Expert workshop on combination effects of chemicals, 28-30 January 2009, Hornbæk, Denmark

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Workshop Report

Professor Andreas Kortenkamp
The School of Pharmacy, University of London
Centre for Toxicology
29-39 Brunswick Square
London WC1N 1AX

Dr Ulla Hass
National Food Institute
Danish Technical University
Mørkhoj Bygade
DK 2860 Søborg

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Summary

An expert workshop on effects of combined exposure to chemicals, with special emphasis on chemicals with endocrine activity was held under the auspices of the Danish Ministry of the Environment. The aim of the workshop was to examine existing scientific knowledge on combination effects of endocrine disrupters, with a focus on regulatory aspects. The workshop participants considered the state of the science of mixtures risk assessment for endocrine disrupters, and discussed the feasibility of approaches to cumulative risk assessment.

A consensus about a number of important issues could be formulated, and this included a series of recommendations:

Cumulative risk assessment (CRA) for endocrine disrupters was seen as both necessary and feasible. The predominant chemical-by-chemical approach in risk assessment was regarded as insufficiently protective against the possibility of mixture effects/ effects of combined exposure.

The application of dose (or concentration) addition as an assessment method was recommended as a default, until evidence as to the suitability of alternative assessment concepts emerges.

A pre-occupation with mechanisms or modes of action as the starting point for the grouping of endocrine disrupters into classes to be subjected to mixtures risk assessment was seen as not practical and scientifically hard to justify. Instead, grouping criteria should focus on common health related effects and the likelihood of co-exposures.

The full potential of CRA for endocrine disrupters cannot be reached without filling a number of data gaps, most importantly in the area of mixtures exposure assessment.

An enhancement of the legal framework in Europe with a view to mandating CRA should be given serious consideration.

Abbreviations

ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
BBP	Benzyl butyl phthalate
CA	Concentration addition
CERCLA	Comprehensive Environmental Response Compensation and Liability Act
CMG	Common mechanism group
CRA	Cumulative risk assessment
DA	Dose addition
DBP	Dibutyl phthalate
DEHP	Diethyl hexyl phthalate
DEPA	Danish Environmental Protection Agency
ER	Estrogen receptor
FQPA	Food Quality Protection Act
GHS	Global Harmonisation System
HI	Hazard index
IA	Independent action
MOE	Margin of exposure
NOEC	No observed effect concentration
NOEL	No observed effect level
NOAEL	No observed adverse effect level
NRC	National Research Council
PBDE	Polybrominated diphenyl ether
PCDD/F	Polychlorinated dibenzo-p-dioxin/furan
PODI	Point of departure index
REACH	Registration Evaluation and Authorisation of Chemicals
RfD	Reference dose
TCDD	Tetra chloro dibenzo-p-dioxin
TDI	Tolerable daily intake
TEF	TCDD equivalency factor
TEQ	TCDD equivalent
UF	Uncertainty factor
UVBC	Unknown or variable composition complex reaction products or biological materials

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Appendix

1. Terms of reference and workshop aims

The Danish Environment Minister authorized the Danish Environmental Protection Agency (DEPA) to host an expert workshop on combination effects of chemicals, with special emphasis on endocrine disrupters. This workshop took place on 28 – 30 January 2009 in Hornbæk, Denmark.

The aim of the workshop was to examine existing scientific knowledge on combination effects of endocrine disrupters, with a focus on regulatory aspects. The following questions were to be addressed:

- What is the state-of-the-science on combination effects at present – for chemicals in general and specifically for endocrine disrupters?
- Which problems can be identified on the basis of the existing knowledge – in relation to health and in relation to the environment?
- What are the challenges the regulatory authorities have to face?
- How can these challenges be met and the existing knowledge be taken into account within the existing regulation?
- What are suggestions for actions with a focus on regulatory aspects on a global, a regional (EU) and national (DK) level?

2. The workshop programme, resource materials

To realize the workshop aims, five different sessions were set up.

Session 1, "Mixtures risk assessment – is it necessary?" was intended as a first step towards defining the issues of the workshop. A second goal was to review the experimental evidence for mixture effects when chemicals are combined at low doses, close to levels that are "points of departure" for risk assessment and regulation (e.g. benchmark doses or NOAELs).

The plan for *Session 2*, "A basis for combined risk assessment – case study: phthalates and other anti-androgens" was to summarize the experimental evidence for combination effects of antiandrogens, to review criteria for grouping these substances for purposes of mixtures risk assessment and to gain an overview of risk assessment methods for mixtures.

Session 3, "The basis of combined risk assessment for other classes of endocrine disrupters and other chemicals" aimed to consider topics for mixture risk assessment relevant to other endocrine disrupting chemicals, such as: What are effect outcomes or mechanisms on which mixtures risk assessment should be based? What is the evidence for combination effects?

Session 4, "From mixtures risk assessment to regulation" was set up for a more general treatment of the mixtures risk assessment, relevant to other groups of chemicals, by summarizing approaches to mixtures regulation, also in ecotoxicology, including an analysis of uncertainty factors and their suitability for dealing with mixture effects.

Sessions 1 – 4 consisted of a series of formal talks, followed by discussions. The talks were based on resource material which was distributed in advance to all participants.

Finally, *Session 5* "Looking forward – what can/should be done?" was conducted in the form of a structured discussion among workshop participants, with the aim of drawing up recommendations for risk assessment, regulation and research.

The workshop programme together with bibliographic references for the resource material, and the list of participants can be found in the appendix. Since most formal talks during the workshop were based on published scientific articles, their content is accessible through the resource list. For this reason, the workshop talks will not be summarized in chronological order in this report. Rather, a structured digest of the presentations, discussions and recommendations of the workshop will be given.

