ‘combination’, or ‘mixture’, or ‘co-exposure’, and ‘low dose’.
- The MEDLINE database was also searched in March 2012, using the following search terms:
  - One of the keywords: ‘interaction’, ‘combination’, ‘mixture’, ‘co-exposure’, ‘cumulative’, ‘additivity’, ‘synergy’.
  - AND keyword ‘toxic’.
  - Limited to 2011 publications only.

- This search resulted in 4150 hits. To reduce numbers the following criterion was added: AND ‘chemical’, ‘pharmaceutical’, ‘toxin’ included in any of the above keywords. This reduced the number of hits to 759 papers.

This last search illustrates the difficulties inherent in finding a comprehensive search strategy that identifies a manageable number of papers. It is impossible in this case, because of the diversity of terminology used, yet narrower searches with manageable numbers of hits might miss some valuable papers. However, based on discussions with international experts in the field of combined toxicity, the Task Force is confident that this review covers more than 95% of the publically available literature relevant for this review, as a result of the variety of search approaches used.

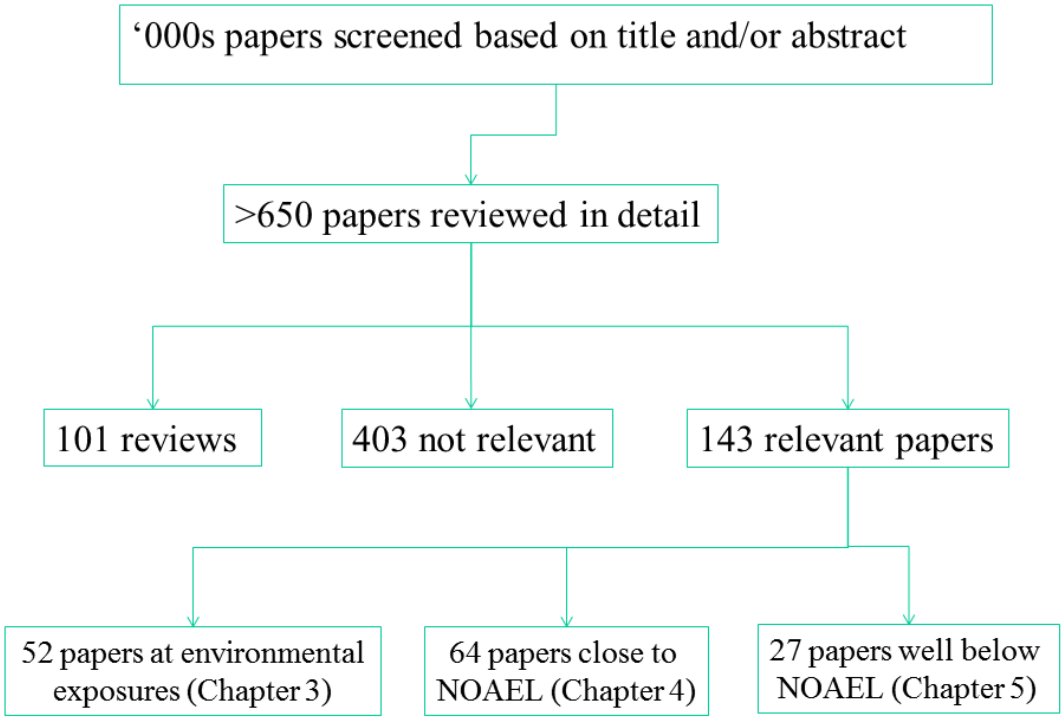
As expected, an overwhelming number of potential primary articles were initially identified based on the title of the paper and the abstract. To further refine our search a number of criteria (described in Section 2.1), were used to identify those articles which were considered acceptable for the purposes of the Task Force.

For transparency and ease of reference Appendix A lists mixture toxicity papers identified in three recent major documents (Boobis et al, 2011; SCHER, SCCS, SCENIHR, 2012; Kortenkamp et al, 2012), and states their position in relation to the review presented in this report.

Starting from the summaries and title / abstract information of the article, each paper identified via the different searches was evaluated in depth to determine whether it was fulfilling the relevance criteria. Whilst this reduced the number of primary articles to only those that were pertinent to the Task Force’s terms of reference, several of the papers were rejected upon evaluation because there was either insufficient information or one or more of the components in the mixture was present at clear effect levels. Standardised proformas were prepared for each relevant reference and are compiled in Appendix B. Further papers were obtained from evaluating these primary references using the ‘reference explosion’ technique.

From the large number of papers examined only a small proportion met the selection criteria used in this review. These are described as ‘relevant’ papers in this review, which is not intended to discredit those papers that did not meet the selection criteria, as all the papers had value in the context of their own objectives.

Figure 2: The numbers of papers reviewed and examined by the Task Force, with those finally identified as relevant divided into the three categories considered in Chapters 3, 4 and 5



3. STUDIES AT ENVIRONMENTAL CONCENTRATIONS AND MULTIPLES, INCLUDING HUMAN STUDIES

The majority of studies reviewed by the Task Force involved the testing of mixtures at concentrations for which there were toxicity data available in the same system for each component of the mixture. This chapter reviews studies which do not meet this criterion, but which tested mixtures that are representative of realistic environmental exposures. For example, if tap water is dosed to animals and compared to ultrapure water, then this is a study of interest in the context of mixtures. The fact that the individual components were not tested, or even identified, does not negate such a study. Society expects that environmental exposures be safe, i.e. without toxicological effect. Regulation seeks to ensure that this is the case by setting standards based on single chemical contaminants. If the resulting whole mixture is tested, then it is interesting to see if it is without any effect, which would be reassuring, or if it produces toxicity. Such toxicity might represent a single component present at a toxic concentration, i.e. a failure of regulation. However, it might instead represent a synergistic toxicological interaction between two or more contaminants, which would be critical information for this review. The main question to be addressed is: if an environmental sample complies with regulations based on single chemical mammalian toxicity data, is there any evidence that the whole sample still produces toxicity?

Animal studies are reviewed, firstly evaluating the exposure basis of the various studies and its relationship to human exposure, and secondly reviewing the results of these studies. Finally, human studies are discussed, including epidemiology studies; this is appropriate, since the human population is exposed to mixtures of chemicals every day.

3.1 Exposure in the animal studies

The studies in this chapter are concerned with the testing of environmental exposures via food, water and soil. The mixtures may be ones taken directly from the environment, e.g. fish from a lake or water from a tap, or the mixtures may be produced in the laboratory to simulate an environmental exposure situation, e.g. adding pesticides to rodent diet to represent concentrations found in food monitoring studies. *In vitro* studies are not included. It is not a test of a realistic environmental exposure if a liver cell culture (for example) is exposed to tap water, since animal livers will never be exposed directly to tap water. Similarly, only relevant routes of exposures are considered. For example, an intra-peritoneal dose is not relevant for assessing human health hazards from water exposure.

In many cases the mixtures are not just tested at environmentally relevant exposure concentrations but are also tested at multiples of environmental exposures. For example, tap

water may be concentrated one hundred-fold (100x) before being given to animals. In a few studies only high multiples of environmental exposures are used (e.g. > 100x); these studies have been excluded because no test of the mixture as present in the environment has been made (i.e. 1x), which would be necessary for interpretation. The 1x concentration in each study represents the environmental exposure situation, but this does not necessarily represent an acceptable situation for human exposure. Some studies included here investigated overtly contaminated tap waters or mixtures simulating contaminated groundwater at hazardous waste sites. In such cases, an attempt has been made to compare the 1x exposure to the acceptable regulatory limits for single chemicals in order to aid interpretation.

In cases where toxicity is seen there is sometimes investigative work performed to seek to establish the cause. This investigative work is discussed in this review, since it could help to determine if the toxicity of the mixture is expected on the basis of the toxicity of individual components or if it represents a synergistic interaction.

A single study concerned the exposure of mice to an environmental mixture of chemicals via soil (Silkworth et al, 1984). The soil concerned was from the Love Canal chemical waste dump, and mice were exposed to the fumes from the soil either with or without direct contact with the soil itself. In this case, it is clear that humans should not be chronically exposed to such an environmental mixture in this way. However, this study used a real mixture found in the environment, and the potential for human exposure to such a mixture clearly exists, irrespective of the regulatory or legal aspects of the situation.

3.2 Animal study results

There are several studies available investigating specific environmental mixtures, some of them representing realistic human worst-case exposures or multiples thereof. Quite a few of these *in vivo* studies were conducted according to (or comparable to) internationally accepted guidelines (OPPTS, OECD). Real drinking water, tap water and reclaimed water proposed for drinking were studied in rat and mouse carcinogenicity studies and in a rat multigeneration study. Three series of NTP (US National Toxicology Program) studies with simulated environmental exposures were conducted investigating subchronic toxicity in rats and mice, reproduction toxicity in mice, subacute and subchronic studies in mice, mostly focused on haematopoiesis, immunology and a genotoxicity battery. The mixtures contained a simulated groundwater contamination near a hazardous waste site and two pesticide / fertiliser mixtures representing Iowa and California groundwater. Three further subchronic rat and mice studies with simulated Denver drinking water mixtures were reviewed. Besides drinking water, some mixtures potentially entering the human food chain were tested in high level animal studies: contaminated fish from the Great Lakes were fed to mink or rat with specific focus on the observed

reproductive failure seen in mink, and there are repeated-dose toxicity studies of several pesticide mixtures representing dietary intakes. Mixtures of PCBs representing worst-case human breast milk contamination were fed to neonatal rats.

Many of these studies showed no effects, even up to high multiples of realistic or worst-case environmental exposures. Effects were rarely seen at realistic worst-case human exposure concentrations; however at concentrations of about 100x or 1000x regulatory limits for human exposures, in some cases, adaptive or adverse effects were described.

Drinking water

All of the studies found under this heading are *in vivo* studies. Many of the identified studies concerned testing environmental exposures in water. Studies based on real environmental samples have the advantage of testing whole mixtures, including all contaminants at relevant concentrations. On the other hand, if concentrations of contaminants change over time, the environmental mixtures tested may not represent a worst-case or a realistic situation.

Other water studies have used artificial mixtures, i.e. mixtures made in a laboratory to simulate an environmental exposure, or some multiple of such an exposure. Such studies are not simple to interpret in terms of relevance to human exposure for a number of reasons:

- The basis for setting the regulatory standard is unclear, especially after a long passage of time.
- Regulatory standards may be set on the basis of protecting wildlife, rather than man (e.g. Great Lakes Water Quality Objectives).
- Regulatory standards change over time, as new data become available, complicating the interpretation of the relevance and acceptability of the exposures tested.
- If multiple chemicals are included at concentrations equal to their allowed maxima, the probability or frequency with which this combination of concentrations might occur in real life is not known.
- Since only selected contaminants are included in the mixture, it is clear that many other contaminants which could co-occur with these in the environment are excluded.

The largest collection of studies of environmental mixtures is that run by the NTP on mixtures of groundwater contaminants. Three different mixtures were tested, each in a range of long-term toxicity studies (further described later in this section):

- A mixture of 25 frequently detected groundwater contaminants (19 organics and 6 metals), representing groundwater concentrations near hazardous waste disposal sites (1x concentration).

- Pesticide / fertiliser mixture, including 5 pesticides, representing Iowa groundwater (1x concentration).
- Pesticide / fertiliser mixture, including 6 pesticides, representing California groundwater (1x concentration).

In each case, multiples of the 1x concentration were also tested. The interpretation of the exposure basis of these studies is complicated due to the factors referred to previously. In the case of the pesticide / fertiliser mixtures, the concentrations were based on the median concentrations found in groundwater samples in a large 1980s survey, including only those samples in which the contaminant was detected. The authors state that the combination of these values for multiple contaminants represents a 'theoretical worst-case scenario' not likely to occur in a real sample (Yang, 1992). Using current drinking-water standards, the California mixture exceeded the allowed standards in the case of aldicarb and ethylene dibromide, whilst the Iowa mixture did not exceed any standard (see Table 7).

A small set of recently conducted studies used clearly contaminated water sources, either water collected at wells and boreholes near a large Nigerian landfill (Adeyemi et al, 2010), mixtures of chlorinated hydrocarbons similar to concentrations in the groundwater near a Taiwanese electronics factory (Wang et al, 2002), or Nanjing city tap water which was known to be contaminated (Zhao et al, 2011a).

The Adeyemi et al (2010) study investigated kidney toxicity in rats caused by a Nigerian contaminated groundwater. Rats were administered drinking water, a leachate simulate and water samples obtained from four different wells near the landfill. Increased Na^+ concentration, necrosis of muscle fibres and cellular infiltration by macrophages are observed in the leachate simulate group. In all groups, increased K^+, urea and creatinine concentration and changes in the kidney and serum ALP, ACP, AST and ALT were observed. The findings in the water groups are considered to be of questionable relevance, as only biochemical parameters are given. No information on kidney weights or any details of macroscopy or histopathology for these groups is presented. The leachate simulate certainly does not represent a human-relevant exposure situation and the level of contamination of the wells is unclear.

A contaminated groundwater source was investigated in a chronic mouse study (Wang et al, 2002). A mixture of representative chlorinated hydrocarbons was tested on male and female ICR mice via drinking water. The concentrations of the test compounds were considered to represent measured groundwater concentrations of 20 wells, which were 1 and 1.5 km from a manufacturer of electric appliances in Taoyuan. In male mice, tail alopecia and deformation were seen at the high dose. In the medium and high dose, increased absolute liver and lung weights, but no changes in relative weights were seen, as well as increased BUN, and serum creatinine values. In female mice increased absolute liver, kidney, uterus and ovary weights were observed without

changes in relative weights. All these changes are considered to be of low relevance as they are probably related to lower body weights and/or lower water intakes (no data available), as previously shown by Simmons et al, 1994. However, in the high dose group a higher incidence of mammary adenocarcinomas is described. Five mammary gland adenocarcinomas were observed in the high dose group compared to 0/1/0 in the control/low/medium dose group. It should be noted that the contaminated groundwater represented by the mixtures in this study represents a realistic contamination and is unacceptable on a single chemical basis.

Juvenile mice were administered drinking water collected from the Nanjing Beihekou water plant for 90 days (Zhao et al, 2011a). Water consumption, body weight, spermatology and testis histopathology were investigated. The number of elongated spermatids was decreased and the number of abnormal sperm was increased. Upon histopathological examination, the interstitial space seems to be expanded, with a decreased number of Leydig cells. As no historical data are discussed in this examination and no further reproduction parameters were investigated, the biological relevance of these findings is unclear. It has to be mentioned, that the tap water used seems to represent contaminated water compared to drinking water standards.

In mouse and rat developmental toxicity studies, rat reproduction, or mouse and rat carcinogenicity studies no effects were observed after administration of tap water, bottled water, drinking water, or reclaimed water from different US States (Chernoff et al, 1979; Staples et al, 1979; Keen et al, 1992; Uriu-Hare et al, 1995). In a series of almost identical subchronic mouse studies with a focus on immune, endocrine and nervous system endpoints, three chemicals were dosed, each at 3x their maximum contaminant level in water (Porter et al, 1999). Sporadically effects (spleen weights, body mass, plaque forming, free thyroid index, aggression score at day 14) were seen, which were rarely repeated over the different studies. Overall the findings are considered to be of unknown toxicological relevance. The publication itself is difficult to follow, some of the effects themselves are of questionable relevance and there is an overall lack of reproducibility across the studies.

In a further small series of studies, mixtures of seven chemicals frequently detected in US groundwater associated with hazardous waste sites were tested at 1x and 10x concentrations in subchronic rat studies (Constan et al, 1995, 1996). The focus was on liver toxicity. No changes were observed at the 1x concentration, however at the 10x concentrations increased hepatocellular labelling indices, increased apoptosis rates and increased BrdU labelling in liver perivenal areas were observed. An initiation promotion experiment (following the Ito et al, 1995 protocol) has been conducted with the above mentioned 7-chemical mixture and with submixtures containing some of the chemicals at different multiples. There were no consistent findings in numbers of liver foci, liver foci areas, or BrdU labelling that would indicate an evidence for hepatocarcinogenicity (Benjamin et al, 1999). Specifically, increased total areas of GST-P positive foci are only seen for the 7-chemical 1x mixture, not for the 7-chemical 10x

mixture and decreased total foci areas were detected in submixtures (10-fold concentrations of the organic and the inorganic compounds included in the original mixture).

A mixture of eight metals found in contaminated Indian groundwater was tested in a 90-day study in rats at different concentrations: at the maximum permissible level (MPL set as drinking water guideline by the WHO), 1x (i.e. at the concentration found in groundwater), 10x and 100x (Jadhav et al, 2007a,b). No effects were seen at the MPL and the 1x concentration. At the ≥ 10x concentration there were decreased body weights, decreased water consumption, increased brain, kidney and liver weights, which were accompanied at the next higher dose (100x) by significant histomorphological changes in brain, kidney and liver.

The NTP has conducted a large set of mixture studies, testing a mixture of chemicals and metals representing contaminated groundwater near hazardous waste disposal sites. Different dilutions of a mixture (between 0.2x and 20x) have been dosed at various combinations in a range of different toxicological studies: reproduction and developmental toxicity studies in rats and mice, subchronic toxicity studies in rats and mice including specific immunotoxic and hematopoietic endpoints (NTP 35, 1993a; Chapin et al, 1989; Heindel et al, 1995; Simmons et al, 1994; Germolec et al, 1989; Hong et al, 1991, 1992; Shelby et al, 1990). In addition, a range of *in vitro* and *in vivo* genotoxicity studies were conducted. The 1x concentration level in the series of these studies is taken to be similar to an average contaminated groundwater, but no specific quantitative rationale or actual measurements are provided in the papers. At the 1x concentration, no effects were observed in either of these very comprehensive studies. However, at the 10x concentration some effects on liver, kidney, testes and blood system were seen (NTP 35, 1993a) as well as decreased live pup weights at term, decreased mating index in mice, decreased number of live pups per litter and sperm concentration / count in rats (Heindel et al, 1995). A subacute study in male Fischer rats gives some evidence, that at least some of the effects on liver and kidney after administration of the contaminated groundwater mixture and multiples thereof might be related to the effects of restricted food and water intake (Simmons et al, 1994). All findings with regard to immunotoxicity are considered to be of low relevance (Germolec et al, 1989; Hong et al, 1991, 1992). With regard to genotoxicity, at all doses in these studies there were increased numbers of sister chromatid exchanges (SCEs) in the bone marrow of mice, with overall low toxicological and biological relevance. Increased mitotic indices in bone marrow were observed in micronucleus studies in male and female rats and mice at the 20x dose only (Shelby et al, 1990). No effects were observed in developmental toxicity studies (Heindel et al, 1995).

In two further sets of large studies, rats and mice were exposed to pesticide / fertiliser mixtures representing 1980s groundwater concentrations in two US States (California and Iowa) in a set of reproduction and developmental toxicity, immunotoxicity and subchronic toxicity studies (NTP 36, 1993b; Kligerman et al, 1993; Heindel et al, 1994). The two pesticide / fertiliser mixtures were administered via drinking water in concentrations of 1x, 10x and 100x (sometimes 0.1x was

also dosed). In addition, a range of genotoxicity assays have been conducted at the same concentrations. The only findings at the 1x dose were increased SCEs in splenocytes without a dose-response relationship, and increased relative liver weights in male and female rats after 6-month exposure time without histopathological correlate. These findings are either of questionable toxicological significance based on the absence of a dose-response relationship or of doubtful biological relevance as the SCE assay is a secondary genotoxicity assay and the relative liver weights were without histopathological correlate (NTP 36, 1993b; Kligermann et al, 1993). Subchronic toxicity studies in mice with the same chemical mixtures were also without any adverse effects at the 1x dose. Increased frequencies of micronuclei in blood (but not in splenocytes) were observed at 10x and 100x, but without a clear dose response (NTP 36, 1993b; Kligermann et al, 1993). With regard to developmental and reproduction toxicity, the only finding which could be observed in well conducted studies in rats and mice were decreased seminal vesicle weights, without effects on sperm parameters, at the 100x concentration dose only (Heindel et al, 1994).

A mixture of inorganics, mainly metal salts, was dosed to rats in a 90-day study with each component present 1x, 5x and 25x the 1978 Great Lakes Water Quality Objective (Chu et al, 1981). All but two of the components in the 25x mixture were present above the Canadian drinking water standard of the time, yet no effects were seen at any dose.

More details on all studies are included in Table 3.

Contaminated soil

One 90-day study has been conducted in female mice to directly study highly contaminated Love Canal soil. The mice were exposed to the soil, with additional direct vapour exposure to the most volatile compounds of the mixture. Some effects were observed in liver, spleen and kidney in this repeated-dose toxicity study (Silkworth et al, 1984). It should be noted again that this contamination does not represent realistic environmental or human exposure, but a mixture exposure of a highly contaminated site in the US.

Table 3: Summary of mixture studies on environmental exposures via water and soil

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Adeyemi et al, 2010			
65 days with focus on kidney toxicity, male and female Albino rats	Experimental water samples obtained from two different wells located at about 1 and 1.5 km from the landfill, and two boreholes located at about 1 and 1.5 km from the landfill	6 dose groups: controls, simulated leachate, well (1 km), well (1.5 km), borehole (1 km), borehole (1.5 km)	Increased Na^+ concentration, necrosis of muscle fibres and cellular infiltration by macrophages is observed in the leachate simulation. In all groups increased K^+, urea and creatinine concentration and changes in the kidney and serum ALP, ACP, AST and ALT were observed. The findings in the water groups are considered to be of questionable relevance, as only biochemical parameters are given. No information on kidney weights or any details on macroscopy or histopathology for these groups. The samples probably do not represent environmentally or human-relevant exposure levels
Reference: Wang et al, 2002			
Chronic study (16-18 months), mouse	Different doses of mixtures of chlorinated hydrocarbons were used similar to concentrations in the underground water near the electronics factory site in Taoyuan	Cyanuric acid mixtures (low, medium, high dose, underground water in µg/ml): Chloroform 5.8, 7.6, 14.0, n.d. - 3.0, 1,1-Dichloroethane 5.8, 12.8, 41.3, n.d. - 36.1, 1,1-Dichloroethylene 1.2, 4.1, 10.6, n.d.- 82.4, 1,1,1-Trichloroethane 2.0, 3.4, 11.9, n.d. - 10.1, Trichloroethylene 44.1, 106.0, 471.2, n.d. - 87.0, Tetrachloroethene 36.0, 90.3, 606.5, n.d. - 210.6. The concentrations of compounds in the underground water represent the results of GC-MS analysis of water samples from 20 wells near a manufacturer of electric appliances in Taoyuan	- Male mice: high dose: tail alopecia and deformation; medium and high dose: increased liver and lung absolute weights (no changes in relative weights), BUN, serum creatinine values increased, trend of increased frequency of hepatocellular neoplasms - Female mice: high dose: increased liver, kidney, uterus and ovary absolute weights (no changes in relative weights), higher incidence of mammary adenocarcinoma (5 mammary gland adenocarcinoma in the high dose group compared to 0/1/0 in the control/low/medium dose groups. The mixtures contained high doses of a mixture of chlorinated alkanes, not representing environmentally or human-relevant exposure levels. Increased mammary adenocarcinoma seem to be the only relevant treatment-related finding
Reference: Zhao et al, 2011a			
90 days in 4-week old mice; with focus on reproduction toxicity (spermatology, testis histopathology	Simulated mixture representing Nanjing city tap water	One-dose group receiving simulated tap water	- No effects on water consumption and body weights; significant reduction in the relative percentages of elongated spermatids in the tap water group. The ratios 4C:2C and 1C:2C (where 1C-round spermatid, 2C is diploid germ cells and 4C - tetraploid germ cells) were claimed to be changed and this was interpreted as an acceleration of the transformation of spermatogonia to primary spermatocytes and spermatogonia to round spermatids ($p > 0.05$). The number of abnormal sperms was increased as indicated by microscopy (1000 sperm smears were investigated, no grading presented) in the

Study type	Mixtures tested	Doses / Basis of exposure	Results
			tap water compared to controls. - Histopathology: Expanded interstitial space and decreased number of Leydig cells. No significant difference in the number of sperm between the treatment and control mice. - Limited study design. The decreased number of elongated spermatids and the increased number of abnormal sperm seem to be treatment related; however the biological relevance is unclear, as no further reproduction parameters are investigated. The tap water used seems to represent contaminated water compared to drinking water standards
Reference: Chernoff et al, 1979			
Mouse teratology study	Durham (NC) municipal drinking water over 8 months	1x, i.e. tap water was used directly	The only apparently treatment-related effect was an increase in the incidence of supernumerary ribs compared to the purified water control
Reference: Staples et al, 1979			
Mouse teratology study	Durham (NC) municipal drinking water over 10 months	1x, i.e. tap water was used directly	No treatment-related effects
Reference: Keen et al, 1992			
Rat developmental toxicity study	1 tap water and 1 bottled water	Only raw waters were tested	No treatment-related effects
Reference: Uriu-Hare et al, 1995			
Rat teratology study	1 tap water and 1 bottled water	Only raw waters were tested	No treatment-related effects, though some residual questions about resorptions caused by an unbalanced design due to an earthquake
Reference: Porter et al, 1999			
Subchronic mouse studies with specific endpoints: endocrine, immune, nervous system function (3 repeats with one strain, 4 repeats with another)	3 chemicals (including 2 pesticides) found in Wisconsin groundwater	3x maximum contaminant level in water (individual chemicals, 2-compound and 3-compound mixtures)	Effects (aggression score at day 14, body mass, free thyroid index, plaque forming (antibody production), final spleen weight) sporadically seen, but rarely repeated, and lacking plausibility
References: Constan et al, 1995, 1996			
6-month study in rats investigating 5 different time points (3, 10 days, 1, 3, 6 months) with specific focus on liver toxicity	Mixtures of 7 chemicals frequently detected in groundwater associated with hazardous waste sites	1x, 10x mixture of chemicals	Increased hepatocellular labelling indices at the 10-day and the 1-month time point in the 10x mixture and 10x submixtures dose groups. No changes at 1x. Increased apoptotic rates and increased BrdU labelling in liver perivenular areas at the 10x mixture

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Benjamin et al, 1999			
Initiation - Promotion experiment (placental glutathione-S-transferase (GST-P) preneoplastic liver cell foci after diethylnitrosamine initiation and partial hepatectomy)	Mixtures of 7 chemicals frequently detected in groundwater associated with hazardous waste sites	1x and 10x mixture of chemicals: 7-chemical-1x, 7-chemical-10x Submixtures of organic and inorganic chemicals (each 10x): Organic-10x, inorganic-10x 10x and 100x of a 3-compound chemical mixture: TLC-10x, TLC-100x	Inconsistent body weight and liver weight changes (decrease or increase) without a dose-relationship; increased total foci area/liver area only seen in the 7-chemical-1x dose group not in the 7-chemical-10x group. In the submixture groups (organic-10x and inorganic-10x) decreased foci number and total foci area were observed. Liver changes are not considered to be treatment related
References: Jadhav, 2007a,b			
90-day study in rats	Mixture of 8 metals found in contaminated Indian groundwater	MPL, 1x, 10x, 100x mixture of 8 metal (salts)	MPL, 1x: no organ weight changes ≥ 10x: decreased body weights, decreased water consumption, increased relative brain weight, increased absolute and relative kidney weight, increased relative liver weights 100x: increased absolute brain weight; statistically significant histomorphological changes in kidney, liver and brain
Reference: NTP Report 35, 1993a			
Subchronic rat study	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Four dilutions of a concentrated mixture. The dose groups are 0.3x, 1x, 3x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Various effects seen at the highest dose tested only (10x) - including liver, kidney, testes and blood systems. No treatment-related effects at 3x or lower doses
References: NTP Report 35, 1993a; Chapin et al, 1989			
Subchronic mouse study	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Four dilutions of a concentrated mixture. The dose groups are 0.3x, 1x, 3x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Disturbed oestrus cycles and decreased water consumption (presumably taste-related) at the highest dose only (10x)
References: Heindel et al, 1995; preliminary results in NTP Report 35, 1993a			
Rat reproductive toxicity study	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 1x, 5x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Decreased water consumption at all doses. At 5x dose and higher there was decreased pup weight development during lactation. At 10x dose there were decreased body and organ weights without histopathology, decreased live pup weights at term, and decreased mating index

Study type	Mixtures tested	Doses / Basis of exposure	Results
References: Heindel et al, 1995; preliminary results in NTP Report 35, 1993a			
Mouse reproductive toxicity study	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 1x, 5x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Decreased water consumption at 5x dose and higher. At 10x dose there were increased organ weights without histopathology, and decreased number of live pups per litter, and sperm concentration/count
Reference: Simmons et al, 1994			
Subacute study rat; liver, kidney and general toxicity	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites and different concentrations of carbon tetrachloride	Animals received for 13 days either *ad libitum* water, or restricted water or the 1x groundwater mix and then at day 14 one gavage dose of carbon tetrachloride (CCl_4): 15 groups tested: *Ad libitum* water group: Controls, 0.0375, 0.050, 0.075, 0.150 mg/kg bw CCl_4 Water restricted group: Controls, 0.0375, 0.050, 0.075, 0.150 mg/kg bw CCl_4 10% Mix group: Controls, 0.0375, 0.050, 0.075, 0.150 mg/kg bw CCl_4	- When comparing the influence of groundwater mix to CCl_4 induced liver toxicity, it has been observed that a small but statistically significant effect attributable to groundwater mix was detected for hepatocellular necrosis but not for hepatocellular degeneration. - Kidney toxicity: No renal lesions occurred that could be attributed to any water treatment or water treatment + CCl_4 combination. CCl_4 had no significant effect on kidney weight, relative kidney weight, CREAT, or BUN/CREAT ratio in any water treatment group. In *ad libitum* water rats, BUN was increased only at the highest dosage level, 0.150 mg/kg bw CCl_4. CCl_4 had no effect on BUN in groundwater mix or water restricted rats. - No hepatic or renal lesions occurred that could be attributed to groundwater mix alone. Conclusion: The response to CCl_4 in the restricted water rats was similar to that of groundwater mix rats, indicating that a substantial portion of the effect of groundwater mix on CCl_4 hepatotoxicity is due to decreased water and feed intake
Reference: NTP Report 35, 1993a; Germolec et al, 1989			
Subacute mouse study with a focus on the immune system	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 0.2x, 2x and 20x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Only effect seen was at the 10x dose, and was a decrease bone marrow granulocyte macrophage progenitors
Reference: NTP Report 35, 1993a; Germolec et al, 1989			
Subchronic mouse study with a focus on the immune system	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 1x, 5x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	A decrease in bone marrow granulocyte macrophage progenitors was seen at the 5x and 10x doses. Only effects seen at the 10x dose were decreased haematopoietic stem cells, and antigen-induced antibody forming cells

Study type	Mixtures tested	Doses / Basis of exposure	Results
References: NTP Report 35, 1993a; Hong et al, 1991, 1992			
Subacute to subchronic mouse study with recovery groups, and specific haematopoietic endpoints	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 1x, 5x and 10x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Effects were only seen at the 10x dose, and were increased relative kidney weight, decreased relative thymus weight, decreased MCV and decreased bone marrow granulocyte macrophage progenitors
Reference: NTP Report 35, 1993a; Shelby et al, 1990			
Battery of *in vitro* and rodent *in vivo* genotoxicity tests	25 most frequently detected groundwater contaminants (19 organics and 6 metals) representing groundwater concentrations near hazardous waste disposal sites	Three dilutions of a concentrated mixture. The dose groups are 1x, 10x and 20x, with the 1x taken to be similar overall to an average contaminated groundwater (but no specific quantitative rationale)	Increased mitotic index in bone marrow in micronucleus studies in males and female rats and mice at the 20x dose only. Increased sister chromatid exchange in bone marrow in male mice at all three doses
References: NTP Report 36, 1993b; Kligerman et al, 1993			
Subchronic rat study with additional genotoxicity endpoints	Pesticide/fertiliser mixture, including 5 pesticides, representing Iowa groundwater	Four concentrations of a mixture, 0.1x, 1x, 10x and 100x, where 1x represents median concentrations found in Iowa groundwater	At concentrations 1x and higher in both sexes, there was an increase in absolute and relative liver weights (all > 10% except relative weights at 1x dose). No histopathological findings in liver. Authors conclude this effect to be of doubtful biological significance
References: NTP Report 36, 1993b; Kligerman et al, 1993			
Subchronic mouse study with additional genotoxicity endpoints	Pesticide/fertiliser mixture, including 5 pesticides, representing Iowa groundwater	Four concentrations of a mixture, 0.1x, 1x, 10x and 100x, where 1x represents median concentrations found in Iowa groundwater	At 10x and 100x concentration, there was an increase in frequency of micronuclei in blood (but not in splenocytes), without a clear dose response
References: NTP Report 36, 1993b; Kligerman et al, 1993			
Subchronic rat study with additional genotoxicity endpoints	Pesticide/fertiliser mixture, including 6 pesticides, representing California groundwater	Four concentrations of a mixture, 0.1x, 1x, 10x and 100x, where 1x represents median concentrations found in California groundwater	At 1x and higher concentration, there was an increase in sister chromatid exchanges in splenocytes, without a clear dose response
References: NTP Report 36, 1993b; Kligerman et al, 1993			
Subchronic mouse study with additional genotoxicity endpoints	Pesticide/fertiliser mixture, including 6 pesticides, representing California groundwater	Four concentrations of a mixture, 0.1x, 1x, 10x and 100x, where 1x represents median concentrations found in California groundwater	At the 100x concentration only, there was an increase in sister chromatid exchanges in splenocytes, and a decrease in absolute and relative liver weights in male mice
Reference: Heindel et al, 1994			
Rat developmental toxicity study (GD6-GD20)	Pesticide/fertiliser mixture, including 5 pesticides, representing Iowa groundwater	Three concentrations of a mixture, 1x, 10x and 100x, where 1x represents median concentrations found in Iowa groundwater	No treatment-related effects

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Heindel et al, 1994			
Mouse reproductive toxicity study	Pesticide/fertiliser mixture, including 5 pesticides, representing Iowa groundwater	Three concentrations of a mixture, 1x, 10x and 100x, where 1x represents median concentrations found in Iowa groundwater	No treatment-related effects
Reference: Heindel et al, 1994			
Rat developmental toxicity study (GD6-GD20)	Pesticide/fertiliser mixture, including 6 pesticides, representing California groundwater	Three concentrations of a mixture, 1x, 10x and 100x, where 1x represents median concentrations found in California groundwater	No treatment-related effects
Reference: Heindel et al, 1994			
Mouse reproductive toxicity study	Pesticide/fertiliser mixture, including 6 pesticides, representing California groundwater	Three concentrations of a mixture, 1x, 10x and 100x, where 1x represents median concentrations found in California groundwater	At 100x concentration, there was a decrease in seminal vesicle weights (but sperm parameters were normal), increased kidney/adrenal weights in female F_1 mice and decreased gestation duration, though these effects were not considered by the authors as being significant
Reference: Chu et al, 1981			
90-day rat study	10 metal salts and fluoride	1x, 5x and 25x the 1978 Great Lakes Water Quality Objectives (which were based on ecotoxicity)	No treatment-related effects
Reference: Silkworth et al, 1984			
90-day study in female CD-1 mice including interim sacrifices	Contaminated soil	- Exposure to vapours of contaminated soils (love canal) in 4 exposure groups: Contaminated soil vapours Contaminated soil vapours with additional direct contact to soil Contaminated soil vapours with lid on top of the cage (increases the vapour exposure to factor 4-10) Contaminated soil vapours with additional direct contact to soil with lid on top of the cage (increases the vapour exposure to factor 4-10) - Components which were detected in mean concentrations in µg/m^3: m-Dichlorobenzene: 18, p-Dichlorobenzene: 27, o-Dichlorobenzene: 22, Hexachloroethane: 8, 2,5 + 2,6 Dichlorotoluene: 121, 1,3,5-Trichlorobenzene: 16, 3,4-Dichlorotoluene: 59, a,a-Dichlorotoluene: 21, 1,2,4-Trichlorobenzene: 24, o,o-Dichlorotoluene: 8, 1,2,3-Trichlorobenzene: 66,	- Without direct soil contact: Increased relative thymus and spleen weights after 4 weeks of exposure, but not after 8 or 12 weeks. Mice exposed to 5- to 10-fold elevated concentrations of volatiles had increased body weights and increased relative kidney weights. - With direct soil contact: Increased body weights (10%) and increased relative liver weights (169%) with centrolobular hepatocyte hypertrophy in 40-70% of the lobules in all mice. Mice exposed to elevated concentrations of volatiles had increased relative liver (184%) with centrolobular hypertrophy, spleen (125%) and kidney (114%)

Study type	Mixtures tested	Doses / Basis of exposure	Results
		1,2,4,5-Tetrachlorobenzene: 106, Pentachlorobenzene: 126, Hexachlorobenzene: 3, a-Benzenehexachloride: 23, Lindane: 27, d-Benzenehexachloride: 55, Chloroform: 7, 1,1,1-Trichloroethane: 4.1, Carbon tetrachloride: 1.0, Trichloroethylene: 1.3, Tetrachloroeethylene: 16.9, 1,1,2-Trichloroethane: 5.7, Bromodichloromethane: 1.0, 1,2-Dibromoethane: 2.2	

Food – dietary intake

The other studies in this section have used artificial mixtures, i.e. mixtures made up in a laboratory to simulate an environmental exposure via food, or some multiple of such an exposure. The use of pesticides to protect crops can result in pesticide residues in food products. One study used pesticide residue monitoring data as the basis for exposure (Dolara et al, 1993). Residue analyses from 1989-1991 in Italy were combined with information on the amounts of each food in the diet to estimate the chronic average dietary intake of 15 pesticides, and these were incorporated into rodent diets accordingly. Various multiples of the environmental exposure were tested, 1x representing average chronic dietary exposure. Pesticide residues in food are regulated to ensure that human chronic dietary exposure does not exceed the acceptable daily intake (ADI). In another study, the basis for test animal exposure was '1x' which represented dietary exposure at the ADI, i.e. at the permitted limit for man, and various multiples of this level were tested (Perez-Carreon et al, 2009).

