‘Mixture effects of chemicals’ ‘The Cocktail Project’ Fødevarekemisk indsats under Fødevareforlig II 2011-2015

Vinggaard, Anne Marie; Hadrup, Niels; Petersen, J.H.; Petersen, Annette; Rasmussen, Peter Have; Dybdahl, Marianne; Frandsen, H.; Taxvig, Camilla; Hass, Ulla; Pedersen, Gitte Alsing; Lykkeberg, Anne Kruse; Boberg, Julie; Sharma, Anoop Kumar; Binderup, Mona-Lise; Granby, Kit; Trier, Xenia; Rosenmai, Anna Kjerstine; Kongsbak, Kristine Grønning; Bengtström, Linda; Skov, Kasper; Hansen, Nadja Lodberg; Svingen, Terje

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‘Mixture effects of chemicals’

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AM Vinggaard, N Hadrup, JH Petersen, A Petersen, PH Rasmussen, M Dybdahl, H Frandsen, C Taxvig, U Hass, GA Pedersen, A Lykkeberg, J Boberg, AK Sharma, ML Binderup, Kit Granby, Xenia Trier, AK Rosenmai, KG Kongsbak, L Bengtstrøm, K Skov, NL Hansen, T Svingen

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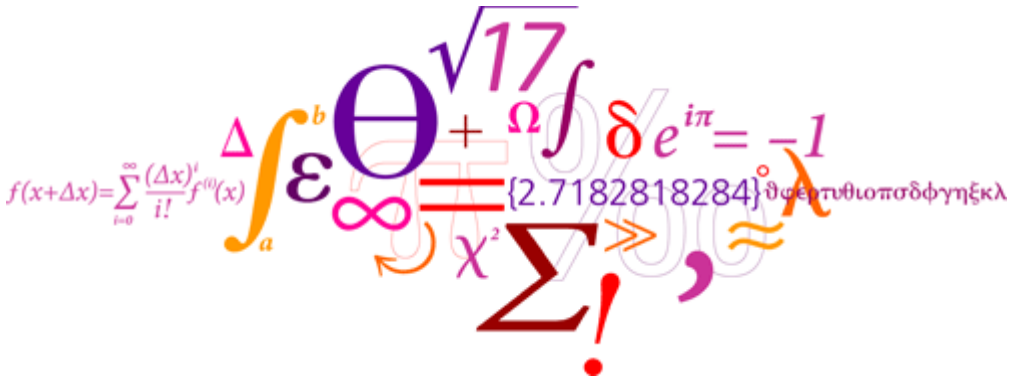

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Cocktail Projektet – et resumé

I 2011 blev et 4-årigt forskningsprojekt om cocktaileffekter af kemikalier i fødevarer iværksat - finansieret af Fødevareministeriet. Projektet Cocktail har været Danmarks hidtil største forskningsprojekt om cocktaileffekter i fødevarer. Forskningsresultaterne bliver formidlet i denne rapport, i en version til den generelle befolkning og ved en endagskonference i marts 2015.

De seneste tyve år har mangelfuld viden om cocktaileffekter og fraværet af pålidelige værktøjer til at risikovurdere kemikalieblandinger været en kilde til bekymring. Bekymringen har været, at den traditionelle tilgang med at risikovurdere ét stof ad gangen ikke tager højde for de sundhedsskadelige effekter, der kan opstå på mennesker, når stoffer optræder sammen (cocktaileffekter).

Projektets mål har været at øge vores viden på området og omsætte denne viden til pragmatiske værktøjer til risikovurdering af kombinationer af kemikalier til brug for regulerende myndigheder i fremtiden.

Projektet havde flere formål:

- At teste beregningsmodeller til at vurdere cocktaileffekter.
- At udvikle metoder til gruppering af stoffer med samme virkning med henblik på at beregne cocktaileffekter.
- At kortlægge den danske befolknings udsættelse for kemikalier gennem fødevarer.
- At få mere sikker viden om enkelte kemikaliers skadelige effekter.

Resultater

Cocktail projektet har tilvejebragt en række leverancer, der bringer Danmark helt i front, når det gælder viden om cocktaileffekter af kemikalier og den eksponering, mennesker udsættes for gennem indtagelse af fødevarer. Heriblandt:

- Test af pålidelig metode til at beregne cocktaileffekter (dosis addition).
- Udvikling af ny strategi til vurdering af sikkerheden af fødevareemballage mht. indhold af kemikalier.
- Udvikling af flere alternative systemer til gruppering af kemikalier (computerbaserede og eksperimentelle).
- Udvikling af metode til bestemmelse af mange små molekyler i én prøve (metabolomics).
- Udvikling af en multimetode til samtidig påvisning af mange kemikalier i en fødevare.
- Brugervenligt værktøj til forskere og myndigheder, der samler eksisterende viden fra databaser i hele verden om stoffers effekter og menneskers eksponering for dem.