3. State of the science on combination effects of endocrine disrupters

Over the past decade, mixture toxicology has undergone a remarkable and productive development. While earlier experimental studies focused mainly on mixtures composed of only two chemicals, the planning, conduct and assessment of multi-component mixtures with up to 50 chemicals is now state of the art. This has extended from in vitro assays to in vivo studies, although scientific data about in vivo combination effects are less prevalent than in vitro studies.

Most mixture studies with endocrine disrupters published in the peer-reviewed literature have been conducted with the aim of explaining the joint action of selected pure compounds in terms of their individual effects (component-based approach).

3.1 Definitions and terms

It is noted that the terms "mixture effects" and "effects of combined exposure" (to more chemicals) are used without discrimination here and that the term "mixture" thus has a broader meaning in this context than when used in chemicals legislation including guidance (e.g. REACH and GHS). The field of mixture toxicology is notorious for its use of poorly defined terms. Depending on context, there are many synonyms, and some terms are uncritically used with entirely different meanings. For this reason, workshop participants agreed on tentative definitions for a number of frequently used terms:

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

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

Additivity: In the context of mixture toxicology, additivity cannot be equated with "additivity" in the mathematical sense. It refers to a situation, termed "non-interaction" (and often used synonymously with "additivity"), where the toxicity of a mixture resembles the effects expected to occur when all mixture components act without diminishing or enhancing each others effects. Additivity expectations for mixtures can be derived from the concepts of dose (or concentration) addition and independent action (see 3.2.1 and 3.2.2). In certain situations, valid expectations for additive combination effects can also be calculated by building the arithmetic sum of the individual effects of all mixture components ("effect summation").

Synergism, antagonism: When an observed combination effect is larger or smaller than expected according to an additivity assumption (based either on dose addition or independent action), there is synergism or antagonism, respectively.

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

Mode of action: A sequence of key cellular and biochemical events with measurable parameters that result in a toxic effect. Mode of action considerations are used to decide whether an effect observed after administration of a chemical in animals has relevance for humans. Mode of action is not intended to build a comprehensive model of a chemical's actions. It is often confused with mechanism of action, or used in overlapping ways. Mode of action can include mechanisms of action, but is considered to be broader.

Cumulative risk assessment (CRA), mixtures risk assessment: The terms are used synonymously. They denote risk assessment approaches that consider the impact of multiple chemical exposures, from multiple sources, routes and pathways, over multiple time frames. It is worth noting that the European use of the term "cumulative risk assessment" encompasses multiple sources, routes and pathways, but restricts considerations to one chemical, not multiple chemicals. For the purposes of this report, the European use of the term is ignored. Toxicity assessments of multi-constituent substances (e.g. technical solvents) or UVBC (unknown or variable composition, complex reaction products or biological materials) also do generally not fall under mixtures risk assessments of the kind discussed during the workshop. The reason is that multi-constituent substances and UVBCs often are treated in the same way as a single chemical entity would be dealt with; no attempts are made to explain mixture effects in terms of the activity of the constituents.

There are various approaches to chemicals risk assessment (Suter and Cormier 2008), and these also impact on CRA. First, risk assessment can be carried out in order to provide trigger values for regulatory action to protect humans or wild life from harm ("protective" risk assessment). In this case, a bias towards conservatism and worst case assumptions is essential. Second, there is risk assessment aimed at quantifying the magnitude of impact resulting from certain exposures to chemicals. Such approaches ("quantitative" risk assessment) need to be as accurate as possible in their risk estimates and tend to utilize probabilistic methods. This report is mainly concerned with protective risk assessment, and less so with quantitative risk assessment.

3.2 Prediction and assessment of mixture effects

When several chemicals occur together in a mixture, they may influence each others effects by enhancing or diminishing their action. In mixture toxicology, such situations are described as toxic interactions. More frequently however, chemicals act together without influencing each others actions. In such cases, it is possible to anticipate quantitatively the effects of a mixture from knowledge about the effects of its individual components. This phenomenon is called non-interaction or additivity. Two concepts are available for the formulation of the null hypothesis of additivity: *dose (*or *concentration) addition* and *independent action*.

These concepts are based on two entirely different ideas about how the joint action of chemicals can be perceived.

3.2.1 Dose addition

Dose addition (DA) is based on the idea that all components in the mixture behave as if they are simple dilutions of one another, which is often taken to mean that the concept describes the joint action of compounds with an identical mechanism of action. When these chemicals interact with an identical, well-defined molecular target, it is thought that one chemical can be replaced totally or in part by an equal fraction of an equi-effective concentration (e.g. an EC50) of another, without changing the overall combined effect.

A widely used application of this approach is the “toxic equivalence factor” (TEF) concept for the assessment of mixtures of polychlorinated dioxins and furans (PCDD/F) (van den Berg et al. 1998). Here, doses of specific PCDD/F isomers are all expressed in terms of the dose of a reference chemical, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), needed to induce the same effect (“equivalent” or “equi-effective” dose). The assessment of the resulting combined effect is obtained simply by adding up all equivalent TCDD doses. The application of TEF only holds when the underlying dose-effect relationships are linear. If this pre-condition is violated, TEFs vary with the effect level that is considered for analysis.

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

3.2.2 Independent action (response addition, effect multiplication)

Independent action (IA) conceptualises mixture effects in a different way. It assumes that the joint effect of a combination of agents can be calculated from the responses of individual mixture components by adopting the statistical concept of independent events. The resulting combined effect can be calculated from the effects caused by the individual mixture components by following the statistical concept of independent random events (Bliss, 1939).