Some studies specifically concerned the exposure of human infants to environmental mixtures via breast milk (Desaulniers et al, 2003; Gyorkos et al, 1985). In these studies, the exposure of infants to specific chemicals was estimated, and this dose (1x) and various multiples of it were given to laboratory animals.

Many of the caveats concerning the interpretation of studies of environmental mixtures in water in the context of human exposure also apply to studies in diet. For example, dietary environmental mixtures will change over time, as will the regulatory limits for specific chemicals. There are also a number of papers available studying mixtures of compounds (potentially) present in food at human relevant concentrations in animal studies. A mixture of 15 pesticides representing chronic dietary intakes in Italy (based on monitoring data) was tested in an *in vivo*

micronucleus test at 0.1x, 1x and 10x doses (Dolara et al, 1993), and produced no toxicity. A mixture of 15 organic chemicals present in the Great Lakes were incorporated into rat diets to give doses equivalent to 1x, 10x, 100x and 1000x the 1978 Great Lakes Water Quality Objectives for each component (Côté et al, 1985). After 90 days of dietary dosing, no effects were seen.

A few studies are available investigating varying mixtures of polychlorinated organics at doses representing those received by infants via human breast milk and multiples thereof. Female rats were dosed during a 20-day period after birth, and liver enzyme induction together with organ weights were determined (Desaulniers et al, 2003). No effects were seen at the 1x and 10x mixtures, very few effects were seen at the 100x concentration (BROD only), but clearly induced liver enzymes and increased liver and decreased adrenal and thymus weights were observed at 1000x concentrations. At the 100x concentration, decreased T4 (thyroxine) and increased TSH were also determined. Basically the same results have been observed in an earlier study of Gyorkos (Gyorkos et al, 1985). In this study immature male rats have been dosed via gavage on two occasions three days apart.

An initiation promotion experiment has been performed with a 12-pesticide mixture each at 1x and 10x the ADI concentration without any observed effects (Perrez-Carreon et al, 2009). The pesticides included in the experiment were all suspected carcinogens

The evaluated studies are summarised in Table 4.

Table 4: Summary of mixture studies on environmental exposures via food

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Dolara et al, 1993			
Rat micronucleus study	Mixture of 15 pesticides representing average chronic dietary exposure in Italy based on monitoring data	Doses were 0.1x, 1x and 10x, where 1x represents average chronic dietary exposure	No effect
Reference: Perez-Carreon et al, 2009			
Initiation-promotion study of liver carcinogenesis in rats	Mixture of 12 pesticides (all carcinogenic)	1x and 10x ADI	No treatment-related effects
Reference: Desaulniers et al, 2003			
Prepubertal female rat study with specific endpoints (hepatic enzyme activity, organ weights and hormones)	Mixture of 16 polychlorinated organics (sum of the three above)	1x, 10x, 100x and 1000x estimated typical human exposure via breast milk in the first 24 days (based on 1989-90 data from southern Quebec)	No treatment-related effects at 1x and 10x. At 100x BROD was affected, and at 1000x BROD, PROD and EROD activities were increased. At 1000x only, there was increased relative liver weight, and decreased adrenal and thymus weight, thyroxine concentration was decreased and TSH was increased

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Gyorkos et al, 1985			
Neonatal male rat study with specific endpoints (liver enzyme activity and liver weight, and pathology in liver, thyroid, trachea, lungs, thymus, bronchial node and spleen)	Mixture of 7 organochlorine pesticides representing the composition found in human breast milk	1x, 2x, 10x and 100x the estimated dose received by a suckling human infant over 180 days	Liver enzyme induction at 10x and higher doses. Liver weight increase (adverse) and mild histopathological changes in liver and thyroid at 100x only[2]
Reference: Gyorkos et al, 1985			
Neonatal male rat study with specific endpoints (liver enzyme activity and liver weight, and pathology in liver, thyroid, trachea, lungs, thymus, bronchial node and spleen)	Mixture of 14 PCBs representing the composition found in human breast milk	1x, 2x, 10x and 100x the estimated dose received by a suckling human infant over 180 days	Liver enzyme induction at 2x and higher doses. Liver weight increase (adverse) and mild histopathological changes in liver and thyroid at 100x only
Reference: Gyorkos et al, 1985			
Neonatal male rat study with specific endpoints (liver enzyme activity and liver weight, and pathology in liver, thyroid, trachea, lungs, thymus, bronchial node and spleen)	Mixture of 7 organochlorine pesticides and 14 PCBs representing the composition found in human breast milk	1x, 2x, 10x and 100x the estimated dose received by a suckling human infant over 180 days	Liver enzyme induction at 2x and higher doses. Liver weight increase (adverse) and mild histopathological changes in liver and thyroid at 100x only
Reference: Côté et al, 1985			
90-day rat study with dietary dosing	15 chemicals found in the Great Lakes	1x, 10x, 100x and 1000x the 1978 Great Lakes Water Quality Objectives (which were based on ecotoxicity)	No treatment-related adverse effects

Great Lakes fish studies

A number of studies have tested environmental mixtures of chemical contaminants in food. The only studies of this type are the large number of studies of fish taken from the Great Lakes. These studies incorporated fish from the Great Lakes into the diets of test animals, and compared the results to control animals fed a diet incorporating fish from the ocean, on the basis that ocean fish would be free of contamination.

Short-term feeding studies in rats in the late 1970s showed effects on the thyroid and adrenals, but in these studies the rats were fed only fish, so nutritional differences between the diets might

have caused the effects rather than contamination (Sonstegard and Leatherland, 1979; Leatherland and Sonstegard, 1980). In a subchronic study in which fish made up 61% of the rat diet, some thyroid effects were also seen, though less clearly than in the earlier work (Leatherland and Sonstegard, 1982). At a similar period, a subacute rat study in which fish made up to 5.8% of the diet resulted in an effect on hormone metabolism in female adrenals, without histopathology, and mild histopathological changes in the liver, whilst a subchronic study by the same authors resulted only in liver enzyme induction (Villeneuve et al, 1981; Chu et al, 1984). A subchronic study with fish as 33% of the diet of mice resulted in liver weight increases, liver enzyme induction and some body weight reduction (Cleland et al, 1987). In the late 1980s, a series of behavioural studies in rats fed on 30% fish showed effects on various aspects of feeding responses and activity patterns, in the absence of body weight effects (Daly et al, 1989; Hertzler, 1990; Daly, 1991); these findings are hard to interpret in a human context. Beattie et al (1996) found no or very limited effects on rat behaviour when they were fed diets of 100% fish, though nutritional effects and design flaws limit the interpretability of these studies. In the mid-1990s, a multigenerational rat study with concurrent clinical, immunological and neurobehavioural assessments, was performed with the inclusion of 5 or 20% fish in the diet (Arnold et al, 1998a,b; Feeley and Jordan, 1998; Tryphonas et al, 1998a,b; Pappas et al, 1998; Seegal et al, 1998; Iverson et al, 1998; Feeley et al, 1998). The only clear treatment-related effects were liver weight increases and enzyme induction (with a secondary effect on vitamin A stores), and subtle effects on the immune system of F_2 males, all without adverse functional consequences or histopathology.

The reproductive success of mink in fur farms around the Great Lakes fell in the late 1960s. Since then, a number of studies have been carried out to evaluate the effects of contaminated fish on the health of mink being grown for fur (Aulerich et al, 1971; Aulerich and Ringer, 1977; Hornshaw et al, 1983; Heaton et al, 1995a,b; Tillit et al, 1996). As far as this review is concerned, these mink studies are treated as if mink were just another test species for the evaluation of human health; in other words the objectives of the mink studies are not relevant to this review, but the data from the studies remain relevant. Studies of contaminated fish have incorporated various amounts of fish into the diet, up to 100% but typically 5-30%. It is clear that some adjustment needs to be made when considering such exposures in the context of the human diet, even for the most highly exposed subpopulation, i.e. in this case people who do a lot of leisure fishing in the Great Lakes. Controlled reproduction studies established that this was caused by the inclusion of fish from the Great Lakes in their diet (Aulerich et al, 1971). This was established by comparison to diets containing (less contaminated) ocean fish. In some cases, adult mink survival was also affected when 30% Great Lakes fish were included in the diet, and the effects could be reproduced by substituting ocean fish plus PCBs for the Great Lakes fish (Aulerich and Ringer, 1977). It was established that mink have a peculiar sensitivity to PCB toxicity. Although contamination declined, up to the 1990s there was still enough contamination

to affect pregnancy and kit survival in mink fed Great Lakes fish (Hornshaw et al, 1983; Heaton et al, 1995a,b; Tillitt et al, 1996).

The contamination of the Great Lakes, especially by PCBs, had clear and major effects on mink fed fish from these lakes. However, there were only minor effects on rats. It has been estimated that a rat diet including 20% fish represents 60x the average consumption of all fish and seafood by the Canadian public, so the exposure in these studies clearly does not represent a realistic human exposure situation.

An overview of these Great Lakes fish studies is included in Table 5.

Table 5: Summary of mixture studies on environmental exposures via Great Lakes fish

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Sonstegard and Leatherland, 1979			
Rat feeding study (1-2, 1 and 2 months duration, respectively), focused only on the thyroid. Males only	Exposure to diets consisting of fish from either the ocean (control) or from the Great Lakes.	Three studies, with the diet being only coho salmon. In the first study, groups were fed salmon from the Pacific (control), Lake Ontario, Lake Michigan or Lake Erie. In the second study, groups were fed salmon from the Pacific (control), Lake Ontario, or from Lake Ontario with the addition of an iodine supplement. In the third study, groups were fed salmon from the Pacific (control), Lake Ontario or Lake Michigan	The groups fed Great Lakes salmon had lower serum T4 concentrations, T4/T3 ratios, increased thyroid weight and in some cases greater epithelial cell height than those fed Pacific salmon
Reference: Leatherland and Sonstegard, 1980			
4 and 8-week rat dietary studies focused only on thyroid and adrenals	Exposure to diets consisting of fish from either the ocean (control) or from the Great Lakes	Two very similar studies differing primarily in their endpoints. The diet consisted of coho salmon. Different groups were fed salmon from the Pacific (control), Lake Ontario, Lake Michigan or Lake Erie	Thyroid epithelial cell weight was increased in rats fed Lake Ontario fish compared to groups fed Pacific salmon. Changes were seen in cells from thyroids of rats fed Lake Ontario and Lake Michigan fish, but not those fed Lake Erie fish. The rats fed Great Lakes salmon had increased relative thyroid weight compared to those fed Pacific salmon. Only the rats fed Lake Erie or Lake Michigan salmon had increased relative adrenal weight compared to those fed Pacific salmon. Swollen sinusoidal complexes in adrenals of rats fed Lake Ontario salmon

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Leatherland and Sonstegard, 1982			
90-day rat dietary study focused only on thyroid	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	The diet included 61% coho salmon. Different groups were fed salmon from the Pacific (control), Lake Ontario, Lake Michigan or Lake Erie	The males fed Great Lakes salmon had increased thyroid weight compared to those fed Pacific salmon, though when corrected for body weight this was only significant for the Lake Michigan group. No effect on thyroid weight in females. Thyroid epithelial cell height was increased only for those females fed Lake Ontario and Lake Michigan fish, and those males fed Lake Erie fish, compared to same sex groups fed Pacific salmon. The only effect seen on T3 and T4 was decrease in T4, T3 uptake and free T4 in females fed Lake Ontario salmon
References: Villeneuve et al, 1981; Chu et al, 1984			
28-day rat dietary study, followed by a very similar 90- day rat dietary study with 90-day untreated recovery groups	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	Adult coho salmon from either Lake Ontario or the Pacific (control) were incorporated into rat diet at 1.45%, 2.9% or 5.8% by weight	The 28-day study showed altered steroid hormone metabolism in female adrenals at all fish doses, without corresponding histopathology. Also mild dose-related histological changes in liver. However, in the 90-day study the only effect that seemed to be treatment related was the induction of one specific hepatic enzyme
Reference: Cleland et al, 1987			
Four-month dietary study in male mice of two strains (C57Bl/6 and DBA/2), focused narrowly on liver and thyroid	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	Different groups were fed salmon from the Pacific (control), Lake Ontario, Lake Michigan or Lake Erie. Fish was 33% of the diet on a dry weight basis	Lake Ontario salmon were the most contaminated and resulted in most effects (10% reduction in body weight in DBA/2 mice, increase in % liver weight, EROD induction and T3 and T4 suppression in both strains). Additionally, EROD was induced in all Great Lakes salmon-fed groups of C57Bl/6 mice, and T3 (and in some cases T4) was supressed for all Great Lakes salmon-fed groups of DBA/2 mice
Reference: Daly et al, 1989			
Four 20-day behavioural studies, with various endpoints and using male rats of various ages	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	Different groups were fed salmon from the Pacific (control) or Lake Ontario. The diet included 30% salmon	The rats fed Lake Ontario salmon developed a preference for predictable food rewards more quickly than rats fed ocean salmon. Mild electric shocks suppressed response to food more in rats fed Lake Ontario salmon compared to rats fed ocean salmon. The authors conclude that the results are consistent with Lake Ontario fed groups having increased reactivity to adverse events
Reference: Hertzler, 1990			
Six 20-day behavioural studies, with various endpoints	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	Different groups were fed salmon from the Pacific (control) or Lake Ontario. The diet included 30% salmon (or 8% in one experiment)	The various experiments examined males and females, adults and young animals and postweaning dams, and examined handled and non-handled rats and food deprivation. A fairly consistent pattern of reduced open field activity, rearing and nosepoke behaviour was seen in Lake Ontario salmon fed rats compared to ocean salmon fed rats

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Daly, 1991			
20 days of feeding test or control diets followed by 8 days on restricted control diet to lower body weight to 80% of ad libitum control, followed by 27 days of testing (still on control diet)	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	Different groups were fed salmon from the Pacific (control) or Lake Ontario. The diet included 30% salmon	No effect on weight gain. When trained using a large food reward there was no effect of diet on response, but when then switched to a small food reward, the rats previously fed Lake Ontario salmon took longer to reach the reward than those previously fed ocean fish. The author concluded that the results are consistent with Lake Ontario fed groups having increased reactivity to an adverse event
Reference: Beattie et al, 1996			
Two subchronic behavioural/neurotoxicity studies, the first with 45 days treated and 45 days untreated diet given to male rats, and the second with 90 days treated diet given to female rats	Exposure to diets consisting of various fish from the Great Lakes	Two studies, the first with diets consisting of 100% potatoes, rat chow, walleye, whitefish or lake trout from Lake Superior, or carp from Little Lake Butte des Morte (LLBM) for 45 days, followed by 45 days on control diets. The second study with diets of 100% rat chow, walleye, whitefish (used as control for walleye group) or lake trout from Lake Superior, carp from LLBM, or Pacific salmon (control for trout and carp groups) for 90 days	The first study showed minimal behavioural effects but was in any case not interpretable due to absence of an uncontaminated fish control. In the second study the use of whitefish as control for the walleye was not sufficiently justified. There was a reduction in accelerating rotarod score with apomorphine challenge for the group fed carp (compared to salmon control), which was only apparent towards the end of the study
References: Arnold et al, 1998a,b; Feeley and Jordan, 1998; Tryphonas et al, 1998a,b; Pappas et al, 1998; Seegal et al, 1998; Iverson et al, 1998; Feeley et al, 1998			
Two-generation toxicity study with concurrent clinical, immunological and neurobehavioural assessments (10 weeks before mating, 1:1 mating, appr. 70 days postweaning, one F_1 male and one F_1 female from 24 litters were mated within the diet groups to produce F_2 litters). Some F_1 animals were switched to control diet for 3 months at 13 weeks old	Exposure to diets containing various amounts of Great Lakes fish	5 dose groups, whose dietary protein consisted of casein and/or salmon from a river flowing into Lake Ontario (LO) or from a part of Lake Huron (LH): 1: 20% casein (controls) 2: 15% casein + 5% LO salmon (LO-5%) 3: 20 % LO salmon (LO-20%) 4: 15% casein + 5% LH salmon (LH-5%) 5: 20% LH salmon (LH-20%) The fresh fish did not violate any guidelines for contaminants, but once prepared for inclusion in the diet they did. The 5% and 20% fish diets were about 15x and 60x the estimate of Canadian public consumption of all fish and seafood	There were positive effects of fish on growth and reproduction - these are not discussed further here. With multiple generations, two sexes and two dose levels, there is an opportunity to identify consistent adverse effects, but few were seen, most effects being inconsistent and so not clearly treatment-related. Clear liver enzyme induction (all fish fed groups), liver weight increases (LO-20% and LH-20%) and reduced vitamin A stores (LO-20%) were clear treatment-related effects. The reduced vitamin A store was due to liver enzyme induction, and no adverse consequence was seen. In addition there seems to be a real immune-system effect in F_2 males (increased leukocytes and lymphocytes in spleen and increased thymus weight), but immune system functional assays showed no effect. The authors proposed behavioural effects, but these are unconvincing

Study type	Mixtures tested	Doses / Basis of exposure	Results
Reference: Aulerich et al, 1971			
Mink reproduction studies, 11, 6 and 6 months duration respectively	Exposure to diets containing fish from either the ocean (control) or various groups fed fish from the Great Lakes	There was 30% fish inclusion in the diet. In experiment 1, the groups were ocean perch (control) and 1967 Lake Michigan coho salmon. In experiment 2, the groups were ocean whiting (control), 1968 Oregon coho salmon, 1967 Lake Michigan coho salmon, 1968 Lake Michigan coho salmon and 1968 Lake Michigan coho salmon canning by-products. In experiment 3, the groups were ocean whiting (control), 1969 Lake Michigan or Lake Erie coho salmon, 1969 Lake Michigan bloater chub and 1969 Lake Michigan or Lake Erie yellow perch	Kits born, kit survival and average weight were reduced in all experiments in all groups fed fish from Lake Michigan, and those fed coho salmon from Lake Erie. Mating success and adult survival was less consistently reduced
Reference: Aulerich and Ringer, 1977			
Mink reproduction study of 6 months duration	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	There was 30% fish inclusion in the diet, either ocean fish (control), coho salmon, or ocean fish plus 30ppm PCBs	There was adult mortality in the coho salmon group. There were similar clinical signs and lesions in the groups fed ocean fish plus PCBs, and the dead mink from these two groups contained similar PCB concentrations
Reference: Hornshaw et al, 1983			
Mink reproduction studies of 12 months duration	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	In experiment 1 there was 30% fresh fish (or equivalent) inclusion in the diet, either Atlantic fish (control), or Great Lakes carp, white sucker, yellow perch, lake whitefish or alewife. In experiment 2 the control was standard mink diet (includes 12.5% ocean fish), which was compared to a diet with 20% perch and 10% sucker (from the Great Lakes)	No effects on adult body weight, though the carp diet resulted in increased liver weight compared to the control. The carp diet in experiment 1 and perch+sucker diet in experiment 2 resulted in reduced pregnancy and no kit survival, whilst the perch diet in experiment 1 resulted in reduced kit growth
References: Heaton et al, 1995a,b; Tilitt et al, 1996			
Six-month mink reproduction study (single study presented in three papers)	Exposure to diets containing fish from either the ocean (control) or from the Great Lakes	The diet included 40% fish, i.e. 0, 10, 20 or 40% carp from Lake Michigan, made up to 40% fish in total using ocean fish	Three adult deaths, probably treatment related. Dose-related reduction in food consumption in females of all groups fed carp, but no effect on body weight. Reduction in live kits and no kit survival in 40% carp group. Reduction in kit weight, growth and survival in 10 and 20% carp groups. Many organ weights as % bodyweight were increased in a way related to carp in the diet. Fewer red and more white blood cells in groups fed carp

3.3 Human studies

Since this report is focused on the effects of mixtures which are of potential relevance to human health risk assessment, any human data which could be used to clarify interactions of chemicals at dose levels below effect thresholds for single chemicals is of particular relevance. Such data fall broadly into three categories: descriptive, observational and experimental. The usefulness of the data for the purpose of addressing low-dose effects of mixtures is determined by the availability of adequate exposure information, in particular dose-response data of the individual components and the mixture, as well as sufficient information that would allow an assessment of cause-effect relationships. This section focuses on descriptive and observational human studies at environmental concentrations. Experimental studies at doses below and around effect levels are reviewed in Section 4.6.

Standard approaches for statistical analysis follow a certain sequence. First, the unadjusted correlations between all single exposures (= risk factor) and the disease under investigation are analysed and tested for their statistical significance. The correlation is expressed in metrics such as Relative Risks or Odds Ratios, depending on the underlying study design. The Relative Risks are adjusted for the confounding effects of other factors that have an effect on the occurrence of disease, such as age, gender, smoking, alcohol consumption, etc. Next, adjustments are made for the potential confounding effect of other exposures, e.g. to other chemicals. The convention is to include so-called interaction terms into the statistical models in order to assess whether the combined exposure shows a different effect compared to the expected effects from single exposures. For example, there is evidence that cigarette smoking and exposure to asbestos in combination impose a larger lung cancer risk to humans than the sum of the single risk factors. Similar observations have been made for smoking and diesel exhaust, smoking and household exposure on respiratory symptoms, smoking and alcohol consumption on laryngeal cancer. However, interactions between risk factors are rare, and unfortunately investigators rarely report the results of these interaction tests, when they have been proven not to exist. Furthermore, all of the described interactions were in the exposure range where the single exposure was already impacting the health status.

Data of a descriptive nature include single or multiple case reports, reports of adverse reactions and studies which investigate a correlation between the occurrence of illness and the existence of occupational or environmental hazards in a population (cross-sectional study). Although some exposure information is usually presented, it is often difficult to estimate dose-response relationships. Furthermore, there are usually no data on exposure to individual components in studies concerning mixtures. An illustrative example of the challenges is a cross-sectional study of renal biomarkers and biological monitoring of four metals at environmental exposure levels in 800 children from different European countries (de Burbure et al, 2006). The authors looked for possible metal interactions in multiple regression analyses by grouping individual metal levels in

urine or blood into quartiles and then adding other metal levels to the analysis. Some significant interactions were found, e.g. increasing levels of mercury appeared to attenuate a possible effect of lead on serum creatinine levels. However, the data did not allow the establishment of no-effect levels, and the effects of individual metals could not be studied separately. Descriptive human studies are therefore not suited to investigate a possible interaction of chemicals present at or below a NO(A)EL.

The two main categories of observational epidemiological investigations are case-control and cohort studies. The former study type classifies subjects by the presence or absence of illness and tries to correlate this with previous exposure to specific agents, whereas the latter investigates exposed and non-exposed groups and looks for the development of disease. A major challenge for both study types is to identify reliable markers for exposure to chemical substances which may date back months or years. Although there are many studies which include biological monitoring of a variety of chemicals, these are either focused on single chemicals or, where they include chemical mixtures, they generally do not look for interactions between the individual constituents. For example, Main et al (2006) studied the correlation of six phthalate monoesters in breast milk of mothers of infant boys with or without cryptorchidism. Positive correlations were found between the concentration of different esters and certain hormone levels or ratios. However, there was no association between phthalate ester levels and cryptorchidism, and no interactions between the phthalate esters were studied. In the extensive literature available in the open literature, no example could be found where a chemical at a concentration below a threshold was exerting an effect on health status only in the presence of another substance.

In conclusion, in the vast body of epidemiological studies it is customary to analyse the data for the presence of interaction between risk factors. Unfortunately, the results of many of these analyses go unreported, because the findings were deemed less relevant than the findings on the single exposures. The interactions reported in the epidemiological literature are mostly with cigarette smoking or alcohol use, and not between exposures to chemical compounds.

3.4 Summary

The majority of this report reviews studies, which were specifically designed to investigate mixture toxicity: exact composition of the mixture was known, toxicological data of single compounds were known and individual effect levels and no effect levels were known. This is a classical approach to addressing, whether expected or unexpected mixture effects are observed.

In this chapter, the Task Force takes a different, more phenomenological approach. Those mixture studies, which are considered to represent environmentally or human-relevant exposure situations are summarised. Chemical regulation is intended to prevent toxicity arising from

exposure to chemicals in the environment. Therefore, if any toxicity is seen, it should be considered whether this is due to mixture effects.

This process starts with the testing of a real environmental mixture (e.g. 1x tap water), or a concentrated mixture (e.g. 1000x), and an observation that it does or does not have an adverse effect when compared to a relevant control (e.g. ultra-purified water). If a highly concentrated mixture is toxic, then this may not be surprising and may not be explored further. However, if the mixture shows toxicity in laboratory animals at a dose close to or at a dose representing realistic human exposure, then this is worthy of further investigation. This investigation would typically seek to discover whether the observed toxicity can be explained on the basis of its individual components, whether the dose-additivity approach for a large number of compounds in a mixture might lead to unwanted effects in animals, or whether there is evidence of a synergistic interaction. It might not be possible to answer these questions due to lack of reliable information about the nature and toxicity of the individual components, in which case a synergistic interaction is one possible explanation, but is not proven. Scientific interest and public-health concern in such a situation would tend to result in this unsatisfactory system being resolved, one way or the other, by the generation of new data. Another consequence of such observations could be to reconsider the practice of setting standard health advisories.

In this chapter, many toxicity studies have been reviewed. They are *in vivo* studies, and include some very detailed long-term studies. The studies will now be summarised in terms of whether treatment-related effects were seen, and whether these effects were at doses representing realistic human exposure.

Of the studies which tested drinking water, no treatment-related effects were seen. This includes a range of detailed long-term studies using samples concentrated by up to 3000x compared to the original water sample. The remaining water studies used artificial mixtures of contaminants designed to simulate contaminated water (1x), or concentrated contaminated water (up to 1000x). Many of these studies showed no convincing treatment-related effects even for concentrated contaminated water, and no studies showed a convincing treatment-related effect at 1x. In some studies, treatment-related toxicity was seen at 10x and higher concentrations of artificially contaminated water samples than might be expected. The majority of water studies do not provide evidence for the occurrence of synergistic interactions. The observed safety margins are in line with what would be expected based on the independent action of chemicals. In some of the more recently conducted studies, water samples or concentrates thereof from contaminated sources in different areas of Africa and Asia were used in animal studies. As the water sources are contaminated, the effects observed in these studies should be interpreted with great care.

The NTP water studies were conducted in the 1980s, and health-water standards may have changed. To get a more reliable basis from which to answer the question whether concentrations

of the single components in the different contaminated, real or artificial water samples are below current health-based water standards or not, Tables 6 and 7 compare drinking water standards (MCL, maximum contaminant levels; CHA, chronic health advisories; RfD, reference doses) set by either the US EPA or the Californian EPA and compares these values with the concentrations used in the 1x mixture of the respective study.

Table 6: Comparison of health-based drinking water standards with concentrations used in NTP mixture studies for groundwater contaminants near hazardous waste sites (Yang and Rauckmann, 1987)

Component	Health-based value (source)		Concentration in tested mixture
Acetone	100 µg/kg bw	(IRIS RfD)	6.90 ppm
Arochlor 1260	0.0005 ppm for total PCBs (MCL - EPA)		0.21 ppm
Arsenic	0.010 ppm	(MCL - EPA)	30.60 ppm
Benzene	0.005 ppm	(MCL - EPA)	5.00 ppm
Cadmium	0.005 ppm	(MCL - EPA)	0.85 ppm
Carbon tetrachloride	0.005 ppm	(MCL - EPA)	0.54 ppm
Chlorobenzene	0.100 ppm	(MCL - EPA)	0.10 ppm
Chloroform	0.080 ppm	(MCL - EPA)	1.46 ppm
Chromium	0.100 ppm	(MCL - EPA)	0.69 ppm
DEHP	0.006 ppm	(MCL - EPA)	0.13 ppm
1,1-Dichlorethane	0.005 ppm	(MCL - California EPA)	0.31 ppm
1,2-Dichlorethane	0.005 ppm	(MCL - EPA)	6.33 ppm
1,1-Dichlorethylene	0.007 ppm	(MCL - EPA)	0.24 ppm
1,2-trans-Dichlorethylene	0.1 ppm	(MCL - EPA)	0.73 ppm
Ethyl benzene	0.7 ppm	(MCL - EPA)	0.65 ppm
Lead	15 ppb	(Action level MCL - EPA)	37.00 ppm
Mercury	0.002 ppm	(MCL - EPA)	0.34 ppm
Methylene chloride	0.005 ppm	(MCL - EPA)	11.20 ppm
Nickel	0.1 ppm	(CHA - EPA)	0.50 ppm
Phenol	2 ppm	(CHA - EPA)	3.27 ppm
Tetrachloroethylene	0.005 ppm	(MCL - EPA)	9.68 ppm
Toluene	1 ppm	(MCL - EPA)	5.18 ppm
1,1,1-Trichloroethane	0.2 ppm	(MCL - EPA)	1.25 ppm
Trichloroethylene	0.005 ppm	(MCL - EPA)	3.82 ppm
Xylenes	10 ppm	(MCL - EPA)	4.07 ppm

MCL: Maximum contaminant level; CHA: Chronic health advisory

Sources for drinking water limits:

US EPA: http://water.epa.gov/action/advisories/drinking/upload/dwstandards2012.pdf

California EPA: http://www.cdph.ca.gov/certlic/drinkingwater/Documents/DWdocuments/EPAandCDPH-11-28-2008.pdf

IRIS: http://cfpub.epa.gov/ncea/iris/index.cfm?fuseaction=iris.showSubstanceList

Table 7: Comparison of health-based drinking water standards with concentrations used in NTP mixture studies for pesticide/fertiliser mixtures (Yang, 1992)

Component	Health-based value (source)		Concentration in tested mixture
Aldicarb	3 ppb	(MCL - EPA)	9 ppb
Atrazine	3 ppb	(MCL - EPA)	0.5 ppb
DBCP	0.2 ppb	(MCL - EPA)	0.01 ppb
1,2-Dichlorpropane	5 ppb	(MCL - EPA)	4.5 ppb
Ethylene dibromide	0.05 ppb	(MCL - EPA)	0.9 ppb
Ammonium nitrate	10000 ppb	(nitrate MCL - EPA)	10000 ppb
Simazine	4 ppb	(MCL - EPA)	0.3 ppb
Alachlor	2 ppb	(MCL - EPA)	0.9 ppb
Cyanazine	1 ppb	(CHA - EPA)	0.4 ppb
Metolachlor	700 ppb	(CHA - EPA)	0.4 ppb
Metribuzin	70 ppb	(CHA - EPA)	0.6 ppb

MCL: Maximum contaminant level; CHA: Chronic health advisory
Source for US EPA drinking water limits:
US EPA: http://water.epa.gov/action/advisories/drinking/upload/dwstandards2012.pdf

Although the health-based standards are from 2012 and the NTP mixture studies were conducted in the early 1990s, it is clear that the pesticide / fertiliser mixtures (Table 7) contain components in the actually administered mixtures mostly at or below their individual allowed concentrations. However, for the mixture representing groundwater contamination near hazardous waste sites, almost all compounds included in this mixture are present in concentrations far above their individual health values (Table 6), which could explain slight effects seen in these animal studies.

The same exercise has been conducted with the mixture studies conducted with contaminated water or using artificial mixtures representing actual river water concentrations. The results are shown in Table 8.

Table 8: Comparison of health-based drinking water standards with concentrations used in studies with contaminated water or using artificial mixtures representing actual river water concentrations

Component	Health-based value (source)		Concentration in tested mixture	Reference
Chloroform	80 ppb	(MCL - EPA)	5.8 ppb (low dose)	Wang et al, 2002
1,1-Dichlorethane	5 ppb	(MCL - California EPA)	5.8 ppb (low dose)	
1,1-Dichloroethylene	7 ppb	(MCL - EPA)	1.2 ppb (low dose)	
1,1,1-Trichloroethane	200 ppb	(MCL - EPA)	2.0 ppb (low dose)	
Trichloroethylene	5 ppb	(MCL - EPA)	44.1 ppb (low dose)	
Tetrachloroethene	5 ppb	(MCL - EPA)	36.0 ppb (low dose)	

***Table 8: Comparison of health-based drinking water standards and concentrations used in studies with contaminated water or using artificial mixtures representing actual river water concentrations** (cont'd)*

Component	Health-based value (source)		Concentration in tested mixture	Reference
Hexachlorocyclopentadiene	50 ppb	(MCL - EPA)	0.015 ppb	Zhao et al, 2011b
Dimethyl phthalate	No data found		0.021 ppb	
2,6-Dinitrotoluene	1 µg/kg bw	(IRIS RfD)	0.226 ppb	
Acenaphtylene	60 µg/kg bw	(IRIS RfD)	0.004 ppb	
2,4-Dinitrotoluene	2 µg/kg bw	(IRIS RfD)	0.02 ppb	
Diethyl phthalate	800 µg/kg bw	(IRIS RfD)	0.027 ppb	
Fluorene	No data found		0.020 ppb	
Phenanthrene	No data found		0.028 ppb	
Anthracene	300 µg/kg bw	(IRIS RfD)	0.029 ppb	
Dibutyl phthalate	100 µg/kg bw	(IRIS RfD)	4.932 ppb	
Pyrene	No data found		0.007 ppb	
Benzyl butyl phthalate	200 µg/kg bw	(IRIS RfD)	0.007 ppb	
Bis(2-ethylhexyl) adipate	400 ppb	(MCL - EPA)	0.733 ppb	
Benzo(a)anthracene	500 µg/kg bw	(IRIS RfD) o, m	0.002 ppb	
Chrysene	5 µg/kg bw	(IRIS RfD) p	0.026 ppb	
Bis(2-ethylhexyl) phthalate	6 ppb	(MCL - EPA)	1.874 ppb	
Benzo[b]fluoranthene	No data found		0.045 ppb	
Benzo[k]fluoranthene	No data found		0.010 ppb	
Benzo[a]pyrene	0.2 ppb		0.005 ppb	
Indeno[1,2,3-cd]pyrene	No data found		0.001 ppb	
Benzo[ghi]perylene	No data found		0.001 ppb	
Arsenic	0.01 ppm	(MCL - EPA)	1.17 ppm	Benjamin, 1999; Constan, 1995, 1996
Benzene	0.005 ppm	(MCL - EPA)	5 ppm	
Chloroform	0.08 ppm	(MCL - EPA)	1.5 ppm	
Chromium	0.1 ppm	(MCL - EPA)	0.14 ppm	
Lead	15 ppb	(Action level MCL - EPA)	2.0 ppm	
Phenol	2 ppm	(CHA - EPA)	3.4 ppm	
Trichloroethylene	0.005 ppm	(MCL - EPA)	3.8 ppm	
Arsenic	0.01 ppm	(MCL - EPA)	2.14 ppm	Jadhav et al, 2007a,b
Cadmium	0.005 ppm	(MCL - EPA)	0.61 ppm	
Lead	0.015 ppm	(Action level MCL- EPA)	24.30 ppm	
Mercury	0.002 ppm	(MCL - EPA)	1.00 ppm	
Chromium	0.1 ppm	(MCL - EPA)	0.95 ppm	
Nickel	0.1 ppm	(CHA - EPA)	0.85 ppm	
Manganese	0.3 ppm	(CHA - EPA)	2.72 ppm	
Iron	0.3 ppm	(SDWR - EPA)	11.11 ppm	

MCL: Maximum contaminant level; RfD: Reference dose; SDWR: Secondary Drinking Water Regulation

Sources for drinking water limits:

US EPA: http://water.epa.gov/action/advisories/drinking/upload/dwstandards2012.pdf

California EPA: http://www.cdph.ca.gov/certlic/drinkingwater/Documents/DWdocuments/EPAandCDPH-11-28-2008.pdf

IRIS: http://cfpub.epa.gov/ncea/iris/index.cfm?fuseaction=iris.showSubstanceList

In the only soil study, toxicity was seen when animals were exposed to soil from the Love Canal waste dump, as expected.

Several studies that were examined involved artificial food-based chemical mixtures. No effects were seen at the 1x dose (representing either typical or maximum allowable human exposure, depending on the study). One study demonstrated adaptive effects (liver enzyme induction) at 2x and 10x whilst no effects at all were seen in any other study at concentrated doses of < 100x. At 100x and higher concentrations (up to 1000x), treatment-related adverse toxicity was increasingly observed. Most of the studies of artificial food-based mixtures involved the testing of 'similar' chemicals. The maximum allowable exposure was typically one hundredth of the NOEL for that chemical, so effects due to single chemicals could be expected to occur at doses of 300x concentrations and higher. The single instance where a mixture effect for dissimilar chemicals was observed was at a 1000x concentration. For similar chemicals, additivity could result in effects at lower concentrations, e.g. 100x, as seen in some of the studies reviewed here. For these reasons, the data reviewed are consistent with that which would be expected on the basis of the independent action of chemicals.

Studies that involved feeding fish from the Great Lakes to mink frequently resulted in severe toxicity. Little was known about the nature of the contaminants present in the early years of this work, and one possible explanation considered was a synergistic interaction between the few contaminants that could be measured. However, further investigation uncovered the full extent of contamination, and found that the additive effect of multiple PCBs could explain the observed toxicity. Mink were also found to be a peculiarly sensitive species with regard to PCBs, especially for reproductive effects. Effects on rodents were far less severe and only occurred at dietary inclusion levels that provided a good safety margin for people consuming fish. The evidence from the Great Lakes fish studies shows that lack of knowledge can result in contamination and subsequent mixture toxicity which might seem to be unexpected. But, the toxicity observed can be explained by the additive effects of similar chemicals, specifically PCBs and dioxins.