Bag om resultaterne: Hvad har Cocktail undersøgt?

Cocktail projektet har bestået af syv delprojekter, hvor forskningen i særligt grad har været rettet mod fem fokusområder eller udfordringer:

1 Matematiske modeller til beregning af cocktaileffekter
2 Hvordan grupperer vi kemikalier?
3 Befolkningens eksponering for cocktails af kemikalier fra fødevarer
4 Identifikation af problemstoffer i komplekse blandinger (emballage, fødevarer)
5 Pragmatisk metode til håndtering af cocktaileffekter

Cocktaileffekter og matematiske modeller

Hvad sker der egentlig, når mennesker udsættes samtidigt for to eller flere kemikalier? Frygten har været, at stofferne kan forstærke hinanden (dvs. forårsage synergi), så deres samlede effekt bliver større end den der kan forudsiges ud fra enkeltstoffernes isolerede effekt. I Cocktail projektet har vi undersøgt, hvordan stofferne typisk virker sammen, og forsøgene viser, at kemikalier oftest virker additivt. Det vil sige, at man så at sige kan lægge effekten af stofferne i blandingen sammen, når der samtidig tages højde for stoffernes styrke. Med andre ord virker stofferne typisk ikke forstærkende (synergistisk) på hinanden.

Forsøgene peger dog samtidig klart på, at hvis der er mange kemikalier til stede i selv små mængder, kan det have en markant skadelig effekt, uden at der er tale om synergi. Med andre ord kan 'mange bække små udgøre en stor å', og meget tyder på, at den nuværende risikovurderingsprocedure ikke i tilstrækkelig grad beskytter mennesker. Der er meget der tyder på, at det totale kemikaliepres vi mennesker er udsat for, kan påvirke vores sundhed specielt for de allerhøjst eksponerede grupper.

Når vi ved, hvordan samspillet mellem kemikalier påvirker mennesket eller en anden organisme, kan vi begynde at beregne cocktaileffekter. En del af Cocktail projektet har således haft som formål at undersøge, hvilken matematisk model, der bedst kan bruges til at beregne cocktaileffekter, når de enkelte kemikaliers effekt og dosis er kendt. Den matematiske beregningsmodel, der bedst og mest pålideligt kunne forudsige kemikaliers cocktaileffekt viste sig at være 'dosis-additions-modellen'. Anvendeligheden af denne beregningsmetode understøtter resultaterne, der peger på, at kemikalier virker additivt.

Gruppering af kemikalier

For at dosis additions-modellen skal kunne bruges optimalt, kræver det, at kemikalierne der indgår i beregningen forårsager den samme effekt. Kemikalier skal med andre ord grupperes efter deres skadevirkning for at kunne give pålidelige beregninger. Da der mangler data fra dyreforsøg for at kunne gruppere kemikalier, har det været nødvendigt at finde alternative løsninger på den problematik.

Pålidelige metoder til at gruppere kemikalier har været en del af Cocktail-projektets fokus. Forskerne har blandt andet etableret en teknologiplatform, metabolomics, der kan identificere en meget stor del af alle små organiske molekyler i én prøve. Metabolomics-platformen viste sig at være i stand til at opdage molekylære ændringer i blodprøver fra rotter, selv når kemikalierne var blandet ved lave doser. Denne teknologiplatform kan i fremtiden bruges til at gruppere stoffer efter deres skadevirkning – dvs. om stoffet eksempelvis giver leverskade, reproduktionsskader, etc.

Udover laboratorieforsøg på celler og dyr, har forskerne i Cocktail projektet fundet en ny anvendelse af en computerbaseret tilgang, integrativ systembiologi, til at gruppere kemikalier efter de effekter, der forudsi-

ges at forekomme i mennesker. Metoden bygger på data fra tidligere laboratorieforsøg, som er tilgængelige via offentligt databaser. Denne metode forventes i fremtiden at komme til at stå endnu stærkere efterhånden som flere data bliver tilgængelige.

Endelig har vi testet nogle computerbaserede modeller til at gruppere kemikalierne på baggrund af deres kemiske struktur ((Q)SAR modeller). Disse modeller kan bruges til at forudsige stoffers egenskaber og gruppere stofferne i tilfælde af, at der ikke er eksperimentelle data tilgængelige for stoffet.