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

3.2.3 Choosing between dose addition and independent action for the purpose of assessment and prediction

A question of fundamental importance to risk assessment and regulation is which of the two concepts, DA or IA, should be exclusively chosen for the interpretation of empirical data, or for anticipating mixture effects of untested combinations. As a way of resolving the issue, DA and IA have been allied to broad mechanisms of combination toxicity, with DA thought to be applicable to mixtures composed of chemicals with a similar mode of action, with the corresponding mechanistic model of "simple similar action", and IA for chemicals with diverse modes of action, and the mechanistic model of "independent joint action".

The issue of distinguishing between these mechanistic models becomes especially important, when DA and IA predict different mixture toxicities. In such cases it is important to realize that the prediction differences or similarties stem from the mathematical features that form the basis of DA and IA (Drescher and Boedeker 1995). Prediction differences are not driven by the biology or toxicology of combinations of chemicals with similar or diverse mode of actions.

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

Conversely, IA is widely held to be appropriate for mixtures of agents with diverse or "dissimilar" modes of action. Although rarely stated explicitly, this presumably stems from the stochastic principles that underpin this concept. The idea that chemicals act independently is equated with the notion of action through different mechanisms. By activating differing effector chains, so goes the argument, every component of a mixture of dissimilarly acting chemicals provokes effects independent of all other agents that might also be present, and this feature appears to lend itself to statistical concepts of independent events. However, theoretically, the stochastic principles of IA are also valid when one and the same agent is administered sequentially. This can be illustrated by using cytotoxicity as an example. Because cells cannot die twice, the probabilistic principle of IA applies, even though the precise mechanisms that underlie the cytotoxic action of the chemical are identical in sequential administration. In the case of simultaneous administration of many chemicals however, the principle of independent

events only applies when the additional assumption is made that all mixture components act strictly independently, through different mechanisms.

The practical relevance of IA for the assessment of mixture effects has been called into question on the basis of considerations of biological organisation. The principle of strictly independent events may rarely apply due to converging signalling pathways and inter-linked subsystems. For these reasons, DA is seen as more broadly applicable, and has been termed the "general solution" for mixture toxicity assessment (Berenbaum 1985).

However, the few studies that were specifically designed for a comparative evaluation of both concepts for mixtures composed of strictly dissimilarly acting substances, demonstrated that IA provides a better prediction of the observed mixture toxicities (Backhaus et al. 2000; Faust et al. 2003). These observations argue against DA as the "general solution" for mixture assessments.

It appears that theoretical considerations are not decisive in answering the question of choice between DA and IA as assessment concepts for endocrine disrupter mixtures. To resolve the issue, it is therefore necessary to consider the empirical evidence.

3.3 Dose addition or independent action? - Experimental evidence with mixtures of endocrine disrupters

The study of mixtures composed of chemically pure endocrine disrupters, in laboratory settings, has yielded a considerable body of evidence showing that concentration (dose) addition provides a sound approximation of experimentally observed additive combination effects (see the review by Kortenkamp 2007). However, due to a predilection of researchers to combine endocrine disrupters of the same type (e.g. estrogenic, antiandrogenic or thyroid-disrupting chemicals), in many of the published studies IA could not have been expected to produce valid additivity expectations.

Even so, there are recent indications that DA gives better approximations of combination effects of endocrine disrupters with diverse modes of action. For example, Rider et al. (2008) conducted mixture experiments with the three phthalates BBP, DBP, and DEHP in combination with the antiandrogens vinclozolin, procymidone, linuron, and prochloraz. Its components have a variety of antiandrogenic modes of action. Vinclozolin and procymidone are AR antagonists, and linuron and prochloraz exhibit a mixed mechanism of action: inhibiting steroid synthesis and blocking the steroid receptor. DA gave predictions of combined effects of the mixed-mode antiandrogens that agreed better with the observed responses than did the expectations derived from IA.

Mixtures of thyroid disrupting chemicals with diverse modes of action also showed combination effects that were approximated better by DA, not IA (Kevin Crofton, workshop presentation of unpublished data).

No case has yet been identified, where IA yielded predictions of endocrine disrupter combination effects larger than those derived from DA, and at the same time were in agreement with experimental data. Taken together, the determinants of additive joint action of endocrine disrupters are fairly well established, and it appears that DA provides good approximation of combination effects. Therefore, until evidence to the contrary

emerges, DA can be adopted as the default concept for the assessment and prediction of endocrine disrupter mixture effects.

Factors that might lead to deviations from expected additive effects, indicative of synergisms or antagonisms, are beginning to emerge and require further research. The magnitude of such deviations cannot be predicted quantitatively. Toxicokinetic interactions are one established cause of deviations from additivity. A notable example of such deviations is the synergism that was observed with a mixture of vinclozolin, prochloraz, finasteride and DEHP with respect to hypospadias and genital malformations among male offspring of female rats (Ulla Hass, workshop presentation of unpublished data).

4. Cumulative risk assessment – is it necessary?

Many experimental studies of mixture effects have been motivated by understanding determinants of additivity and predictability. Inevitably, this has meant that chemicals had to be combined at doses considerably higher than those encountered by the general population. Two issues need to be addressed to judge the relevance of combination effects for risk assessment: Do combination effects occur when chemicals are combined at low doses? Are the uncertainty factors used to translate apparently safe dose levels derived from animal experiments into acceptable exposures for humans insufficiently protective to take account of mixture effects?