4. STUDIES WITH MIXTURES ONLY TESTED AT OR NEAR NO(A)EL OF SINGLE COMPONENTS

Despite many studies being described as investigations performed at low doses, closer inspection of the data revealed that the term 'low dose' was often used to describe dose levels (or concentrations) around the NO(A)EL. Clearly, such studies did not examine the effects of dose levels close to expected human exposures (i.e. at least 10-fold below NO(A)EL) such as the studies described in Chapter 5, nor did they investigate environmentally relevant exposures such as those discussed in Chapter 3. However, these so-called low dose investigations represented a significant proportion of the available and reviewed 'low dose' literature. Moreover, a number of these studies have been cited in the recent expert reviews and opinions (Boobis et al, 2011; Kortenkamp et al, 2009; Kortenkamp et al, 2012; SCHER, SCCS, SCENIHR, 2012) as being of critical importance for driving conclusions concerning mixture toxicity. Consequently, this chapter gives an overview of those studies (*in vitro* and *in vivo*) that tested mixtures in which all the individual components were present at or around their NO(A)ELs. Studies were excluded if any individual component exceeded their NO(A)EL in the mixture.

With respect to this chapter it is of particular importance to note that a NO(A)EL does not equate to a no effect level, especially as the variability in a given endpoint can mean that a NO(A)EL may in fact represent a response of up to 20% over controls (Kortenkamp et al, 2009). Subsequently, toxicological responses could be expected in the mixture studies reviewed in this section, in the apparent absence of effects for the individual components, particularly if each component has a similar mode of action or exerts its toxicity on the same tissue. Unfortunately, several studies investigated only a single dose level for both the individual components and for the mixtures or only limited data were provided (Kacham et al, 2006; Sagai and Ichinose, 1991; Mayura et al, 1984; Soto et al, 1994; Wade et al, 1997; Gaido et al 1997a; Birkhøj et al; 2004; Takayama et al, 1989). Consequently, for such studies no conclusions could be drawn about whether the mixture effects were as expected, or whether the effects exceeded expectations. Furthermore, several studies reviewed in this chapter claimed synergy (e.g. Ito et al, 1991; Casey and co-workers[2]). However, in some cases this finding could not be substantiated by the Task Force either because the data were limited or because a definition describing the magnitude of deviation from additivity was not given.

A problem occasionally faced in the studies reviewed was that the dose levels used in the mixtures were based on NO(A)ELs derived from parameters that were not measured in the mixture studies. For example, two papers are included in this chapter, which investigated dieldrin in combination with either endosulfan (Wade et al, 1997) or toxaphene (Gaido et al, 1997a) in the female rat. The doses used in these studies represented approximately 1/10 of their maximum

[2] Casey et al, 2004, 2005; Gennings et al, 2004a; Moser et al, 2005, 2006; Stork et al, 2006.

tolerable doses (MTDs); however the parameters investigated included uterine weight, hormone measurements, progesterone and oestrogen receptors and uterine peroxidase activity for which the NO(A)ELs were not given.

The chapter is divided into sections based on either acute exposure to mixtures or according to the different toxicity parameters investigated (toxicity in specific organs, developmental toxicity, endocrine toxicity, carcinogenicity). Each section is then further subdivided into two subsections. The first subsection discusses those studies where the parameters assessed were affected by the mixture investigated, but where no effects were induced by the individual components when tested individually at the same dose levels as those used in the mixture. The second subsection addresses those studies where no effects were observed for both the mixture and its individual components. In both cases, studies were only included in this chapter if the components of a mixture did not themselves affect the parameters measured when tested individually at the same dose levels used in the mixture.

4.1 Single dose studies

Three *in vivo* studies were considered suitable for inclusion in this section, one examined multiple parameters of kidney toxicity and one investigated hepatotoxicity. The third study evaluated lethality as the endpoint. Despite this endpoint being rather crude, the study was, nonetheless still considered relevant as it was well conducted and non-lethal doses for the individual components were established. One study, describing acute hepatotoxicity after single dosing of carbon tetrachloride and trichloroethylene, was discounted based on the above criteria for inclusion in this chapter as carbon tetrachloride was always administered at effect levels (Steup et al, 1993). Two studies investigating cholinesterase inhibitors considered mortality as an endpoint (Frawley et al, 1957; Kacham et al, 2006). Frawley et al (1957) was excluded as other, more appropriate and more sensitive endpoints such as cholinesterase inhibition were not addressed, and Kacham et al (2006) was discounted because, although chlorpyrifos and parathion did not individually induce lethality, relevant biochemical changes due to treatment with the individual components were observed.

Ten *in vitro* studies examined mainly cytotoxicity as an endpoint; six of them did not record any effect on the parameter investigated when the mixture consisted of components at their individual no observed effect concentrations (NOECs) and four studies described mixture effects but did not allow any conclusion on the type of interaction due to their limited experimental design. In addition, such a small part of the work in Rouimi et al (2012) was relevant that it is not included below (one relevant part showed an effect of a mixture at the single component NOEL, and the other did not).

4.1.1 Studies showing effects induced by mixtures in the absence of effects for their individual components

Jonker and co-workers have published a series of papers determining whether similarly or dissimilarly acting chemicals show evidence for additive, synergistic or antagonistic effects, or act independently (Jonker et al, 1990, 1993a,b; 1996). Most of these studies are not relevant for this chapter but are discussed in Chapter 5; however one study (Jonker at al, 1993a) was considered pertinent for this chapter as the authors focused on mixtures that were based around the minimum nephrotoxic effect level (MNEL) and the no nephrotoxic effect level (NNEL). The authors examined a multitude of nephrotoxicity parameters in rats treated with a single dose of potassium dichromate, mercury dichloride, hexachloro-1:3-butadiene (HCBD) or d-limonene, or a mixture of all four. The NNEL and MNEL of the individual components were first identified in a dose-ranging study and the main study used NNELs that were a factor of 10 (potassium dichromate), 20 (d-limonene), 10 (HCBD), and 4 (mercuric chloride) below the MNELs, which seemed relatively conservative for the first three substances. The NNEL-mixture did not cause any signs of impaired renal function or renal damage, *"suggesting absence of both dose additivity and potentiating interaction at the tested subeffective levels of the individual nephrotoxicants"*. Assessment of hyaline droplet formation was not reproducible across the various experiments. Interpretation of the findings with the MNEL-mixture is difficult, as the dose-response data from the individual range-finding studies in part only covered doses up to the component dose level applied in the mixture experiment. The authors claimed additive, synergistic and antagonistic effects depending on the endpoint examined, but they failed to provide any definition of terms, nor any mathematical interaction-type analysis to support the hypothesis. The incomplete individual substance dose-response data from the range-finding study indicate that some of the parameters measured did not follow simple linear dose relationships, but in part resulted in seven-fold higher response when the dose was only doubled (see mercuric chloride data for lactate dehydrogenase [LDH]). For the dose range around the NNEL, which is presumed to be close to or equivalent to the NOEL for nephrotoxicity, the study confirmed the absence of unexpected mixture effects.

Single dose lethality after i.p. administration to mice was investigated by Sansing et al (1976) for citrinin, ochratoxin A and penicillic acid and their binary mixtures. Either two or three dose levels for each mixture were at individual substance effect levels, meaning only one or two were at NOELs. Of the 18 groups dosed at or below the NOELs for each substance, 16 resulted in no lethalities, whilst two resulted in a single mortality each: 20 mg/kg citrinin + 3.8 mg/kg ochratoxin A (0.8x NOEL + 0.62x NOEL); 20 mg/kg citrinin + 0.6 mg/kg ochratoxin A (0.8x NOEL + 0.1x NOEL). Taking into account the crude nature of lethality as an endpoint, and the implicitly high doses and interindividual variation, these singular findings are unlikely to represent any unexpected interaction.

Similar conclusions can be drawn from the study by Kacham et al (2006), which examined lethality in neonate rats after p.o. administration of chlorpyrifos and parathion. Co-administration resulted in significant mortality although substances were administered at their assumed individual LD1. The type of interaction remains unknown, as no dose responses were established.

Stacey (1987) investigated *in vitro* rat hepatocyte viability (potassium loss, AST release, lactate / pyruvate ratio) after exposure to cadmium chloride, chloroform and their binary mixtures for different durations. A mixture effect when combining NOECs of individual substances was observed for one endpoint and at a single time point (one-hour potassium levels). Due to a lack of dose-response and interaction modelling, the type of interaction is unknown. The biological significance of this singular finding in a whole set of *in vitro* experiments is also unclear. The same author (Stacey, 1989) examined *in vitro* rat hepatocyte viability (potassium loss, AST and LDH release) for trichloroethylene, tetrachloroethylene, 1,1,1-trichloroethane and their binary and ternary mixtures. Two dose levels were used, a subthreshold dose and a lower dose. No effects were seen in mixtures containing only the lower doses, whilst almost all mixtures containing at least one of the higher doses produced effects. The type of interaction is unknown, but certainly does not suggest synergism. In the same paper dosing the same chemicals *in vivo* at a single subthreshold dose level resulted in effects for all mixtures – the nature of the interaction cannot be defined.

Cell viability via the MTT assay in the rat dopaminergic cell line 1RB3AN27 after exposure to paraquat and $FeCl_2$ was examined by Peng et al (2007). The authors claimed findings of 'synergy', as the combination of $FeCl_2$ with paraquat decreased cell viability compared to paraquat alone. The data are not interpretable as presented in the publication, as no dose response was established for $FeCl_2$, and control data without $FeCl_2$ and paraquat are not shown. Assuming that concurrent control data were employed adequately in the analyses, it would still not be possible to come to a conclusion on the type of interaction due to the lack of dose-response data and interaction modelling.

Tagliaferri et al (2010) reported data on cell viability via the MTT assay in the human neuroblastoma SK-N-MC cell line exposed to BDE-47 and BDE-99. Most of the data is not relevant for this review due to effect concentrations applied and lack of dose-response data. The MTT work was carefully designed and shows some interesting results. It is difficult to judge how the statistical analyses were performed in detail, especially how variation was taken into account. Even if those analyses and the findings of more than additive effects at dose levels around the BDE-47 NOEC and less than additive effects at higher doses were robust, those departures from additivity are marginal and would most probably not be of biological significance. The relevance of a decreased cell viability in an *in vitro* assay of e.g. around 60% versus predicted 70% is questionable.

4.1.2 Studies showing an absence of effects for both mixtures and their individual components

Three publications by Ceccatelli and co-workers (Vettori et al, 2006; Johansson et al, 2006; Tofighi et al, 2011) investigating the effects on cell viability following *in vitro* exposure of the cells to a binary combination of PCBs (PCB 126 or PBC 153) with methyl mercury were considered relevant for this chapter. Specifically, Vettori et al (2006) showed that there were no effects on cell viability (MTT) of PC12 cells when exposed to a combination of methyl mercury and PCB 153 at their individual NOECs. Similarly, binary combinations of either PCB 126 or PCB 153 with methyl mercury had no effect on the viability of AtT20 pituitary cells (Johansson et al, 2006) or hippocampal neurons (Tofighi et al, 2011) when each component was present at its NOEC. Testing at higher, moderately toxic concentrations led to dose additivity / slight synergism or antagonism being observed.

A restricted number of concentrations and combinations of methyl mercury, PCBs and PCDDs/PCDFs were tested *in vitro* in rat splenocytes and leukocytes for cell viability and other parameters by Omara et al (1997, 1998). Neither the mixture experiments employing NOECs of the components, nor those using effect concentrations of methyl mercury and NOECs of the other components, detected any influence of the mixture.

In addition to the studies described in Table 9, a small part of the study of Marinovich et al (1996) can be considered relevant.

Table 9: Summary of acute exposure studies investigating mixtures at or around the individual component NO(A)ELs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Jonker et al, 1993a				
In vivo: nephrotoxicity in rats	Quaternary mixtures: Potassium dichromate, Mercury dichloride, Hexachloro-1:3-butadiene, d-Limonene	Two quaternary mixtures were tested as determined by a range-finding study, representing the 'minimum-nephrotoxic effect-level' (MNEL) and the 'no-nephrotoxic-effect-level' (NNEL) of the individual components	Mixture and individual compounds at the NNEL did not induce effects. Individual components and mixture at MNEL except d-Limonene induced tubular necrosis and changes in clinical chemistry and urine analysis	No effects at NNEL, neither by individual compounds nor by quaternary mixture. At the MNEL, effects by the mixture in part lower or higher than of individual components, but due to lack of dose-response data, interactions cannot be evaluated

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Sansing et al, 1976				
In vivo: Lethality in mice after i.p. injection	Binary mixtures of Citrinin, Ochratoxin A and Penicillic acid	4 dose levels of each of 4 different ratios of each pair of mycotoxins were tested at levels below and above the LD_{50}s of the individual components	Of the 18 groups dosed at or below the NOELs for the individual components, 16 resulted in no lethalities, whilst two resulted in 1/6 mortalities each	No effects, although the study is limited by the crude endpoint of lethality and the associated high doses. Mixture results in the NOEL range do not point towards unexpected effects
Reference: Stacey, 1987				
In vitro: Rat hepatocyte viability (potassium loss, AST release, lactate/pyruvate ratio)	Binary mixtures of Cadmium chloride and Chloroform	All combinations of 25, 50 and 100 µM $CdCl_2$ and 15, 30 and 60 mM Chloroform were tested. Non-relevant aspects: AST release and the lactate/pyruvate ratio were only affected at chloroform effect doses (and were not influenced by adding Cd)	Potassium levels were significantly decreased 1 hour after exposure to 25 µM $CdCl_2$ (individual NOEC) in combination with 15 and 30 mM Chloroform (individual NOECs)	No clear conclusions can be reached. No effect by mixture at individual substances NOECs. Mixture effect at NOECs of individual substances observed for one endpoint and time point. Due to a lack of dose-response and interaction modelling, the type of interaction is unknown. The biological significance of this singular finding in a whole set of *in vitro* experiments is also unclear
Reference: Stacey, 1989				
In vitro: Rat hepatocyte viability (potassium loss, AST and LDH release)	Binary and tertiary mixtures of Trichloroethylene, Tetrachloroethylene, 1,1,1-Trichloroethane	Exact doses used are not meaningful as the cultures were exposed via the vapour phase, but a subthreshold dose of each chemical and a lower dose (approx. half) were used in all combinations	Similar effects were seen with all three endpoints. For the two and three component mixtures, none containing only the lower doses produced effects, whilst almost all containing at least one higher dose did produce an effect.	The type of interaction is unknown due to the lack of more single chemical dose-response data, but certainly does not suggest synergism
In vivo: Rat single dose hepatotoxicity study (ALT, SDH sorbitol dehydrogenase, urea)	Binary and tertiary mixtures of Trichloroethylene, Tetrachloroethylene, 1,1,1-Trichloroethane	Trichloroethylene: 10 mmol/kg bw Tetrachloroethylene: 15 mmol/kg bw 1,1,1-Trichloroethane: 15 mmol/kg bw	Increase in all endpoints for all binary and tertiary mixtures	Single sub-threshold dose only was used, so no conclusion about the type of combination effect can be drawn
Reference: Peng et al, 2007				
In vitro: Rat dopaminergic cell line 1RB3AN27 cell viability (MTT assay)	Binary mixtures of Paraquat and $FeCl_2$	100, 200, 300 and 400 µM Paraquat were tested with or without one concentration of $FeCl_2$ (80 µM). Control data not shown, but according to text, $FeCl_2$ and 100 and 200 µM Paraquat were statistically not different from controls (for $p<0.01$; $p<0.05$ not given)	At all 4 Paraquat dose levels, addition of $FeCl_2$ led to a decrease in cell viability compared to Paraquat alone. No dose response established for $FeCl_2$, so that type of interaction, if any, is unknown	No clear conclusions can be reached. The claim of synergy is not backed by data. Dose response for $FeCl_2$ not examined or presented, not known if the $FeCl_2$ concentration applied was really a NOEC in this experiment. Even if it were, the interaction type could have been anything from less-than additive to more-than additive

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Tagliaferri et al, 2010				
In vitro: Human neuroblastoma SK-N-MC cell line cell viability (MTT assay)	Binary mixture: BDE-47 and BDE-99 (which are themselves congener mixtures)	BDE-99 tested at NOEC (5 µM) and above, BDE-47 at the NOEC and 2.5-fold below (2.5 and 1 µM)	At and around the NOECs, cell viability by the mixtures was reported to be lower than predicted based on Loewe addition or Bliss independence models	More than additive effect reported, but of low magnitude and questionable biological relevance
Reference: Vettori et al, 2006				
In vitro: PC12 cell viability (MTT) and TBARS and dopamine levels	Binary mixture: Methyl mercury and PCB 153	For cell viability, a multitude of different concentration combinations were tested throughout the dose-response curve	No effect by mixture at individual substances NOECs. Some data points of mixtures at individual substance effect concentrations showed antagonism, mostly dose additivity	No effect by mixture at individual substances' NOECs
Reference: Johansson et al, 2006				
In vitro: AtT20 pituitary cells (apoptosis and necrosis)	Binary mixture: Methyl mercury with PCB 153 or PCB 126	Dose responses established for individual components for % cell death. Combinations tested only at two concentrations (NOEC and 2 x NOEC – described as being moderately cytotoxic)	No effect by mixture at individual substances' NOECs. Mixtures at 2 x NOEC for individual substances: slight synergy (Methyl mercury/ PCB 126) or additivity (Methyl mercury/PCB 153) were observed	No effect by mixture at individual substances' NOECs
Reference: Tofighi et al, 2011				
In vitro: Hippocampal neurons (apoptosis and necrosis)	Binary mixture: Methyl mercury with PCB 153 or PCB 126	PCBs tested at three concentrations and Methyl mercury tested at four concentrations across different experiments for % cell death. Combinations tested only at two concentrations (NOEC and 2 x NOEC – described as being subcytotoxic)	No effect by mixture at individual substances' NOECs. Mixtures at 2 x NOEC for individual substances: an antagonistic interaction was observed for necrotic cell death and synergy was recorded for apoptosis (Methyl mercury/ PCB 153 combination only)	No effect by mixture at individual substances' NOECs
Reference: Omara at al, 1997				
In vitro: Rat splenocyte viability	Ternary mixtures of Methyl mercury, PCBs and PCDD/PCDFs	The 4 relevant mixtures tested all contained 1x the NOEL of Methyl mercury, and 0.07 or 1x the NOEL of the PCDD/PCDF mixture, and 0.02x or 1x the NOEL of the PCB mixture. Other endpoints and concentrations investigated in the study are not relevant here, as they only involved effect concentrations	Cell viability was decreased at Methyl mercury effect concentrations only, with no influence of the other mixture components	No effect by mixture at individual substances' NOECs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Omara et al, 1998				
In vitro: Rat splenocyte viability and immunological assays	Ternary mixtures of Methyl mercury, PCBs and PCDD/PCDFs	The 4 relevant mixtures tested all contained 1x the NOEL of Methyl mercury, and 0.07 or 1x the NOEL of the PCDD/PCDF mixture, and 0.02x or 1x the NOEL of the PCB mixture. Other endpoints and concentrations investigated in the study are not relevant here, as they only involved effect concentrations	Cell viability was decreased at Methyl mercury effect concentrations only, with no influence of the other mixture components. Immunological assays showed no effect in any treatment group	No effect by mixture at individual substances' NOECs

4.2 Studies on organ toxicity

This section is concerned specifically with organ toxicity generally resulting from subacute exposure to mixtures. Mixture studies concerning carcinogenicity / preneoplastic lesion formation, endocrine toxicity, developmental toxicity and acute toxicity are discussed in separate sections.

Seventeen *in vivo* studies were considered suitable for inclusion in this section, which addressed a range of different organ toxicities including thyroid, liver and lung.

4.2.1 Studies showing effects induced by mixtures in the absence of effects for their individual components

Of the seventeen studies included in Section 4.2, fourteen of them described mixture effects. Several of these studies indicated a deviation from the expected responses. For example, Crofton et al (2005) recorded a greater than additivity effect on thyroid hormones when testing various dilutions of a complex mixture of 18 PAHs in the rat. In this study, the concentrations of the individual components in the stock mixture were below those found to have significant biological activity. Only dilutions (33, 22, 11, 3.3, 1.1 and 0.33%) of the stock mixture were tested and effects, which were greater than expected, were recorded for the 11, 22 and 33% dilutions.

Van Birgelen et al (1996) tested binary mixtures of TCDD and various PCBs, demonstrating a significant increase in porphyrin accumulation when testing mixtures of TCDD/PCB 153 in the absence of effects for PCB 153 on its own.

The papers by Casey et al (2004, 2005), Gennings et al (2004a), Moser et al (2005, 2006) and Stork et al (2006) describe investigations of neurochemical (blood and brain ChE) and neurobehavioural (motor activity, gait score, tail pinch response) effects in adult and preweanling rats who were dosed with a mixture of 5 (acephate, diazinon, chlorpyrifos, dimethoate, malathion = full ray) or 4 (same except malathion = reduced ray) organophosphates which were administered over a range of doses in a fixed proportion according to their estimated presence in diet. Deviation from additivity was calculated based on mathematical predictions of response from individual dosing studies. Synergy was seen for most parameters with effects more pronounced at lower dose levels. However, the deviation from additivity was small (mostly within a factor of 2). Removal of malathion had variable effects depending on the parameter studied.

Seven additional studies showed mixture effects (Wade et al, 2002a; Sagai and Ichinose, 1991; Institóris et al, 2006; Merhi et al, 2010; Groten et al, 1997; Gilbert et al, 2011; Chaturvedi, 1993, only a small fraction of the latter is relevant, hence it is not tabulated). However, no conclusion could be drawn about whether the effects could be predicted by CA or IA models or even if they deviated from the models as dose responses for the individual compounds were not conducted. In the studies of Sagai and Ichinose (1991) and Institóris et al (2006), the components were only tested at their NOELs.

4.2.2 Studies showing an absence of effects for both mixtures and their individual components

Three studies in this section described an absence of effects when the mixture consisted of compounds at their individual NOELs (Akay et al, 1999; Wade et al, 2002b; McLanahan et al, 2007). In the study of Akay et al (1999), binary and tertiary mixtures of endosulfan, carbaryl and dimethoate were evaluated for their effects on the rat immune system following treatment for 3.5 months. Each compound was present in the mixture at 100x or 1000x its ADI, where 100x ADI was described as the NOEL. At 100x ADI the mixtures produced no apparent treatment-related effects. Additional studies have been conducted by this group but these were not considered relevant for this chapter as the dose levels investigated were well below the NOEL. Consequently, these studies are further discussed in Chapter 5.

In Wade et al (2002b) sexually mature male rats were exposed for 70 days via oral gavage to a mixture of ubiquitous persistent organochlorines as well as metals (lead and cadmium) and effects on liver, kidney, immunological and reproductive systems were evaluated. Chemicals were included in the 1x mixture at their MRL or TDI (as determined by the US EPA or ATSDR) or, in the case of TCDD at the NOEL. Mixtures of 10x, 100x and 1000x were also tested. At the 1x mixture dose (which is of relevance to this chapter) an absence of effects in the systems examined indicates that toxicity is not enhanced by co-exposure.

McLanahan et al (2007) investigated sequential co-exposure to PCB 126 and perchlorate on thyroid axis parameters, and did not detect any mixture effects at individual NOELs.

In addition to the tabulated studies, a limited dietary rat study of Frawley et al (1957) can also be considered relevant.

Table 10: Summary of organ toxicity studies investigating mixtures at or around the individual component NO(A)ELs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Crofton et al, 2005				
In vivo: Thyroid hormones in the rat following 4-day treatment	Complex mixture of all 18 polyhalogenated aromatic hydrocarbons (PHAHs). The concentrations of each component in the stock mixture are given below: TCDD 0.013µg/ml PCDD 0.013µg/ml TCDF 0.019 µg/ml 1-PCDF 0.006 µg/ml 4-PCDF 0.02 µg/ml OCDF 0.065 µg/ml PCB 28 78.600 µg/ml PCB 52 155.200 µg/ml PCB 77 2.000 µg/ml PCB 101 153.800 µg/ml PCB 105 76.700 µg/ml PCB 118 381.100 µg/ml PCB 126 0.610 µg/ml PCB 138 380.900 µg/ml PCB 153 382.200 µg/ml PCB 156 13.100 µg/ml PCB 169 0.400 µg/ml PCB 180 377.900 µg/ml	Mixture ratio based on ratios of PHAHs found in breast milk, fish and other sources of human exposure. Concentrations of the individual compounds in the undiluted mixture were below those found to have significant biological activity. The following dilutions of the stock mixture were tested: 33, 22, 11, 3.3, 1.1 and 0.33%	No deviation from additivity at the lowest dose, but greater-than-additive effect at the 3 highest mixture doses (33, 22, 11% stock)	No effects. Deviation from expectation (up to 2.5-fold) only at higher mixture concentrations
Reference: van Birgelen et al, 1996				
In vivo: Liver (hepatic porphyrin levels, CYP P450 measurements, CYP 1A2 activity) following 13	Binary mixtures: TCDD + PCB 153 TCDD + PCB 156 TCDD + PCB 126	TCCD was near NO(A)EL in most mixtures. PCB 156 and PCB 126 were presented in the low-dose mixture near their respective NO(A)ELs. PCB 153	TCDD/PCB 153 Porphyrin levels were significantly accumulated (> additive action) for all mixtures in absence of accumulation by	Deviation from expectation for TCDD/PCB 153 mixture

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
weeks dietary treatment. Only the porphyrin accumulation data were relevant for the Task Force		did not induce porphyrin accumulation. TCDD/PCB 153 (/kg): 33.9 ng/0.68 mg 32.6 ng/1.95mg 32.0 ng/6.40 mg 318 ng/0.64 mg 301 ng/1.81 mg 293 ng/5.85 mg TCDD/PCB 126 (/kg) 27 ng/0.48 µg 26 ng/3.25 µg 23 ng/10.4 µg 315 ng/0.44 µg 306 ng/3.06 µg 126 ng/9.68 µg TCDD/PCB 156 (/kg) 317 ng/76 µg 305 ng/366 µg 290 ng/696 µg	PCB 153 alone. TCDD/PCB 126 TCDD/PCB 156 Co-administration of these PCBs with TCDD yielded no further hepatic porphyrin accumulation compared to the highest single dose of PCB or TCDD congeners	
References: Casey et al, 2004, 2005; Gennings et al, 2004a; Moser et al, 2005, 2006; Stork et al, 2006				
In vivo: Adult and preweanling Long-Evans rats. Neurochemical: Blood and brain ChE. Neurobehavioural: Motor activity, gait score, tail pinch	(1) 5-component mixture of OP pesticides (2) 4-component mixture of OP pesticides (no Malathion)	Ratios: Acephate - 0.04; Diazinon - 0.002; Chlorpyrifos - 0.031; Dimethoate - 0.102; Malathion - 0.825. Based on relative human exposure estimates in US diet. Acephate - 0.2286; Diazinon - 0.0114; Chlorpyrifos - 0.1767; Dimethoate - 0.5833 Adults: Mixtures tested at 10; 55; 100; 200; 300; 450 mg/kg for full ray; and 1.75; 9.6; 17.5; 35; 52.5; 78.8 for reduced ray. Pups: Mixtures tested at 10; 29; 40; 60; 100; 165 mg/kg for full ray; and 1.75; 3.5; 7.0; 10.5; 17.5; 28.9 for reduced ray	Adults: Some components of the mixture were present at effect levels, others below effect levels for all parameters. Results showed significant deviation from additivity (= synergism) for all parameters except for tail-pinch. The effect was greater at lower dose levels, but the ratio of predicted: actual values was not higher than 2.1 (brain ChE). Pups: Results were generally similar to those seen in adults but ratios at lower and higher doses were similar (1.3 – 3.0). The largest deviation from additivity was seen for tail-pinch (ratio 3.5 for full ray); this disappeared when Malathion was removed	Synergy was seen for most parameters with effects more pronounced at lower dose levels. However, the deviation from additivity was small (mostly within a factor of 2). Removal of Malathion had variable effects depending on the parameter studied
Reference: Wade et al, 2002a				
In vivo: Thyroid parameters (hormones, histopathology and liver enzyme induction) in the rat	Complex mixture: Aldrin, p-p'-DDT, p-p'-DDE, DIELD, ENDO, Hepatochlor, HCB, HCH, Mirex, MCX, 1,2,3-Trichloro-benzene, 1,2,4-Tri-	Individual compounds were present in mixture at concentrations that represented 1x, 10x, 100x, 1000x minimal risk levels or tolerable daily intakes (MRL/TDI/RfD/NOEL)	Adverse thyroid effects observed only at the high dose (1000x) mixture, where frank toxicity occurred. Thyroid hormones were affected and UDPGT enzyme induction was observed	No conclusion can be derived concerning additivity etc. as dose responses for the individual chemicals were not conducted

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
	chlorobenzene, 1,2,3,4-Tetrachlorobenzene, Pentachlorobenzene, TCDD, PCB (as Arochlor 1254), $CdCl_2$; $PbCl_2$	depending on compound	from the 100x dose	
Reference: Sagai and Ichinose, 1991				
In vivo: Studies on lung effects in male rats following a 22-month inhalation study. Relevant parameter was changed in lipid peroxidation by measuring lung thiobarbituric acid (TBA) reactant	Binary mixtures: Ozone Nitrogen dioxide (NO_2)	Binary combinations tested each component at its NOEL: Ozone/NO_2 (ppm): 0.05 / 0.04	TBA reactant increased following treatment	No conclusion can be derived concerning additivity etc
Reference: Institóris et al, 2006				
In vivo: Studies on general toxicity; haematological and immune system function in rat following 4- and 12-week dosing periods	Binary and tertiary mixtures: Pb-acetate + $HgCl_2$; Pb-acetate + $CdCl_2$; $HgCl_2$ + $CdCl_2$ Pb-acetate + $HgCl_2$ + $CdCl_2$	NOELs following 4-week exposures established in separate studies. Mixtures based on combination of NOELs: Pb-Acetate: 20 mg/kg $HgCl_2$: 0.4 mg/kg $CdCl_2$: 1.61 mg/kg	Binary mixtures: Significant changes to organ weights in all three mixtures; Pb+Cd resulted in red and white blood cell count and haematocrit. Tertiary mixture: No effects on immune function at either time-point. Significant increase in lung weight; decrease in PLN at 4 weeks' treatment	No conclusion can be derived concerning additivity, etc
Reference: Merhi et al, 2010				
In vivo: Studies on gender-linked haematopoiesis and metabolic disturbances in mice dosed for 4 weeks	Six-component mixture: Alachlor, Captan, Diazinon, Endosulfan, Mancozeb, Maneb	Individual components were not tested in this study, instead authors relied on ADIs established by Joint FAO/WHO Meeting on Pesticide Residues (2005) extrapolated to mice on basis of body weight. The mixture was based on these values	Mixture resulted in significant effects: Females - spleen weight increased; males - liver weight decreased. Variations in hepatic metabolism mostly in males indicative of neoglucogenesis. Significant (non-pathological) changes to platelet count (30% increase, males); 8% decrease in red blood cell count; haemoglobin count and haematocrit (males); 1.4-fold increase in white blood cells (females)	No conclusion can be derived concerning additivity, etc

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Groten et al, 1997				
In vivo: haematology, clinical chemistry, biochemistry, pathology after combined oral and inhalation subacute exposure of male rats	9-substance mixture: Aspirin, Cadmium chloride, Stannous chloride, Loperamide, Spermine, BHA, DEHP, Dichloromethane, Formaldehyde. 16 other mixtures with each 5 of the above substances tested is not relevant, as only tested at effect levels	Individual compounds were present in mixture at concentrations that represented the MOAEL, NOAEL or 1/3 of NOAEL	Very few effects with mixture composed of individual substances at their NOAELs, no treatment-related effects at 1/3 of NOAELs apart from a non-specific slight increase in relative kidney weight.	No deviation from the expectation that combined exposure to partly similarly acting substances at their NOAELs can cause effects. Due to experimental design, it is not possible to conclude on the type of interactions or non-interactions
Reference: Gilbert et al, 2011				
In vivo: 8-week mouse liver and immunotoxicity study with female autoimmune prone mice	Binary mixture: Trichloroethylene (TCE) and Mercuric chloride	TCE was dosed at 9.9 or 187 mg/kg/day in drinking water; mercuric chloride was dosed s.c. twice a week equivalent to 0.26 mg/kg/day	Significant reduction in water consumption for mercury alone, the higher TCE dose and for both mixture groups. All doses of single chemicals produced effects on one or more of the immune-related endpoints. They also seemed to have an increased liver histopathology score, but it was not stat sig. Significant increase in liver histopathology for both mixture groups, though the increase was only slightly higher than for single chemicals	Without stat sig histopathology, the other effects are not adverse, so a NOAEL was achieved for single chemicals, whilst the mixtures produced effects. Cannot be interpreted re nature of combination effect
Reference: Akay et al, 1999				
In vivo: Studies on the immune system (IgG and IgM and haematology) in the rat following treatment for 3.5 months	Binary and tertiary mixtures: ENDO + CARB ENDO + Dimethoate CARB + Dimethoate ENDO + CARB + Dimethoate	Each component tested at 100x and 1000x ADI both individually and in the mixture, where 100x ADI = NOEL	Single chemical data indicated no effects at the NOEL (i.e. 100 x ADI). Similarly the 100x ADI mixture produced no effects that appear to be treatment related.	No effect
Reference: Wade et al, 2002b				
In vivo: Studies on subchronic effects on hepatotoxicity, renal effects, immunotoxicity and reproductive toxicity	Complex mixture: Aldrin p-p'-DDT, p-p'-DDE DIELD, ENDO Hepatochlor HCB, HCH Mirex, MCX 1,2,3-Trichlorobenzene 1,2,4-Trichlorobenzene 1,2,3,4-Tetrachlorobenzene Pentachlorobenzene	Individual compounds were present in mixture at concentrations that represented 1x, 10x, 100x, 1000x minimal risk levels or tolerable daily intakes (MRL/TDI/RfD/NOEL) depending on compound	No effects with 1x mixture. Effects on liver and reproductive parameters at >100x. Renal and immunotoxic effects at the high dose	No effect at mixture level where each component was present at or below individual NOEL

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
	TCDD PCB (as Arochlor 1254) $CdCl_2$; $PbCl_2$			
Reference: McLanahan et al, 2007				
In vivo: Thyroid function endpoints in SD rats	(only dosing study II relevant:) Subsequent exposure to PCB 126 and perchlorate	Single gavage of 0, 0.075, 0.75, 7.5 µg/kg PCB 126, one day later 0.01mg/kg bw perchlorate per drinking water for one to four days	Lowest PCB 126 dose and the perchlorate individual doses were NOELs. No significant differences from control for co-exposed animals were detected	Near the NOEL for PCB 126 and ClO_4, no interactions between the chemicals occur

4.3 Studies on developmental toxicity

Eight studies are included in this section in which the effects of various chemical combinations on developmental parameters were investigated in the rat (Narotsky et al, 1995; Mayura et al, 1984; Zajac and Abel, 1990; Reinstein et al, 1984) and the mouse (Teramoto et al, 1980; Lee et al, 2006).

As for the other sections in this chapter, changes were observed when investigating the effects of mixture on developmental parameters; however in some cases there remains some doubt as to whether the mixtures consisted of individual compounds at their true NOELs (e.g. Mayura et al, 1984).

4.3.1 Studies showing effects induced by mixtures in the absence of effects for their individual components

In the Narotsky et al (1995) rat developmental study, trichloroethylene (TCE), di(2-ethylhexyl) phthalate (DEHP) and heptachlor (HEPT) were tested in linear regression from single substances doses inducing 2 g weight loss during GD6-8. NOELs were achieved for DEHP, HEPT, but not for TCE. The authors claimed synergistic effects for maternal mortality in DEHP/HEPT. HEPT potentiated the effect of TCE and DEHP for full litter resorption and prenatal loss. Response- and dose-additivity effects were also recorded; however only two of the three compounds were tested at their respective NOELs. Similarly, the investigation of a combination of ochratoxin A (1 mg/kg, NOEL) and citrinin (30 mg/kg, NOEL) in a rat subcutaneous developmental toxicity study indicated increases in resorptions and pup mortality as well as significant increases in malformations (Mayura et al, 1984). Although the authors state that both compounds were present in the mixture at their respective NOELs, teratogenicity has been reported for both compounds at doses, which were slightly higher than those tested singly (1.75 mg/kg/day for ochratoxin A, Mayura et al [1982]; and 35 mg/kg/day for citrinin, Reddy et al [1982]). The

authors described their choice of dose levels for the combined treatment as being subthreshold teratogenic levels but the proximity of the doses to those that induce frank developmental toxicity brings into question the applicability of the term subthreshold in this case.

Teramoto et al (1980) demonstrated dose additivity when a combination of ethylenethiourea (ETU) and $NaNO_2$ were investigated in a single dose mouse developmental toxicity study via oral gavage on GD6 or 8, 10 or 12. Doses given were 400 mg/kg ETU combined with 50/100/200 mg/kg $NaNO_2$. Here, a special case of chemical reaction between components of a mixture is assumed as dosing of the two components separated in time for 400 mg/kg ETU and 200 mg/kg $NaNO_2$ ($NaNO_2$ dosed two hours after ETU) did not induce toxicity.

A less clear picture comes from an i.p. mouse study where a combination of cadmium chloride and all-trans-retinoic acid (RA) was investigated at doses of: $CdCl_2$ - 0.5, 1, 2, 3, 5 mg/kg and RA - 1, 3, 5, 7.5 mg/kg, with respective NOELs being 0.5 mg/kg $CdCl_2$; 1 mg/kg RA (Lee et al, 2006). The dose response from RA is somewhat unclear, and the paper lacks sufficient dose-response information to fully investigate interactions. Reproductive effects seen were lower mean foetal weight than in controls in all mixtures. RA had no effect on reproductive endpoints at all doses. $CdCl_2$ induced lower foetal weight at 1, 2, 3, 5 mg/kg. Lowest tested doses of individual substances caused no effect on forelimb defects, again noting some uncertainties around the RA dose response, but all mixtures induced defects. In the case of the subthreshold combination (0.5 mg/kg $CdCl_2$ with 1 mg/kg RA) forelimb ectrodactyly occurred in 19% of foetuses opposed to 0% expected on the basis of the simple arithmetic sum of the chemicals' individual responses, which the authors termed synergism. Based on the lack of dose-response information, and the lack of knowledge about the mode of action of both chemicals, it is not possible to exclude the possibility that the combination effects could be predicted by independent action or dose addition. In addition, it appears that mixtures were dosed at double the volume of that administered to animals receiving single substances.