Befolkningens eksponering for kemikalier

For at kunne vurdere risikoen ved forskellige kemikalier er det afgørende at vide, hvor meget mennesker typisk er udsat for af kemikaliet. Derfor har en del af projekt Cocktail handlet om at få et overblik over mængden af pesticider og andre forureninger i fødevarer. Projektet har med udgangspunkt i resultater fra den danske fødevarekontrol og andre undersøgelser dannet et billede af udsættelsen for kemikalier i fødevarer i perioden 2004-2011. Undersøgelsen har blandt andet vist, at der, når det handler om fødevareforureninger, kan være behov for at begrænse udsættelsen for stoffer som bly, cadmium, PCB og dioxiner. Det er dog vigtigt at pointere, at når det gælder fx hormonforstyrrende effekter, er kemikalier generelt ikke tilstrækkeligt undersøgt, men i de tilfælde, hvor vi har viden, viser resultaterne, at der er behov for at reducere indtaget af hormonforstyrrende kemikalier. Kortlægningen viste også, at menneskers indtag af pesticider via fødevarer generelt er begrænset. De fødevarer der bidrager mest til vores eksponering for pesticider, er den frugt og de grøntsager som vi indtager mest af, f.eks. æbler. Det foreslås, at de indsamlede data om befolkningens udsættelse for kemikalier gennem fødevarer løbende bliver opdateret.

Identifikation af problemstoffer i fødevarer og emballage

Fødevarer indeholder ofte en kompleks blanding af kemikalier. Cocktail projektet har også haft fokus på at udvikle såkaldte multimetoder, som giver mulighed for samtidig måling af en række vidt forskellige stoffer i fødevarer, som f.eks. pesticider og giftstoffer fra skimmelsvampe (mykotoksiner). Metoden har afsløret helt nye, ukendte mykotoksiner fra svampe i korn, som vi ikke vidste fandtes i kornet. Dette ændrede mykotoksin forureningsmønster er formentlig et resultat af de klimaforandringer, der pågår for øjeblikket.

Fødevareemballage af papir og pap er på grund af deres komplekse sammensætning en anden udfordring at teste i forhold til indhold af kemikalier. Derfor har vi udviklet en alternativ metode, hvor vi tester emballagen vha. biologiske tests for hormonforstyrrende og kræftfremkaldende effekter og derefter identificerer de problematiske kemikalier. Metoden kan bruges til at give et fingerpeg om emballagen er sundhedsmæssig forsvarlig.

Pragmatisk metode til håndtering af cocktaileffekter

Et klart mål med Cocktail projektet har været at lave en værktøjskasse, der kan bruges, når man fremadrettet skal tage højde for cocktaileffekter som en del af risikovurderingen.

På baggrund af resultaterne i Cocktail projektet anbefaler forskerne en pragmatisk tilgang til risikovurdering af kemikalie-cocktails. Der er udarbejdet et flow-diagram, der trin for trin skitserer fremgangsmåden, som munder ud i beregning af en Hazard Index, der er et kvantitativt mål for risikoen for en cocktaileffekt. Beregningen kan foretages på flere niveauer alt efter hvor mange data der er til rådighed for de enkelte kemikalier i blandingen. På det laveste niveau f.eks. vil alle kemikalier grupperes sammen i én gruppe uafhængig af effekt og de fleste data vil være grove skøn. På det højeste niveau er beregningen af Hazard Index baseret på faktiske, målte værdier og kemikalierne vil være grupperede efter deres effekt. Beregningen på dette trin er gennemskuelig, konsistent og data-drevet og resulterer i et mere præcist estimat af risikoen for cocktaileffekter. På denne måde kan beregningen forfines alt efter hvor mange data der er til rådighed.

DTU Fødevareinstituttet har udviklet et software – 'Cocktail Effect Calculator' - til dels at søge efter informationer om de enkelte kemikalier i en given blanding og dels til at foretage beregninger af f.eks. et Hazard Index, der er baseret på den matematiske beregningsmetode Dosis Addition.

Perspektiver

Cocktail projektets resultater understøtter arbejdet med at tilpasse lovgivningen, så den tager højde for cocktaileffekter. Det er dog vigtigt at understrege, at vi nok er nået et skridt videre i den rigtige retning, men at vi ikke er i mål endnu, og der stadig er behov for en vægtig fremadrettet indsats for at cocktaileffekter kan implementeres fuldt ud i risikovurderingen af kemikalier. Blandt andet peger projektet på, at der i fremtiden vil være behov for en styrket risikovurdering i form af at:

- undersøge effekter af "real-world mixtures" dvs. i relevante sammensætninger af kemikalier og mængder for mennesker
- generere mere viden om effekter af enkeltstoffer til brug ved beregning af risiko for cocktaileffekter (fx bisphenol A alternativer og fluorkemikalier)
- at finde alternative metoder til at farevurdere enkelte kemikalier
- generere overblik over menneskers eksponering til kemikalier generelt
- opnå mere viden om fødevareemballagers betydning for forurening af fødevarer
- videreudvikle de eksisterende tilgange fra dansk og europæisk side ved vurdering og håndtering af cocktaileffekter

Toksikologiske data for kemiske forureninger er ofte mangelfulde og for at kunne beregne cocktaileffekter er der behov for mere viden om enkeltstoffers farlighed og indtagsniveauer.