4.1 Mixture effects at low doses of mixture components

Certain experimental mixture studies have been designed to assess whether combination effects occur when chemicals are combined at low doses, here defined as being sufficiently low to be without observable effects when tested on their own (i.e. below the sensitivity of the chosen experiment to be measurable). Often, these doses were in the range of those commonly used to derive estimates of safe human exposures (so-called points of departure, usually no-observed-adverse-effect-levels, NOAELs, or benchmark doses). The review by Kortenkamp et al. (2007) summarizes the evidence for endocrine disrupters and other types of chemicals, and an update was provided by Michael Faust (workshop presentation).

For combinations composed of chemicals that interact with the same molecular receptor or molecular target in an organism, there is good evidence that mixture effects can arise at doses around, or below, points of departure. Considering the main assumptions underlying the concept of dose addition, this is to be expected (see 3.1.1).

In contrast, theory predicts that mixtures which follow IA should not yield a combination effect as long as all components are present at doses associated with zero responses. This is widely held to mean that mixtures of dissimilarly acting chemicals are safe, as long as exposure to each component does not exceed its individual point of departure (COT 2002, VKM 2008). With reference to the apparent diversity of chemical exposures in the "real world", IA is taken as the default assessment concept in human toxicology, when strict similarity criteria of dose addition appear to be violated or if specific evidence for

the compounds of a given mixture is lacking. Implicitly taking "dissimilar action" or "independent joint action" as the negation of "simple similar action" it is then assumed that IA must hold, even without further proof that the underlying mechanisms indeed satisfy any explicit dissimilarity criterion. This is then taken to mean that combined exposures are without risk as long as all components stay below their points of departure. Consequently, possible mixture effects are considered an irrelevance for chemicals risk assessment.

In apparent contradiction to this view, there is good evidence that combinations composed of chemicals with diverse modes of action also exhibit mixture effects when each component is present at doses equal to, or below points of departure (Kortenkamp et al. 2007, and updates in Michael Faust's workshop presentation).

The flaw in the above line of thinking is two-fold:

First, when chemicals cannot be shown to interact with the same molecular targets, it does not follow, that they must act in a dissimilar fashion. It is conceivable that diverse modes of action lead to similar adverse outcomes – dissimilar action is not the simple negation of similar action.

Second, points of departure, and particularly NOAELs, are confused with with true zero effect levels. Under IA, combination effects cannot arise when the individual responses of each component in the mixture are zero. With large numbers of chemicals however, even very small individual effects will lead to considerable combined responses. For example, 100 chemicals that each produce 0.1% of a maximal effect, are expected to yield a response of 9%, according to IA. However, the resolving power of most testing methods in regulatory use is far too low to demonstrate such small effects. Far from signifying zero effect levels, NOAELs describe a grey zone, where the presence of effects can neither be proven, nor ruled out with confidence. NOAELs are frequently associated with effects of between 5 and 10% (Kortenkamp et al. 2007, Scholze and Kortenkamp 2007).

Taken together, there is good evidence to show that the implicit null-model of many regulatory assessments, namely, that only the most potent compound determines the toxicity of the mixture, is usually wrong. Instead, more than one chemical in the mixture contributes to the observed effects (either according to DA or IA) in contradiction to the regulatory default model of "only the most toxic compound counts".

The demonstration that mixtures of dissimilarly acting chemicals are not without effect when they are combined at doses around points of departure, does say little about whether or not risks are present in "real world" exposure settings. The decisive factor for such risks to occur lies in the number of chemicals, and their levels: Only if sufficient numbers of chemicals of sufficient potency and at sufficiently high exposure levels are present, are combination effects to be expected. The issue can only be decided on the basis of better information about relevant combined exposures of human populations and wild life. This information is currently missing, and this knowledge gap presents a major challenge to risk assessment.

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

Although observations of combination effects of endocrine disrupters at low doses have lent urgency to calls to account for such effects in chemicals risk assessment and regulation, the need for doing so is often disputed with the argument that the conventional chemical-by-chemical risk assessment is sufficiently protective. The Uncertainty Factors (UF) usually applied to translate apparently safe dose levels derived from animal experiments into acceptable exposures for humans, so goes the argument, already cover the possibility of combination effects. The issue was examined by Martin Scholze (workshop presentation).

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

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

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

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

Finally, the issue of UF's and mixture effects can be approached from a different direction by asking the question: how large would an additional assessment factor have to be to take account of mixture effects? For a combination of chemicals that follows dose addition, it can be shown that the RfD's for each individual chemical would have to be divided by the number of chemicals that contribute to an overall mixture effect. For example, if a combined effect from simultaneous exposure is due to 5 chemicals, then the RfD of every chemical has to be divided by 5, which is equivalent to saying that an

additional assessment factor of 5 is needed to cover mixture effects (NRC 2008). Correspondingly larger factors are needed if more chemicals can be shown to contribute to a common adverse outcome. However, choices about sufficiently protective factors cannot be made without better information about the number of relevant chemicals, their levels and potency, and how they contribute to human exposures.

To summarize, a specific "mixtures assessment factor" is currently not employed in the traditional chemical-by-chemical risk assessment, and there is little to suggest that commonly used UF are overly protective. There is not much "room" to allow for mixture effects.

5. Approaches to Cumulative Risk Assessment

The practice of Cumulative Risk Assessment (CRA) is furthest developed in the USA, where the US EPA is by far the most important authority for mixtures risk assessment and regulation. Until recently, a common application of mixtures risk assessment in the USA was to Superfund waste sites. The Comprehensive Environmental Response Compensation and Liability Act (CERCLA) which came into force in 1980 specifically calls for mixture risk assessment during the evaluation of risks that stem from hazardous waste sites and chemical accidents. An additional stimulus for CRA was the passage of the Food Quality Protection Act (FQPA) in 1996 which required the estimation of health risks from combinations of pesticides with a common mode of action, from any exposure source.