In addition to the studies in Table 11, a small fraction of the study of Chandra et al (1983) can be considered relevant; the relevant mixture resulted in an effect, but the data did not support an interpretation of the nature of the combination effect.

4.3.2 Studies showing an absence of effects for both mixtures and their individual components

The rat developmental toxicity study (GD10-20) of Zajac and Abel (1990) examined combinations of lead acetate and ethanol at doses of 0.2 and 0.67 x NOEL of both compounds, 0.4 and 0.89 x NOEL, 0.6 and 1 x NOEL, respectively. The NOEL of lead acetate was 75 mg/kg and 5 g/kg for ethanol. When compared to the 'lead only' group, no statistically significant effects were seen at the two lowest dose mixtures while the third lowest dose caused reduced

maternal weight gain (adverse). However, when compared to the 'ethanol only' group, none of the three mixtures were statistically different, which suggests that ethanol was driving the observed effects. Similarly, a combination of zinc and copper at or below their respective NOELs (1 x NOEL Zn plus 1/19 x or 1 x NOEL Cu) administered in the diet to pregnant rats from day 0-21 gave no developmental or maternal toxicity (Reinstein et al, 1984).

In addition to the studies in Table 11, a small fraction of the study of Khera and Iverson (1981) can be considered relevant; the relevant mixture produced no toxicity.

Table 11: Summary of developmental toxicity studies investigating mixtures at or around the individual component NO(A)ELs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Narotsky et al, 1995				
Developmental toxicity study in rats, day 6-15, oral gavage	Trichloroethylene (TCE), Di(2-ethylhexyl) phthalate (DEHP), Heptachlor (HEPT)	Linear regression from single substances doses inducing 2 g weight loss on GD6-8; NOEL achieved for DEHP, HEPT, not for TCE	Synergistic developmental toxicity for several endpoints. The DEHP-HEPT, were synergistic for maternal mortality. HEPT potentiated the effect of TCE and DEHP for full litter resorption and prenatal loss	Beside response additivity also examples of dose additivity were found. Very complex study design, adequate dose groups, NOEL only for 2 of 3 chemicals, therefore the final outcome remains unclear
Reference: Mayura et al, 1984				
Developmental study, s.c. in rats, single dose on GD5, 6, 7, 8, 10, 11, 14	Ochratoxin A, Citrinin	Single dose 1 mg/kg Ochratoxin / 30 mg/kg Citrinin, being single components NOEL	Mixture induced increase resorption and reduction in number live foetuses, which were significant if dosed on GD5 and 7 (+ reduced implants on GD7). The mixture caused significant increased malformations at most dosing times, including malformed ribs, hydronephrosis, hydrocephalus and spinal defects	No clear conclusions can be reached. Indications of an additive effect but lack of lower doses is a limitation. The individual components also increased skeletal malformations but did not reach statistical significance. NOELs used in this study were close to clear teratogenic effect levels established by the same group in separate studies
Reference: Teramoto et al, 1980				
Developmental single dose, oral gavage, mice	Ethylenethiourea (ETU); Sodium nitrite ($NaNO_2$)	Dosing on GD6 or 8, 10 or 12 of 400 mg/kg ETU combined with 50/100/200 mg/kg $NaNO_2$. Investigation developmental effects	Increased foetal deaths, reduced foetal weight and abnormalities in group 400 mg/kg ETU and 200 mg/kg $NaNO_2$	Additive action concluded for mixture. This is a special case of chemical reaction between components of a mixture. Proximity of the single component doses to the LOEL is not clear. Authors report earlier experiment with dosing

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
				of 2 components separated in time for 400 mg/kg ETU and 200 mg/kg $NaNO_2$. $NaNO_2$ dosed 2 hours after ETU did not induce toxicity
Reference: Lee et al, 2006				
Postaxial forelimb ectodactyly, i.p. in pregnant mice. GD9.5, collection of foetuses GD18	Cadmium chloride ($CdCl_2$), all-trans-Retinoic acid (RA)	Doses : $CdCl_2$: 0.5, 1, 2, 3, 5 mg/kg; RA: 1, 3, 5, 7.5 mg/kg; NOELs are 0.5 mg/kg $CdCl_2$ and 1 mg/kg RA	Reproductive effects: mean foetal weight lower than controls in all mixtures. RA had no effect on repro endpoints at all doses. $CdCl_2$ induced lower foetal weight at 1, 2, 3, 5 mg/kg. Forelimb defects: lowest tested doses of individual substances caused no effect. RA dose response not clear. All mixtures induced defects. In case of subthreshold combinations, induced significant increases in mean litter % with forelimb defects	No clear conclusion can be drawn. Authors claim synergy, but cannot exclude additivity based on lack of dose-response information. Authors cite other publication demonstrating clear dose response between 12.5 and 75 mg/kg. Mixtures are dosed as double volume of single doses, which was not controlled for
Reference: Zajac and Abel, 1990				
Developmental toxicity study, oral gavage in rats, GD10-20	Lead acetate, Ethanol	Doses: 0.2x and 0.67x NOEL of both compounds; 0.4x and 0.89x NOEL; 0.6x and 1x NOEL. NOEL of Lead acetate = 75 mg/kg and Ethanol = 5 g/kg	No statistically significant effects of the two lowest dose mixtures, third lowest dose caused reduced maternal weight gain when compared to 'lead only' group, i.e. effect driven solely by ethanol	No effect seen with mixtures. Second experiment reported is not relevant
Reference: Reinstein et al, 1984				
Developmental toxicity study in rats, day 0-21, diet	Cu, Zn	1x NOEL Zn plus 1/19x or 1x NOEL Cu	No effects for single compounds and combinations for general and reproductive toxicity	No effects were induced by exposure to mixture. Complex nutritional design, groups receiving diets deficient in Zn or Cu not considered, no proper evaluation of maternal clinical chemical data (haematocrit, haemoglobin, total plasma cholesterol, plasma triglycerides)

4.4 Studies on the endocrine system or models of endocrine activity

Concern that alterations to the endocrine system by chemicals can result in adverse health effects in the human population and wildlife has led to increased scrutiny in both Europe and the USA of endocrine disrupters (EDs). Consequently, endocrine disruption is now considered as a cut-off criterion in the recently revised Plant Protection Products Legislation in Europe (EU, 2009), and in America the mandatory testing of many registered compounds has come into force with the launch of EPA's Endocrine Disrupter Screening Program (US EPA, 1998). The possibility that EDs can cause effects at doses below the NO(A)ELs or at environmentally relevant concentrations and that such compounds could interact at low-dose levels to induce significant effects has been and continues to be a topic of much debate. Consequently, the number of publications addressing low-dose mixture effects of EDs was much greater than for any of the other effects addressed in this report, clearly reflecting the current trends and concerns in toxicology. Surprisingly, however, in contrast to what one would expect given all the discussions about the potential for EDs to interact at low or environmentally relevant doses there were actually relatively few publications (7 *in vitro* and 2 *in vivo*) that were considered acceptable for inclusion into Chapter 5 (studies with mixtures tested well below NO(A)EL of single components). A greater number of studies tested mixtures which contained each of the individual components at or around their NO(A)ELs (6 *in vivo* and 8 *in vitro*), which again reflects the fact that the term 'low dose' is used in the sense of testing combinations around individual NO(A)ELs and has nothing to do with human- or environmentally relevant doses.

A number of the studies highlighted pertinent aspects concerning mixture toxicology and how data can be interpreted. For example, predicting mixture effects when the dose responses are non-monotonic is difficult and neither concentration addition nor independent action can accurately determine the final outcome (Ohlsson et al, 2010b). Furthermore, when investigating potential low-dose interactions it is imperative that the NO(A)EL / NOEC for the individual components of the mixture are adequately described. This is particularly important when investigating multiple parameters as was the case in the *in vivo* study described by Birkhøj et al (2004), where effects were recorded for luteinising hormone (LH) measurements following treatment with one of the mixture components (methiocarb) when tested on its own.

4.4.1 Studies showing effects induced by mixtures in the absence of effects for their individual components

As could be expected when investigating mixtures comprising of components around their NO(A)ELs / NOECs, the majority of the studies in this section demonstrated mixture effects. The mixture data from six of the eight *in vitro* studies could be predicted by concentration addition (CA) and/or independent action (IA); however there were cases where neither of these models could predict the data. For example, in the studies of both Charles et al (2007) and Kunz

and Fent (2006) the observed responses of the complex mixtures were greater than expectations and, in the case of Kunz and Fent, the observations were described as synergistic effects based on the isobole method of Kortenkamp and Altenburger (1998). The inhibition of oestradiol secretion in H295R cells following exposure to a mixture of three phytoestrogens similarly deviated from expectations (Ohlsson et al, 2010a); however in this example this could be due to the low basal levels of oestradiol generally associated with this cell line rather than a true deviation from expectation. A second study by Ohlsson et al (2010b) highlighted a limitation in the CA and IA models for mixtures in that these models assume monotonic dose responses and, without modification, cannot be used to predict non-monotonic effects.

Kjaerstad et al (2010) describe *in vitro* studies on androgen receptor (AR) antagonism using an AR reporter gene assay. Four mixtures were studied: AR antagonists (flutamide, vinclozolin and procymidone); anti-androgens (finasteride, MEHP, prochloraz and vinclozolin); parabens; and azoles (epoxiconazole, propiconazole and tebuconazole). In all cases, a NO(A)EL was determined; however cells were exposed to equimolar mixtures instead of equipotent mixtures. It must be noted that for each mixture, individual NO(A)ELs were similar, so effectively equimolar and equipotent mixtures were also rather similar. Exposure to mixtures of similarly acting AR antagonists, anti-androgens and azoles (three separate mixtures) caused anti-androgenic effects in the AR reporter gene assay, which could be considered additive. Exposure to a mixture of parabens only caused effects at cytotoxic concentrations.

Two well conducted studies (Birkhøj et al, 2004; Payne et al, 2001) looked at downstream transcriptional effects following hormone receptor activation (AR and ER [oestrogen receptor], respectively) and demonstrated mixture effects were dose or response additive. Soto et al (1994) also reported additivity of cell proliferation effects following exposure of MCF-7 breast cancer cells to a mixture of 10 oestrogenic chemicals, tested only at one concentration.

Rider and co-workers (2008, 2009, 2010) investigated the effects on the male rat reproductive tract following *in utero* exposure to several doses of various combinations (complex and binary) of pesticides, dioxins and phthalates (vinclozolin, procymidone, linuron, prochloraz, BBP, DBP, DEHP, DiBP, DiHP, DPP, TCDD). For the two complex mixtures investigated (one of 7 compounds and the other of 10 compounds; see Table 12 for details), changes in the parameters measured were dose dependent and the outcomes could be predicted by the dose addition model. Similarly, a Hershberger type assay investigating a mixture of five pesticides indicated that the weak effects on the weights of the adrenals and the LABC muscle were additive (Birkhøj et al, 2004); however whether the effects could be predicted by CA is difficult to determine as the mixture was tested at only one dose level.

A single *in vivo* study investigating the effects of a binary mixture of TCDD and DBP indicated a deviation from response additivity, where mixture responses were greater than predicted

(Rider et al, 2010). However, the authors acknowledged that the data were preliminary as the group sizes were low and because liver toxicity and malformations of external genitalia are not typical effects for TCDD and required further clarification. Similarly, a preliminary study by Jacobsen et al (2010) recorded effects following *in utero* exposure of rats to various concentrations of a complex mixture consisting of five anti-androgens. Although anti-androgenic effects were observed at all dose levels (likely additive) the study was limited as the group sizes were low and many of the parameters that were affected due to the mixture were not evaluated for the individual components at the same dose levels.

4.4.2 Studies showing an absence of effects for both mixtures and their individual components

Two papers are included in this section, which investigated dieldrin in combination with either endosulfan (Wade et al, 1997) or toxaphene (Gaido et al, 1997a). The two binary mixtures with endosulfan / dieldrin (3 mg/kg for each compound) and toxaphene / dieldrin (30 µmol/kg/d for each compound) were investigated in an uterotrophic assay, focusing on uterine weight, progesterone receptor concentration and uterine peroxidase activity. In both studies, no effects were recorded in any of the parameters investigated when either compound was tested on its own or in its respective combination. Unfortunately, in both papers no indication is given as to the proximity of the doses used in the study to the NO(A)ELs for each component; however for dieldrin and endosulfan the doses used represented approximately 1/10 of their MTDs.

Table 12: Summary of studies on the endocrine system or models of endocrine activity investigating mixtures at or around the individual component NO(A)ELs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Charles et al, 2007				
In vitro: ER alpha reporter assay using MCF-7 cells	Complex mixture of 6 synthetic chemicals: OP, BPA, MXC, o,p-DDT, HCH, DPN. In presence or absence of 2 phytoestrogens: Genistein + Diadzein	Chemicals: A fixed ratio mixture was used with the dose range spanning very low subthreshold levels to concentrations at which each component was present at its individual oestrogenic threshold. 5 mixture concentrations were investigated (0.02, 0.2, 1.0, 2.0, 3.0 µM) corresponding to 1/100, 1/10, 1/2, 1, 1.5 x NOEC for each component. Phytoestrogens: Final concentrations of 0.01, 0.025, 0.05, 0.1 and 0.15 µM were tested	No effects observed for lower concentrations of synthetic chemicals on phytoestrogen response. Effects were observed at ≥ 0.5x NOEC	Deviation from expectation proposed for only the higher concentrations of the synthetic chemicals in combination with effective phytoestrogen levels

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Kunz and Fent, 2006				
In vitro: Yeast oestrogen assay	Complex (one 4 components and one 8 components) mixtures of UV filters: 3BC; BP1; BP2; BP3; 4DHB; Et-PABA; BS; PS. Additional work on binary mixtures was all at effect levels	Complex mixtures: based on BC10 (10% basal activity) and NOEC	Combinations of 4 or 8 pure and partial agonists showed synergistic effects at BC10 and NOEC levels. Synergism based on isobole methods and deviation from CA	Deviation from expectation of CA model for the complex mixtures
Reference: Ohlsson et al, 2010a				
In vitro: Hormone secretion in H295R cells (cortisol, aldosterone, testosterone, oestradiol)	Ternary mixture: DIAD + GEN + API	Mixtures contained compounds at equimolar concentrations. Six mixtures were assessed with total flavonoid concentrations of 0.03, 0.09, 0.3, 0.9, 3 and 9µM	With the exception of oestradiol secretion, the effects on hormone secretion were predicted by both CA and IA models. The inhibition of oestradiol secretion was underestimated by both models (IA > CA)	Dose additivity and independent action predicted effects for all hormones except for oestradiol where data deviated from expectation
Reference: Ohlsson et al, 2010b				
In vitro: Hormone secretion in H295R cells (Cortisol, aldosterone)	Binary and ternary mixtures*:* KETO + PRO KETO + IMA IMA + PRO KETO + PRO + IMA	- Mixture exposures performed with fixed ratio design and equimolar ratios of compounds. - Ternary mixture final imidazole concentrations: 0.003, 0.009, 0.03, 0.09, 0.3, 0.9, 3, 9µM. - Binary mixtures final imidazole concentrations (concentrations represented divergent effects for hormone secretion based on individual and ternary mixture data): 0.06 and 0.2µM	- Cortisol secretion: Additivity was observed for the binary and ternary mixtures which could be predicted by CA and IA models. - Aldosterone secretion: Biphasic dose responses were recorded for this compound. Consequently modified CA and IA models were developed. - Binary mixtures: Effects dependent on the constituents in the mixture. KETO + IMA had no effect on aldosterone secretion as predicted by modified CA and IA models. - Ternary mixture: CA models (modified or standard) slightly underestimated the effects. The modified IA model markedly over- and under-estimated the stimulatory and inhibitory effects respectively	Dose additivity and independent action predicted effects on cortisol secretion. The non-monotonic effects on aldosterone could not be predicted highlighting a limitation of the CA and IA models

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Kjaerstad et al, 2010				
In vitro: AR reporter gene activation	4 separate mixtures: 'AR antagonists' (Flutamide, Vinclozoline, Procymidone); 'Anti-androgens' (Finasteride, MEHP, Prochloraz, Vinclozolin); 'Parabens' (Methyl paraben, Ethyl paraben, Propyl paraben, Butyl paraben, Isobutyl paraben); 'Azole fungicides' (Epoxiconazole, Propiconazole, Tebuconazole)	Single compounds: Dose-response curves established. Only one of the parabens was antagonistic at doses tested. Mixture: Equimolar mixtures tested using fixed mixture ratio	For 'AR antagonists', 'Anti-androgens' and 'Azole fungicide' mixtures, results were predicted by CA model. For the 'Parabens' mixture antagonism seen at lower concentrations than predicted by CA model. Authors postulate this is due to cytotoxicity.	CA predicted mixture effects at low doses.
Reference: Birkhøj et al, 2004				
In vitro: AR transcriptional activity	Complex mixture of 5 pesticides: DELTA, METH, PRO, SIMA, TRIB	Single compounds: Dose-response curves established Mixture: Equimolar mixture constructed with the 5 compounds	IC_{25} values established for individual compounds and the mixture. Results were predicted by CA model	CA predicted mixture effects
Reference: Birkhøj et al, 2004				
In vivo: Hershberger type study (castrated rats; 7 days dosing, SAT weights plus adrenal, kidney, liver and thyroid weights plus hormone levels)	Complex mixture of 5 pesticides: DELTA, METH, PRO, SIMA, TRIB	Single doses: DELTA and METH: 2.5 mg/kg bw PRO, SIMA, TRIB: 25 mg/kg bw Mixture: Same doses	- Adrenal/LABC weights: statistically significant changes for mixture in absence of effect for individual compounds - Kidney and thyroid weights: Unaffected for both mixture and individual compounds. - Hormone levels: No effects observed for mixture or for pesticides alone except METH and LH measurements. All other parameters: pesticides not included in the mixture at their individual NO(A)ELs	Weak additive effect for LABC and adrenal weights. Study highlights the need to confirm that the NOEL is correctly identified in multi-parameter investigations
Reference: Payne et al, 2001				
In vitro: MCF7 cell proliferation	Complex mixture of 4 compounds: o-p'-DDT p-p'-DDT p-p'-DDE β-HCH	Full concentration response curves with two mixture types: a: molar mixture ratios of 1:1:1:1 b: 1:10:5:4 o,p'-DDT; p,p'-DDE; β-HCH; p,p'-	Cell proliferation was significantly increased for mixture with compounds around their NOECs	Dose additivity and independent action predicted effects

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
		DDT, representing the range of the relative abundance of each of the four organochlorines in human serum in Western industrialised countries		
Reference: Soto et al, 1994				
In vitro: MCF7 cell proliferation	Complex mixture of 10 compounds: ENDOβ, ENDOα, TOXA, DIELD, TCB, p,p'-DDT, HCB, p,p'-DDD, p,p'-DDE, MXC	All compounds tested at 1µM which was considered as NOEC but dose responses for individual compounds were derived using widely spaced concentrations	Cell proliferation was observed for mixture (weakly additive) in the absence of effects for the individual compounds at the same concentration	Weak additivity was observed for the mixture but no clear conclusion can be derived as the mixture was tested at a single concentration. All compounds individually appeared to have the same NOEC (1µM), however this could be due to the use of widely spaced concentrations in the dose-response curves
Reference: Rider et al, 2008; 2009				
In vivo: *In utero* exposure examining male rat reproductive tract malformations (hypospadias, epididymal agenesis, undescended testes). Androgen sensitive tissue weights, AG distance, nipple retention	Complex mixture of 7 compounds: VINC,PROC, LIN, PRO, BBP, DBP, DEHP	Mixture dose levels based on dose-response curves for male reproductive tract malformations for the individual compounds. Each compound present in mixture at 100, 75, 50, 25% of top dose. Compounds were present in mixture at or below their NO(A)ELs in the two lowest mixture doses	All androgen sensitive endpoints were affected in a dose dependent manner	Outcome could be predicted by dose addition model
Reference: Rider et al, 2009; 2010				
In vivo: *In utero* exposure examining male rat reproductive tract malformations (hypospadias, epididymal agenesis, undescended testes). Androgen sensitive tissue weights, AG distance, nipple retention	Complex mixture of 10 compounds: VINC,PROC, LIN, PRO, BBP, DBP, DEHP, DiBP, DiHP, DPP	Complex mixture: Dose levels based on dose-response curves for male reproductive tract malformations for the individual compounds. Each compound present in mixture at 100, 80, 60, 40, 20, 10% of top dose. Compounds were present in mixture at or below their NO(A)ELs in the two lowest mixture doses	Complex mixture: Incidence of retained nipples for doses ≥ 20%. All other parameters / tissues were affected for doses ≥ 40%. Data indicated additivity	Complex mixture: Study is an extension of Rider et al, 2008. As with the first investigation, the data in this study could be predicted by dose addition model
	Binary mixture: TCDD, DBP	Binary mixture: Mixture dose levels based on dose-response curves for male reproductive tract	Binary mixture: Various parameters were affected for both mixtures (testicular and epididymal weights,	Binary mixture: Deviation (i.e. synergism) from response additivity was recorded although no conclusion was given

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
		malformations for the individual compounds. Both compounds present in mixture at 100 or 65% of the dose known to induce male reproductive tract malformations	malformations, hypospadias, vas deferens malformations). In addition, liver effects were observed in the absence of any known effects by the individual compounds. Mixture responses exceeded response addition expectations	concerning dose addition. Authors describe these data as preliminary and require clarification
Reference: Jacobsen et al, 2010				
In vivo: *In utero* exposure of rats. Parameters studied were maternal body weight, gestational length, pup mortality and weights, male reproduction organ weights, uterus weights, pup liver weights; AGD, nipple/areola retention, malformations of male external genitalia, steroid hormone levels in pups, progesterone in dams	Complex mixture of 5 compounds: PROC, MAN, EPOX, TEBU, PRO	Mixture combined compounds as %, of the dose that caused no major effects on pregnancy length and pup survival in earlier studies for each compound. Mixture concentrations: 25%, 50%, 75%, 100%, 125%. These equated to the following doses (in mg/kg bw): EPOX: 3.75, 7.50, 11.25, 15.00, 18.75, MAN: 6.25, 12.50, 18.75, 25.00, 31.25 PRO: 8.75, 17.50, 26.25, 35.00, 43.75 TEBU: 12.5, 25.00, 37.50, 50.00, 62.50 PROC: 12.5, 25.00, 37.50, 50.00, 62.50	Effects were observed even at the lowest mixture doses (25, 50%): increased gestation length, decreased prostate and epididymides weights; increased AGD index in female pups, decreased AGD index in male pups; increased nipple retention in male pups; increased numbers of dysgenesis of the external male genitalia. At 50% there was increased pup mortality, decreased birth weights of female pups, increased liver weights of male and female pups. Pmix75% and Pmix100% could not be evaluated due to pup mortality. No effects on hormone levels (however large standard variation)	Anti-androgenic effects likely to be additive, but study is limited in group sizes. In addition, the anti-androgenic parameters were not measured for individual compounds in the study. Data are described as preliminary and require further investigation
Reference: Wade et al, 1997				
In vivo: Immature rat uterotrophic assay (incl. PR, ER, peroxidase activity and hormone measurements)	Binary mixtures: ENDO + DIELD	ENDO and DIELD present at 3 mg/kg in mixture. This dose did not induce any changes when given alone for either compound	No effects observed for any parameter either as a mixture or individually	No effect; but study limited as only one dose level tested and no indication how dose relates to the NOEL for ENDO and DIELD
Reference: Gaido et al, 1997a				
In vivo: Immature mouse uterotrophic assay (incl. PR, ER, peroxidase activity and hormone measurements)	Binary mixtures: TOXA + DIELD	Both compounds present in mixture at 30 µmol/kg/d. Compounds tested individually at 60 µmol/kg/d	No effects observed for any parameter either as a mixture or individually	No effect; but study limited as only one dose level tested and no indication how dose relates to the NOELs for TOXA and DIELD

4.5 Studies on carcinogenicity

Although methods for establishing the carcinogenic risk of individual compounds are well established there has always been a concern that the risk associated with mixtures would be greater than that of individual compounds even when each component is strictly regulated through the application of safety factors. Consequently, many studies have been conducted to study the potential carcinogenic / mutagenic hazard posed by exposure to such mixtures (Ashby et al, 1978; Feron et al, 2001; Lagorio et al, 2000; Ito et al, 1996; Takayama et al, 1989). Several of the published studies were not evaluated as they were either outside the scope of this Task Force's remit, for example exposure to particles (wood or textile dust; Feron et al, 2001) or air pollution (Lagorio et al, 2000) or did not fulfil the other criteria described in Chapter 2 (e.g. mixtures evaluated at doses where one or more of the components were present at an effect level; Ito et al, 1996). However, a number of studies were found to be acceptable for inclusion in either the present or the following chapter. Specifically, this section comprises of six acceptable *in vivo* studies (including Hasegawa et al, 1994a; Hooth et al, 2002; Ito et al, 1991; Takayama et al, 1989; Kroes et al, 1974) and three acceptable *in vitro* studies (including Mondal et al, 1978; Kang et al, 1996).

4.5.1 Studies showing effects induced by mixtures in the absence of effects for their individual components

None of the *in vivo* studies described effects which were demonstrably predicted by dose / concentration addition. One study described the observed mixture effects (development of liver foci in DEN initiated rats) as being synergistic (Ito et al, 1991); however, the approach taken to calculate synergy was unorthodox. When tested individually at 1/25 of their carcinogenic dose, the compounds (Trp-P-1; Glu-P-2; IQ; MeIQ and MeIQx) gave no statistically significant changes in liver foci incidence compared to the controls although intergroup variability (both decreases and increases compared to the controls) was recorded. The addition (by means of simple effect summation) of these minor effects by the authors led them to arrive at a value indicating the overall effect caused by the individual compounds. This was then compared to the increase in liver foci observed due to treatment of the rats with the mixture where each component was present at 1/25 their carcinogenic dose. The ratio between these two numbers led the authors to conclude that a synergistic effect had occurred; however without the use of appropriate models to predict the final outcome and in the absence of appropriately conducted statistics it is difficult to determine whether the mixture effects were truly synergism or merely concentration addition or indeed only chance findings.

Similarly, Takayama et al (1989) investigated a mixture of forty carcinogens combined at one-fiftieth of their individual TD_{50} (tumorigenic dose causing tumours in 50% of treated F344 rats). The TD_{50}s were based on separate oral carcinogenicity study data in the literature. Target sites of the 40 carcinogens included, liver, thyroid, urinary bladder, skin, Zymbal's gland, and

20 carcinogens exhibited two or more target sites. Despite the mixture containing eight urinary bladder carcinogens, there were no treatment-related changes in tumour incidence in this tissue. Of the other target organs examined, only the liver and thyroid revealed statistically significant increases in preneoplastic and neoplastic lesions: markedly increased incidence of neoplastic nodules in livers of treated rats, and combined increased incidence of follicular cell adenomas (n = 2) and follicular cell carcinomas (n = 3) of the thyroid. The authors conclude that, in the absence of data on the individual carcinogen tested at the dose present in the mixture, it is not possible to conclude whether the effects on the liver and thyroid were a result of synergistic or additive effects.

Although carcinogenicity cannot be fully studied *in vitro*, certain aspects of tumour formation or MOA can be investigated using *in vitro* assays. Consequently, three *in vitro* studies were considered acceptable for inclusion in this section all of which showed mixture effects in the absence of effects for the individual compounds at their mixture concentration. Mondal et al (1978) studied the development of preneoplastic cell transformation in C3H/10T1/2 mouse embryo cells following treatment with TPA, impure or pure saccharin, methylcholanthrene or binary combinations of the individual compounds. All combinations, with the exception of impure saccharin / TPA, gave an increased incidence of preneoplastic cell transformation in the absence of effects for the individual compounds at the same concentrations (considered as NOECs). Unfortunately, the dose responses for the individual compounds and the mixtures were limited (only one or two concentrations were investigated) so interpretation of the data was difficult as far as additivity or otherwise is concerned. Kang et al (1996) investigated the inhibition of gap junctional intercellular communication in human breast epithelial cells following exposure to DDT, dieldrin, 2,3,4-HCB, 2,4,5-HCB, 2,4,5-HBB or binary combinations of the individual compounds. Concentration response curves were established for this parameter for the individual compounds and the binary mixtures were tested at concentrations which were non-cytotoxic and which represented the no-effect concentration for each of the components. Some of the mixtures led to an inhibition of gap junctional intercellular communication even though the individual components were at their NOECs; however no evaluation could be made of whether the effects were additive or deviated from additivity due to the limited dose levels used for the mixtures. A part of the untabulated study of Roloff et al (1992) can be considered relevant. The relevant mixture produced toxicity but the data did not support an interpretation of the nature of a combination effect.

Guigas et al (1993) describe mutation effects of the triazine herbicides atrazine (active ingredient and formulated) and cyanazine alone and in combination with quercetin in two *in vitro* tests (HPRT-test and sister chromatid exchange, SCE, in Chinese hamster ovary cells). Only two concentrations were tested (different by a factor of 10), both did not show any effect in the SCE test for either single compounds or combinations with and without metabolic activation. In the HPRT-test, single compounds did not increase mutant frequency without metabolic activation

whereas there was an increase at the high concentration with metabolic activation. In combination with quercetin and without metabolic activation there was a small increase in mutation frequency at the high dose for both herbicides which was consistent with an additive effect. In contrast, there was an antagonistic effect in the herbicide / quercetin combination at the high dose with metabolic activation.

4.5.2 Studies showing an absence of effects for both mixtures and their individual components

In a similar study design to that of Ito et al (1991), Hasegawa et al (1994a) found no effect on liver foci formation in initiated rats when testing PhIP, Glu-P-1, Glu-P-2, IQ and MeIQ in a mixture where each component was present at 1/25 their carcinogenic dose.

Hooth et al (2002) investigated the effect on the rodent kidney of a mixture of drinking water disinfection by-products with dissimilar modes of action (MOA) with respect to the renal effects (potassium bromide: renal toxicant and carcinogen; MOA: oxidative DNA damage; chloroform: renal toxicant and carcinogen; MOA: cytotoxicity / regenerative cell proliferation; bromodichloromethane: renal toxicant and carcinogen (vehicle dependent); MOA: cytotoxicity / regenerative cell proliferation and Mutagen X 3-chloro-4-dichloromethyl-5-hydroxy-2(5H)-furanone: renal toxicant but not carcinogen; MOA: direct acting mutagen). Eker rats (a rat strain that is predisposed to kidney tumours) were exposed to the individual compounds (three dose levels) and either a low-dose or high-dose mixture of them. Renal adenomas were increased in male and female rats dosed for 10 months with either the high dose of mutagen X or the high dose mixture. None of the other individual compounds or the low-dose mixture had any effect on the kidney parameters leading the authors to conclude that the combined carcinogenic risk for exposure to this mixture of drinking water disinfection by-products was less than additive.

Kroes et al (1974) investigated the effects of mixtures of lead arsenate or sodium arsenate and diethylnitrosamine (DEN) in a 120 week carcinogenicity study. Whereas the high dose of lead arsenate and the only dose of sodium arsenate tested were generally toxic doses (decreased body weight gain), the low dose of lead arsenate and the only dose of DEN investigated represented NO(A)ELs. Likewise, no adverse effects for the mixture of DEN and the low dose of lead arsenate were observed. For the mixture of sodium arsenate and DEN general toxicity, but no carcinogenic effects were observed.

In addition to the tabulated studies, a fraction of the study of Berry et al (1979) can be considered relevant; the relevant mixtures resulted in no toxicity.

Table 13: Summary of carcinogenicity studies investigating mixtures at or around the individual component NO(A)ELs

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Ito et al, 1991				
In vivo: Short-term carcinogenicity in DEN initiated rats	5-component mixture: Trp-P-1, Glu-P-2, IQ, MeIQ, MeIQx	Equipotent based on tumour-inducing dose: 1x, 1/5x, 1/25x tumorigenic dose for individual compounds. Mixture: 1/5 or 1/25 individual tumorigenic doses	Synergy claimed for liver foci development at 1/25 mixture but unorthodox methods used to arrive at this conclusion	No conclusion can be derived. Author's conclusion on synergy is questioned, as simple effect summation was used to arrive at expectation of mixture effect
Reference: Takayama et al, 1989				
In vivo: Two-year carcinogenicity in F344 rats	40-component mixture: Acetamide; 3-amino-9-ethylcarbazole HCl; 4-Amino-2-nitrophenol; 2-amino-5-nitrothiazole; 2-Aminoanthraquinone; aniline HCl; Anisidine HCl; Azobenzene; p-Benzoquinone dioxime; 4-Chloro-o-phenylenediamine; p-Chloroaniline; Clofibrate; p-Cresidine; Cupferron; Dapsone; 2,4-Diaminoanisol sulphate; 4,4'-Diaminodiphenyl ether; 2,4-Diaminotoluene; N,N'-Diethylthiourea; 2,4-Dinitrotoluene; Hydrazobenzene; Michler's ketone; Nafenopin; Nitriloacetic acid trisodium salt I; 5-Nitro-o-ansidine; 5-Nitroacenaphthene; N-Nitrosodiphenylamine; Reserpine; 4,4'-Thioaniline; o-Toluidine HCl; 2,3,6-Trichlorophenol; tris(2,3-Dibromopropyl)phosphate; 2-Amino-1,4-dimethyl-5H-pyridol[4,3-b]-indole; 3-Amino-1-methyl-5H-pyridol[4,3-b]indole; 2-Amino-6-methyldipyridol[1,2-a:3',2'-d]-imidazole; 2-Aminodipyridol[1,2-a:3',2'-d]-imidazole; 2-Amino-3-methylimidazo[4,5-f]quinolone; N-[4-(5-Nitro-2-furyl)-2-	TD_{50} estimated from published reports of 2-year oral carcinogenicity tests. Mixture tested at 1/50th of TD_{50}s	Increased incidence of preneoplastic and neoplastic changes in liver and thyroid of treated animals	No conclusion can be derived concerning additivity, etc

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
	thiazolyl]formamide; 2-Acetylaminofluorene; 3'-Methyl-4-dimethylaminoazobenzene			
Reference: Mondal et al, 1978				
In vitro: Neoplastic cell transformation in C3H/10T1/2 cells	Binary mixtures: MCA/TPA MCA/pure Saccharin MCA/impure Saccharin TPA/pure Saccharin TPA/impure Saccharin	Each component tested at NOEC in mixture. Concentrations tested: Saccharin (pure and impure): 100 µg/ml MCA: 0.1 µg/ml; 1.0 µg/ml TPA: 0.1 µg/ml Mixtures : Saccharin (pure and impure): 100 µg/ml MCA or TPA: 0.1 µg/ml	Increased neoplastic cell transformation observed for all combinations except TPA/impure Saccharin	No conclusion can be derived concerning additivity, etc
Reference: Kang et al, 1996				
In vitro: Normal human breast epithelial cells – gap junctions	Binary mixtures: DDT / 2,4,5-HCB DDT / 2,3,4-HCB DDT / 2,4,5-HBB DIELD / 2,4,5-HCB DIELD / 2,4,5-HBB DIELD / 2,3,4-HCB	Based on NOECs Mixtures tested at 0.25xNOEC DDT/ 0.5xNOEC HCB 0.5xNOEC DIELD/ 0.5xNOEC of HCB. Mixtures tested at 0.25xNOEL of DDT + 1xNOEL of HBB, and 0.5xNOEL of DIELD + 1xNOEL of HBB	Inhibition of gap junctional intercellular communication for these mixtures only. No effects for these mixtures	No conclusion can be derived concerning additivity, etc
Reference: Guigas et al, 1993				
In vitro: HPRT test and SCE in CHO cells (clone K1-BH4)	Quercetin / Gesamprim (formulation of Atrazin), Quercetin / Atrazin, Quercetin / Cyanazin; all experiments with/without metabolic activation	2 concentrations were tested with a factor of 10 difference: 'low' combines the low concentrations of the individual compounds; 'high' combines the high concentrations	No effect in the SCE test for either single compounds or combinations with and without metabolic activation. In the HPRT-test, there was a small increase in mutation frequency at the high dose for both herbicides in combination with quercetin and without metabolic activation	The high-dose finding in the HRPT test was consistent with an additive effect. In contrast, there was an antagonistic effect in the herbicide / quercetin combination at the high dose with metabolic activation
Reference: Hasegawa et al, 1994a				
In vivo: Short-term carcinogenicity in initiated rats	5-component mixture: PhIP, Glu-P-1, Glu-P-2, IQ, MeIQ	Equipotent based on tumour-inducing dose: 1x, 1/5x, 1/25x tumorigenic dose for individual compounds. Mixture: 1/5 or 1/25 individual tumorigenic doses	No effects observed for incidence of liver foci	No effects

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Hooth et al, 2002				
In vivo: Short-term carcinogenicity in Eker rats (Tsc2 tumour-suppressor genetically modified strain: predisposition to develop renal tumours)	4-component mixture: $KBrO_3$ Mutagen X $CHCl_3$ BDCM	2 doses of each component and mixture evaluated with low dose being non-neoplastic and high dose being neoplastic $KBrO_3$: 0.02, 0.4 g/l Mutagen X: 0.005, 0.07 g/l $CHCl_3$: 0.4, 1.8 g/l BDCM: 0.07, 0.7 g/l	Low-dose mixture had no effect on kidney parameters compared to the controls. Significant increases in renal adenomas and overall tumours recorded for the high dose mixture and for the high dose Mutagen X groups	No effects. Authors concluded that the combined carcinogenic risk for exposure to this mixture of drinking water disinfection by-products was less than additive due to the absence of effects for the low-dose mixture
Reference: Kroes et al, 1974				
In vivo: 120 week carcinogenicity in Wistar rats	Binary mixtures: Lead arsenate, diethyl-nitrosamine (DEN)	Lower dose of lead arsenate and tested dose of DEN re-presented NO(A)Els	No effects for this mixture	No effects
	Sodium arsenate, diethyl-nitrosamine	Tested dose of sodium arsenate was generally toxic, but not carcinogenic.	General toxic effects (bw changes) No carcinogenic effects	No carcinogenic effects

4.6 Human experimental studies

Experimental studies in humans of chemical mixtures are constrained by ethical concerns about exposing volunteers to potentially harmful substances and have also practical limitations such as small group sizes which may preclude statistical evaluation. Therefore, only a small number of compounds have been investigated and where interactions between chemicals have been studied this has been usually done at effect levels. The series of studies by Cometto-Muniz et al (1997, 1999, 2001, 2004), however, investigated the dose-dependent interactions of various mixtures of alcohols, esters, ketones and alkyl benzenes using odour perception, nasal pungency and eye irritation as endpoints. In the first experiment, they showed that thresholds for all sensory responses declined with increasing number of components in the mixture. Lipophilicity also enhanced the chemosensory potency of mixtures. For odour, partial agonism was the most frequent interaction, for nasal pungency both partial and complete agonism were present, for eye irritation synergistic action was seen for the most lipophilic and for the most complex mixture. The later studies focused on binary mixtures and the investigations went beyond establishment of simple thresholds since probabilities of detection for specific concentrations of single chemicals were calculated. The binary mixtures were then tested at various levels of probability of detection for the single compounds. For 1-butanol and 2-heptanone the detectability followed a straightforward dose addition model for all endpoints, whereas for the butyl acetate / toluene mixture complete agonism was seen at lower levels, and partial agonism at higher levels. Finally,

the mixture of ethyl propanoate and ethyl heptanoate again showed complete agonism at the lower probability of detection for the single compounds (0.40). At the higher level (probability of detection 0.80) the mixtures showed complete agonism for nasal pungency, but only partial agonism for eye irritation. These studies show that for trigeminal responses to solvent mixtures complete additivity is the norm at lower concentrations (= lower probability of detection). At higher concentrations (= higher detectability) the response is somewhat more variable; however, studies including a complete dose-response range show no evidence of supra-additive responses.