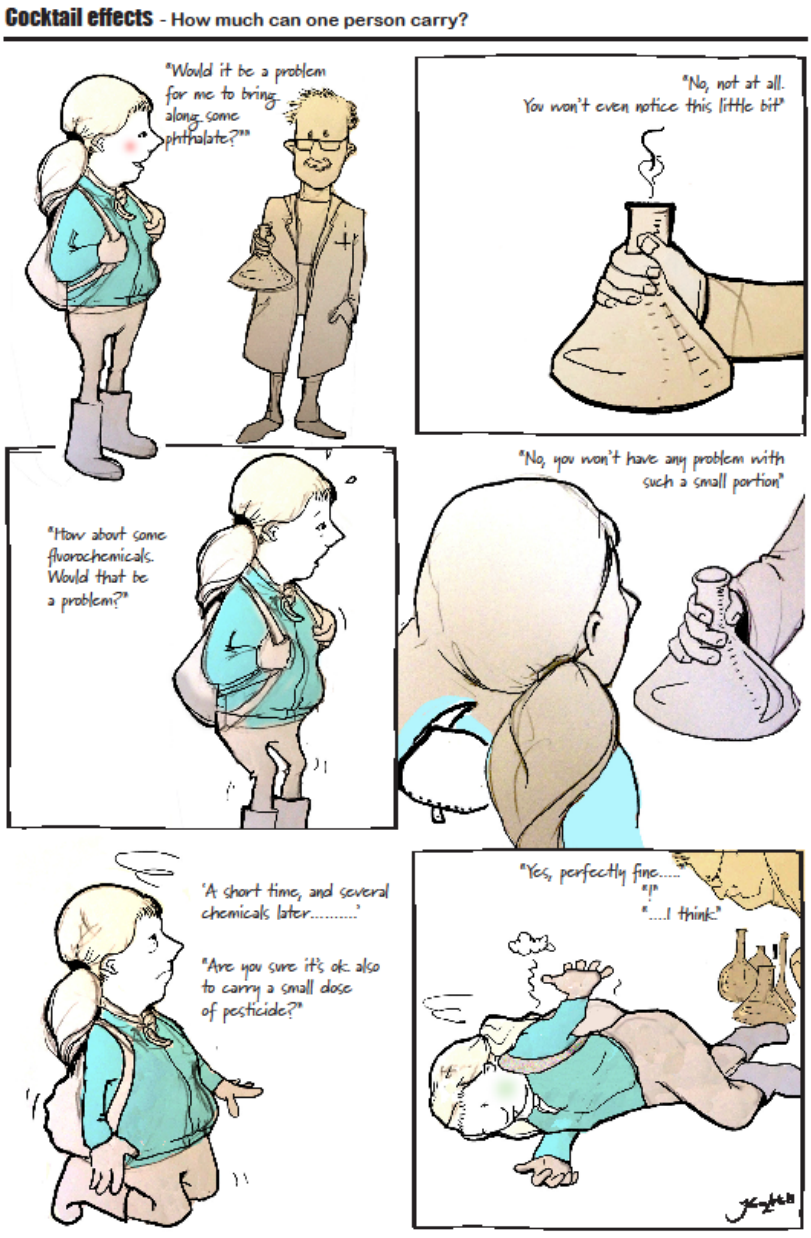
Værktøj til risikovurdering bør testes og videreudvikles

Med Cocktail projektet anbefaler forskerne en pragmatisk, enkel og foreløbig metode til at belyse cocktaileffekter. Denne bør videreudvikles og forfines. En naturlig fortsættelse af arbejdet vil være at teste værktøjets brugbarhed i den daglige risikovurdering af cocktaileffekter. Samtidig skal de eksisterende danske og europæiske risikovurderingsmetoder af cocktails videreudvikles.

Værktøjet bør forbedres ved, at:

- omfatte andre uønskede kemikalier end dem i fødevarer, som f.eks. kemikalier fra kosmetik, forbrugerprodukter og miljøpåvirkning
- samle den tilgængelige information om toksicitet og eksponeringsniveauer for et bredt udsnit af kemiske stoffer

Cocktail projektet har bragt os et skridt videre i den rigtige retning imod en robust og realistisk human risikovurdering af kemikalie-cocktails i fødevarer, men vi er ikke i mål endnu. Videreudvikling og forbedring af den foreslåede tilgang vil føre til, at risikovurdering af kemikalie-cocktails vil kunne føres ud i livet og dermed føre til en højere grad af beskyttelse af befolkningen.



The Cocktail Project – a summary

In 2011, a four-year research project on cocktail effects of chemicals in foods funded by the Danish Ministry of Food was launched. The Cocktail project has been Denmark's largest research project on cocktail effects in food so far. The research results are disseminated in this report, in a version for the general public and at a one-day conference in March 2015.

Concern has been growing during the last 20 years regarding the cocktail effect of chemical mixtures from our foods due to our inadequate knowledge of these effects and the absence of reliable tools to assess the risk from chemical mixtures. The traditional approach to risk assess one chemical at a time does not take into account the adverse health effects that may occur in humans when substances occur together (cocktail effects).