Several workshop presentations have dealt with existing approaches and practices of CRA (presentations by Linda Teuschler, Rolf Altenburger and Henrik Tyle), and one workshop aim was to evaluate whether these approaches can be used productively to deal with endocrine disrupter mixtures.

5.1 The grouping of chemicals for the purpose of cumulative risk assessment

CRA begins with the identification of chemicals that should be grouped together and subjected to joint risk assessment. In Superfund site assessments this is driven by considerations of joint exposures. In contrast, CRA for pesticides begins with the identification of a group of chemicals that are considered to induce a common toxic effect by a common mechanism, a so-called common mechanism group (CMG). The criterion proposed by US EPA (2000) for grouping chemicals for cumulative risk assessment is "toxicological similarity".

Extensive guidance exists about how this should be implemented (US EPA 2000). Pesticides and other chemicals are considered to qualify for inclusion in a CMG when their mechanism of toxicity shows similarities in both nature and sequence of major biochemical events (workshop presentations by Linda Teuschler and Rolf Altenburger).

The use of toxicological similarity based on mechanisms, however, may lead to overly narrow groupings. For example, organophosphate pesticides and carbamates inhibit acetylcholinesterase, and this is shown to be a relevant step in the manifestation of toxicity. Because the mechanism of inhibition by carbamates is via carbamylation, and

that of organophosphates by phosphorylation, and because this is judged to represent different molecular mechanisms, the two types of pesticides are not assessed together, but included in separate CMGs for the purpose of mixtures risk assessment. Such narrow groupings ignore that joint effects can also occur from combined exposures with other than common mechanisms (workshop presentation by Rolf Altenburger).

5.1.1 Grouping for antiandrogens

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

The NRC therefore recommended a broader based move towards establishing grouping criteria for phthalates and other antiandrogens. With this type of endocrine disrupter, a case can be made for adopting a physiological approach to analyzing toxic mechanisms of action with respect to similarity or dissimilarity. If it is recognized that the driver of male sexual differentiation during development is the effect of androgen action, it is irrelevant whether the hormones' effects are disrupted by interference with steroid synthesis, by antagonism of the androgen receptor, or by some other mechanism (for example, affecting consequences of androgen receptor activation). The resulting biological effects with all their consequences for male sexual differentiation are similar, although the molecular details of toxic mechanisms - including metabolism, distribution and elimination - differ profoundly in many respects. Judged from such a perspective, a focus on phthalates to the exclusion of other antiandrogens not only would be artificial and lack credibility, but could imply serious underestimation of cumulative risks posed by agents for which there is simultaneous exposure (workshop presentations by Ulla Hass and Andreas Kortenkamp).

5.1.2 Thyroid disrupting chemicals

Similar considerations may apply to the group of thyroid disrupting chemicals which affect multiple targets through a variety of mechanisms. In an echo of the situation with antiandrogenic chemical mixtures, the question is: which level of biological complexity should be used to cumulate joint effects? If an endpoint representative of a specific mode of action is chosen (e.g. variations in T4 levels), then certain chemicals might be left out of a common grouping. On the other hand, if the endpoint chosen for integration is at a very high level of complexity (e.g. changes in cognitive function), not only a very large number of chemicals but also a variety of non-chemical stressors will have to be taken into account. This may become difficult to handle in risk assessment settings (workshop presentation by Kevin Crofton).

5.1.3 Dioxin-like endocrine disrupters

Dioxins and dioxin-like compounds represent a group of endocrine disrupters where key events of toxicity are thought to be mediated by binding to the arylhydrocarbon receptor (AhR). These chemicals were first grouped according to descriptors of chemical structure (to include only polychlorinated dibenzo-p-dioxins and –furans, PCDD, PCDF), but insights into their biological activity led to the incorporation of co-planar PCBs and other poly-halogenated polycyclic hydrocarbons (workshop presentation by Martin van den Berg). By using the criterion of AhR activation, polybrominated diphenyl ethers (PBDE) were not included in the group of dioxin-like chemicals. It turned out that pure PBDE were devoid of AhR activity, and that earlier reports of AhR activation could be ascribed to contamination with dioxin-like chemicals, most importantly polybrominated dibenzodioxins and –furans. The most potent PCDD, 2,3,7,8 TCDD, is selected as the reference chemical, and the potency of all other dioxin-like chemicals is expressed in terms of TCDD effect concentrations, so-called TCDD equivalents, with TCDD equivalency factors (TEF) (van den Berg et al 2006). The use of TEF for the assessment of mixtures of dioxin-like chemicals is an application of the concept of dose addition, and is a widely accepted risk assessment method.

5.1.4 Estrogenic chemicals

In many ways, estrogenic chemicals resemble dioxin-like chemicals: Their activity is thought to be mediated by binding to estrogen receptors (ER alpha or beta), which suggests itself as a straightforward grouping criterion (workshop presentation by Andreas Kortenkamp). Furthermore, there are reference agents of high potency (17-beta-estradiol, DES) and there is good evidence that mixtures of estrogenic chemicals follow dose addition when the assessment is based on events relatively close to receptor activation (Kortenkamp 2007). Consequently, it has been suggested that this group of endocrine disrupters should be assessed just like dioxin-like chemicals, by using the toxicity equivalency concept. However, this suggestion has been called into question by Safe, with reference to the complexity of estrogen signaling (discussed in Kortenkamp 2007). Nearly 20 years ago, evidence has emerged that ER activation is possible without binding to the binding pocket of the steroid hormone, e.g. by phosphorylation through activation by growth factors. This opens the possibility that estrogen action can be substantially modulated by chemicals interfering with other phosphorylation events. Should such agents be subjected to CRA with estrogenic chemicals? Furthermore, more research is needed to elucidate the toxicological relevance of ER activation. Although chemicals such as DES are potent disrupters of male and female sexual differentiation, it remains to be seen whether these effects are mediated by ER activation. Similarly, the mechanisms by which estrogens play a role in breast cancer are not entirely resolved.