Table 14: Human experimental studies

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Cometto-Muniz et al, 1997				
Olfactory nerve stimulation (odour), trigeminal nerve stimulation (nasal pungency, eye irritation). Eight osmic subjects (4m, 4f), age 21-60 y Four anosmic subjects (2m, 2f), age 20-66 y	2 three-component mixtures, 2 six-component mixtures, 1 nine-component mixture. Alcohols: 1-Propanol, 1-Butanol, 1-Hexanol Esters: Ethyl acetate, Hexyl acetate, Heptyl acetate Ketones: 2-Pentanone, 2-Heptanone Alkyl benzenes: Toluene, Ethyl benzene, Propyl benzene	Mixtures were prepared in proportion of the odour thresholds of individual components; concentrations were varied in three-fold steps above and below the reference concentration	Thresholds for all sensory responses declined with increasing number of components in the mixture. Lipophilicity also enhanced the chemosensory potency of mixtures. For odour, partial agonism was the most frequent interaction, for nasal pungency both partial agonism and agonism were present, for eye irritation synergistic action for the most lipophilic and for the most complex mixture was seen	This is an exploratory study which showed that for sensory irritation effects of mixtures (partial) agonism is the norm, with some synergism suggested for eye irritation. Reliance on odour thresholds for single compounds established in previous studies is a limitation, as is the use of a threshold concept rather than concentration-detection functions (see later studies for details)
Reference: Cometto-Muniz et al, 1999				
Olfactory nerve stimulation (odour) trigeminal nerve stimulation (nasal pungency, eye irritation) Eighteen osmic subjects, age 18-54 y Seven anosmic subjects, age 28-59 y	Binary mixtures of 1-Butanol (99.8%) and 2-Heptanone (98%) 5 dilutions each of single compounds; 16 binary mixtures (4 concentration levels each for the two compounds), 1 dilution with both compounds at zero probability of detection	Probabilities (p) of detection (0.20, 0.40, 0.60 and 0.80) for specific concentrations were calculated. Binary mixtures were established in a 4 x 4 matrix where each p of one chemical was combined with each level of the other	Detectability of binary mixtures followed a straightforward dose addition model, most closely for odour perception, but also for nasal pungency and eye irritation	In contrast to the previous investigations, this study went beyond establishment of simple thresholds to include a range of responses from chance detection to virtually perfect detection. No significant variation from dose addition was seen. However, only binary mixtures were tested

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Cometto-Muniz et al, 2001				
Trigeminal nerve stimulation (nasal pungency, eye irritation). Nasal pungency: Six anosmic subjects, Age 34–74 y Eye irritation: twelve normosmic subjects, age 19-51 y	Binary mixtures of Butyl acetate (99+%) and Toluene (99.8%). 3 detection probability levels for eye irritation; 4 for nasal pungency. Each level consisted of 5 tests (2 for the single substances, 3 mixtures)	Probabilities (p) of detection for specific concentrations were calculated: for eye irritation 0.50, 0.75, and 1.00, for nasal pungency also 0.25. Binary mixtures included p of 0.25, 0.50 and 0.75 of one chemical combined with each level of the other (i.e. 3 mixtures).	For both endpoints similar findings were seen: At relatively low levels of detection for single compounds (< 0.50) complete agonism was seen for the mixtures. At higher levels (p of detection > 0.50) the mixtures showed partial agonism	The same strengths and weaknesses as for the 1999 study apply: reasonable number of subjects and replicates, use of detectability (psychometric functions), but only binary mixtures were tested
Reference: Cometto-Muniz et al, 2004				
Trigeminal nerve stimulation (nasal pungency, eye irritation). Nasal pungency: Five anosmic subjects, age 20-64 y Eye irritation: 20 normosmic subjects, age 18-54 y	Binary mixtures of Ethyl propanoate (97+%) and Ethyl heptanoate (98+%). 2 detection probability levels for both eye irritation and nasal pungency. Each level consisted of 5 tests (2 for the single substances, 3 mixtures)	Two levels of probability (p) of detection for specific concentrations were calculated for both eye irritation and nasal pungency (0.40 and 0.80). Binary mixtures included 1/4, 1/2 and 3/4 of a p of 0.80 or 0.40, respectively, of one chemical combined with each level of the other (i.e. 3 mixtures)	For both endpoints at the lower level of detection for single compounds (0.40) complete agonism was seen for the mixtures. At the higher level (p of detection 0.80) the mixtures showed complete agonism for nasal pungency, but only partial agonism for eye irritation	As in the previous investigation (2001), the response to the binary mixtures was agonistic at the lower end of detection, but only partially agonistic (for eye irritation) at the higher end

4.7 Summary

This chapter focused on studies in which the individual components were present in the mixtures at or around their individual NO(A)ELs for the parameters measured. A total of 60 papers encompassing *in vitro* and *in vivo* rodent studies were considered relevant. Four additional studies conducted using human volunteers were also included in this chapter. As the NO(A)EL can represent a response of up to 20% compared to the controls (Kortenkamp et al, 2009), responses were expected due to the mixture in the apparent absence of effects for the individual components. Consequently, only just above a quarter of the papers described an absence of effects upon exposure to a mixture. In contrast, about three quarters (46 papers) reported mixture effects in the absence of effects for the individual components (however, this figure may be inflated by publication bias). In well-designed studies, where robust dose responses were established for both the mixture and its individual compounds, it was possible to determine whether or not the mixture effects represented additivity or even a deviation from additivity. However, 25 of the papers reporting mixture effects were assessed to be rather limited in study

design due to either insufficient dose levels investigated for the mixture and its components (e.g. Gaido et al, 1997a; Sagai and Ichinose, 1991), limited group sizes (e.g. Jacobsen et al, 2010) or insufficient evaluation of the NO(A)ELs for the mixture components (e.g. Wade et al, 1997 and Gaido et al, 1997a based the dose levels of the individual components in the mixtures tested on parameters which were not measured in the study). Thus, based on the Task Force's assessment of these studies no conclusion could be drawn concerning the nature of the mixture effects. Of the remaining papers, 10 of them demonstrated mixture effects that clearly showed no deviation from additivity, which the Task Force considered robust. A further 11 papers (7 studies) described effects that appeared to deviate from additivity. However, in the case of 8 of these papers, the use of the term synergy is inappropriate in the view of the Task Force as deviation from expectations in these cases was small (mostly in the order of 2-2.5 fold difference from additivity expectations; e.g. the series of 6 publications by Casey and co-workers), or was not supported by more detailed later studies (Cometto-Muniz et al, 1997). This analysis highlights an aspect of mixture investigations which is crucial to allow a thorough evaluation of the data generated. Specifically, there is a clear need to not only describe the predicted outcome of the mixture investigations (by applying, for example, dose addition) but also to clearly define the term synergy before the conduct of any study investigating mixtures and their components. Finally, the claim of synergy was supported by the data from the studies of Kunz and Fent (2006), van Birgelen et al (1996) and from the preliminary binary mixtures reported by Rider et al (2010).

5. STUDIES WITH MIXTURES TESTED WELL BELOW NO(A)EL OF SINGLE COMPONENTS

This chapter includes *in vivo* and *in vitro* studies that were conducted with test doses (or concentrations) well below the NO(A)ELs of single mixture components. For this report, 'well below the NO(A)EL' is defined as at least 10-fold below the NO(A)EL. Studying these relatively low exposure levels is relevant and important, as real-life exposures often occur at these levels or at even lower levels (see Chapter 3). Mixtures consisting of independently acting components, at concentrations well below individual NO(A)ELs should, in theory, not elicit any adverse toxic effect as they do not exceed their individual NO(A)ELs. However, if interaction between components occurs, unexpected toxicological effects might be observed.

In some cases, mixtures consisting of components at concentrations well below individual NO(A)ELs do elicit an adverse effect. If the individual components have a similar mode of action, it can be expected that the components will act in an additive manner. Thus, the mixture effect can be predicted by adding the concentrations of all individual components.

If the individual components have a similar mode of action, and the toxic effect is larger than expected based on additivity, one can speak of synergy. Finally, if one component of a mixture does not have any effect on the endpoint of interest but does modulate the severity of effect of one of the other components, one can speak of potentiation.

In this chapter, selected studies have been grouped firstly by toxicological endpoint (endocrine system, genotoxicity, carcinogenicity and general toxicity) and secondly by type of combination effect.

5.1 Studies on the endocrine system or models of endocrine activity

A significant proportion of the publications identified, which studied doses / concentrations of individual mixture components well below NO(A)ELs, were investigations of effects on the endocrine system or in *in vitro* models assessing potential hormonal activity. This probably reflects the interest and concern, across many sectors (industry, regulators, NGOs and academia), that so-called 'endocrine disruption' required special consideration from a risk assessment perspective, especially if combined exposures to such chemicals at low effect (approximating environmentally relevant) doses resulted in measurable effects. The, now infamous, publication of Arnold and colleagues reporting 1000-fold synergy in a yeast-based oestrogen receptor activation assay when binary combinations of endosulfan, dieldrin and toxaphene were tested, was also a significant stimulus for others to move into studying mixtures of oestrogenic and anti-

androgenic chemicals (Arnold et al, 1996; 1997). This study was later discredited and withdrawn.

The studies identified fall into two broad categories:

- Studies, both *in vitro* (n = 4) and *in vivo* (n = 2), demonstrating additivity when mixture components are present below their individual NO(A)ELs.
- *In vitro* studies (n = 4) where no effects were observed in mixtures tested at concentrations where components are present below their individual NO(A)ELs.

5.1.1 Studies showing additivity

Table 15 provides an overview of the studies which provide sufficient evidence to conclude whether or not the mixture effects are additive when tested at doses where each constituent is present below its individual no effect level.

The publications of Silva et al (2002) and Rajapakse et al (2002) studied mixture effects of oestrogen receptor agonists in a yeast reporter gene assay (transfected with the human oestrogen receptor alpha). The former investigated eight xenoestrogens, mixed in proportion to their individual EC_{01}s and tested this 'fixed mixture ratio' across the full concentration-response curve (10 concentrations). Dose additivity expectations were confirmed for all 10 tested concentrations, including where individually the xenoestrogens were present at < 0.5x EC_{01}s. Although the main focus of the latter publication was to demonstrate that 'weak' xenoestrogens could, when present at low concentrations, modulate the effect of the potent endogenous oestradiol, the combination effects were also in line with dose additivity expectations even when all components were included in the mixture at < 0.5x EC_{01}s.

Dose additive oestrogenic effects of mixtures of UV-filters have also been demonstrated by Heneweer and colleagues (2005), even when individual components were present below NO(A)ELs. These studies evaluated the ability of binary and quaternary mixtures of UV-filters to induce oestrogen-regulated pS2 gene transcription in MCF-7 breast cancer cells. In the case of the quaternary equipotent mixture, where each UV-filter was present in proportion to the concentration increasing basal gene transcription by 50% (EC_{50}), mixtures were tested down to 0.01-fold of their EC_{50}s with clear evidence of dose additivity at these levels.

Most recently, a study by Kolle and colleagues (2011) has been published that describes dose additive anti-androgenic effects in an *in vitro* yeast assay, stably transfected with a human androgen receptor and lac-Z reporter gene. Full dose-response curves were obtained for flutamide and vinclozolin, ranging from 100-fold below NOEC to 100-fold above NOEC. At

concentration levels which did not elicit a response of the individual compounds, the combination of these compounds also did not elicit a response. Concentration additivity was observed in the linear, non-receptor-saturated concentration range. Interestingly, at higher concentrations combination effects are less than additive, probably due to receptor saturation.

Two *in vivo* studies on the endocrine system were identified which meet the relevance criteria and demonstrate additivity at doses below NO(A)ELs (Tinwell and Ashby, 2004; Howdeshell et al, 2008).

Tinwell and Ashby (2004) investigated the effects of seven oestrogenic chemicals (nonylphenol, bisphenol A, methoxychlor, genistein, oestradiol, diethylstilbestrol and ethinyl oestradiol) in two- and seven-component mixtures in the immature rat uterotrophic assay. Mixtures where individual substance doses were selected such that they would be non-uterotrophic or weakly uterotrophic demonstrated that the combined effect was significantly higher than the highest effect of ethinyl oestradiol. The authors state that these data "clearly established the potential of the effects reported by Silva et al (2002) *in vitro* to be seen also *in vivo*". The publication also clearly indicates that for effects which follow sigmoidal dose-response relationships, a gross over-estimate of combination effects will occur in the low-dose region through the concept of effect summation. Unfortunately, the data available in the publication do not allow an evaluation of the observed mixture effects against predictions of dose addition.

Howdeshell et al (2008) demonstrated that prenatal exposure to a mixture of five phthalate esters inhibited rat foetal testicular testosterone production in a dose-additive manner, which, in turn, is hypothesised to induce a range of reproductive malformations (the 'phthalate syndrome'). It should be noted that this study focused on determining slope and ED_{50} values of the individual phthalates and a mixture of phthalates and not on establishing NO(A)ELs. It is clear from the data that dose additivity accurately predicted the combination effect of the five phthalates. There is little information on the mixture effects in the low-dose region but there is no indication of deviation from additivity at doses down to 0.5x NOEL.

Table 15: Endocrine studies showing additivity

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results
Reference: Silva et al, 2002			
In vitro (yeast hER activation assay)	One multi-component mixture of 8 'oestrogens': OH-PCB 61; DCBP; CBP; GEN; 2DHB; BHP; BPA; RMB	Equipotent - based on EC_{01}s Concentrations tested: 10 nM - 100 µM; Mixture: 0.4 - 40 µM total 'oestrogens'. 3 concentrations of tested mixture were below individual substance EC_{01}s	Dose addition predicted combined effects
Reference: Rajapakse et al, 2002			
In vitro (yeast hER activation assay)	3 mixtures of 'oestradiol + a mixture of 11 xenoestrogens	Equipotent - based on EC_{01}s 11XE mix based on EC_{01}s. Mixtures	Dose addition predicted combined effects

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results
	(11XE)': Oestradiol OH-PCB 61; DCBP; CBP; GEN; 2DHB; BHP; TCB; BPA; RMB; TriCB	with E2 were 1:25000; 1:50000 and 1:100000 E2:11XE mix Concentrations tested: 10 nM - 100 µM; (E2: 10pM - 1 nM); Mixture: 0.4 - 40 µM total 'oestrogens' 3 concentrations of tested mixture were below individual substance EC_{01}s	
Reference: Heneweer et al, 2005			
In vitro, pS2-gene transcription in MCF-7 breast cancer cells	Binary mixture: BP-3 and BP-1. Quaternary mixture of 4 UV-filters: BP-1, BP-3, OMC and 4-MBC	Equipotent-based on pS2 gene transcription EC_{50}. Quaternary mixture tested down to 0.01-fold of individual EC_{50}s	Dose addition predicted combined effects. (No departure from dose additivity in both mixture experiments)
Reference: Kolle et al, 2011			
In vitro, yeast based hAR transcriptional activation assay	Binary mixture: Vinclozolin and Flutamide	Full dose-response curves of individual components and combinations of all tested concentrations	Dose addition predicted combined effects at concentrations around EC_{50}. Combined effects were less than additive at higher concentrations, due to receptor saturation
Reference: Tinwell and Ashby, 2004			
In vivo (rat uterotrophic assay)	2 mixture ratios referred to as alpha and alpha*; dependent on single substance inducing a detectable effect (alpha*) BPA; GEN; NP; MXC; 17b E2; DES; EE	7 dose levels ; s.c. injection. Mixture based on 1xLOEL and 0.5; 0.2; 0.1; 0.05; 0.02; 0.01 x LOEL	Low doses - no effects on uterine weight; clear statistically significant effect (increased) uterine weight at 0.1x LOEL (increase)
Reference: Howdeshell et al, 2008			
In vivo (SD rat) Maternal body weight gain, whole litter loss, foetal mortality, foetal testicular testosterone production, testicular testosterone levels	5-component mixture of phthalate esters: BBP; DBP; DEHP; DiBP; DPP	Mixture based on equipotency in reduction of testosterone production. Top dose: 300 mg/kg/day of BBP, DBP, DEHP and DiBP each and 100 mg/kg/day of DPP. Dilutions of top dose: 100; 80; 60; 40; 20; 10; and 5%	Maternal body weight gain was reduced at 520 mg/kg/d of the mixture (40% of top dose), foetal testosterone production was the most sensitive endpoint (reduction at 260 mg/kg/d; 20% of top dose) and was reduced at 1/2 to 1/3 of dose levels that increased foetal mortality. The effects seen closely matched a dose-addition model

5.1.2 Studies showing no mixture effects

The Task Force literature review only identified three relevant publications on endocrine activity of mixtures that reported no-combination effects below NO(A)ELs of individual substances. This may be explained by the fact that the majority of studies on endocrine active chemicals were not

focused on investigating interactions in the low-dose / effect region and did not put particular effort into determining NO(A)ELs or points of departure.

The report from Wade and colleagues (1997) presents data on binary mixtures of endosulfan and dieldrin in: the *in vitro* MCF-7 breast cancer cell line proliferation assay; an *in vitro* rat uterine oestrogen receptor binding assay, and; the *in vivo* rat uterotrophic assay. The results from the MCF-7 proliferation and receptor-binding assays appear, on inspection of the data presented in the publication, to support a conclusion of dose additivity when each organochlorine (OC) is present above its NOEC. There is, however, no evidence of effects, additive or otherwise, when each OC was present at or below its NOEC. The rat uterotrophic assay studies presented by Wade and colleagues are discussed in Section 4.2.

In an evaluation of the ability of binary mixtures of OC pesticides to activate the oestrogen receptor transfected into HeLa cells (a transformed human ovarian carcinoma cell line), Tully et al (2000) did not find any induction of transcriptional activation using individual substances or binary combinations where components were present around or below their NOECs. It can be concluded that in this experiment combinations of chemicals did not show any interaction between chemicals at any dose level resulting in increased activity. In addition to the tabulated studies, Ashby et al (1997) found no oestrogenicity of endosulfan and dieldrin singly or in a mixture.

Table 16: Endocrine studies showing no mixture effects

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results
Reference: Wade et al, 1997			
In vitro, MCF-7 cell proliferation assay	Binary combinations of ENDO and DIELD: 2 equipotent mixtures (on basal medium). Lowest concentrations were 0.02x + 0.1x NOEC for DIELD and ENDO, respectively	With basal medium the NOEC was 10 µM for DIELD and 2 µM for ENDO. Additional study with a subthreshold concentration of oestradiol did not establish a NOEC for either (therefore data not relevant)	Reported mixture effects only when individual substances present at or above NOECs
In vitro, rat uterine oestrogen receptor binding	Binary combinations of ENDO and DIELD: 4 (equipotent) mixtures at high receptor concentration (lowest concentration was 0.0008x + 0.004x the NOEC for DIELD and ENDO respectively) and 3 (equipotent) mixtures at low receptor concentration (lowest concentration was 0.02x the NOEC for both compounds)	NOEC was 10E-5 and 2x10E-6 M for DIELD and ENDO, respectively, at the high concentration of receptors. In case of low concentrations of receptors, the NOEC for both DIELD and ENDO was 4x10E-7 M	Reported mixture effects only when individual substances present at or above NOECs
Reference: Tully et al, 2000			
In vitro (ER transfected HeLa cells)	Multiple binary mixtures of organochlorine pesticides	Individual components: 1 nM and 10 µM Mixtures equimolar (1:1) covering same concentration range	No effects observed for individual or mixtures

5.2 Studies on genotoxicity

In addition to studies reported in the previous section on the endocrine system, numerous reports have been published describing genotoxic effects of combined exposure to substances. However, when applying the criteria set to this chapter, only a very small number of papers (n = 3) fulfilled the inclusion criteria of this report (see Chapter 2).

5.2.1 Studies showing additivity or deviation from additivity

None of the papers described effects that could be characterised as being additive. However, studies performed by Ashby and Styles (1980) and Donnelly et al (1998) showed an effect that could be described as a deviation from additivity which can be explained by pharmacokinetic interaction. Pharmacokinetic interaction occurs when one substance alters the concentration of another substance by affecting absorption, distribution, or clearance of the other substance.

The *in vitro* mammalian study (Baby Hamster Kidney [BHK] transformation assay) performed by Ashby and Styles (1980) describes effects that indicate potentiation. In this study, cells were exposed to single concentrations of 2-acetylaminofluorene (2-AAF) and acetanilide (0; 0.025; 0.25; 2.5 and 25 µg/ml), as well as, binary mixtures in a 1:1 ratio. Data showed that 2-AAF increased the numbers of transformants at the three highest tested concentrations, resulting in a NOEC of 0.025 µg/ml. In contrast, acetanilide did not cause an increase in numbers of transformants in any of the tested concentrations, when tested alone.

When 2-AAF and acetanilide were added simultaneously, a mixture effect was observed which was approximately two-fold higher than the effect of 2-AAF alone. The NOEC remained at 0.025 µg /ml, resulting in a steeper concentration-response curve than the one obtained when 2-AAF was tested alone. However, this potentiating effect was only observed when concentrations of 2-AAF were above NOEC. When testing the mixture at NOEC for both components, no potentiating effects were observed. Furthermore, testing a mixture of the same components *in vivo* generated an entirely different result. *In vivo* 2-AAF alone acts as a clear liver carcinogen in rats, whereas acetanilide does not. When administered simultaneously, no tumours were formed and a clear case of anticarcinogenesis was presented. It should therefore be noted that *in vivo* and *in vitro* studies could generate non-equivalent results which should be carefully looked out.

In the same study by Ashby and Styles (1980), a mixture of two optical isomers of ethoxymethoxymorpholinophosphine oxide (EMMPO) was tested in the BHK transformation assay. Individually, both isomers were inactive at doses up to 25 µg/ml. However, when tested in 1:1 mixtures, an increased number of transformations were observed at a concentration of

0.25 µg/ml and higher. This observation could be explained by one of the isomers being activated to a DNA-reactive species. The other isomer contributes by acting as a competitive substrate for the enzyme that detoxifies the active isomer.

Donnelly et al (1998) performed an Ames test for bacterial mutagenicity using known genotoxicants (benzo[a]pyrene [BaP], pentachlorophenol [PCP] and trinitrotoluene [TNT]). Exposure to some combinations resulted in fewer revertant numbers than would have been expected based on additivity. The protocol included five levels of a fixed ratio of all components (0.2, 20, 50, 100 and 200 µg/plate for B[a]P and PCP and 0, 40, 400, 1000, 2400 and 4800 µg/plate for TNT). Metabolic activation via addition of S9 mix was included in the protocol.

Individually, B[a]P was mutagenic with metabolic activation, TNT showed increased numbers of mutations without metabolic activation and PCP did not show any mutagenic effect at all. When PCP was added to B[a]P, there was no change in effect of B[a]P at any concentration. However, when TNT was added to B[a]P, an inhibition of the effect of B[a]P was observed, but only at B[a]P concentrations above NOEC. Finally, a tertiary mixture without metabolic activation was tested and showed slightly lower revertant numbers than that observed for the individual chemicals. Effects observed for the tertiary mixture with metabolic activation are approximately half the value predicted based on simple additivity of the effects observed with the individual compounds.

From the study by Donnelly et al (1998), it can be concluded that the bacterial mutagenicity of B[a]P was reduced when TNT was added. The tertiary mixture (B[a]P, TNT and PCP) also showed reduced mutagenicity compared to B[a]P alone, but to a lesser extent. It has been postulated that TNT would be able to reduce metabolic activation of B[a]P or to reduce cellular uptake or binding with DNA.

5.2.2 Studies showing no interaction effects

The study by Wang and colleagues (1987) did not describe any interaction effects when substances were tested in binary mixtures. An *in vitro* sister chromatid exchange test and chromosomal aberration tests in Chinese Hamster ovarian cells were carried out. Eight different pesticides were included in this study. Mixtures tested were based on formulations which were available on the market in China. However, for this report the focus is on the mixtures of kasugomycin / carbendazim and captafol / polyoxin. Therefore, only test concentrations for these combinations which were below NOEC of the individual compounds have been included.

When tested alone, exposure of cells to carbendazim resulted in a dose-response curve in the chromosomal aberration test with a NOEC of 33 µg/ml. None of the tested concentrations of

kasugomycin elicited a response. A mixture of kasugomycin / carbendazim (ratio 1:12) showed a dose-response curve which had the same shape as carbendazim alone.

In the sister chromatid exchange test, exposure to captafol alone resulted in a dose-dependent increase of sister chromatid exchanges, with a NOEC between 0.02 and 0.1 µg/ml. None of the tested concentrations of polyoxin induced exchanges in this assay. A mixture of captafol / polyoxin (ratio 10:1) showed a dose-response curve with a NOEC slightly lower, but in the same range as for captafol alone. The maximum effect elicited by the mixture was slightly lower than the one with captafol alone.

The studies performed by Wang et al do not give any indication of significant interactions between chemicals tested at concentrations below NOEC.

Table 17: Genotoxicity studies

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Ashby and Styles, 1980				
BHK (baby hamster kidney) transformation assay	2-AAF + ACTLD	0.025; 0.25; 2.5 and 25 µg/ml and binary mixtures in a 1:1 ratio	Indications of potentiation, but only at concentrations above NOEC	Pharmacokinetic interaction
BHK (baby hamster kidney) transformation assay	2 optical isomers of EMMPO	0.025; 0.25; 2.5 and 25 µg/ml and binary mixtures in a 1:1 ratio	Results indicate potentiation of activation of one isomer by competitive inhibition of the detoxifying enzyme by the other isomer	Pharmacokinetic interaction
Reference: Donnelly et al, 1998				
Bacterial mutagenicity (Ames) test. Metabolic activation via addition of S9 mix was included in the protocol	BaP, PCP and TNT	0.2, 20, 50, 100 and 200 µg/plate for B[a]P and PCP and 0, 40, 400, 1000, 2400 and 4800 µg/plate for TNT	Addition of TNT to B[a]P decreased bacterial mutagenicity of B[a]P	Pharmacokinetic interaction
Reference: Wang et al, 1987				
Sister chromatid exchange and chromosomal aberration in CHO cells	KSM, BCM, CAP and PO	Kasugomycin (1.6-1000 µg/ml), Carbendazim (6.6-4156 µg/ml), Captafol (0.005-3.1 µg/ml) and Polyoxin (0.26-161.3 µg/ml)	No effect caused by addition of Kasugomycin to Carbendazim or Polyoxin to Captafol	No effect

5.3 Studies on carcinogenicity

Although not supported by evidence from epidemiological and experimental studies, there is increasing public concern that trace levels of chemicals in food and water may pose a carcinogenic risk to humans. An example of these chemicals is pesticides, which are regulated through acceptable daily intakes (ADIs). However, concern is raised that these expected safe intake levels do not take into account the possibility of toxic effects via additive or synergistic interactions of these chemicals present in a mixture at their individual ADIs. Only few studies (n = 3) were identified, and all these involved pesticides.

5.3.1 Studies showing additivity or deviation from additivity

None of the identified studies focusing on the carcinogenic potential of an interaction between pesticides at their individual dose well below no effect levels, described effects following an additive mode of action, nor did they show deviation from additivity.

5.3.2 Studies showing no mixture effects

The three studies, that were identified focusing on the potential carcinogenic effect of combined exposure to pesticides, described mixtures in which the individual compounds were present at their ADI level or a factor of the ADI (i.e. 1x, 10x and 100x ADI). None of the mixtures showed any effects on the studied endpoints in the described bio-assays for carcinogenicity in any of these studies (Table 18).

Studies by Ito et al (1995 and 1996) studied the combined effect of pesticides on rat carcinogenesis. The mixture investigated in the 1995 study comprised 20 most likely dissimilar acting chemicals, although all but one, i.e. endosulfan, are organophosphates. Each chemical was present in the mixture at its individual ADI level or 100x the ADI. NO(A)ELs were not established, and doses were based on previously established ADI levels. In DEN induced animals, the number and area of GST-P positive liver foci were significantly increased in the 100x ADI group only, by 34% and 52% respectively. No changes were observed in the non-initiated group or in the DEN induced animals receiving the ADI mixture (a no-initiation / no treatment group was not run). Assuming that the 100x ADI level is approximately the NO(A)EL, this study might indicate a possible mixture effect around the NO(A)EL level, but not at ADI level, and only in the sensitive (initiated) model. However, it is difficult to be conclusive since no single compounds were tested in the models and dose-response curves / NO(A)EL levels were not established in this study.

In a follow-up study, Ito et al (1996) tested the potential carcinogenic effect of two mixtures of 20 or 40 pesticides given in the diet using a multi-organ medium term bioassay (DMBDD model[3]). Pesticides selected for the mixtures were chemicals of high volume production (40) or with reported or suspected carcinogenicity (20), all most likely dissimilar acting chemicals. No tumour promoting potential in any tissue or organ was observed with both the 40 and 20 pesticide mixtures in the DMBDD initiated animals. In the non-initiated animals, neither pre-neoplastic nor neoplastic lesions were observed (a no-initiation / no-treatment group was not run). Only captafol, used as a positive control, increased the number or area of GST-P positive liver foci and had apparent tumour promoting effects in the liver, thyroid and kidney; the dose level used for captafol (1500 mg/kg in the diet) is however not comparable to the mixtures. It is difficult to draw definitive conclusions from this study, since no single compounds were tested and there was no dose response / NO(A)EL level established. However, the study shows that two mixtures at ADI levels of 40 or 20 pesticides did not exert tumour modulating potential, even though the latter comprised 20 pesticides for which carcinogenicity has been reported or suspected.

In addition to these two studies by Ito and co-workers, Perez-Carreon et al (2009) also investigated the tumour promoting and co-carcinogenic effects of pesticides at their individual ADI levels in a mixture. 12 commercially available dissimilarly acting pesticides, each at their 1-fold and 10-fold ADI levels, were investigated for their hepato-carcinogenic potential by co-administration in an alternative Solt-Farber protocol. No changes in the number of altered hepatocyte lesions or GST-P expression compared to control animals were observed in rats receiving the 1x- and 10x ADI pesticide mixtures. The authors conclude that the selected pesticide mixture is deprived of additive / synergistic effects on tumourgenesis at comparative low doses tested in a medium-term hepatocarcinogenesis model (Perez-Carreon et al, 2009). It should be noted however, that no dose responses or NO(A)EL levels were established, no single chemicals were tested but ADI levels were based on literature data (and were not available for all compounds), the test groups were relatively small (5-9 animals) and not well described.

The overall conclusion from the three studies is that the ADI provides a safe intake level to humans even in chronic co-exposures to multiple substances, and the results provide support for the current safety factor approach. However, for reasons described above, the results of these studies have to be treated with care and may not provide definitive evidence to support these conclusions.

[3] Abbreviation explained and references to DMBDD model given in Ito et al, 1996.

Table 18: Carcinogenicity studies

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results
Reference: Ito et al, 1995			
In vivo (rat; dietary exposure; 2-week DEN initiation + 6-week treatment)	2 mixtures equivalent to 1x ADI and 100x ADI	ACE, BMF, CVP, CPF, DCV, DDVP, EDDP, ENDO, ETF, FT, IBP, IX, MALA, DMTP, PPHM, PTP, PF, TFM, TCF, VT	Uninitiated: no effects in GST-P foci Initiated: 1x ADI no effects; 100x significant increase in number and area of foci
Reference: Ito et al, 1996			
In vivo (rat; dietary exposure; 4 weeks' initiation; 28-week exposures to 40 HPV substances and 20 substances [suspected carcinogens])	2 mixtures: (1) 40 HPV substances (2) 20 substances (suspected carcinogens) but not considered further as not relevant. Both mixed in proportion to ADI	40-pesticide mixture: ACE, BC, BS, BTZ, CM, CB, CP, CPP, CF, CYF, CYH, CYP, DFB, FEN, FBO, FV, FLUC, FL, GLYPH, IMA, MALA, MN, MCl, META, METO, MB, MYCLO, oxamyl, PDM, PM, PPHM, PPC, PF, QC, SXD, TBC, TDM, TClF, VINC, zineb. 20-pesticide mixture: ACE, AMI, CAP, CLOF, CYP, 2,4-D, DClV, DClB, DICO, Foset, GLYPh, MAN, MN, MEFO, PM, PHOS, PCZ, PPX, TDF, TF. Captafol (1500 mg/kg) as positive control. Purities or CAS numbers were not given	Non-initiated - no effects Initiated - only positive control; decreased bw; increased rel. liver / kidney weight; increased number or area of GST-P foci
Reference: Perez-Carreon et al, 2009			
In vivo, oral gavage Modified Solt-Farber protocols for medium term rat hepatocarcino-genesis. 8-week exposure to pesticides, with induction (DEN +/– 2-AAF +/– partial hepatectomy) at 4 weeks. Co-carcinogenic effect and promoting activity evaluated using gamma-glutamyl transpeptidase (GGT) positive altered hepatocyte foci, as well as protein and mRNA levels of glutathione S-transferase P in liver extracts	Mixture of 12 pesticides (all carcinogenic)	2 dose groups containing the individual compounds at the following doses (representing 1x or 10x ADI): AlaCl: 0.0002/0.002 mg/L (ADI ND), ATRA: 0.0001/0.001 mg/L (ADI ND), CARBO 0.0008/0.008 mg/L (ADI: 0.002 mg/kg bw), CPP 0.004/0.04 mg/L (ADI 0.01 mg/kg bw), DIA 0.0008/0.008 mg/L (ADI 0.002 mg/kg bw), DICO 0.001/0.01 mg/L (ADI 0.002 mg/kg bw), ENDO 0.0024/0.024 mg/L (0.006 mg/kg bw), ID 0.024/0.12 mg/L (ADI 0.06 mg/kg bw), MAN 0.02/0.1 mg/L (ADI 0.03 mg/kg bw), MN 0.02/0.1 mg/L (ADI 0.03 mg/kg bw), PROC 0.04/0.2 mg/L (ADI 0.1 mg/kg bw), ROTE 0.05/0.25 mg/L (ADI ND)	No tumour-promoting activity or co-carcinogenic effect in the rat medium term liver carcinogenesis model. Compared to controls (vehicle) same number of hepatocyte lesions and equal GST-P expression on liver extracts

5.4 Studies on general toxicity

5.4.1 Studies showing additivity

The series of studies performed by Jonker et al (1990, 1993a,b, 1996) are a good example of increasingly targeted study design to answer the question whether similarly or dissimilarly acting chemicals show evidence for additive, synergistic or antagonistic effects, or act independently. The investigation of acute effects of dissimilarly acting nephrotoxic chemicals (Jonker et al, 1993a) has already been discussed in Section 4.1.1. Details of the experimental design of the other studies are shown in Table 19. The results for both randomly chosen mixtures as well as mixtures of chemicals with the same target organ but different modes of action demonstrate absence of additivity (i.e. independent action) at doses below 'no nephrotoxic effect levels'. In contrast, for chemicals with a similar mode of action at the target organ, additivity was evident when combinations were dosed at corresponding fractions of the 'no nephrotoxic effect level'. Neither synergism nor antagonism was observed.