The project aimed to increase our knowledge in this field and to translate that knowledge into pragmatic tools for risk assessment of mixtures of chemicals for future use by regulatory authorities.

The project had several purposes:

- To test mathematical models to assess cocktail effects.
- To develop methods for grouping of substances with the same effect in order to calculate cocktail effects.
- To map the Danish population's exposure to chemicals through foods.
- To obtain more detailed knowledge on harmful effects of individual chemicals.

Results

The Cocktail project has provided findings and developed tools that bring Denmark to the forefront when it comes to knowledge of cocktail effects of chemicals and the exposure to humans through ingestion of foods. These results include the following:

- Testing of reliable methods to calculate cocktail effects (dose addition).
- Development of a new strategy for assessing the safety of food packaging.
- Development of alternative systems for grouping chemicals (computational and experimental).
- Development of a method for simultaneous determination of many small molecules in one sample (metabolomics).
- Development of a multi-method for simultaneous detection of many chemicals in foods.
- A user-friendly tool for scientists and governmental institutions for mixture risk assessment that brings together existing knowledge from databases worldwide about substance effects and human exposure to them.

Behind the results: What did the Cocktail project study?

The Cocktail project consisted of seven sub-projects, in which research targeted five focus areas or challenges:

1 Mathematical models for calculating cocktail effects
2 How do we group the chemicals?
3 Human exposure to cocktails of chemicals from food
4 Identification of problem substances in complex mixtures (packaging, foods)
5 Pragmatic approach for tackling cocktail effects

Cocktail effects and mathematical models

What actually happens when people are simultaneously exposed to two or more chemicals? The concern has been that the substances can reinforce each other (synergy) so that their combined effect is greater than what can be predicted from the isolated effect of single substances. In the Cocktail project, we studied how chemicals are usually operating together, and the experiments show that chemicals typically act additively. This means that the efficacies of the chemicals in the mixture can be added, when taking appropriate account of the potency of the chemicals. In other words, the chemicals typically do not act synergistically or antagonistically. The experiments indicated, however, that if many chemicals are present in even small amounts, it can have a significant detrimental effect without such synergy. In other words, 'the straw may break the camel´s back'. This suggests that the current risk assessment process does not adequately protect people, and there are many indications that the total chemical pressure to which humans are exposed, may affect our health especially for the highest exposed groups.
When we know how the interaction between chemicals affects humans or other organisms, we can calculate cocktail effects. Part of the Cocktail project has had the objective to study which mathematical model that can best be used to calculate cocktail effects when the effect and the dose of the chemicals are known. The mathematical calculation model that best and most reliably predicts the chemicals' cocktail effect was the dose addition model. Application of this method of calculation supported that chemicals typically act additively and therefore neither reinforce nor weaken each other.

Grouping of chemicals

A requirement for the dose addition model to be applied is that the effects of the chemicals included in the calculation are of the same nature. Chemicals should, in other words, be grouped according to their adverse effects in order to make reliable calculations. In the absence of animal data for grouping of chemicals, it was necessary to find alternative solutions to the problem.

Reliable methods for grouping chemicals have been a focal point in the Cocktail project. The researchers included the establishment of a technology platform 'metabolomics' to identify a large proportion of most small organic molecules in one sample. The metabolomics platform was able to detect molecular changes in blood samples from rats even when the chemicals had been mixed at low doses. This technology platform can in the future be used to group substances by their harmful effect, e.g. liver damage, reproductive damage, etc.

Besides laboratory experiments on cells and animals, we identified a new use of a computational approach, integrative systems biology, to group chemicals by the effects that are predicted to occur in humans. The method is based on data from previous laboratory experiments, which are available via publicly available databases. This method is expected to become even more powerful in the future as more data become available.

Finally, we tested various computer-based models for grouping chemicals based on their chemical structure ((Q)SAR models). These models can be used to predict the properties of substances and to group the substances in cases, where no experimental data are available for the substances.

Human exposure to chemicals

In order to assess the risks of various chemicals, it is essential to know how much people are typically exposed to the chemical. In the Cocktail project, we provided an overview of the amount of pesticides and other contaminants in foods. The results were obtained from Danish food surveys and other studies from 2004-2011 and formed a picture of the chemical contamination of foods. The study showed that when it comes to food contaminants, it may be necessary to limit exposure to substances such as lead, cadmium, PCBs and dioxins. It is important to stress that regarding for instance endocrine disrupting effects, chemicals are in general not adequately investigated, but in the cases where we have some knowledge, the results show that there is a need of reducing the exposure to endocrine disrupting chemicals. The surveys showed that the human intake of pesticides through food is generally limited. The foods that contributed the most to our pesticide exposure are the types of fruits and vegetables that we consume the most, such as apples. It is suggested that data on human exposure to chemicals through foods will be continually updated.