Considerations of mode of action as a grouping criterion are often of little use in ecotoxicological mixtures risk assessment, because each chemical usually exhibits multiple modes of action. The solution to this problem is to take account of sensitivity differences in various receptors and species (Leo Posthuma, workshop presentation).

5.2 Mixtures risk assessment methods

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

Considering that experimental studies with endocrine disrupters showed that dose addition is a useful concept for the approximation of combination effects, component-based methods derived from dose addition suggest themselves as risk assessment approaches. These include the Hazard Index (HI), Point of Departure Index (PODI) and the TEQ concept (Linda Teuschler and Henrik Tyle, workshop presentations).

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

5.2.1 Tiering

Depending on the quality of the data that are available for CRA (data poor or data rich), tiering methods might be very productive to explore the problem, and refine (with more sophisticated models and associated supporting data) when needed (Leo Posthuma, workshop presentation). At the lowest tier (tier 0), it may become apparent that the situation to be evaluated does in fact not present an issue for mixtures risk assessment. In the next higher tier (tier 1), termed “simple generic”, data about mixed exposures may not be present, but it may be deemed desirable to safeguard against the possibility of joint effects by adopting a specific mixtures assessment factor. In tier 2, “moderately simple generic”, sufficient data may be available to warrant the assumption of dose addition throughout, in which case variants of this concept could be applied, even though independent action may produce less conservative estimates. In a quite data rich situation (tier 3, “complex specific”) sufficient information about various modes of action may be available, such that mixed mixtures assessment models (DA within groups of compounds perceived to follow simple similar action, followed by IA across groups) can be applied. Finally, in the highest tier 4 (“highly specific”) it would be possible to address both issues of modes of action and differences in the vulnerability of various species or risk receptors.

In the light of the data situation typical for many endocrine disrupters, it would appear that assessments at tier 1 and tier 2 are currently possible.

5.2.2 Validation

With the aim of putting CRA methods on a sound footing, it is important to seek situations where the outcome of specific assessments can be validated. While this is achievable in ecotoxicology (presentation Leo Posthuma), the situation is much more complicated in the arena of human toxicology.

5.3 Regulation and risk management

CRA for endocrine disrupters and other chemicals can yield important stimuli for regulation and risk management, by providing the basis for a procedure of relative ranking, e.g. according to the most potent chemicals. This would offer the possibility of strictly regulating, or even eliminating those chemicals that are shown to have the greatest impact on a combination effect. Other rankings could be performed in terms of the most problematic exposure settings, or the most vulnerable population subgroups (Leo Posthuma, workshop presentation).

6. Consensus formulation and recommendations

The workshop participants reached a consensus on a number of specific issues relevant to CRA of endocrine disrupters. The participants also made certain recommendations concerning risk assessment methods, research needs, and legislative requirements.

6.1 Mixtures risk assessment is necessary

In view of the evidence about mixture effects at low experimental doses (see 4.1) and the uncertainty of commonly employed UF in single-chemical risk assessment (see 4.2) a disregard for combination effects was considered undesirable and not in line with currently available empirical evidence. Any CRA method, even one that employs the narrowest possible toxicological grouping criteria, was deemed to represent an improvement compared to the current pre-occupation of conventional risk assessment with chemical-by-chemical approaches. Moreover, an extended look at simultaneous or sequential exposure issues was deemed crucial, to add to the classical toxicological approaches.

6.2 The assumption of independent action as a default for “real world” mixtures is not tenable

As discussed in 4.1, the absence of proof of “similarity” in the mode of action of mixture components cannot be taken to indicate applicability of IA as a default, with the implicit assumption that combination effects are not to be expected if all chemicals are present at doses below their individual points of departure or NOAELs. The workshop participants recognized that the available empirical data do not support this widely held view. Instead, there is good evidence that mixtures that follow IA exert effects even when all mixture components are present at doses below their NOAELs.

6.3 The application of dose addition is recommended as a default, until evidence to the contrary appears

The empirical evidence with endocrine disrupter mixtures (see 3.3) shows that DA yields reasonable approximations of observed combination effects. There are many examples where IA has produced underestimations of observed joint effects. Crucially, no case could be identified, where IA afforded a more conservative mixture effect prediction that was at the same time in agreement with the experimental mixture effects. It is conceivable that such evidence appears in the future, but until this is the case, DA was recommended as the default assessment method, irrespective of presumed modes of action. This modus operandi has the additional advantage of requiring fewer data than the alternative concept of IA, with the consequence that it can serve as lower-tier approach in many circumstances.

6.4 Steps towards CRA: criteria for the grouping of chemicals, assessment methods

Grouping criteria that are driven exclusively by thoughts about mechanisms or the key events for a mode of action were seen as problematic by the workshop participants (see 5.1), and it was recommended that grouping should be dissociated from mechanistic considerations. For risk assessment, phenomenological grouping criteria, based on common adverse health outcomes, were seen as a more useful starting point for groupings. Nevertheless, it was recognised that toxic effects become less specific for the initiating event, as one moves further down-stream of an effector chain. This loss of specificity may lead to the inclusion of an ever wider array of chemicals into a grouping for CRA, ultimately blurring all distinctions, with the need to include all chemicals. However, this was not perceived to be a critical problem for endocrine disrupting chemicals.