5.4.2 Studies showing deviation from additivity

In the study by Kakko et al (2000) the effect of pyrethrin alone and in combination with piperonyl butoxide (PBO) on total and Mg^{2+}-activated ATPase was investigated. This endpoint is not recognised as a major target for toxicity of pyrethrin / pyrethroids which are primarily ion channel toxins. However, the authors suggest that ATPases may be involved in ion homeostasis secondary to pyrethrin effects on sodium channels. The two compounds were tested in a fixed ratio of pyrethrin: PBO of 1:4 at concentrations from 0.1 - 1000 μM for pyrethrin and 0.4 - 4000 μM for PBO. The effect on total ATPase was more pronounced than that on Mg^{2+}-activated ATPase. Whereas total ATPase was inhibited by pyrethrin at concentrations of 100 μM and above, the effect of PBO alone was significant only at 4000 μM. In combination, there was a log-linear decrease in total ATPase activity starting at 0.1/0.4 μM pyrethrin / PBO which became significant at 10/40 μM. The statistics are not entirely clear in the paper, but the potentiating effect in the mixture is at least 10-fold and possibly 100-fold. The authors point out that PBO is used in pyrethrin formulations precisely because of its potentiating action which is due to its inhibitory effect on the microsomal mixed functional oxidase (MFO) system which is involved in pyrethrin detoxification. The deviation from additivity in this study is therefore an expected effect based on pharmacokinetic interactions.

Choi et al (2010) studied the comparative nephrotoxicity of melamine (MEL, 1x = 0.315 mg/kg), cyanuric acid (CA, 1x = 0.25 mg/kg) and a mixture of both (MC, 1x = 0.315 mg/kg MEL + 0.25 mg/kg CA) at 5 dose levels ranging from 0.1X to 1000X in adult male Sprague-Dawley rats. Neither compound showed nephrotoxic effects on its own at any dose tested, as evidenced by total

and relative kidney weight, BUN and creatinine. The NO(A)EL for nephrotoxicity of the individual compounds was therefore above the highest dose tested, i.e. > 315 mg/kg for MEL and > 250 mg/kg for CA. In contrast, the NO(A)EL for the combined administration was 3.15 + 2.5 mg/kg MEL + CA, suggesting that treatment with the combination was at least 100x more toxic than with each chemical individually. Effects on white blood cells were already seen at all dose levels of the single compounds and showed some evidence for additivity at higher doses.

In a similar experiment, Jacob et al (2011) studied the nephrotoxicity of a MEL + CA mixture given over seven days in the diet to adult F344 rats at rising concentrations of both MEL and CA in equal proportion, compared to a high dose of MEL and CA alone. The top doses were calculated to achieve a target dose of 200 mg/kg bw/day for the individual compounds and 100 mg/kg bw/day for each mixture component; however, due to reduced food consumption, the effective daily doses were markedly reduced in the two highest dosing groups for the mixture and the MEL-only group. No treatment-related effects were seen in either MEL-only or CA-only treated rats except for scattered crystals in renal tubules in 5 out of 12 animals of the MEL group. In contrast, there was a marked increase in kidney weight as well as BUN and creatinine levels in rats treated with the MEL + CA mixture starting at 229 ppm of each compound. The NO(A)EL of the mixture exposure was therefore approximately 10x lower than would be expected from simple dose additivity. Both sets of authors attribute the renal findings to the formation of high-molecular weight MEL-CA complexes which are excreted as crystals and destroy renal tubular structures. These studies are therefore an example of synergy due to chemical interaction of the individual compounds. The difference in synergistic response in the two studies may be related to the different dosing regimen (sequential gavage dosing vs. dietary mixture).

Two additional investigations in cats (Puschner et al, 2007) and fish and pigs (Reimschuessel et al, 2008) did not include dose-response information and are therefore not discussed in detail here. However, they showed similar findings with regard to the combined nephrotoxic effects of MEL and CA to the two studies mentioned above.

5.4.3 Studies showing no mixture effects

The publications by Akay et al (1999) and Selmanoglu and Akay (2000, 2001) represent a single body of work using the same experimental model and may in fact be a single study, although the results are not interpreted as a whole in these papers. In every case, male Swiss Albino rats were treated for 3.5 months with endosulfan, carbaryl and dimethoate. Depending on the endpoints investigated, various doses for pairs of substances and/or combinations of the three chemicals were chosen. These were always based on multiples of the ADI for the individual substances ranging from 1x to 1000x. The first study of this series (Akay et al, 1999) dealing with haematological and immunological endpoints has already been discussed in Section 4.2.2. The

results from the histopathology studies (Selmanoglu and Akay, 2000) indicate variable effects of the mixtures at 10x or 100x ADI. However, as acknowledged by the authors, these are difficult to interpret with regard to synergistic or antagonistic effects, not least because there are no data for individual substances, therefore no NOELs could be established. Furthermore, it is stated in Selmanoglu and Akay (2001) that at 1x to 100x ADI there were no effects on liver enzymes or biochemical endpoints. Although the authors state that the biochemical data are consistent with the histopathology findings, this appears to be the case only for the 1000x ADI studies, i.e. those conducted at effect levels for single substances, but not for the 10x and 100x ADI studies. Taking all three reported studies together, the overall conclusion is that there appears to be no discernible effects of various combinations of the three pesticides at dose levels below the NOELs of individual substances. These studies do not fit perfectly into this chapter in terms of study design, but neither would they fit well into Chapter 3. Therefore they are included here.

Vitalone et al (2008) describe a well conducted and documented study on the effects of binary exposure to methylmercury (MeHg) and PCB 126 on CNS functions in developing rats. In this study, pregnant Wistar were dosed (from GD7 to PND21; day of birth was denoted as PND0) with 0.5 mg/kg/day MeHg, 100 ng/kg/day PCB 126 or both. Effects on pregnancy (number of pregnancies, lengths of pregnancy, litter size, sex ratios) and body weight were studied, as were effects on pre- and post-weaning neurobehaviour. Dose selection was based on previously published reports. 0.5 mg/kg bw MeHg was chosen as a low dose based on Coccini et al (2000) and Roegge and Schantz (2006). The 100 ng/kg/day dose of PCB 126 was based on the observation that the ten-fold higher dose of PCB 126 (1 µg/kg bw) caused body weight reductions in dams, prolongation of length of pregnancy, reduction of litter size, and a significant decrease in the number of alive pups. The only effects, where the single component groups represented individual NOAELs were: Pregnancy length, sex ratio at birth, physical development (at weaning), attention, coordination and balance, object discrimination, spatial and conditioned learning. No effects were seen in the mixture group, when these parameters were evaluated. Although some limited effects on studied parameters were observed, the results do not support the hypothesis that co-exposure to MeHg and PCB 126 results in additive or synergistic effects.

Rudzok et al (2010) studied the effect of binary combinations of diclofenac, irgasan and nickel on cell viability/cytotoxicity in HepG2 cells. Using mathematical models described by Jonker et al (2005) the authors demonstrated antagonism when the components were present at or below their individual EC_{50}s in the mixture. However, whether this deviation from both the IA and CA models occurred at concentrations below the NOAECs for each component is difficult to elucidate from the data as they are presented in the paper.

Table 19: General toxicity studies

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
Reference: Jonker et al, 1990; 1993a,b; 1996				
In vivo, oral gavage	4 associated publications. 1990 study: 8-compound mixture. Choice of compounds was fully arbitrary wrt target organs, MOA and (un)expected interaction. 1993 studies: 4 nephrotoxicants with dissimilar modes of actions. 1996 study: 4 nephrotoxicants with similar modes of action	- 1990 study: mixture of compounds at their individual MOAEL*, their individual NO(A)EL or at 1/10 or 1/3 of their NO(A)EL - 1993 studies: mixtures of compounds at their individual NNEL*, MNEL* or ¼ NNEL - 1996 study: compounds in mixture at their individual NONEL* (¼ LONEL*) and ½ LONEL (all 4) or 1/3 LONEL (3 compounds). Individual compounds at NONEL and LONEL	- 1990 paper: independent action of dissimilarly acting chemicals in a 28-day study (all endpoints included). - Acute (1993) and subacute (1993) renal toxicity with dissimilarly acting chemicals: independent action at doses below MNEL. - Subacute renal toxicity (1996) on kidney with similar acting chemicals: evidence for dose additivity	Additivity. 1996 study (dose-additivity was shown based on the application of the same 'toxicity units' for all groups [individual compounds = 1 TU, 4-compound mixture = 4*1/4 = 1 TU, 3-compound mixture = 3*1/3 = 1 TU]
* MOAEL = minimum observed adverse effect level; NNEL = no nephrotoxic effect level; MNEL = minimum nephrotoxic effect level; NONEL = no observed nephrotoxic effect level; LONEL = lowest observed nephrotoxic effect level				
Reference: Kakko et al, 2000				
In vitro (total and Mg^{2+} ATPase activity in synaptosome of cerebrum)	Binary mixture of PYR and PBO (1:4 mixture ratio - based on marketed formulation)	0.1 - 1000 µM PYR 0.4 - 4000 µM PBO Mixture 1:4 ratio at same concentrations as above	Total ATPase: NO(A)EL based on authors stats: 100 µM PYR; 400 µM PBO; 1:4 mixture NO(A)EL at 1µM PYR + 4µM PBO, i.e. NO(A)EL shifted 100-fold lower = synergy	Clear synergy. (PBO inhibits detoxifying enzymes [MFO] and is used specifically as a synergist for PYRs. No additivity expectations in the paper)
Reference: Choi et al, 2010				
In vivo, oral gavage, Sprague-Dawley rats dosed for 7 days	Binary mixture (MC) of MEL and CA	0.0315 - 315 mg/kg MEL 0.025 - 250 mg/kg CA Mixture: 1:1 ratio at the same concentrations	MEL and CA had no effect on absolute and relative kidney weight, BUN and CREAT at all doses (NO(A)EL for MEL > 315 and for CA > 250 mg/kg). All parameters were significantly increased in MC at doses > 3.15 + 2.5 mg/kg, i.e. NO(A)EL was reduced > 100x. Histopathology: crystal formation in renal tubules	Clear synergy. (MEL and CA form high molecular weight complexes which accumulate as crystals in renal tubules)
Reference: Jacob et al, 2011				
In vivo, dietary administration F344 rats dosed for 7 days	Binary mixture (MC) of MEL and CA	0 (control), 7, 23, 69, 229, or 694 ppm of both MEL and CA, 1388 ppm MEL, or 1388 ppm CA alone	CA alone: no treatment-related effects. MEL alone: scattered crystals in renal tubules in 5 out of 12 animals. MEL + CA: kidney effects at 694 (sacrificed moribund at day 3) and 229 ppm. NOEL	Clear synergy. The NO(A)EL of the mixture exposure was appr. 10x lower than would be expected from simple dose additivity.

Study type	Mixtures tested (excluding controls)	Doses / Basis of exposure	Results	Conclusion
			69 ppm (calc. NO(A)EL approx. 8 mg/kg bw)	MOA: same as Choi et al (2010)
Reference: Akay et al, 1999 ; Selmanoglu and Akay, 2000 ; 2001				
In vivo, oral gavage, albino rats, dosed for 3.5 months. - 1999 study evaluated IgG, IgM and haematology. - 2000 study evaluated liver, kidney and testes weights and histopath. - 2001 study evaluated serum liver enzymes; pseudocholine sterase activity and some clin. chemistry	Binary and tertiary mixtures of ENDO, DIM and CARB	Dependent on publication, mixtures composed of 1x; 10x; 100; and 1000x ADIs. ADIs were: 0.00612 mg/kg/day - ENDO; 0.0204 mg/kg/day - DIM; 0.0101 mg/kg/day - CARB	- 1999 study: Single substances induced effects only at 1000x ADI (decreased effects on WBC count). →100x ADI: ENDO + DIM - increased WBC, and DIM + CARB - increased RBC. →1000x ADI: effects seen on IgG, IgM and cell counts. - 2000 study: Dose responses were not supportive of there being treatment-related effects on organ weights at 10x or 100x ADI. - 2001 study: No effects of any single, two-way or three-way mixtures at 1x, 10x and 100x ADI. Effects of single chemicals and mixtures at 1000x ADI	No effects
Reference: Vitalone et al, 2008				
In vivo, dietary exposure, Wistar rats, perinatal dosing	Binary mixture of Methyl mercury and PCB 126	NOELs based on previously published data. 0.5 mg/kg bw MeHg was chosen as a low dose based on Coccini et al (2000) and Roegge and Schantz (2006). The 100 ng/kg/day dose of PCB 126 was based on the observation that the 10-fold higher dose of PCB 126 (1 µg/kg bw) caused body weight reductions in dams, prolongation of length of pregnancy, reduction of litter size, and a significant decrease in the number of alive pups	Subtle and transient effects observed for some endpoints in some dose groups, but their biological significance is questionable	No effects
Reference: Rudzok et al, 2010				
In vitro, cell viability / cytotoxicity in HepG2 cells using MMT, NRU and AlamarBlue methods	Binary mixtures of Nickel (Ni), Diclofenac (DCF), Irgasan (IRG)	Full dose-response curves established for each component. Fixed ratio and full factorial designs used for binary mixtures	For each of the combinations, antagonism was recorded at dose-levels below the individual component EC_{50}s and synergism at concentrations higher than the EC_{50}s. In the case of the nickel/IRG combination, synergism was only observed when IRG was present at a toxic concentration	Using the mathematical models of Jonker et al (2005) authors established that both CA and IA models gave similar predictions for the combinations

5.5 Summary

Twenty-seven papers were identified and reviewed in this chapter; between them they tested 41 chemical mixtures. In twelve of the papers, the mixtures tested did not produce effects. Eleven papers demonstrated mixture toxicity which could be explained by dose additivity for similar chemicals. The remaining four papers described clear synergy or potentiation, either attributable to pharmacokinetic interaction or to chemical interaction.

These are studies in which there were mixture doses which were well below NO(A)ELs for each component, and which resulted in clear synergy or antagonism:

- A mixture of pyrethrin and piperonyl butoxide (PBO) showed substantial synergy for total ATPase in an *in vitro* system (Kakko et al, 2000). Observed synergy was not unexpected as PBO is often used in pyrethrin formulations because of its potentiating action which is due to its inhibitory effect on the MFO system, capable of detoxifying pyrethrin.
- In a baby hamster kidney transformation assay, two optical isomers of ethoxy-methoxymorpholinophosphine oxide showed potentiation of the toxicity. It was postulated that one isomer competitively inhibited the detoxifying enzyme of the other isomer (Ashby and Styles, 1980).
- A mixture of melamine and cyanuric acid showed decreased NO(A)ELs compared to the individual components alone (Choi et al, 2010; Jacob et al, 2011; Puschner et al, 2007; Reimschuessel et al, 2008). This observation was explained by chemical interaction of the two components resulting in formation of a large complex, capable of destroying renal structures.

6. DISCUSSION

This chapter brings together the various aspects of the Task Force's work, summarises the evidence from the literature with respect to low-dose interactions and draws conclusions.

Quite a number of papers claim the occurrence of unexpected mixture effects or synergy, when chemicals are tested in combinations below NO(A)ELs and resulted in toxicity. It has been observed that many such papers do not provide evidence that could support such a claim (CoT, 2002; Boobis et al, 2011; SCHER, SCCS, SCENIHR, 2012), often due to the lack of suitable single chemical dose-response data. Mixture effects observed for combinations of chemicals below their NO(A)ELs are presented as if they would be totally unexpected and of high public concern. However, knowing of the existence of dose-response curves and subthreshold effects and assuming dose additivity in case of similar modes of actions, these effects are not necessarily surprising and should not lead to a general concern about combination toxicity. When it comes to claims of a synergistic interaction, the nature and magnitude of the claimed synergy is frequently not stated. For example, synergy is often viewed as being the production of a toxic mixture effect more severe than would be expected on the basis of the toxicities of the individual components of the mixture. But what is the basis for the size of effect that would be expected? It is certainly not enough to test a mixture of two similar chemicals at doses that would each cause a 10% effect, to observe an effect of 30% and then to claim synergy. Assuming dose addition, the mixture effect might be from 11% to 100% or more, depending on the shape of the dose response, without the need to invoke a synergistic interaction. To expand on this point, the case of two similar chemicals is considered, each tested at a dose that would cause a 10% effect. Each chemical is considered to have a dose response such that twice the dose causing a 10% effect results in a 40% effect. In this case, dose addition means that the mixture would cause an 80% effect. To interpret mixture effects of similar chemicals more elaborate dose-response data are needed, including at effect levels, especially to support claims of synergy.

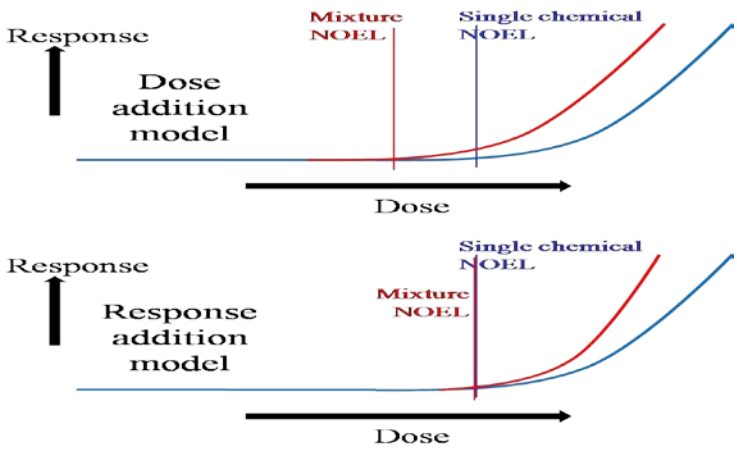
In this report, the Task Force has considered and presented authors' conclusions in the relevant chapters and tables. In addition, the Task Force tried to draw its own conclusions for each of the studies reviewed by inspection of the toxicity data presented by the authors. Firstly, mixture doses are ignored where one or more components were tested at an effect level. Then, it was identified whether or not there was toxicity seen for any of the remaining mixture doses. A comparison of the observed results against expectations was sought as shown below. Using such an approach led to some discrepancies between published conclusions and the conclusions of the Task Force as highlighted in Table 20 and discussed later in this chapter.

- For similarly acting substances, dose / concentration addition is expected. This can result in toxic effects for mixtures where a number of components are close to or at their NOELs. Any significant deviations from this expectation are deemed to be antagonism or synergy.

Sometimes there is not enough information to determine whether the results represent dose addition or an interaction (antagonism or synergy).

- For dissimilarly acting substances, response additivity is expected. Therefore, mixtures where each component is at a NOEL are not expected to produce toxicity. If toxicity is seen, then it will represent either synergy or chance variability.
- For human- or environmentally-relevant concentrations of combinations of chemicals (each present within their individual reference values or health-based maximum concentrations), effects are not expected to be observed, as these concentrations should represent safe human levels.

Figure 3: Expected combination effects: *for similar chemicals, dose addition can result in mixtures that cause effects even when each component is present below a NOEL; for dissimilar chemicals, response addition does not result in mixtures that cause effects when each component is present below a NOEL*



6.1 Studies at environmental concentrations

Studies of real or artificial mixtures representing environmental exposures were reviewed in Chapter 3. These included many high quality long-term studies focusing on exposures via water, food or in one case soil. If each component of these mixtures is present at a safe level, based on single-chemical regulation, then it might be expected that the mixture is without toxic effect, and any evidence of toxicity would be of great interest. In several cases, mixtures were tested that represent contamination in excess of regulatory limits for single chemicals. For this reason, an important part of the interpretation of the studies in Chapter 3 was a comparison between the exposure concentrations and established regulatory limits for single chemicals. As well as testing environmental exposure concentrations (1x), other usually higher multiples of environmental exposure concentrations were also tested (e.g. 100x).

For those studies where the exposures were acceptable or close to acceptable on a single chemical basis, there was no convincing toxicity seen at 1x exposure concentrations (Tables 3 and 4). This includes studies of real drinking water in two mouse teratology studies and a rat developmental toxicity study (see Table 3). Sometimes adaptive effects were seen at 10x or 100x and sometimes toxicity at 100x and higher multiples of environmental exposure concentrations. For example, Jadhav et al (2007a,b) found no toxicity for a mixture of eight metals present at their MPL in water, but found body and organ weight effects at 10x and significant histopathology at 100x. Studies in which polychlorinated organics were fed to immature rats at doses representing those received by infants via human breast milk showed no effect at 1x and 10x doses, limited liver enzyme induction at 100x, and clear liver enzyme effects and liver, adrenal and thymus weight changes at 100x (Desaulniers et al, 2003, for females; Gyorkos et al, 1985, for males). A particular highlight in this category were the NTP studies of pesticide / fertiliser mixtures, representing 1980s groundwater concentrations in two US States, in a set of reproduction and developmental toxicity, immunotoxicity, subchronic toxicity and genotoxicity assays. Across this large battery of well conducted studies there were no convincing treatment-related adverse effects at environmentally relevant concentrations (1x, see Table 3). Increased frequencies of micronuclei in blood (but not in splenocytes) were observed at 10x and 100x in subchronic mouse toxicity studies with mice, without a clear dose response, whilst there were decreased seminal vesicle weights, without effects on sperm parameters, at the 100x concentration dose only.

In mixture studies conducted around the acceptable exposure limit for single chemicals (e.g. Jadhav et al [2007a,b] and the NTP pesticide / fertiliser mixtures, see Tables 3 and 4), the exposures were typically one hundredth of the lowest NOEL for each chemical. Therefore, effects due to single chemicals could be expected to occur at doses of 300x concentrations and higher (or even 100x on occasions as the NOEL does not necessarily represent a dose where there is zero effect). For similar chemicals, dose additivity could result in effects at lower concentrations, depending on the number of chemicals in the mixture. The results of the studies reviewed are consistent with these expectations, and therefore are consistent with what would be expected on the basis of the independent action of chemicals.

For those studies where the exposures were clearly unacceptable on a single chemical basis, toxicity was in some instances seen at 1x exposure concentrations, and at least some effect was always seen at 10x and higher multiples of environmental exposure concentrations (see Table 3). Studies of well-water near a Nigerian landfill and of Nanjing drinking water suggest toxicity at 1x, though the scope of the studies does not permit a full interpretation of the significance of the findings. A study of exposure to soil from the Love Canal waste dump shows clear toxicity (Silkworth et al, 1984). These studies show the importance of procedures to regulate chemical exposures in order to protect human populations. A highlight amongst the studies in this category was the NTP hazardous waste site mixture studies. In this extensive series of rodent studies,

exposures represented contaminated groundwater with concentrations far in excess of single chemical standards. Despite this, there was no convincing treatment-related toxicity at 1x, but toxicity in some studies at 10x and 20x exposure concentrations.

A case that does not fit easily into either of the above two scenarios, i.e. clearly acceptable or unacceptable exposure on a single chemical basis, is the case of the Great Lakes fish mixture studies (Table 5). In this case, the exposure was initially assumed to be acceptable by the standards at the time, but later proved to be unacceptable. In the early 1970s, the severe reproductive toxicity seen in mink was completely unexpected on the basis of the concentrations on known contaminants that could be measured. For this reason, synergistic interaction was a possible explanation that was proposed. After much detailed investigation, it became clear that the fish from the Great Lakes were highly contaminated with PCBs and dioxins, the toxicity of these chemicals was recognised, and it became clear that their additive effects were able to explain the mixture toxicity seen. This case is a famous one and had an important role in stimulating research and better regulation of chemicals in the environment. Such chemicals are now regulated together on the basis of dose additivity. The toxicity of these chemicals to rodents was far less severe than their toxicity to mink. Additionally, unlike rodent, mink are able to eat a diet consisting entirely of fish. Toxicity to mink only occurred at dietary inclusion levels (i.e. fish eaten in g/kg/day) that also provided a good safety margin for people consuming fish.

It might be thought that human epidemiology studies would be an ideal way to study mixture toxicity, but exposures and dose responses are notoriously difficult to quantify for single chemicals in such studies, let alone for multiple chemicals simultaneously. No human epidemiology study passed the strict relevance criteria for this review.

6.2 Studies with mixtures only tested at or near NO(A)ELs

There were 60 papers on studies with mixtures only tested at or near NO(A)ELs that were reviewed in Chapter 4 (in some cases different ratios of the same set of chemicals were tested but these have not been counted as distinct mixtures in deriving this total) either *in vitro* or *in vivo* using rodent models. There were also four papers reporting human studies.

In 18 of the papers the mixtures tested around the NO(A)EL did not produce effects. Since the NO(A)ELs for single chemicals can represent up to a 20% difference from the control response, it is to be expected that a relatively high proportion of mixture studies conducted at or around the NO(A)EL of single compounds will produce effects. In fact, 10 papers demonstrated mixture toxicity which could be explained by additivity, whilst a further 25 papers demonstrated mixture toxicity but the study design and/or data presented did not allow a conclusion to be drawn about the presence or nature of any possible interaction. Specifically, many of the authors did not

describe their expectations and, thus, did not identify whether their data were as expected or otherwise. Coupled with the fact that there was often only a single dose level investigated for the mixture, this meant that often the only conclusion that could be drawn by the Task Force was that there was or was not an effect induced by the mixture in the absence of apparent effects induced by the individual components.

In the remaining 11 papers, deviations from additivity appeared to occur for some of the mixtures tested and this was often termed synergy. The data described by Casey and co-workers[4] in six papers falls under this category; however, deviations from additivity were only a factor of about two. One *in vitro* investigation and two *in vivo* studies are sufficiently robust in their designs and resulting data to allow a conclusion of synergy (effects greater than expected on the basis of concentration addition or response addition). These are:

1. van Birgelen et al (1996) reported a significant accumulation of hepatic porphyrin in animals treated with mixtures of TCDD and PCB 153 (over 800-fold over control levels) in the absence of accumulation for PCB 153 or TCDD alone. An underpinning mechanistic understanding of how this apparently strong synergy might be explained is currently absent, and it is not possible to quantify the magnitude of the reported synergy.
2. Kunz and Fent (2006) observed significant synergistic effects in the yeast oestrogen assay when testing 2-, 4-, and 8-component mixtures of UV-filters. For example, in the case of the 4-component mixtures the observed effects were seen at concentrations at least two orders of magnitude lower than predicted using dose addition.
3. The *in vivo* study detailed in Rider et al (2010) reports, amongst others, results from binary combinations of TCDD and dibutyl phthalate which indicated effects on the developing male reproductive tract and liver pathology which occurred at lower doses than expected on the basis of response addition, i.e. synergy. The authors highlight that these studies were preliminary (having small group sizes) and that for some effects, e.g. liver malformations, they were not elicited by either chemical when dosed alone and therefore require further work to be fully rationalised.

A significant number of studies in Chapter 4 described findings which the authors described as synergy, but there was often insufficient justification for this conclusion, or erroneous assumptions had been made regarding the shape of the dose-response relationships. The conclusion from Chapter 4 is that robust instances of synergy are relatively rare. These few instances are supported by having, at the outset, a clear hypothesis as to what mathematical model (concentration addition or response addition) is most appropriate for the mixture of interest (based on e.g. mode of action), and generating sufficient statistical power to demonstrate any observed interaction is significant.

[4] Casey et al, 2004, 2005; Gennings et al, 2004a ; Moser et al, 2005, 2006; Stork et al, 2006.

The work of Fears and co-workers (Elashoff et al, 1987; Fears et al, 1988, 1989) are interesting, although not strictly relevant for the remit of this Task Force ('low-dose interactions') due to the choice of dose levels investigated. This group studied the effects of binary combinations of rat carcinogens with different target organs following two-year treatment. The objective of this series of studies was to determine if chemical carcinogens acted additively, synergistically or antagonistically to produce tumours. Using the independent action model, synergism was defined as being greater than the expected outcome and antagonism was defined as being less than the expected outcome based on the model. Each compound was investigated at three dose levels and the binary mixtures covered all possible combinations of dose levels and compounds. Unfortunately, and perhaps because the authors were investigating antagonism, the dose levels used for most of the compounds were tumorigenic in the target organ. From a total of 24 possible pairwise combinations, twenty gave no evidence of interactions between the two components, and the remaining four combinations resulted in measurable interactions. Specifically, the combinations of NTA/MNNG (nitriloacetic acid / N-methyl-N′-nitro-N-nitrosoguanidine) and NTA/NBBN (N-butanol-N-butylnitrosamine) resulted in an antagonism of the MNNG-induced stomach tumours and of the NBBN bladder tumours and, when combinations of the liver carcinogens AFLA/DPN (aflatoxin B1 / dipentylnitrosamine) and LAS/CYC (lasiocarpine / cycad flour) were investigated, a synergistic interaction was observed. These studies demonstrate how applying mode of action knowledge to choose the appropriate mathematical model to estimate additivity can reduce the likelihood of inappropriately assuming that mixture effects are due to interaction (be it synergistic or antagonistic).

One peculiarity was a single paper in which a non-monotonic dose response was observed (*in vitro* study using H295R cells and measuring hormone secretion; Ohlsson et al, 2010b). This non-monotonicity could be a peculiar aspect of the cell system used – it is a dynamic system in which cascades of hormone synthesis are involved. In this study the standard predictive models of dose addition and response addition were unable to adequately describe the outcome, a phenomenon which is widely acknowledged, but not yet overcome.

Also reported in Chapter 4 are the few relevant instances the Task Force identified of experimental low-dose mixture studies conducted in humans (Cometto-Muniz et al, 1997, 1999, 2001, 2004). This body of work investigated various multi-component mixtures of alcohols, esters, ketones and alkyl benzenes on olfactory and trigeminal nerve stimulation in respect of establishing thresholds and, in the later studies, also probabilities of detection. Mathematical transformation of the threshold effect in the first study showed that dose addition (partial or complete) was the most frequent response with the exception of eye irritation where synergy was reported for the most lipophilic and most complex mixtures. On the basis of the presented data it was not possible for the Task Force to quantify the magnitude of the synergies reported. In the later experiments, which included full dose responses, complete dose addition adequately predicted mixture effects at lower concentrations (i.e. lower probability of detecting a response in

the test subjects). Where probabilities of detection were higher, both partial and complete dose additions were seen, but no synergy was observed.

6.3 Studies with mixtures tested well below NO(A)ELs

In Chapter 5, studies were reviewed which investigated combinations of chemicals well below their individual NO(A)ELs. A threshold of ≥ factor 10 below the NO(A)EL was defined to be the inclusion criterion in this chapter. Twenty-seven papers were identified and reviewed, which between them tested 41 chemical mixtures. In twelve of the papers the mixtures tested did not produce effects. Eleven papers demonstrated mixture toxicity which could be explained by independent action (dose additivity for similar chemicals and response additivity for dissimilar chemicals). In the remaining four papers, two showed evidence of a synergistic interaction and one of potentiation:

- A mixture of pyrethrin and piperonyl butoxide showed a synergistic effect in the mixture of at least 10-fold and possibly 100-fold on total ATPase in an *in vitro* system (Kakko et al, 2000).
- Two optical isomers of ethoxymethoxymorpholinophosphine oxide showed a 100-fold potentiation of the toxicity of one isomer by competitive inhibition of the detoxifying enzyme by the other isomer in an *in vitro* system (Ashby and Styles, 1980).
- In two *in vivo* studies of the comparative nephrotoxicity of melamine and cyanuric acid, Jacob et al (2011) found a synergistic effect of approximately 10-fold after dietary administration of the combination compared to each chemical individually, whereas the synergistic effect in the oral gavage study of Choi et al (2010) was at least 100-fold.

A common feature of the first two studies is that they can be explained by known toxicological modes of enzyme interactions. As both studies are investigating specific modes of actions *in vitro* which do not necessarily lead to adverse *in vivo* findings, the human relevance of the data is unknown. Kakko et al (2000) point out that PBO is used in pyrethrin formulations precisely because of its potentiating action which is due to its inhibitory effect on the microsomal mixed functional oxidase (MFO) system which is involved in pyrethrin detoxification. The deviation from additivity in this study is therefore an expected effect, based on pharmacokinetic interactions. Such *in vitro* findings of synergy are of great interest; however, it would be extremely valuable to examine these specific effects *in vivo,* enabling their practical relevance to be fully evaluated. A well-known example of a synergistic interaction that has been established *in vivo* is the case of the combined effects of pyrethroids and azoles on honey bees (Pilling et al, 1995); these mixtures therefore fall under strict regulatory measures. It is also worth noting that piperonyl butoxide is included within formulations of pyrethrins and

pyrethroids specifically as a synergist, and that the acute toxicity of each of these formulated products is specifically tested.

Ashby and Styles (1980) postulated that the observed synergy in their mixture of two optical isomers of ethoxymethoxymorpholinophosphine oxide could be explained by one of the isomers being activated to a DNA-reactive species, where the other isomer contributes by acting as a competitive substrate for the enzyme that detoxifies the active isomer. Again, this is a pharmacokinetic interaction based on the similarity of the two isomers.

A direct chemical interaction between two closely related molecules is at the heart of the effect seen in the studies by Jacob et al (2011) and Choi et al (2010). Cyanuric acid is an analogue of melamine and the two molecules form self-assembling, high-molecular weight complexes via organised intramolecular networks of hydrogen bonds and π–π aromatic ring stacking which are excreted as crystals and destroy renal tubular structures.

6.4 Overall synthesis of evidence on low-dose interaction

A number of relevant higher-tier studies on complex mixtures representing relevant drinking water or food concentrations (or multiples thereof) were found. The evaluation of the *in vivo* studies on mixtures of compounds, at human- or environmentally-relevant concentrations, gave no convincing evidence that combined exposures to substances at individual reference dose levels represent a human health concern. Effects were observed in the mixture studies at 1x concentrations only in cases where unacceptable maximum permissible limits or exceeded reference dose levels were dosed in the mixtures (compiled in Chapter 3). This is especially evident when comparing the large NTP studies: Effects in the 1x exposure group were more frequently observed in the chemicals study (representing mixtures near hazardous waste sites), where the individual concentrations were clearly above individual reference values. Whereas in the pesticide mixture studies – in which reference values were only exceeded for aldicarb, but were below reference values for the other compounds – no convincing treatment-related effects were seen.

Table 20 compiles the findings from the tables in Chapters 4[5] and 5, showing cases where mixture toxicities were seen, which are greater than would be expected on the basis of independent action. The division of results between different types of toxicity, and between *in vitro* and *in vivo* studies is shown. (The single case of potentiation is included within the synergy cases in the analysis from here onwards.)

[5] This excludes five papers from Chapter 4 for which there were not sufficient relevant data to tabulate. For this reason the figures shown in Table 20 do not exactly match those shown in Chapter 4.

Table 20: Summary of findings with respect to low-dose interactions

Toxicity	Study type	Number of papers	Number of mixtures tested	Number of mixtures showing low-dose interactions*	Papers including mixtures showing low-dose interactions*
Single dose toxicity	*in vitro*	9	14	0	
	in vivo	7	16	0	
General / Organ toxicity	*in vitro*	2	4	1	Kakko et al, 2000
	in vivo	26	31	3	van Birgelen et al, 1996 Choi et al, 2010 Jacob et al, 2011
Developmental toxicity	*in vivo*	6	6	0	
Endocrine toxicity	*in vitro*	15	28	2	Kunz and Fent, 2006
	in vivo	8	9	1	Rider et al, 2010
Genotoxicity and Carcinogenicity	*in vitro*	6	19	1	Ashby and Styles, 1980
	in vivo	9	12	0	
All	*in vitro*	32	65	4	
	in vivo	56	74	4	
	both	86**	139	8	

* Mixtures showing toxicity that was more than expected based on independent action (i.e. more than additivity in the case of dissimilar chemicals, and more than response addition in the case of dissimilar chemicals), for mixture doses where each component was at or below its NO(A)EL.

** Two papers contained both *in vivo* and *in vitro* components that met the acceptability criterion, hence this total is 86 not 88.

Eight mixtures out of 139 tested showed convincing evidence of synergy, i.e. less than 6%. Since two of these eight mixtures were the same (melamine and cyanuric acid), only seven distinct mixtures demonstrated synergy (5%), of which only three did so in *in vivo* studies. The *in vitro* findings of synergy are very interesting, but it would be extremely valuable to examine these specific effects *in vivo*, to enable their practical relevance to be fully evaluated.

There is no indication that the frequency of synergy differs between different types of toxicity.

6.5 Implications for risk assessment

Like the remainder of this report, this section focuses exclusively on mammalian studies and human risk assessment. The critical question that has been addressed is whether mixture toxicity challenges the current chemical regulatory framework which is based largely on single chemical regulation. In other words, if each chemical is regulated singly then does this result in adequate safety margins when considering exposure to mixtures in the environment? In at least one specific respect it is clear that single chemical regulation can be insufficient, i.e. the case of significant exposure to a large number of similar chemicals. Dose additivity is expected in these

circumstances, which means that exposure to 10 chemicals each at half of their maximum acceptable concentration would only have a safety margin of 20x rather than the 200x that would be expected for each chemical singly. The worst-case scenario for this situation is when considering products which are themselves mixtures of similar chemicals (e.g. commercial PCB mixtures) or which co-occur due to a shared propensity to accumulate in fat (e.g. dioxins and PCBs in fish in the Great Lakes, Table 5). This situation is already addressed in regulation in the case of dioxin-like chemicals by the use of toxicological equivalence factors and dose addition in their risk assessment, or in the case of cumulative residue assessments conducted for pesticides with common modes of action. Commercial products which consist of mixtures are also specifically tested for toxicity in some sectors.

In the case of chemicals that do not bioaccumulate and which are not found together in the same product, there is recent evidence that the toxicity of real environmental mixtures is dominated by often one and rarely more than three chemicals (ECETOC, 2011b). Assuming that each chemical in such a mixture is at an acceptable concentration based on its own toxicity, the toxicity of the mixture would in most cases also be acceptable as long as there is no synergistic interaction. Also the evaluation of the large body of relevant animal studies on complex mixtures at environmentally- or human-relevant exposures gave no evidence for such a safety concern. No effects were observed, when the single substances in the mixtures were present at or below their individual reference dose levels. In animal studies it is possible to test multiple chemicals each at or close to its own NO(A)EL, and many such studies have been undertaken (Chapter 4). It is not surprising to find that in quite a number of cases this resulted in mixtures which produced toxicity. However, in the context of risk assessment it is important to note that equipotent chemical mixtures exist only in the laboratory.