Identification of problem substances in food and packaging

Food often contains a complex mixture of chemicals. The Cocktail project has focused on developing so-called multi-methods, which allows simultaneous measurement of a number of different substances in foods such as pesticides and toxins from molds (mycotoxins). The method has revealed new, unknown mycotoxins from fungi in cereals. This new contamination pattern of foods by mycotoxins is thought being a result of on-going climate changes.

Food packaging made from paper and board is another challenge to test in relation to the content of chemicals because of the complex composition. We have developed an alternative method in which we investigate the packaging material by integrating selected biological tests for endocrine disrupting and carcinogenic effects with analytical identification of the problematic chemicals. The method can be used to give an indication of the safety of existing and new packaging material.

Pragmatic approach for tackling cocktail effects

One of the goals of the Cocktail project was to create a tool that can be used for risk assessment of chemical mixtures. Based on the results of the Cocktail project and earlier projects, we recommend a pragmatic approach for risk assessment of chemical cocktails. A flow chart that outlines a step-by-step procedure for mixture risk assessment has been generated. The output of this procedure is the calculation of a Hazard Index, which provides a quantitative value about the risk of a cocktail effect. The calculation can be made at several levels depending on how much data are available for the individual chemicals in the mixture. At the lowest level, all chemicals are grouped together in one group irrespective of their individual effect and most of the data will be rough estimates. At the highest level, the calculation of the Hazard Index is based on actual measured values, and the chemicals will be grouped according to their effect. The calculation at this level is transparent, consistent, and data-driven, resulting in a more precise estimate of the risk of a cocktail effect. In this way the calculation is refined depending on how much data is available.

DTU Food has developed a user interface - 'Cocktail Effect Calculator ' - partly to serve as an information source on individual chemicals in a given mixture and partly to enable Hazard Index calculations, which is based on the mathematical dose addition model.

Perspectives

The results of the Cocktail project support efforts to adapt the legislation to take cocktail effects into account. It is important to stress that the results of this project is an important step forward in the right direction, but we haven't yet fully achieved the aim of being able to implement cocktail effects in chemical risk assessment. Among other things, the project points out that in the future, there will be a need for the following:

• Investigation of the effects of 'real-world mixtures', i.e. relevant chemical mixtures at dose levels close to those humans are exposed to.
• Increased knowledge of the effects of individual substances for use in calculating the risk of cocktail effects (e.g. bisphenol A alternatives and fluorochemicals).
• An overview of human exposure to chemicals in general from many sources.
• Further development of the existing approaches from the Danish and European sides in the assessment and management of cocktail effects.

Toxicological data are often inadequate for chemical contaminants and to calculate a cocktail effect, there is a need for more knowledge on hazards and levels of individual chemicals.

Tools for mixture risk assessment should be tested and further developed

As a result of the Cocktail project, we can provide a rough, preliminary method to estimate cocktail effects. This method should be further developed and refined. A natural continuation of this work will be to test the utility of the tool in the daily risk of cocktail effects.

The tool should be improved by the following:

- Including other undesirable chemicals than those in foods, such as chemicals from cosmetics, consumer products and dust.
- Gaining more knowledge on the significance of food packaging materials' contamination of foods.
- Collecting the available information on toxicity and exposure levels for a wide range of chemical substances.

The Cocktail project has provided the first stepping stones down the path of robust and realistic human risk assessment of chemical cocktails found in food and food packaging, but the full aim hasn't yet been achieved. Further development and improvements to the basic system as listed above would take us closer to the aim of implementing cocktail risk assessments in practice and thus a higher degree of safety and protection for the human population.

1. Introduction

Regulation of chemicals within the EU is typically based on risk assessments of individual chemicals. However, in the modern world, humans are typically exposed to a large number of chemicals simultaneously, often referred to as a *cocktail*. This means that the combined toxicity can exceed the toxicity of the individual chemicals, if they exert similar biological effects. Such cocktail effect thus becomes a regulatory challenge, but should be considered and taken into account in general when devising strategies for risk assessment and regulatory guidelines for chemicals.

The European Parliament has passed several regulations related to foods in the EU, stipulating that cocktail effects of chemical residues should be considered. However, due to the complexity of the issue, the implementation of testing methodologies for risk assessment of chemical cocktails remains a challenge, ultimately hindering urgently needed improvements in food safety.

The purpose of the Cocktail Project was to provide new knowledge about the effects and risks of human exposure to mixtures of chemicals through food sources. This new knowledge has contributed to the development of new tools that can be useful for risk assessment of mixtures of chemicals in foods.

The main focus of the project was risk assessment of mixture effects of endocrine disrupting chemicals (EDCs), both in relation to toxic effects of mixtures and exposure to these substances. Other contaminants such as fluorinated compounds, bisphenols, mycotoxins, and migrants from food contact materials were also assessed in the studies, with additional toxic effects (e.g. carcinogenic effects) other than endocrine disrupting effects also analyzed.