Another useful criterion for groupings is the likelihood with which simultaneous exposures to several chemicals occurs.

A tiered assessment, depending on the extent and quality of existing data about hazards and exposures is recommended. For example, to alleviate concerns about mixture effects, it would be possible to adopt a specific assessment factor in the traditional chemical-by-chemical risk assessment, even without any further data. In more data-rich situations, it is feasible to utilize applications of the dose addition concept to define margins of exposure or other indicators of risk.

6.5 CRA for endocrine disrupters, although feasible, is hampered by important data gaps

Due to significant experimental advances in the last five years, determinants of additive mixture effects of classes of endocrine disrupters are now quite well understood. The prospect of CRA for endocrine disrupters is limited by incomplete information about relevant exposure scenarios. This is particularly critical for human risk assessment: it is not even possible to say with confidence whether there are only a few chemicals that contribute significantly to an overall mixture effect, or whether the number of relevant chemicals is likely to be high. Better knowledge about this aspect of the problem would

have an enormous impact on the prospects of CRA. The issue can only be resolved through dedicated mixtures exposure assessment approaches, where scores of chemicals are measured in one and the same sample. This would also provide information about the feasibility of using certain index chemicals as surrogates for exposure measurements. Furthermore, it was recommended to identify exposure "hot spots" with the aim of using those for targeted monitoring (with associated exposure 'cold spots' as points of reference for interpretation).

Another challenge concerns the issue of dose metrics. The usefulness of data from animal experiments would be enhanced greatly if the internal tissue levels resulting from experimental exposures were known. This would enable a read-across to readily accessible data about human tissue levels.

Further research needs are in the following areas:

The joint effects of different classes of endocrine disrupters need to be evaluated, and a better understanding of hormone systems other than estrogens, androgens and thyroid hormones is urgently required.

Finally, determinants that lead to synergisms between endocrine disrupters need to be investigated.

6.6 A better legislative basis for CRA is needed in Europe

Without the legal mandates laid down in the US American CERCLA and FQPA, cumulative risk assessment would not have been implemented in the USA. With the exception of the recent changes in European pesticides regulations, where mixture risk assessment is mandated, comparative legal frameworks that clearly address CRA do currently not exist in Europe. In REACH for example, CRA for multiple chemicals from multiple sources, routes and pathways is only addressed to a very limited extent in the current guidance. Other relevant European legislations do not contain a mandate for CRA for multiple chemicals from multiple sources, routes and pathways.

Development of a comprehensive implementation of CRA should be given serious consideration in all relevant legislation and guidance dealing with chemicals safety assessment and the establishment of safe emission and exposure levels. It is essential to assess the scope of existing laws and guidance in order to define better whether existing regulation can be amended to accommodate CRA, or whether tailor-made regulations need to be developed.

6.7 Prioritisation

The workshop participants were asked to distill their recommendations into a few main points and to prioritize. Consensus on the following was reached:

- CRA for endocrine disrupters can start immediately – important information necessary to make decisions about groupings of chemicals to be subjected to mixture risk assessment is available.

- Dose addition should be used as the default lower-tier assessment method. It should replace the current risk assessment paradigm that is focused on single chemicals, with its erroneous implicit assumption of “only the most toxic compound counts”.
- The legal basis and/or guidance for CRA in Europe needs to be enhanced further.

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Appendix

Expert workshop on combination effects of chemicals, 28-30 January 2009, Hornbæk, Denmark

Programme outline

Wednesday, 28 January 2009	
12:00	Lunch
13:30	**Henrik Soren Larsen, Andreas Kortenkamp** Welcome and introductory remarks

Session 1: Mixtures risk assessment – is it necessary?

13:45	**Round table opening discussion: Are there examples where combined exposures have proven to pose risks?**

This discussion is intended as a first attempt at defining issues: workshop participants are invited to give their opinions about what, if any, they regard as important examples where mixtures are a problem, in human and/or ecotoxicology.

14:30	**Michael Faust** Low dose mixture effects – a review of experimental evidence

This presentation will review the experimental evidence for mixture effects when chemicals are combined at low doses, close to levels that are "points of departure" for risk assessment (i.e. benchmark doses or NOAELs).

***Resource**: Kortenkamp et al. 2007 EHP 115 Suppl 1 : 106*

15:00	**Discussion**
15:30	**Coffee break**

Session 2: A basis for combined risk assessment – case study: phthalates and other anti-androgens

Beginning with a fairly well-researched group of chemicals, this session is a first attempt at crystallizing issues for mixtures regulation: What is the experimental evidence for combination effects of phthalates and other antiandrogens? How can these data be

assessed? What are criteria for grouping these substances for purposes of mixtures risk assessment?

15:45 **Ulla Hass**
Combination effects of phthalates and other anti-androgens after gestational exposure

***Resource**: Hass et al. 2007 EHP 115, Suppl 1 : 122, Metzdorff et al. 2007 Toxicol Sci 98 : 87, Christiansen et al. 2008 Int. J. Androl. 31: 241*

16:15 **Discussion**

16:30 **Andreas Kortenkamp**
Which chemicals should be grouped to protect against combination effects resulting in disruption of male sexual differentiation? – a discussion of grouping criteria

***Resource**: Summary chapter of US NRC report "Cumulative risk assessment for phthalates – the tasks ahead"*

17:00 **Discussion**

17:15 **Linda Teuschler**
An overview of chemical mixtures risk assessment methods

***Resource**: Teuschler 2007 TAP 223: 139*

Having discussed the specifics of antiandrogen mixtures in some detail, this presentation is intended to broaden the discussion and will summarize the methods that have been used to group other substances for the purpose of mixtures risk assessment. Is it possible to derive generally applicable criteria?