Moving on from dose additivity, the other phenomenon which could compromise a regulatory framework based largely on single chemical regulation is synergy. A very large synergistic interaction would have the potential to compromise risk assessment even when each chemical is well below maximum acceptable exposures. However, after reviewing the evidence for the size of reported synergistic interactions, Boobis et al (2011) found no cases of synergy in which the effect seen exceeded additivity by more than a factor of four. This greatly reduces the potential of synergistic interactions to compromise regulatory safety margins. Our review only identified a single case from relevant *in vivo* studies in which a greater synergy was demonstrated – the special case resulting from the direct chemical reaction between melamine and cyanuric acid.

The review by Boobis et al (2011) was unable to estimate the probability of an interaction being synergistic. Based on our review some attempt can be made to estimate this probability. Our criteria for demonstrating relevant synergy is that a mixture effect was seen that was greater than would be expected on the basis of no chemical interaction – the mixture must also have produced toxicity at doses where the individual components did not produce toxicity. The frequency of

deviations from additivity found was less than 6%. There are numerous factors suggesting that this could be an overestimate (publication bias, chemicals chosen in order to find synergy, findings not independently confirmed) or an underestimate (poorer studies may miss synergy even if it occurs).

The Task Force's findings indicate that chemical interactions are sufficiently uncommon and small, and that a default assumption of non-interaction is reasonable to make when it comes to risk assessment. In other words, dose addition for similar chemicals and response addition for dissimilar chemicals are reasonable default assumptions. The analysis of studies of environmental mixtures also supports this.

For dissimilar chemicals, synergy is presumably the only way that a mixture of such substances can result in toxicity if the individual chemicals are present at or below their zero-effect levels (note this may be below the NO(A)EL). The Task Force's review identified two cases of synergy involving dissimilar chemicals, one *in vitro* (Kakko et al, 2000) and one *in vivo* (van Birgelen et al, 1996).

The DG SANCO Scientific Committee Opinion (SCHER, SCCS, SCENIHR, 2012) indicated that the possibility of synergistic interactions has to be considered on a case-by-case basis using expert judgement. The Task Force believes that the results of this review can help to form a better basis for this expert judgement, by cataloging in a systematic way the synergistic interactions which have been demonstrated to have an impact on risk assessment. Many assume that synergy is likely to occur when one chemical affects the degradation of another, for example. But there are very few cases where it has been demonstrated that this matters, i.e. where it has been demonstrated to result in a reduction of NO(A)ELs and so have an impact on risk assessment. In the Task Force's review, only seven such papers were found, most of which focused on specific modes of interaction, potentially leading to highly increased effects (Kakko et al, 2000; Ashby and Styles, 1980; Choi et al, 2010; Jacob et al, 2011; van Birgelen et al, 1996; Kunz and Fent, 2006; Rider et al, 2010). It would be valuable to have a broader discussion to infer rules which could be used to anticipate cases of synergy. The work of this Task Force can contribute valuable information to inform this discussion, which would be of great use to risk assessors. In this way science and risk assessment practice can improve over time.

The studies reviewed did not show evidence of a different pattern of combination effects according to the type of toxicity examined, for example acute toxicity, organ toxicity, developmental toxicity, endocrine toxicity or carcinogenicity. Deviations from additivity did not seem to be any more prevalent in any one of these domains. Endocrine toxicity is sometimes said to represent a special case in regard to synergy at low doses, but no evidence of this was found.

The terms 'similarly' and 'dissimilarly' acting chemicals have been used in this report, but have not been defined. This is because this consideration was outside the remit of this Task Force. This is an active field of investigation and debate, for which better information on the mode of action of less well-studied chemicals is particularly important (SCHER, SCCS, SCENIHR, 2012). Likewise other aspects of the risk assessment of mixtures are also beyond the remit of the Task Force. The reader is advised to consult the DG SANCO Scientific Committee Opinion (SCHER, SCCS, SCENIHR, 2012) and the report of the 'Workshop on Combined Exposure to Chemicals' (ECETOC, 2011a) for a perspective on these broader issues. In addition there is a recent relevant review for EFSA on specific aspects (Kortenkamp et al, 2012). The ongoing EU project 'ACROPOLIS'[6] is also highly relevant in the field of chemical mixture risk assessment.

6.6 Data gaps

When the Task Force started its work, it wondered how many studies would meet its strict inclusion criteria. Many good studies had to be excluded, usually because one or more chemicals resulted in toxicity at the mixture dose tested. This happened even in several studies which seem to have been designed to target no effect level doses (e.g. Hass et al, 2007), which may reflect study-to-study variability, or in some cases the fact that the NOEL for single chemicals was determined on the basis of different endpoints from those used in the mixture study (e.g. Lehraikia et al, 2011). Despite this, the Task Force was pleasantly surprised to find more studies meeting its inclusion criteria than expected, though many of them were suboptimal for the purpose (e.g. only examining a single dose around the NOEL).

Ideal studies from the point of view of this review would:

- Include a good definition of the dose response of each component and of the mixture, in the same test system using the same endpoints. The response to each component and to the mixture would be characterised at several doses around their own no effect level and into effect levels. This implies approximately equipotent dosing, and dose spacing that is relatively close.
- Either includes a wide range of endpoints, or if the critical adverse effects are already well known then these would be specifically targeted.
- Have a clear hypothesis in terms of the choice of chemicals studied (e.g. similar or dissimilar), and compare the results observed to a default assumption of dose additivity in the case of similar chemicals or response addition for dissimilar chemicals.
- Adequate group size and other usual aspects of good study quality.
- Apply mathematical dose-response modelling to examine whether the hypotheses of interaction were met, including consideration of confidence intervals.

[6] http://www.acropolis-eu.com/

The Task Force is aware of some further studies which are underway which may meet these criteria. Under Cefic-LRI for example, there is one study ongoing, which tests three compounds with a known anti-androgenic mode of action in a generation toxicity study at effect levels, no-adverse-effect-levels and at a reference value (www.cefic-lri.org). Investigated parameters are classical parameters of a reproduction toxicity study (e.g. organ weights, sexual maturation, fertility) but also subthreshold parameters such as metabolomics and transcriptomics. At authority level, a 28-day combination study is ongoing, where different azoles are combined at different dose levels (NOAEL/100, NOAEL/10, NOAEL, 3xNOAEL, 10xNOAEL). In this study classical toxicological and more sensitive parameters (RNA, gene expression, hormones) are being investigated (personal communication M. Wilks, 2012).

There are a number of classic and widely accepted cases of chemical synergy. However, in most cases these synergies have only been studied and demonstrated at effect levels. It would be valuable to examine some of these cases at lower doses in the way described here. Such cases include the interaction of carbon tetrachloride and alcohols in the case of liver toxicity, the interaction between organophosphorous insecticides and their metabolic inhibitors in the case of cholinesterase inhibition, and many classic drug-drug interactions. It is of course natural that the focus in studies of drug-drug interactions is on effect levels, but studies at no effect levels of single drugs would help to shed light on the broader issue of chemical interactions. The Task Force could find no evidence that robustly tests whether these classic cases result in toxicity at doses which are without effects on a single chemical basis, or whether synergy is restricted to the region of effect levels. Thresholds of interactions were discussed, e.g. by Yang and Dennison (2007), but the body of available experimental studies to examine the hypotheses is still small.

6.7 Concluding remarks

Chemical regulation is mainly based on single substances, but exposure is to complex mixtures, which raises the question of whether the regulatory framework is adequate and protective. A thorough literature review was conducted, focusing specifically on where a mixture has been tested at doses which are at or below the NO(A)EL for every component of the mixture. An additional literature review analysed studies in which real or artificial mixtures representing environmental exposures were tested, e.g. tap water.

For studies conducted at or close to the NO(A)EL of each component, toxicity was often seen, which is to be expected on the basis of dose additivity and also considering that a small size of effect for a single chemical can already occur at the NO(A)EL. It should also be noted that the toxicity of real mixtures is often dominated by very few components, whilst equipotent chemical mixtures are found only in the laboratory. Across all the papers reviewed, mixtures producing more toxicity than expected on the basis of independent action were the exception rather than the

rule and the deviation from expectation was in several cases small in magnitude. A few specific cases of synergistic interaction were identified. Small deviations from independent action probably represent natural variability rather than a reproducible deviation. There was no evidence that deviations from independent action were associated with specific types of toxicity, e.g. endocrine effects. Studies in which environmental exposures were tested were consistent with these findings.

Independent action of chemicals was found to be the predominant principle of mixture toxicity, so a default assumption of dose additivity for similarly acting chemicals and response addition for dissimilarly acting chemicals is reasonable. In reality, human exposure will be to complex mixtures of similar and dissimilar chemicals, many without mode of action data that could inform chemical grouping considerations. For this reason, dose addition is a conservative default assumption that could be made in the absence of better information, as part of a tiered assessment process. Synergistic interaction is an exception, and it is hoped that this review can contribute to an improved understanding of when this might be anticipated, for example by identifying demonstrated instances of synergy. This review indicates that prioritisation of mixtures for risk assessment should focus on the presence of multiple similar chemicals each close to their acceptable limit and on situations were synergy can be anticipated. Studies of real or simulated environmental mixtures indicate that effects are seen only in those cases where single chemicals were included in the mixtures at unacceptable concentrations (e.g. above maximum allowable health-based drinking water concentrations). Reduced safety margins generally resulted from situations in which single-chemical regulations were not enforced. Based on our evaluation, there is no evidence that exposure to complex mixtures of components, each well regulated according to established risk assessment approaches (reference value settings, maximum allowable concentrations in drinking water), would pose a health risk to humans.

ABBREVIATIONS

2,4,5-HBB	2,4,5-hexabromobiphenyl
AChE	Acetylcholinesterase
ACP	Acid phosphatase
ADI	Acceptable daily intake
AFLA	Aflatoxin B1
AGD	Anogenital distance
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AR	Androgen receptor
AST	Aspartate transaminase
ATSDR	(US) Agency for Toxic Substances and Disease Registry
ATPase	Adenosine Triphosphatase
BaP	Benzo[a]pyrene
BBP	Benzyl butyl phthalate
BC10	Concentration resulting in 10% basal activity
BDCM	Bromodichloromethane
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BHA	Butylated hydroxyanisole
BHK	Baby hamster kidney
BrdU	Bromodeoxyuridine
BROD	Benzyloxyresorufin-*O*-dealkylase
BUN	Blood urea nitrogen
bw	Body weight
CA	Concentration addition
CA	Cyanuric acid
CAG	Cumulative assessment group
CAS	Chemical Abstracts Service
$CC1_4$	Carbontetrachloride
$CdCl_2$	Cadmium chloride
CHA	Chronic health advisory
$CHCl_3$	Chloroform
ChE	Cholinesterase
CHO	Chinese hamster ovarian (cells)
CoT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CREAT	Creatinine

CT	Combined treatment
CYC	Cycad flour
CYP	Cytochrome P450
DBCP	Dibromochloropropane
DBP	Dibutyl phthalate
DCBP	2,5-dichloro-4‘-biphenylol
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethylhexyl phthalate
DEN	Diethylnitrosamine
DG SANCO	Health and Consumers Directorate General
DiBP	Diisobutyl phthalate
DIELD	Dieldrin
DiHP	Diisohexyl phthalate
DNA	Deoxyribonucleic acid
DPN	Dipentylnitrosamine
DPP	Dipentyl phthalate
EC	Effect concentration
ED	Endocrine disrupter
EFSA	European Food Safety Authority
EPA	(US) Environmental Protection Agency
ER	Oestrogen receptor
EROD	Ethoxyresorufin-*O*-dealkylase
ETU	Ethylenethiourea
EU	European Union
F_1	First generation of offspring
F_2	Second generation of offspring
GC-MS	Gas chromatography-mass spectrometry
GD	Gestation day
Glu-P-1	2-Amino-6-methydipyrido[1,2-α:3',2'-*δ*]imidazole
Glu-P-2	2-Aminodipyrido[1,2-α:3',2'- *δ*]imidazole
GST-P	Placental glutathione-*S*-transferase
HBB	Hexabromobiphenyl
HCB	Hexachlorobiphenyl
HCBD	Hexachloro-1:3-butadiene
HCl	Hydrochloric acid
HEPT	Heptachlor

HPRT	Hypoxanthine phosphoribosyltransferase
IA	Independent action
IC	Inhibiting concentration
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
IPCS	International Programme on Chemical Safety
IQ	2-Amino-3-methylimidazo[4,5*f*]quinoline
IRIS	Integrated Risk Information System
$KBrO_3$	Potassium bromate
LABC (muscle)	Levator ani-bulbocavernous (muscle)
LAS	Lasiocarpine
LD1	Dose resulting in 1% lethality
LDH	Lactate dehydrogenase
LH	Luteinising hormone
LLBM	Little Lake Butte des Morts
LOAEL	Lowest observed adverse effect level
LOEL	Lowest observed effect level
LONEL	Lowest observed nephrotoxic effect level
LRI	Cefic’s Long-range Research Initiative
MC	Binary mixture of MEL and CA
MCA	3-methylcholanthrene
MCL	Maximum contaminant level
MCV	Mean corpuscular volume
MeHg	Methylmercury
MEHP	Monoethylhexylphthalate
MeIQ	2-Amino-3,4-dimethylimidazo[4,5*f*]quinoline
MeIQx	2-Amino-3,8-dimethylimidazo[4,5*f*]quinoxaline
MEL	Melamine
MEL-CA	Melamine-cyanuric acid
METH	Methiocarb
MFO	Mixed functional oxidase
MNEL	Minimum nephrotoxic effect level
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MOA	Mode of action
MOAEL	Minimum observed adverse effect level
MPL	Maximum permissible level / limit
MRL	Minimum risk level

mRNA	Messenger RNA
MTD	Maximum tolerable dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
$NaNO_2$	Sodium nitrite
NBBN	N-butanol-*N*-butylnitrosamine
n.d.	Non detectable
NGO	Non-governmental organisation
NIEHS	National Institute of Environmental Health Sciences
NNEL	No nephrotoxic effect level
NO(A)EC	No observed (adverse) effect concentration
NO(A)EL	No observed (adverse) effect level
NONEL	No observed nephrotoxic effect level
NP	Nonyl phenol
NTA	Nitrilotriacetic acid
NTP	(US) National Toxicology Program
OCDF	Octachlorodibenzofuran
OECD	Organisation for Economic Co-Operation and Development
OP	4-tert-octylphenol
OPPTS	(US EPA) Office of Prevention, Pesticides and Toxic Substances
PAH	Polycyclic aromatic hydrocarbon
PBO	Piperonyl butoxide
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PHAH	Polyhalogenated aromatic hydrocarbon
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5*b*]pyridine
PLN	Phospholamban
Pmix	Pesticide mix
PND	Post-natal day
p.o.	*per os* (administration)
PR	Progesteron receptor
PROD	Pentoxyresorufin-*O*-dealkylase
RA	Retinoic acid
RBC	Red blood cell
RfD	Reference dose
RNA	Ribonucleic acid

SAT	Sex accessory tissues
s.c.	Sub-cutaneous
SCCS	Scientific Committee on Consumer Safety
SCE	Sister chromatic exchange
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SDWR	Secondary Drinking Water Regulation
T3	Triiodothyronine
T4	Thyroxine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCDD	Tetrachlorodibenzodioxin
TCDF	Tetrachlorodibenzofuran
TCE	Trichloroethylene
TD	Tumorigenic dose
TDI	Tolerable daily intake
TLC	TCE, lead, chloroform
TNT	2,4,6-trinitrotoluene
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Trp-P-1	3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
TSH	Thyroid stimulating hormone
TU	Toxicity unit
UDPGT	UDP-glucuronyltransferase
UV	Ultra violet
WBC	White blood cell
WHO	World Health Organisation

List of chemicals abbreviated in Tables 12, 15, 16, 17, 18 and 19

2-AAF	2-acetylaminofluorene
2,4-D	2,4-dichlorophenoxyacetic aid
2DHB	2,4-dihydroxybenzophenone
3BC	3-benzylidine camphor
4DHB	4,4'-dihydroxybenzophenone
4-MBC	3-(4-methylbenzylidene) camphor
ACE	Acephate
ACTLD	Acetanilide
AlaCl	Alachlor
AMI	Amitraz
API	Apigenin
ATRA	Atrazine
β-HCH	Beta hexachlorocyclohexane
BaP	Benzo[a]pyrene
BBP	Benzyl butyl phthalate
BC	Bendicarb
BCM	Carbendazim
BHA	Butylated hydroxyanisole
BHP	Benzyl-4-hydroxyparabene
BMF	Butamifos
BP-1	Benzophenone-1
BP-2	Benzophenone-2
BP-3	Benzophenone-3
BPA	Bisphenol A
BS	Bensulide
BS	Benzyl salicylate
BTZ	Bentazone
CA	Cyanuric acid
CAP	Captafol
CARB	Carbaryl
CARBO	Carbofuran
CB	Chlorobenzilate
CBP	4-chloro-4'-biphenylol
CF	Clofentezine
CLOF	Clofentezine
CM	Chinomethionat
CP	Chlorpropham
CPF	Chlorpyrifos
CPP	Chlorpyriphos
CREAT	Creatinine
CVP	Chlorfenvinphos
CYF	Cyfluthrin
CYH	Cyhalothrin
CYP	Cypermethrin
DBCP	Dibromochloropropane
DBP	Dibutyl phthalate
DCBP	2,5-dichloro-4'-biphenylol
DClB	Dichlobenil
DClV	Dichlorvos
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DDVP	Dimethoate
DEHP	Diethylhexyl phthalate
DELTA	Deltamethrin
DEN	Diethylnitrosamine
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DFB	Diflubenzuron
DIA	Diazinon
DIAD	Diadzein
DiBP	Diisobutyl phthalate
DICO	Dicofol
DIELD	Dieldrin
DiHP	Diisohexyl phthalate
DIM	Dimethoate
DMTP	Methidathion
DPN	2,3-bis(4-hydroxyphenyl)-propionitrile)
DPP	Dipentyl phthalate
E2	Oestradiol-17ß
EDDP	Edifenphos
EE	Ethynyloestradiol
EMMPO	Ethoxy methoxy morpholino phosphine oxide
ENDO	Endosulfan
EPOX	Epoxiconazole
Et-PABA	Ethyl-4-aminobenzoate
ETF	Etrimfos
FBO	Fenbutanatin oxide
FEN	Fenarimol
FL	Flutolanil
FLUC	Flucythrinate
Foset	Foset
FT	Fenitrothion
FV	Fenvalerate
GEN	Genistein
GGT	Gamma-glutamyl transpeptidase
GLYPH	Glyphosate

HCB	Hexachlorobiphenyl
HCH	ß-hexachlorocyclohexane
HEPT	Heptachlor
IBP	Iprobenfos
ID	Prodione
IMA	Imazalil
IX	Isoxathion
KETO	Ketoconazole
KSM	Kasugamycin
LH	Luteinising hormone
LIN	Linuron
MALA	Malathion
MAN	Mancozeb
MB	Metribuzin
MC	Binary mixture of MEL and CA
MCA	3-methylcholanthrene
MCl	Mepiquat chloride
MEFO	Mefolachlor
MeHg	Methyl mercury
MEHP	Monoethylhexylphthalate
MEL	Melamine
META	Metalaxyl
METH	Methiocarb
METO	Metolachlor
MN	Maneb
MXC	Methoxychlor
MYCLO	Myclobutanil
NP	Nonylphenol
OH-PCB-61	2,3,4,5 tetrachloro-4'-biphenylol
OMC	Octyl methoxy cinnamate
OP	4-tert-octylphenol
o,p-DDT	o,p'-dichlorodiphenyltrichloroethane
Oxamyl	Oxamyl
PBO	Piperonyl butoxide
PCP	Pentachlorophenol
PCZ	Propiconazole
PDM	Pendimethalin
PF	Pyrifenox or pyraclofos
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5*b*]pyridine
PHOS	Phosmet
PM	Permethrin
PO	Polyoxin
PPC	Propiconazole
PPHM	Pirimiphosmethyl
PPX	Propoxur
PRO	Prochloraz
PROC	Procymidone
PTP	Prothiophos
PS	Phenyl salicylate
PYR	Pyrethrin
QC	Quinclorac
RMB	Resorcinol monobenzoate
ROTE	Rotenone
Salol	Phenyl salicylate
SDH	Sorbitol dehydrogenase
SIMA	Simazin
SXD	Sethoxidim
TBC	Thiobencarb
TCB	2,3,4,5-tetrachlorobiphenyl
TCDD	Tetrachlorodibenzodioxin
TCF	Trichlorfon
TClF	Trichlorfon
TDF	Triadimefon
TDM	Triadimefon
TEBU	Tebuconazole
TF	Trifluralin
TFM	Tolclofos-methyl
TNT	Trinitrotoluene
TOXA	Toxaphene
TRIB	Tribenuron-methyl
TriCB	2,3,4-trichlorobiphenyl
VINC	Vinclozolin
VT	Vamidothion
XE	Xenoestrogens
Zineb	Zineb

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APPENDIX A: SUMMARY OF KEY REFERENCES

For transparency and ease of reference this Appendix lists those mixture toxicity papers identified in three recent major publications, and states their position in relation to the review presented in this report. The three recent documents concerned are:

- The review of synergistic interactions by Boobis et al (2011). The six key papers identified in this review are included in the following table.
- The DG SANCO Scientific Committee Opinion (SCHER, SCCS, SCENIHR, 2012). All mixture studies cited are included in the following table.
- A report from Kortenkamp et al (2012). All studies identified as being both for dissimilar chemical mixtures and at low doses (so called 'dual relevance' in the report, Section 10.3 and Table 7 in that publication) are included in the following table.

Table A1: Recently published major articles and key references cited therein

Reference (cited in one or more of these reviews)	Boobis et al (2011) Synergy review Key papers	DG SANCO Scientific Committee Opinion (SCHER, SCCS, SCENIHR, 2012)	Kortenkamp et al (2012) 'Dual relevance' papers	Status in our review
Altenburger et al, 1996		yes		Not relevant. A study in a non-mammalian test system
Broderius et al, 2005			yes	Not relevant. A study in a non-mammalian test system
Broerse and van Gestel, 2010			yes	Not relevant. A study in a non-mammalian test system
Charles et al, 2002a		yes		Not relevant. Mixtures were tested around but not below NO(A)EL for each component
Charles et al, 2007		yes		Relevant
Chen et al, 2004	yes	yes		Not relevant. Epidemiological investigation, chemical exposure in combination with cigarette smoking
Crofton et al, 2005	yes	yes	yes	Relevant (relates to Gennings et al, 2007)
Eustache et al, 2009			yes	Not relevant. Mixture was not tested at or below the NO(A)EL for every component
Faust et al, 1994		yes		Not relevant. A study in a non-mammalian test system
Faust et al, 2003			yes	Not relevant. A study in a non-mammalian test system
Groten et al, 1997			yes	Relevant
Hass et al, 2007		yes		Not relevant. Mixture was not tested at or below the NO(A)EL for every component
Hermens et al, 1985		yes	yes	Not relevant. A study in a non-mammalian test system
Ito et al, 1995			yes	Relevant
Jobling et al, 2009		yes		Not relevant. A study in a non-mammalian test system

Table A1: Recently published major articles and key references cited therein

Reference (cited in one or more of these reviews)	Boobis et al (2011) Synergy review Key papers	DG SANCO Scientific Committee Opinion (SCHER, SCCS, SCENIHR, 2012)	Kortenkamp et al (2012) 'Dual relevance' papers	Status in our review
Jonker et al, 1990			yes	Relevant
Kacham et al, 2006		yes		Not relevant. Mixture was not tested at or below the NO(A)EL for every component
Karanth et al, 2001		yes		Not relevant. Individual substances tested at 1x, 0.75x, and 0.5x LD1 doses. Mixtures all assessed at effect doses of the individual substances. Also considered sequential exposures
Karanth et al, 2004		yes		Not relevant. Reports only sequential or concurrent exposures to the two OP insecticides, with no single exposure data
Korsak et al, 1988	yes	yes		Not relevant. Mixture was not tested at or below the NO(A)EL for every component
Korsak et al, 1992		yes		Not relevant. Paper focuses on neurophysiological effects. Lowest tested mixture was 50 ppm xylene + 50 ppm toluene. DNELs for both are 100 ppm, based on a NOAEC for neurophysiological effects of 26 ppm. Therefore, 50 ppm is not low dose
Laetz et al, 2009		yes		Not relevant. A study in a non-mammalian test system
Merino-Garcia et al, 2003			yes	Not relevant. A study in a non-mammalian test system
Meyer et al, 1994	yes	yes		Not relevant. Primary paper on mouse skin tumour co-promotion by mirex and TPA. Mixture was not tested at or below NO(A)EL. NO(A)EL not evaluated
Moser et al, 2005	yes	yes		Relevant
Moser et al, 2006	yes	yes		Relevant
Nesnow et al, 1998		yes		Not relevant. Mixture was not tested at or below the NO(A)EL for every component
Pavlaki et al, 2011			yes	Not relevant. A study in a non-mammalian test system
Payne et al, 2001		yes		Relevant
Rajapakse et al, 2004		yes		Not relevant. Not designed to look at mixture effects in the low-dose region. At higher doses, reported subdose additive effects in mixtures containing NP and OP, hypothesised to be due to toxicity
Tichy et al, 2002		yes		Not relevant. A study in a non-mammalian test system
Wade et al, 2002b			yes	Relevant
Walker et al, 2005		yes		Not relevant. Lowest dose of TCDD had clear effects on the liver, see TR-521
Walter et al, 2002		yes	yes	Not relevant. A study in a non-mammalian test system
Wang et al, 2009			yes	Not relevant. A study in a non-mammalian test system

APPENDIX B: EVALUATION OF PRIMARY REFERENCES CONSIDERED RELEVANT FOR THIS REPORT

Summaries of the evaluations of these publications are on the CD attached to this Technical Report.

APPENDIX C: PRIMARY REFERENCES CONSIDERED NOT RELEVANT FOR THIS REPORT

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Aguilar et al, 1997	Mixture was not tested at or below the NO(A)EL for every component
Al-Omar et al, 2000	Mixture was not tested at or below the NO(A)EL for every component
Altenburger et al, 2000	Good study, but environmental endpoints rather than human health. Fulfills expectations of dose additivity for similar MOA
Anand et al, 2005	Mixture was not tested at or below the NO(A)EL for every component
Andrews et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Arcaro et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Arnold et al, 1996	Paper later withdrawn. Story described in Carpy et al (2000) review
Arnold et al, 1997	Falsified results; paper was later withdrawn
Aubé et al, 2011	Only mixture was tested. No information on individual components
Axelrad et al, 2002	Mixture was not tested at or below the NO(A)EL for every component
Aylsworth et al, 1989	Mixture was not tested at or below the NO(A)EL for every component
Backhaus et al, 2000	Good study, but environmental endpoints rather than human health. Fulfills expectations of dose additivity for similar MOA
Bae et al, 2001	Single chemical data is not presented in a way such that NOELs can be determined
Bannister and Safe, 1987	No NO(A)EL-mixtures tested
Bannister et al, 1989	No NO(A)EL-mixtures tested
Basketter et al, 2001	Vehicle plus test chemical tested, not mixtures of test chemicals
Batra et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Beckman and Nordenson, 1986	Mixture was not tested at or below the NO(A)EL for every component
Belz et al, 2008	Study was on plant species only
Bemis and Seegal, 1999	Mixture was not tested at or below the NO(A)EL for every component
Benachour et al, 2007	Data presented were inconsistent between the various experiments described (see Fig 2 vs. Fig 9 where 500 µM of each component gave certain % aromatase inhibition in first experiment but in second experiment (dose response), the % inhibitions were significantly different). Thus the data presented for combinations are strongly questioned, particularly when the authors claim synergy, which is more likely due to cytotoxicity. In addition, where synergy is claimed there is already at least 25% inhibition of aromatase with the individual compounds on their own
Bennett et al, 1996	No indication is given as to where on the dose response each of the components lies. For the cytochrome oxydase activity there are already reductions (statistical significance for sodium azide in the synoptic membrane sample) observed with the individual components
Berenblum, 1929	Not at NOEL
Berger et al, 1987	No NO(A)ELs established for individual compounds for several compounds and target organs. Lowest dose of individual compounds not tested individually. Curious effect on haematopoietic system at lowest combination dose
Bertazzi et al, 1993	Single substance only, no human exposure data
Bessi et al, 1995	Mixture was not tested at or below the NO(A)EL for every component in one study. In another study, one component was not tested at the dose contained in the mixture

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Bianchi-Santamaria et al, 1997	Poor reporting makes it difficult to draw conclusions
Biegel et al, 1989a	No NO(A)EL mixtures tested
Biegel et al, 1989b	Mixture was not tested at or below the NO(A)EL for every component
Birnbaum et al, 1986	Mixture was not tested at or below the NO(A)EL for every component
Birnbaum et al, 1989	Mixture was not tested at or below the NO(A)EL for every component
Bishayi and Sengupta, 2006	No information provided on the selection of concentrations of the compounds; however from the primary references given in the experimental design it appears that all of them are at effect levels
Borch et al, 2004	Paper not relevant. Individual components gave effects on foetal hormone production. Dose levels were high. No indication of NO(A)ELs for any of the compounds. Insufficient dose levels used (at best two were used for DEHP)
Bosgra et al, 2009	Mixture was not tested at or below the NO(A)EL for every component
Boyd et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Bull et al, 2004	Mixture was not tested at or below the NO(A)EL for every component
Burrows and Way, 1979	Based on mortality and LD50s only
Cannon et al, 1994	Based on LD50s only
Cao et al, 2011	No primary toxicity data presented
Carfagna et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Carlson, 1975	Mixture was not tested at or below the NO(A)EL for every component
Carpentieri et al, 1988	Study on nutrients and their deficiencies
Cassee and Feron, 1994	Mixture was not tested at or below the NO(A)EL for every component
Cassee et al, 1994	Abstract only
Cassee et al, 1996a	Mixture was not tested at or below the NO(A)EL for every component
Cassee et al, 1996b	Mixture was not tested at or below the NO(A)EL for every component
Cassee et al, 1996c	Mixture was not tested at or below the NO(A)EL for every component
Cassidy et al, 1994	Only chlordane was dosed. But measured were 4 isomers and 2 active metabolites. Not relevant, but raises an interesting issue
Cavalieri et al, 1983	Not relevant as all combinations are above individual NOEL
Cavallo et al, 2006	No dose response. Comparison between 'exposed' and 'not-exposed'. However, non-exposed smokers have the same level of metabolite in urine as the exposed group, but show no effect in the comet assay. Likely that other factors are responsible for the effects in the exposed population
Cavieres et al, 2002	Unreliable paper, as explained in uncontested letters to the editor in the same journal (Env Health Persp. 2003. 111:A450-451)
Chandra et al, 1981	Mixture was not tested at or below the NO(A)EL for every component
Chapman et al, 1994	Neither low dose nor robust interaction assessments
Charles et al, 2002a	Mixtures tested around but not below NO(A)EL for each component
Charles et al, 2002b	Mixtures tested around but not below NO(A)EL for each component
Chaturvedi et al, 1991	Mixture was not tested at or below the NO(A)EL for every component
Chaturvedi, 1993	No single chemical data and no other basis for interpreting the mixture results, e.g. it is not based on a real mixture
Cheever et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Chen and Eastmond, 1995	Only combination of effect doses was tested

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Chen et al, 1990	No data, only theoretical / statistical considerations
Chen et al, 2004	Epidemiological investigation, chemical exposure in combination with cigarette smoking, evaluation based on abstract only
Chen et al, 2009	Only mixtures were dosed
Chmielnicka et al, 1988	Kinetic endpoints only
Christiansen et al, 2008	No combinations at NO(A)EL or below the NO(A)EL tested. Mixture contained chemicals at effect levels for nipple retention, epididymides weights, bulbourethral glands weights and dysgenesis of external reproductive organs
Christiansen et al, 2009	In the mixtures tested finasteride was always at an effect level for nipple retention, the most sensitive parameter
Christie, 1989	Mixture was not tested at or below the NO(A)EL for every component
Chu et al, 1980	Mixture was not tested at or below the NO(A)EL for every component
Chu et al, 1986	Mixture was not tested at or below the NO(A)EL for every component
Cianflone et al, 1980	Kinetic endpoints only
Cifuentes et al, 2009	No mixture testing
Coccini et al, 2011	No overall NO(A)ELS established, only NO(A)ELs or LO(A)ELs for certain tissues
Coffey et al, 2005	Presents data from Gordon et al (2006) paper
Colborn, 2004	No mixture testing
Connell et al, 1985	Biological endpoints plus lethally infected animals as point of departure
Cornish and Adefuin, 1967	Although there was a NOEL for CCl_4 for the main endpoint of the study, the paper says that this dose regime caused liver pathology
Cumberbatch et al, 1993	Mixture was not tested at or below the NO(A)EL for every component
Cunnane, 1981	Zinc deficiency was studied
Dalgaard et al, 2000	Mixture was not tested at or below the NO(A)EL for every component
Davis and Safe, 1989	No NO(A)EL-mixtures tested
de Burbure et al, 2006	Observational epidemiology study, complex interactions, no NO(A)ELs established
de Jongh et al, 1995	Mixture was not tested at or below the NO(A)EL for either component
DeMarini et al, 1993	Complex mixtures only, e.g. coal tar
Dhawan et al, 1995	Only lead was dosed, not mixtures. Kinetics only
Diaz-Barriga et al, 1990	Lethal doses only
Diel et al, 2006	Only levels above NO(A)EL were studied
Diggle and Gage, 1955	Mixture was not tested at or below the NO(A)EL for every component
Dobrev et al, 2001	Kinetic endpoints only
Dobson et al, 2008	No NO(A)EL was established for single compounds or mixtures in the *in vivo* studies
Dolara et al, 1992	Would be relevant except that NOELs are not presented for the single components
Dolara et al, 1994	*In vitro* tests of environmental mixtures
Donnelly et al, 2004	No NO(A)EL mixtures tested
Drake et al, 2009	Only effect levels were tested
Eide, 1996	Single components not tested, and only kinetics shown

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Eide and Johnsen, 1998	No controls
El-Masri et al, 1996	Modelling only
El-Masri et al, 2004	Modelling only
Elsenhans et al, 1987	Kinetics only
El-Sisi et al, 1993	Mixture was not tested at or below the NO(A)EL for every component
Eroschenko and Johnson, 2000	Doses used in combination did represent LOAEL doses for detectable histological alterations *in uteri* and vaginas; no low-dose level was evaluated
Eustache et al, 2009	Individual components of the mixtures often gave similar effects of the same magnitude as those observed in the corresponding mixture
Farant and Wigfield, 1990	Mixture was not tested at or below the NO(A)EL for every component
Faroon and Mehendale, 1990	Mixture was not tested at or below the NO(A)EL for every component
Faroon et al, 1991	Mixture was not tested at or below the NO(A)EL for every component
Faustman-Watts et al, 1985	Mixture was not tested at or below the NO(A)EL for every component
Ferm, 1969	Mixture was not tested at or below the NO(A)EL for every component
Finelli and El-Gazzar, 1977	Mixture was not tested at or below the NO(A)EL for every component
Flora et al, 1991	Mixture was not tested at or below the NO(A)EL for every component
Folland et al, 1976	Human epidemiology study. Insufficient dose-response information etc.
Fowler and Mahaffey, 1978	No NO(A)EL-mixtures tested
Fowler et al, 2008	Only tested effect of couple of chemical mixtures present in sewage sludge applied to pasture. No single substance data presented
Frawley et al, 1957	Only mortality was considered whilst other, more appropriate and more sensitive endpoints such as cholinesterase inhibition, were not addressed
Frawley et al, 1963	Only test substance was a mixture
Frische et al, 2009	Yeast screen with relatively high concentrations of non-oestrogenic test compounds. Looking at interplay between cytotoxicity and interference with oestrogenic effect of 17B-oestradiol
Fujii and Nakatsuka, 1983	Mixture was not tested at or below the NO(A)EL for every component
Fukayama et al, 1999	No testing of individual fragrance raw materials to establish dose response or NO(A)EL, no reference to NO(A)ELs from other sources
Fukuda et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Fukushima et al, 1988a	Mixture was not tested at or below the NO(A)EL for every component
Fukushima et al, 1988b	Mixture was not tested at or below the NO(A)EL for every component
Fukushima et al, 2003	Not at NOEL
Gaido et al, 1997b	No mixtures tested
Gao et al, 2000	Mixture was not tested at or below the NO(A)EL for every component
Gao et al, 2003	Lipopolysaccharide was one of the two compounds tested, i.e. outside the scope of the Task Force
Gardner et al, 1977	Sequential administration of agents
Gaughan et al, 1980	Based on LD50/IC50s only, i.e. no NOELs
Gelzleichter et al, 1992	Mixture was not tested at or below the NO(A)EL for every component
Gennings, 1995	A secondary source only
Gennings, 1996	A secondary source only