In sum the primary focus points were:

- Generation of data for the combined effects of chemicals with different modes of action.
- Modeling of combined effects and realistic exposures.
- Estimate exposure levels to chemical contaminants and EDCs via food for the general Danish population.
- Developing novel strategies for evaluation of food contact materials.
- Identifying new potential EDCs and additional methods for detecting them.
- Generating new technologies for elucidating the mechanisms by which EDCs act, such as metabolomics and bioinformatics.
- Outline specific recommendations for risk assessment of chemical mixtures.

For chemicals with a similar mode- or mechanism-of-action, we have a solid basis for evaluating mixture effects. This information comes from work on dioxins, mutagens, and EDCs. Therefore, the main focus of this project related to chemicals with differing modes or mechanisms of action. The approach was either ‘bottom-up’, where both individual chemicals and mixtures were examined, with data used to construct a model for predicting the effect of the mixture; or ‘top-down’, where a modelled mixture or ‘realistic’ mixtures were investigated and – if relevant - fractionated for further testing and identification of the toxic chemicals.

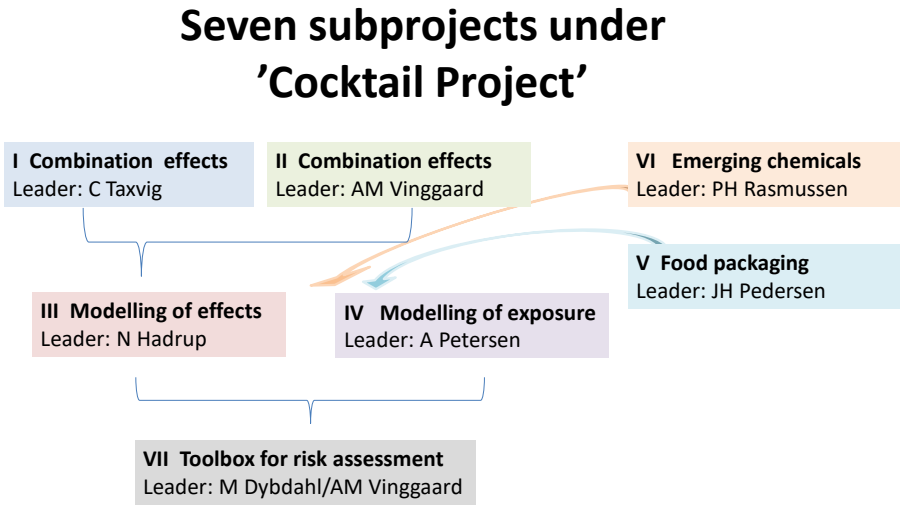
There was also a need to develop a test strategy for the study of complex mixtures of substances from food contact materials that contain a variety of chemical substances with limited regulatory legislations. The goal was to develop an effective strategy for testing undesirable effects and exposure of food contact materials in order to provide a basis for decisions on new regulatory initiatives.

This report presents an overview of the obtained results, the recommendations, and the future perspectives. To get insight into detailed results, the applied methods and other technical details, we refer to the peer-reviewed papers and the PhD reports.

2. Organization of the Cocktail Project

2.1 How was the project structured?

The Cocktail Project was divided into 7 subprojects, denoted by roman numerals. The first two (Cocktail I & II) dealt with obtaining and gathering toxicological data from animal and cell-based studies. Some animal studies have been parts of previous projects, but have in this project been extended with studies on mechanisms of action of the chemicals or mixtures in question. In Cocktail III, toxicological data were modelled by various mathematical models. In Cocktail IV human exposure data for chemical contaminants in foods were collected and presented. Cocktail V focused on complex mixtures obtained from food packaging materials of paper and board. Cocktail VI dealt with complex mixtures of mycotoxins and pesticides in food and methods to detect them. Finally, in Cocktail VII a toolbox for risk assessment of chemical mixtures was developed.
A diagram illustrating the organization of the project can be found in Appendix B.



2.2 Who contributed to the project?

DTU Food was the primary contributor to the Cocktail Project. The persons involved from DTU Food are listed in section 14.2.2. The following research institutions contributed to parts of the project:

- DTU Systems Biology
- DTU Management
- DTU Environment
- DTU Compute
- Brunel University, UK
- University of Alberta, CA
- National Centre of Computational Toxicology, Environmental Protection Agency, US
- The Food and Environment Agency, UK
- University of Rennes, FR

3. How do mixture effects present in experimental systems?

3.1 Toxicodynamic interactions

As part of the Cocktail Project, data from dose-response experiments of individual substances and mixtures in developmental rat and cell studies were obtained (Cocktail I & II). In some cases studies from existing projects financed by other sources were included, and the studies were extended with measurement of additional parameters of varying molecular complexity that were used for modeling purposes. The Cocktail Project has generated comprehensive toxicological data for individual substances, as well as for mixtures, with the end goal of performing mathematical modelling to predict what model best describes a given mixture effect.