17:45 **Discussion**

18:00 **Drinks and dinner**

Thursday, 29 January 2009

Session 3: The basis of combined risk assessment for other classes of endocrine disrupters and other chemicals

The following series of talks will consider topics for mixture risk assessment relevant to other endocrine disrupting chemicals, such as: What are effect outcomes or mechanisms on which mixtures risk assessment should be based? What is the evidence for combination effects?

9:00	**Kevin Crofton** Effect profiles of thyroid-disrupting chemicals and experimental evidence of mixture effects ***Resource**: Crofton 2008 IJA, Crofton et al. 2005 EHP* **Discussion**
9:30	**Martin van den Berg** Dioxins, PCBs and related chemicals – an update on the TEF approach ***Resource**: Van den Berg et al. 2006 Tox Sci 93 : 223* **Discussion**
10:00	**Andreas Kortenkamp** Estrogens and estrogen-like chemicals – an update on combined effects ***Resource**: Kortenkamp 2007EHP 115 Suppl 1: 98* **Discussion**
10:30	**Coffee break**
11:00	**Rolf Altenburger** A brief overview of other efforts of mixtures risk assessment: organophosphates, carbamates, chloroacetanilides, triazines… ***Resource**: EPA guidance documents*

11:30 **General discussion: Is mixtures risk assessment for endocrine disrupters and other chemicals a viable prospect? What are barriers? What are opportunities?**

Suggested topics for discussion include: Are toxicologically relevant endpoints sufficiently well characterized to provide a basis for mixtures risk assessment? What are major sources of uncertainty? Knowledge gaps?

12:30 **Lunch**

Session 4: From mixtures risk assessment to regulation

The scene is set for a more general treatment of the mixtures risk assessment, relevant to other groups of chemicals. The session begins with a brief summary of approaches to mixtures regulation, considers practice in ecotoxicology, and what can be derived for human toxicology and ends with an analysis of uncertainty factors and their suitability for covering mixture effects.

14:00 **Henrik Tyle**
Synopsis of approaches to mixtures regulation (top n, PODI, HI, TEF, relative potency factors, etc)

Resource: VKM report 2008, p 38 – 51, Feron et al 2004, ETAP 18, 215

14:30 **Leo Posthuma**
Practical approaches in ecotoxicological mixture risk assessment in support of urgent policy questions

15:00 **Martin Scholze**
Uncertainty factors in standard setting – are mixture effects covered?

15:30 **Coffee break**

16:00 **General discussion – focus: can existing chemicals regulation be modified to take account of mixtures effects?**

17:00 **Break-out group: Formulation of theses and summary**

Here we are looking for volunteers with extreme stamina: Three to four participants are wanted who are willing to take it upon themselves to distill the discussions so far into a few theses/summary, to be presented the following day.

18:00 **Drinks and dinner**

Friday, 30 January 2008

Session 5: Looking forward – what can/should be done?

9:30 **Break-out group**
Presentation of theses and summary

The break-out group will present their theses and summary for discussion and comment.

10:00 **General discussion and conclusion**

At this stage, this discussion is deliberately left a little unstructured, but the intention is to reflect on the insights from a science perspective with practical steps for risk assessment and regulation in mind.

10:30 **Coffee break**

10:45 **General discussion (continued)**

12:30 **Lunch**

14:00 **General discussion (continued)**

15:30 **Andreas Kortenkamp**
Summing up, conclusion, recommendation and outlook

16:00 **Close**

Resources references

(in the order of the talks)

Michael Faust

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Linda Teuschler

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Kevin Crofton

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Martin van den Berg

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There were no resources circulated for Leo Posthuma's and Martin Scholze's presentations

List of Participants
Expert Workshop on Combinations Effects 28-30 January 2009

Name	Affiliation	e-mail
USA		
Linda Teuschler	U.S. EPA/NCEA-Cin	teuschler.linda@epa.gov
Kevin Crofton	US EPA/RTC-NC	crofton.kevin@epa.gov
EU excl. DK		
Martin van den Berg	University of Utrecht, NL	m.vandenberg@uu.nl
Michael Faust	Faust & Backhaus Environmental Consulting, DE	faust@fb-envico.com
Martin Scholze	University of London, UK	martin.scholze@pharmacy.ac.uk
Leo Posthuma	National Institute for Public Health and the Environment (RIVM), NL	l.posthuma@rivm.nl
Rolf Altenburger	Helmholtz Centre for Environmental Research – UFZ, DE	rolf.altenburger@ufz.de
Thomas Backhaus (absent)	Göteborg University, SE	Thomas.backhaus@dpes.gu.se
Gabriele Schöning	European Chemicals Agency, FIN	Gabriele.Schoening@echa.europa.eu
Andreas Kortenkamp Scientific organiser	University of London, UK	andreas.kortenkamp@pharmacy.ac.uk
DK		
Jens-Jørgen Larsen	National Food Institute, Danish Technical University, DK	jjla@food.dtu.dk
Ulla Hass Practical and co-scientific organiser	National Food Institute, Danish Technical University, DK	ulha@food.dtu.dk

Nina Cedergreen	University of Copenhagen, Dep. of Agricultural Sciences, DK	ncf@life.ku.dk
Henrik Tyle	Danish EPA, Chemicals Division, DK	hty@mst.dk
Marie Louise Holmer (attended the last day)	Danish EPA, Chemicals Division, DK	mlh@mst.dk
Pia Juul Nielsen Workshop co-coordinator	Danish EPA, Chemicals Division, DK	pjn@mst.dk

Location: Sauntehus slotshotel, **e-mail:** sauntehus@royalclassic.dk