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Gennings et al, 2002	Experiment seems suitable, but the data are not presented in a way that enables an analysis within the scope of the Task Force
Gennings et al, 2004a	Secondary publication only; statistical re-evaluation of Moser et al (2005) data
Gennings et al, 2004b	DDT was only tested singly at effect levels, therefore there is no evidence that the mixture was at a NOEL for DDT
Gennings et al, 2007	Secondary publication only; statistical re-evaluation of Crofton et al (2005) data
Gessner and Cabana, 1970	Only ED50s are presented
Goldoni et al, 2008	Only one concentration of PCB was used throughout the experiment and in combinations with methyl-mercury. Whereas for methyl-mercury the lowest concentration was a NOEC, the PCB concentration used caused a reduction of cell viability, i.e. combinations of the compounds at their respective NOECs were not tested
Gomez-Arroyo et al, 1987	No mixtures tested
Gordon et al, 2006	Discrepancies between dose levels given in 'materials and methods' and 'results' do not allow a clear judgement whether NOELs were established for the individual compounds (parameter hypothermia). For the parameter AChE inhibition, at least for one compound no NOEL was established
Graumann et al, 1999	High variability making it difficult to draw clear conclusions
Gregoraszczuk et al, 2008	Mixture was not tested at or below NO(A)EL for every component
Groten et al, 1991	Mixture was not tested at or below the NO(A)EL for every component
Groten et al, 1996	Fairly complex paper describing the fractionated factorial design, using two subchronic toxicity studies as examples; exposures are at effect levels. The Task Force has looked at more relevant primary papers from this group already (the nephrotoxicity series)
Groten et al, 1998	Same data as Tajima et al (2002). Not at a NOEL for all components
Gupta and Kadel, 1989	Mixture was not tested at or below the NO(A)EL for every component
Gupta et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Haake et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Haddad et al, 2000	Kinetic modelling only
Haddad et al, 2001	Modelling only
Haider et al, 2002	All *in vitro* studies of environmental mixtures have now been excluded
Hamm et al, 2005	Models the data of Gessner and Cabana (1970) but there was no NOEL established for ethanol
Haneef et al, 1995	Mixture was not tested at or below the NO(A)EL for every component
Hasegawa et al, 1989	No dose response, above NOELs for all compounds
Hasegawa et al, 1991a	Lowest dose in mixture of two compounds was not an NO(A)EL, no concurrent control in non-initiated animals
Hasegawa et al, 1991b	Some of the individual compounds were administered at effect level only
Hasegawa et al, 1992	Paper is not interpretable because, amongst others, non-monotonic dose response for the individual compounds. Also, high doses of the antioxydants are used (0.5 to 1% in diet) leading in part to liver weight increases
Hasegawa et al, 1994b	One compound of the high dose ('1/10') mixture was also positive when tested alone at the same dose. For the low-dose ('1/100') mixture individual compounds were not tested alone at the respective dose

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Hasegawa et al, 1996	Paper compiles results from 3 other papers, Ito et al, 1991, Hasegawa et al, 1991a, 1994b
Hass et al, 2007	Mixture was not tested at or below the NO(A)EL for every component
Hassan et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Hassoun and Dencker, 1982	Mixture was not tested at or below the NO(A)EL for every component
Hecker, 1976	Article on terminology of cancer aetiology without discussion on doses tested
Hendriksen et al, 2007	Effects were observed at low dose for individual components and changes in mixtures were sometimes no more than those observed for individual components
Hermann, 1981	Mixture was not tested at or below the NO(A)EL for every component
Hertzberg and Teuschler, 2002	Model / calculation considerations only, no data
Hewitt et al, 2007	Mixtures were tested at concentrations which individually caused increases in micronucleus formation
Hirose et al, 1997	Effects seen for individual comments
Hong et al, 1993	Paper is on irradiation, i.e. outside the scope of this Task Force
Howdeshell et al, 2007	Mixture was not tested at or below the NO(A)EL for every component
Howe and Webster, 1990	Mixture was not tested at or below the NO(A)EL for every component
Hrelia et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Hsieh et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Hughes and Phillips, 1990	Mixture was not tested at or below the NO(A)EL for every component
Hughes et al, 1988	Difficult to evaluate, but from figures it seems that all dose levels except controls were effect doses. Also, *in vitro* with questionable *in vivo* relevance
Hurley et al, 1998	Review of mechanisms of rodent thyroid tumorigenesis. Mixtures not addressed
Hussain et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Irato and Albergoni, 2005	Mixture was not tested at or below the NO(A)EL for every component
Ishigam et al, 2001	Kinetics only
Ito et al, 1998	Paper compiles results from other papers: Ito et al, 1991, Hasegawa et al, 1994a, Ito et al, 1995 and 1996
Iversen, 1994	Appropriate control not present
Iwai et al, 2002	No NOELs but enhancement of existing effect
Iyaniwura, 1989	Mixture was not tested at or below the NO(A)EL for every component
Iyaniwura, 1991	Mixture was not tested at or below the NO(A)EL for every component
James et al, 1993	Acetaminophen alone was at an effect level; although a small effect
Jensen and Sleight, 1986	Mixture was not tested at or below the NO(A)EL for every component
Jett et al, 1999	Mixture was not tested at or below the NO(A)EL for every component
Johansen et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Joshi and Thornburg, 1986	Mixture was not tested at or below the NO(A)EL for every component
Juhos et al, 1978	Mixture was not tested at or below the NO(A)EL for every component
Kacham et al, 2006	Although chlorpyrifos and parathion did not individually induce lethality, relevant biochemical changes due to treatment with the individual components were observed

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Kang et al, 2011	Only mixture was tested. No information on individual components
Karanth et al, 2001	Individual substances tested at 1x, 0.75x, and 0.5x LD1 doses, i.e. outside the scope of this Task Force. Mixtures all assessed at effect doses of the individual substances. Also considered sequential exposures
Karanth et al, 2004	Reports only sequential or concurrent exposures to the two OP insecticides, with no single exposure data. Also, combinations based on 1x and 0.5x LD1, which does not meet the Task Force's criteria for relevance
Kavlock et al, 1979	Only high multiples of an environmental mixture were tested
Kayajanian, 1997	Single substance
Kayajanian, 1999	Single substance
Khan et al, 2005	In terms of histopathology, even the lowest dose of sodium chlorate (SC) was not a NO(A)EL, questioning the absence of any significant T4 change in the SC groups
Khera and Iverson, 1981	ETU was already teratogenic at dose level tested
Kholkute et al, 1993	Mixture only tested, no single components, and the concentrations tested do not relate to real mixtures
Kim et al, 2010	Individual components and a mix of them were tested, but only effects for the individual components recorded
Kimmel et al, 1997	Mixture was not tested at or below the NO(A)EL for every component
Kimpe et al, 1999	*In vitro* mechanistic work irrelevant for TF
Kimura et al, 1976	Not at NOEL
Kinoshita et al, 2003	No mixture tested (only phenobarbital in diethylnitrosamine initiated system)
Kitagawa, 1986	Not at NOEL
Kitchin et al, 1994	Initiator promoter studies, but no data presented for initiators alone
Kluwe et al, 1979	Mixture was not tested at or below the NO(A)EL for every component
Kobayashi et al, 2010	One component was not dosed singly
Kohila et al, 2004	Mixture was not tested at or below the NO(A)EL for every component. There were effects of Al alone *in vitro* and *in vivo*
Kondo et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Korsak et al, 1988	Mixture was not tested at or below the NO(A)EL for every component
Korsak et al, 1991	Reports only blood levels of toluene and m-xylene monitored over 7 hours, following vapour exposures, individually (100ppm) or combined (100ppm+100ppm). There is a 'combination effect' but the exposure concentrations are not low dose and the effects are most likely due to induced changes in metabolism
Korsak et al, 1992	Paper focuses on neurophysiological effects. Lowest tested mixture was 50 ppm xylene + 50 ppm toluene. DNELs for both are 100 ppm, based on a NOAEC for neurophysiological effects of 26 ppm. Therefore 50 ppm is not low dose
Krasteva et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Krechniak et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Kuntz et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Kurppa et al, 1981	High exposure levels
Kushida et al, 2005	Diethylnitrosamin was only used at one, clearly effective dose
Lamb et al, 1981	Mixture was not tested at or below the NO(A)EL for every component

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Last and Cross, 1978	Mixture was not tested at or below the NO(A)EL for every component
Last and Warren, 1987	Mixture was not tested at or below the NO(A)EL for every component
Lau and Baird, 1992	Mixture was not tested at or below the NO(A)EL for every component
Lau et al, 1997	Abstract only; no data presented
Lau et al, 2006	Mixture was not tested at or below the NO(A)EL for every component
Lauer et al, 1994	Only high multiples of an environmental mixture were tested
Leavens et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Leavens et al, 1997	Mixture was not tested at or below the NO(A)EL for every component
Lee-Chen et al, 1993	Mixture was not tested at or below the NO(A)EL for every component
Leece et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Lengyel et al, 2006	No NO(A)EL mixture tested
Lesca et al, 1994	Not at NOEL
Li et al, 1989	No NO(A)EL mixture was tested
Lindros et al, 1990	NOELs could not be determined from the information presented
Liu et al, 1999	Only single dose tested which was effect dose for individual substances and mixture
Liu et al, 2006	This paper could be relevant but there are a number of drawbacks / questions: no data are given for the components individually, even though these were tested at 1/30LD50. The use of LD50s rather than NO(A)ELs is unusual. Authors conclude that interactions are either additive, antagonistic or synergistic depending on parameter measured but it is difficult to conclude based on the data presented.
Lodovici et al, 1994	A study using environmental mixtures, but only very high multiples of environmental exposure concentrations were tested
Lodovici et al, 1997	A study using environmental mixtures, but only very high multiples of environmental exposure concentrations were tested
Loeffler and Peterson, 1999	No experimental data were presented for NO(A)EL combinations (studies with individual compounds and with the mixture were done at doses which represented NO(A)EL doses for certain antiandrogenic endpoints (AGD, day of PPS, nipple retention), but insufficient data were presented
Lonky et al, 1996	Human epidemiology only
Lutz et al, 2002	LOEL was used instead of NOEL
Luyendyk et al, 2003	Studies on one chemical plus lipopolysaccharide-induced inflammation
Luyendyk et al, 2004	Lipopolysaccharide as one of the two mixture components, i.e. outside the scope of this Task Force
Maekawa et al, 1992	Not relevant as not simultaneous administered
Mahaffey and Fowler, 1977	Seems to describe the same study as Mahaffey et al, 1981
Mahaffey et al, 1981	Only single dose tested which was effect dose for individual substances and mixture
Main et al, 2006	Lots of individual correlations, but no actual data on mixture effects
Malich et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Marinovich et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Marsee et al, 2006	No correlation of exposure to effects. Estimated pharmacokinetics
Mason and Edwards, 1989	Mixture was not tested at or below the NO(A)EL for every component
Mason et al, 1989	Mixture was not tested at or below the NO(A)EL for every component

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
McDorman et al, 2005	No NO(A)EL mixture was tested
Meadows et al, 2002	Secondary analysis of data from another publication
Meechan et al, 1953	Not at NOEL
Mehendale, 1989	Pre-treatment study
Mejía et al, 1997	Doses near to acute toxicity were tested
Melcangi and Panzica, 2006	Mini-review on the role of neurosteroids. Mixtures not addressed
Menné et al, 1992	Human patch testing without dose response
Messiha, 1978	Mixture was not tested at or below the NO(A)EL for every component
Metzdorff et al, 2007	Mixture was not tested at or below the NO(A)EL for every component
Meyer et al, 1994	Primary paper on mouse skin tumour co-promotion by mirex and TPA. Mixture not tested at or below NO(A)EL. NO(A)EL not evaluated
Mileson et al, 1998	Contains no toxicology data
Miller et al, 1958	Not at NOEL
Moon et al, 1985	No direct comparison of the contribution of combination of metals to test variance; only assessment whether or not interaction of metals was significant
Morris et al, 1999	Mixture was not tested at or below the NO(A)EL for every component
Morrissey et al, 1992	No NO(A)EL mixture was tested
Moser et al, 2003	Very extensive study, but summary statistics only. No information on dose levels at which interactions were seen
Moslen et al, 1977	Mixture was not tested at or below the NO(A)EL for every component
Motomura and Narahashi, 2001	Mixture was not tested at or below the NO(A)EL for every component
Müller et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Murphy and DuBois, 1957	Kinetics only
Murphy et al, 1959	Mixture was not tested at or below the NO(A)EL for every component
Mustafa et al, 1984	Mixture was not tested at or below the NO(A)EL for every component
Mylroie et al, 1986	Mixture was not tested at or below the NO(A)EL for every component
Nagyeri et al, 2012	No information on NO(A)ELs for effects for the single components
Nagymajtényi et al, 1998	Mixture was not tested at or below the NO(A)EL for every component / endpoint
Naraharisetti et al, 2008	Malathion was added to the mixture at an effect level
Nation et al, 1989	Mixture was not tested at or below the NO(A)EL for every component
Nation et al, 1990	Mixture was not tested at or below the NO(A)EL for every component
Nellemann et al, 2003	Only effect levels were tested
Nesnow et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Newsome et al, 1978	Mixture was not tested at or below the NO(A)EL for every component
Nielsen et al, 1988	Mixture was not tested at or below the NO(A)EL for every component
Nordenson and Beckman, 1984	Mixture was not tested at or below the NO(A)EL for every component
Nylén et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Ortiz et al, 1995	Mixture was not tested at or below the NO(A)EL for every component
Pan et al, 2006	High-dose exposure only. Reduced testosterone recorded in workers exposed to DBP and DEHP. However paper probably not relevant as the levels of the metabolites of these phthalates in the urine exceeded the TDI

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Pasquini et al, 1994	Study using environmental mixtures, but only a very high multiple of environmental exposure concentrations was tested
Patel et al, 1998	No single chemical data presented
Patnode and Curtis, 1994	No mixtures tested
Payne et al, 2000	NOECs were not established and dose spacing of mixtures was insufficient to draw conclusions in the low-dose range
Peng et al, 2010	Mixture was not tested at or below the NO(A)EL for every component
Pereira et al, 2007	Qualitative assessment of histopathological effects only. Individually the substances had either some effect on thyroid or adrenal glands at the single dose assessed.
Perry and Erlanger, 1978	Mixture was not tested at or below the NO(A)EL for every component
Phillips et al, 1972	Mixture was not tested at or below the NO(A)EL for every component
Phillips et al, 1973	Mixture was not tested at or below the NO(A)EL for every component
Piatti et al, 1994	Mixture was not tested at or below the NO(A)EL for every component
Pitot et al, 1987	Classical initiation / promotion experiment with sequential application of initiator and promotor. Only figures, but no tables with numeric data given. Lowest dose of initiator diethylnitrosanine given was obviously an effective dose. Promotor phenobarbital applied was effective on its own
Platt and Buesselberg, 1994	Mixture was not tested at or below the NO(A)EL for every component
Poech and Dawson, 1996	Non-mammalian test system
Pohl et al, 2003	Review of the ATSDR (2001) interaction profile work; also they appear to be BINWOE assessments without test data to support the assessments
Poon et al, 2011	No single chemical data. The PBDE mixture cannot be considered an environmental mixture. The PCB mixture is an artificial mixture based on residues in fish in WI, but the lowest dose used is 3mg/kg. In the Canadian multigeneration study the highest dose achieved by feeding PCB contaminated fish to rats directly was 0.08 mg/kg (Feeley and Jordan, 1998). Therefore only very high multiples of environmental exposure were studied
Porter et al, 1993	Mixture was not tested at or below the NO(A)EL for every component
Pott et al, 1998	Not at NOEL
Puschner et al, 2007	Only one animal per group was tested, and the same animals received single chemicals and later the mixture
Qin et al, 2011	Data are from a non-mammalian system (Vibrio)
Raizada et al, 2001	Mixture was not tested at or below the NO(A)EL for every component
Rajapakse et al, 2001	Mixture was not tested at or below the NO(A)EL for every component
Rajapakse et al, 2004	Not designed to look at mixture effects in the low-dose region. At higher doses, reported subdose additive effects in mixtures containing NP and OP, hypothesised to be due to toxicity
Ramamoorthy et al, 1997a	Mixture was not tested at or below the NO(A)EL for every component
Ramamoorthy et al, 1997b	One component of the mixture did not induce effects in the estrogenicity assays
Raymond et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Rebert et al, 1995	Mixture was not tested at or below the NO(A)EL for every component
Reinstein et al, 1984	Zinc deficiency was studied
Reuzel et al, 1990	Mixture was not tested at or below the NO(A)EL for every component

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Richardson et al, 2001	Mixture was not tested at or below the NO(A)EL for every component; data not presented in a way that is amenable to the analysis needed
Rivedal and Sanner, 1980	Mixture was not tested at or below the NO(A)EL for every component
Roney and Colman, 2004	No experimental data
Rouas et al, 2011	No uranium NOEL was tested
Rowles et al, 1989	Mixture was not tested at or below the NO(A)EL for every component
Ruiz et al, 2011a	Mycotoxins are not considered to be chemicals but more protein-like
Ruiz et al, 2011b	Mycotoxins are not considered to be chemicals but more protein-like
Saad et al, 2011	Study on effects of vinclozolin and genistein and a mixture of both compounds on peripheral development. The doses used in the mixture experiment corresponded to individual effect levels for most of the potentially oestrogenic parameters (expression of ERa and ERb, mammary gland histopathology) studied
Safe, 1998	No exposure data. Useful as background to TEF/TEQ approach
Said et al, 1999	Mixture was not tested at or below the NO(A)EL for every component
Sakai et al, 1995	Mixture was not tested at or below the NO(A)EL for every component
Salamone et al, 1979	Mixture was not tested at or below the NO(A)EL for every component
Savolainen, 1980	Only levels above NO(A)EL were studied
Savolainen and Riimäki, 1981	Only levels above NO(A)EL were studied
Savolainen et al, 1980	Only levels above NO(A)EL were studied
Savolainen et al, 1981	Only levels above NO(A)EL were studied
Saxena et al, 1989	Mixture was not tested at or below the NO(A)EL for every component
Schantz and Widholm, 2001	Review on endocrine pathways and endocrine active compounds linked to cognitive effects in animals. Mixtures not addressed.
Schlesinger et al, 1992a	Mixture was not tested at or below the NO(A)EL for every component
Schlesinger et al, 1992b	Mixture was not tested at or below the NO(A)EL for every component
Schmidt et al, 2005	The results in the different experiments are not consistent, and there is only an additive effect by combination treatment, if at all, which cannot be evaluated as the data shown are not sufficiently detailed. In parts, controls are missing, too. Dose levels are in range of LOELs, but with experimental weaknesses and unclear interpretation of data
Schmidt et al, 2006	Dose levels of at least two of the components already affected the parameters investigated when tested individually. The dose levels of all three components and in particular that of BPA (200mg/kg/d) are not considered as low doses
Schmolke et al, 1992	Kinetics only
Schubert et al, 1978	Mixture was not tested at or below the NO(A)EL for every component
Scott et al, 2007	Goal of study was to investigate whether di-(n-butyl)-phthalate induced a reduction of androgen levels in foetal testis and contributed to dysgenetic features, multinucleated gonocytes and sertoli cell numbers and Leydig cell aggregation. No intention to investigate mixture effects, doses well above NOEL
Segal and Fedoroff, 1989	Mixture was not tested at or below the NO(A)EL for every component
Sharonova et al, 2000	No NOEL for Zn, and Cu seems not to have been tested alone
Siraj et al, 1981	Mixture was not tested at or below the NO(A)EL for every component
Skoczynska and Smolik, 1994	Mixture was not tested at or below the NO(A)EL for every component

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Slikker et al, 2004a	Does not contain data on mixture studies. Mechanistic considerations of dose-response relationships
Slikker et al, 2004b	Does not contain data on mixture testing
Smital et al, 2011	Relationship between the concentrations tested and those in the original water samples is not stated
Smyth et al, 1969	LD50s only
Smyth et al, 1970	LD50s only
Snyder and Friedman, 1998	Mixture was not tested at or below the NO(A)EL for every component
Stanko et al, 2010	Only the mixture was tested, not the components. Besides, the lowest dose in this study is equivalent to roughly 900 ppb in drinking water (for example the atrazine parent MCL is 3 ppb)
Stelzer and Chan, 1999	Not relevant as all combinations are above individual NOEL
Steup et al, 1993	Carbon tetrachloride was not used at or around NO(A)EL
Stine and al, 1991	Mixture was not tested at or below the NO(A)EL for every component
Su et al, 1971	Mixture was not tested at or below the NO(A)EL for every component
Sühnel, 1990	Secondary paper only; reassesses data from two publications on interaction of antiviral agents. Conclusion from one *in vivo* example is that at low doses, antagonism dominates while at higher doses synergy occurs. Other *in vitro* example shows synergy at all examined dose levels. Not relevant, as secondary reference only. Need to evaluate primary references to conclude on the dose ranges
Sühnel, 1992a	Not relevant; no data, only theoretical / statistical considerations
Sühnel, 1992b	Not relevant; no data, only theoretical / statistical considerations
Sühnel, 1998	Not relevant; no data, only theoretical / statistical considerations
Swan et al, 2005	Epidemiological study. Criteria cannot be applied. Increased prenatal maternal urinary levels of certain phthalate mono-esters or of all measured phthalate mono-esters were found to be inversely linked to the anogenital distance observed in new-born boys. Suggestion of a more than additive effect
Szabo et al, 1973	Mixture was not tested at or below the NO(A)EL for every component
Tadic, 1992	Mixture was not tested at or below the NO(A)EL for every component
Taets, 1996	Preliminary results presented. The full results are in Taets et al (1998) and are not relevant
Taets et al, 1998	Mixture was not tested at or below the NO(A)EL for every component
Tajima et al, 2002	Mixture was not tested at or below the NO(A)EL for every component
Takahashi et al, 1987	Mixture was not tested at or below the NO(A)EL for every component
Tarantini et al, 2011	Mixture consisted of equimolar binary mixtures of B[a]P and one other PAH. Concentrations used were 1 µM (for Comet assay) or 5 µM (DNA adduct levels experiments) which is not low dose as there was a considerable effect in BaP treated cells at these concentrations. Furthermore, there are no dose-response curves presented for the individual components
Tardif et al, 1996	Kinetics only
Tasker and Strain, 1998	Mixture was not tested at or below the NO(A)EL for every component
Tawfic, 1965	Not at NOEL
Thawley et al, 1977	Mixture was not tested at or below the NO(A)EL for every component
Thiruchelvam et al, 2000a	Mixture was not tested at or below the NO(A)EL for every component

Paper was reviewed vs. the Task Force's criteria and judged not relevant	Why judged not relevant for this Task Force?
Thiruchelvam et al, 2000b	Mixture was not tested at or below the NO(A)EL for every component
Thuvander et al, 1999	Mixture was not tested at or below the NO(A)EL for every component
Tinwell et al, 2000	Single substance study only
Traiger and Plaa, 1971	Mixture was not tested at or below the NO(A)EL for every component
Valko et al, 2006	Review on MOA; no experimental data
Valkusz et al, 2011	Only mixture was tested. No information on individual components
Van Meeuwen et al, 2007	No dose responses performed for most phyto- and synthetic oestrogens. Phyto- and synthetic oestrogens not tested individually, but only as mixtures
van Ravenzwaay et al, 2010	Different combinations of dibutylphthalate and di(2-ethylhexyl)-phthalate were tested in a 28-day study in Wistar rats to study the individual metabolite profiling (metabolomics) compared with the patterns obtained after administration of the different mixtures. The semi-quantitative outcomes of a metabolome analysis are considered a suitable tool to study the nature of the interaction between different compounds. However as one of the compounds used in this study (namely di(2-ethylhexyl)-phthalate) was administered at toxic doses only, this study seemed to be outside the scope of this Task Force
Vassilev et al, 1993	Either Ni or Co was dosed, not both
Verhaar et al, 1997	No experimental data. Proposes a method that uses PBPK modelling for risk assessment of complex mixtures such as petroleum substances
Vijver et al, 2011	Despite the interesting title the paper does not consider mammals
Villeneuve et al, 1974	Mixture was not tested at or below the NO(A)EL for every component
Vodela et al, 1997a	Only single dose tested which was effect dose for individual substances and mixture, also unusual species (chicken)
Vodela et al, 1997b	See Vodela et al, 1997a
Von Nieding and Wagner, 1977	Co-exposures at MAK concentrations, but no dose response and comparable effect already with NO_2 alone, i.e. above NOEL
Voskoboinik et al, 1997	Mixture was not tested at or below the NO(A)EL for every component
Walker et al, 2005	Lowest dose of TCDD had clear effects on the liver, see TR-521
Wang and Kurzer, 1998	NOELs were not established for any of the test substances and the mixtures were only tested at concentrations of the single substances that resulted in effects.
Wang et al, 2011	Only effect levels were tested
Warren and Last, 1987	Mixture was not tested at or below the NO(A)EL for every component
Warshawsky et al, 1993	Mixture was not tested at or below the NO(A)EL for every component
Waters et al, 1990	Review on database, not experimental
Weber et al, 1985	Mixture was not tested at or below the NO(A)EL for every component
Welch et al, 2011	Mixture experiment only at LD50; no low dose
Whittaker et al, 2011	Mixture consisted of individual components at LOEL
Williams et al, 1958	Mixture was not tested at or below the NO(A)EL for every component
Wren, 1991	Epidemiology only
Wu et al, 1996	Mixture was not tested at or below the NO(A)EL for every component
Wysocka-Paruszewki et al, 1980	Mixture was not tested at or below the NO(A)EL for every component
Xie et al, 2010	Only effect levels were tested

Paper was reviewed vs. the Task Force's criteria and judged not relevant	**Why judged not relevant for this Task Force?**
Xu et al, 2010	Environmental mixture was dosed i.p., so the paper was not further reviewed as this is a non-relevant route of exposure to an environmental mixture
Yamamoto et al, 1995	Rather a cancer-promotion assay with sequential admin of high doses of different initiators and cacodylic acid
You and Bartolucci, 2004	No NO(A)EL mixture tested
Yücesoy et al, 1997	Human data. No dose response
Zhao et al, 2011b	Environmental mixture was dosed i.p., so the paper was not further evaluated as this is a non-relevant route of exposure to an environmental mixture

MEMBERS OF THE TASK FORCE

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[a] Responsible for primary peer review.

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ECETOC PUBLISHED REPORTS

Monographs

No.	Title
No. 1	Good Laboratory Practice (Published October 1979)
No. 2	A Contribution to Strategy for Identification and Control of Occupational Carcinogens (Published September 1980)
No. 3	Risk Assessment of Occupational Chemical Carcinogens (Published May 1985)
No. 4	Hepatocarcinogenesis in Laboratory Rodents: Relevance for Man (Published October 1982)
No. 5	Identification and Assessment of the Effects of Chemicals on Reproduction and Development (Reproductive Toxicology) (Published December 1983)
No. 6	Acute Toxicity Tests, LD_{50} (LC_{50}) Determinations and Alternatives (Published May 1985)
No. 7	Recommendations for the Harmonisation of International Guidelines for Toxicity Studies (Published December 1985)
No. 8	Structure-Activity Relationships in Toxicology and Ecotoxicology: An Assessment (Summary) (Published June 1986)
No. 9	Assessment of Mutagenicity of Industrial and Plant Protection Chemicals (Published June 1987)
No. 10	Identification of Immunotoxic Effects of Chemicals and Assessment of their Relevance to Man (Published August 1987)
No. 11	Eye Irritation Testing (Published June 1988)
No. 12	Alternative Approaches for the Assessment of Reproductive Toxicity (with emphasis on embryotoxicity/teratogenicity) (Published November 1989)
No. 13	DNA and Protein Adducts: Evaluation of their Use in Exposure Monitoring and Risk Assessment (Published October 1989)
No. 14	Skin Sensitisation Testing (Published March 1990)
No. 15	Skin Irritation (Published July 1990)
No. 16	Early Indicators of Non-Genotoxic Carcinogenesis (Published June 1991)
No. 17	Hepatic Peroxisome Proliferation (Published May 1992)
No. 18	Evaluation of the Neurotoxic Potential of Chemicals (Published September 1992)
No. 19	Respiratory Allergy (Published August 1993)
No. 20	Percutaneous Absorption (Published August 1993)
No. 21	Immunotoxicity: Hazard Identification and Risk Characterisation (Published September 1994)
No. 22	Evaluation of Chemicals for Oculotoxicity (Published November 1994)
No. 23	Receptor Mediated Mechanisms in Chemical Carcinogenesis (Published December 1995)
No. 24	Risk Assessment for Carcinogens (Published July 1996)
No. 25	Practical Concepts for Dose Selection in Chronic Toxicity and Carcinogenicity Studies in Rodents (Published February 1996)
No. 26	Aquatic Toxicity Testing of Sparingly Soluble Volatile and Unstable Substances (Published September 1996)
No. 27	Aneuploidy (Published August 1997)
No. 28	Dose-response and threshold-mediated mechanisms in mutagenesis - Mutation Research Special Issue (Published January 2000)
No. 29	Skin Sensitisation Testing for the Purpose of Hazard Identification and Risk Assessment (Published September 2000)
No. 30	Genetic Susceptibility to Environmental Toxicants (Published October 2001) Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 482, Issues 1-2, Pages 1-115 www.sciencedirect.com/science/journal/00275107
No. 31	Guidance on Evaluation of Reproductive Toxicity Data (Published February 2002)
No. 32	Use of Human Data in Hazard Classification for Irritation and Sensitisation (Published July 2002)

No. 33 Application of Physiological - Toxicokinetic Modelling to Health Hazard Assessment of Chemical Substances (Published February 2003)
Toxicology Letters, Volume 138, Issues 1-2
www.sciencedirect.com/science/journal/03784274

No. 34 Toxicogenomics in Genetic Toxicology and Hazard Determination (Published August 2005)
Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 575, Issues 1-2
www.sciencedirect.com/science/journal/00275107

No. 35 Biomarkers and molecular epidemiology (Published August 2006)
Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 600, Issues 1-2
www.sciencedirect.com/science/journal/00275107

No. 36 Environmental Genotoxins in Children and Adults (Published August 2006)
Mutation Research/Genetic Toxicology and Environmental Mutagenesis, Volume 608, Issue 2
www.sciencedirect.com/science/journal/13835718

No. 37 Biomarkers in Children and Adults (Published July 2007)
Toxicology Letters, Volume 172, Nos. 1-2
www.sciencedirect.com/science/journal/03784274

No. 38 Toxicity of Engineered Nanomaterials (published May 2009)
Toxicology Letters, Volume 186, Issue 3
www.sciencedirect.com/science/journal/03784274

Technical Reports

No.	Title
No. 1	Assessment of Data on the Effects of Formaldehyde on Humans (Published January 1979) (Updated by TR No. 6)
No. 2	The Mutagenic and Carcinogenic Potential of Formaldehyde (Published May 1981)
No. 3	Assessment of Test Methods for Photodegradation of Chemicals in the Environment (Published August 1981)
No. 4	The Toxicology of Ethylene Glycol Monoalkyl Ethers and its Relevance to Man (Published June 1982) (Updated by TR No. 17)
No. 5	Toxicity of Ethylene Oxide and its Relevance to Man (Published September 1982)
No. 6	Formaldehyde Toxicology: An Up-Dating of ECETOC Technical Reports 1 and 2 (Published September 1982)
No. 7	Experimental Assessment of the Phototransformation of Chemicals in the Atmosphere (Published September 1983)
No. 8	Biodegradation Testing: An Assessment of the Present Status (Published November 1983)
No. 9	Assessment of Reverse-Phase Chromatographic Methods for Determining Partition Coefficients (Published December 1983)
No. 10	Considerations Regarding the Extrapolation of Biological Data in Deriving Occupational Exposure Limits (Published February 1984)
No. 11	Ethylene Oxide Toxicology and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 5 (Published March 1984)
No. 12	The Phototransformation of Chemicals in Water: Results of a Ring-Test (Published June 1984)
No. 13	The EEC 6th Amendment: A Guide to Risk Evaluation for Effects on the Environment (Published March 1984)
No. 14	The EEC 6th Amendment: A Guide to Risk Evaluation for Effects on Human Health (Published March 1984)
No. 15	The Use of Physical-Chemical Properties in the 6th Amendment and their Required Precision, Accuracy and Limiting Values (Published June 1984)
No. 16	A Review of Recent Literature on the Toxicology of Benzene (Published December 1984)

No. 17 The Toxicology of Glycol Ethers and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 4) (Published April 1985) (Updated by TR No. 64)
No. 18 Harmonisation of Ready Biodegradability Tests (Published April 1985)
No. 19 An Assessment of Occurrence and Effects of Dialkyl-o-Phthalates in the Environment (Published May 1985)
No. 20 Biodegradation Tests for Poorly-Soluble Compounds (Published February 1986)
No. 21 Guide to the Classification of Carcinogens, Mutagens, and Teratogens under the 6th Amendment (Published February 1986)
No. 22 Classification of Dangerous Substances and Pesticides in the EEC Directives. A Proposed Revision of Criteria for Inhalational Toxicity (Published January 1987)
No. 23 Evaluation of the Toxicity of Substances to be Assessed for Biodegradability (Published November 1986)
No. 24 The EEC 6th Amendment: Prolonged Fish Toxicity Tests (Published October 1986)
No. 25 Evaluation of Fish Tainting (Published January 1987)
No. 26 The Assessment of Carcinogenic Hazard for Human Beings exposed to Methylene Chloride (Published January 1987)
No. 27 Nitrate and Drinking Water (Published January 1988)
No. 28 Evaluation of Anaerobic Biodegradation (Published June 1988)
No. 29 Concentrations of Industrial Organic Chemicals Measured in the Environment: The Influence of Physico-Chemical Properties, Tonnage and Use Patterns (Published June 1988)
No. 30 Existing Chemicals: Literature Reviews and Evaluations (Fifth Edition) (No longer available) (Published May 1994)
No. 31 The Mutagenicity and Carcinogenicity of Vinyl Chloride: A Historical Review and Assessment (Published July 1988)
No. 32 Methylene Chloride (Dichloromethane): Human Risk Assessment Using Experimental Animal Data (Published May 1988)
No. 33 Nickel and Nickel Compounds: Review of Toxicology and Epidemiology with Special Reference to Carcinogenesis (Published February 1989)
No. 34 Methylene Chloride (Dichloromethane): An Overview of Experimental Work Investigating Species Differences in Carcinogenicity and their Relevance to Man (Published March 1989)
No. 35 Fate, Behaviour and Toxicity of Organic Chemicals Associated with Sediments (Published January 1990)
No. 36 Biomonitoring of Industrial Effluents (Published April 1990)
No. 37 Tetrachlorethylene: Assessment of Human Carcinogenic Hazard (Published May 1990)
No. 38 A Guide to the Classification of Preparations Containing Carcinogens, Mutagens and Teratogens (Published July 1990)
No. 39 Hazard Assessment of Floating Chemicals After an Accidental Spill at Sea (Published July 1990)
No. 40 Hazard Assessment of Chemical Contaminants in Soil (Published April 1992)
No. 41 Human Exposure to N-Nitrosamines, their Effects and a Risk Assessment for N-Nitrosodiethanolamine in Personal Care Products (Published August 1990)
No. 42 Critical Evaluation of Methods for the Determination of N-Nitrosamines in Personal Care and Household Products (Published February 1991)
No. 43 Emergency Exposure Indices for Industrial Chemicals (Published March 1991)
No. 44 Biodegradation Kinetics (Published September 1991)
No. 45 Nickel, Cobalt and Chromium in Consumer Products: Allergic Contact Dermatitis (Published March 1992)
No. 46 EC 7th Amendment: Role of Mammalian Toxicokinetic and Metabolic Studies in the Toxicological Assessment of Industrial Chemicals (Published May 1992)
No. 47 EC 7th Amendment "Toxic to Reproduction": Guidance on Classification (Published August 1992)
No. 48 Eye Irritation: Reference Chemicals Data Bank (Second Edition) (Published June 1998)
No. 49 Exposure of Man to Dioxins: A Perspective on Industrial Waste Incineration (Published December 1992)
No. 50 Estimating Environmental Concentrations of Chemicals using Fate and Exposure Models (Published November 1992)
No. 51 Environmental Hazard Assessment of Substances (Published January 1993)
No. 52 Styrene Toxicology Investigation on the Potential for Carcinogenicity (Published August 1992)

No. 53 DHTDMAC: Aquatic and Terrestrial Hazard Assessment (CAS No. 61789-80-8) (Published February 1993)
No. 54 Assessment of the Biodegradation of Chemicals in the Marine Environment (Published August 1993)
No. 55 Pulmonary Toxicity of Polyalkylene Glycols (Published December 1997)
No. 56 Aquatic Toxicity Data Evaluation (Published December 1993)
No. 57 Polypropylene Production and Colorectal Cancer (Published February 1994)
No. 58 Assessment of Non-Occupational Exposure to Chemicals (Published May 1994)
No. 59 Testing for Worker Protection (Published April 1994)
No. 60 Trichloroethylene: Assessment of Human Carcinogenic Hazard (Published May 1994)
No. 61 Environmental Exposure Assessment (Published September 1994)
No. 62 Ammonia Emissions to Air in Western Europe (Published July 1994)
No. 63 Reproductive and General Toxicology of some Inorganic Borates and Risk Assessment for Human Beings (Published February 1995)
No. 64 The Toxicology of Glycol Ethers and its Relevance to Man (Published August 1995)
No. 65 Formaldehyde and Human Cancer Risks (Published May 1995)
No. 66 Skin Irritation and Corrosion: Reference Chemicals Data Bank (Published March 1995)
No. 67 The Role of Bioaccumulation in Environmental Risk Assessment: The Aquatic Environment and Related Food Webs (Published October 1995)
No. 68 Assessment Factors in Human Health Risk Assessment (Published August 1995) (Updated by TR No. 86)
No. 69 Toxicology of Man-Made Organic Fibres (Published April 1996)
No. 70 Chronic Neurotoxicity of Solvents (Published February 1996)
No. 71 Inventory of Critical Reviews on Chemicals (Only available to ECETOC members) (Published August 1996)
No. 72 Methyl *tert*-Butyl Ether (MTBE) Health Risk Characterisation (Published June 1997)
No. 73 The Value of Aquatic Model Ecosystem Studies in Ecotoxicology (Published December 1997)
No. 74 QSARs in the Assessment of the Environmental Fate and Effects of Chemicals (Published June 1998)
No. 75 Organophosphorus Pesticides and Long-term Effects on the Nervous System (Published December 1998)
No. 76 Monitoring and Modelling of Industrial Organic Chemicals, with Particular Reference to Aquatic Risk Assessment (Published January 1999)
No. 77 Skin and Respiratory Sensitisers: Reference Chemicals Data Bank (Published August 1999)
No. 78 Skin Sensitisation Testing: Methodological Considerations (Published December 1999)
No. 79 Exposure Factors Sourcebook for European Populations (with Focus on UK Data) (Published June 2001)
No. 80 Aquatic Toxicity of Mixtures (Published July 2001)
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