In a previous project ('ReproPestiMix') that overlapped with the Cocktail Project, mixture effects of pesticides were investigated. The main objective of the project was to explore the hypothesis that combined developmental exposure to five endocrine disrupting pesticides (EDCs: epoxiconazole, mancozeb, procymidone, tebuconazole, prochloraz) at dose levels below No Observed Adverse Effect Levels (NOAELs) for the single pesticides, leads to adverse developmental toxicity effects in rats. It was also investigated if mathematical modeling of the expected mixture effects could offer useful estimates of the effects, as compared with the mixture effects observed by experimental means. The rats were exposed to the pesticides during the fetal and suckling period, and mixture effects of the pesticides at dose levels where individual pesticides caused no effects, were observed. Mixture effects were found for several end points, including extended gestational period, increased nipple retention in male offspring, as well as significant effects on male reproductive organ weights (reduced) and genital malformations (increased frequency and severity). These rat studies have shown that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides can cause adverse effects on male sexual development. A comparison of the acceptable daily intakes (ADIs) for the individual pesticides to the mixture's 'no effect levels' indicated that the ADIs are not sufficiently low to protect against potential mixture effects. The results of this project imply that risk assessment based on NOAELs for single chemicals can underestimate the risk.

In another EU project, named 'Contamed', 12 EDCs representing various chemical classes were investigated in developmental rat studies (bisphenol A, linuron, dichlorodiphenyldichloroethylene (DDE), 4-methylbenzylcathinone (4-MBC), Octyl methoxycinnamate (OMC), procymidone, the phthalates: DEHP and DBP, vinclozoline, epoxiconazole, butylparaben, and prochloraz). The chemicals were selected based on known exposure of the European population to EDCs. Doses reflecting 100-, 150-, 200 and 450-fold high-end human exposure levels were given to the rats. In order to investigate the interaction of estrogenic and anti-androgenic chemicals, groups of chemicals considered being estrogenic (bisphenol A, butylparaben, 4-MBC, and OMC) and anti-androgenic (linuron, DDE, procymidone, DEHP, DBP, vinclozoline, epoxiconazole, and prochloraz) were given separately and in combination. The results showed that the four estrogenic chemicals present in the total mix of all 12 chemicals did not make a marked contribution to the effects of the entire mixture on hallmarks of male sexual differentiation.

For the majority of affected end points, pronounced and significant effects were observed at the highest dose level of the total mixture and anti-androgenic mixture, but adverse effects were also seen at the lower mixture doses. These effects included decreased anogenital distance (AGD) and increased nipple retention at 150—200-fold human exposure levels, increased numbers of females with irregular oestrus at 200-fold, and decreased ovary weights at all doses of the total mixture, including the 100-fold. An anti-androgenic effect also appeared to be present in the male rats exposed to the 100-fold human exposure.
In female offspring, a marked reduction in serum levels of prolactin was observed and may be an early biomarker for the observed adverse effects on estrous cycle and mammary gland development (Mandrup et al., in press). Late effects on the offspring observed after a dose corresponding to 100-fold human exposure levels included effects on onset of menopause, ovary weight and sperm quality. These data indicate that the safety margin is less than 100, which is commonly considered necessary in risk assessments. This suggests that highly exposed human population groups may not be sufficiently protected against mixtures of endocrine-disrupting chemicals. Therefore, mixture effects should urgently be included in chemical risk assessment.

In vitro effects on sex hormone synthesis of the 12 individual endocrine disrupting chemicals and their mixtures were also investigated (Hadrup *et al.*, 2013). The data showed that five of the single chemicals (BPA, epoxiconazole, linuron, OMC, and prochloraz) and the total mixture inhibited testosterone levels, and the chemicals acted additively.

The overall conclusion from the discussed and other projects is that in the vast majority of cases, chemicals are acting with an additive effect in experimental systems. Only in rare cases has synergism or antagonism been observed, and in most cases these deviating effects can probably be ascribed to toxicokinetic interactions that occurs at higher doses.

3.2 Toxicokinetic interactions and the effects on mixture predictions

Whereas toxicodynamic interactions describe the way the chemicals treat the body, toxicokinetics is the discipline describing how the body is treating the chemicals. Chemicals may interact by affecting absorption, distribution, metabolism and/or excretion of each other (i.e. toxicokinetic interactions). One classical example is the effect of one chemical on liver enzymes that affect the metabolism, and subsequently the blood levels of another chemical. We have observed three examples of toxicokinetic interactions in our rat studies.

In the 'ReproPestiMix' project, where rat offspring were exposed to five pesticides during the fetal and suckling period, we found additive effects on nipple retention in the male offspring that is an early marker for adverse effects on male reproductive health. The prediction of the mixture effect based on dose-additivity was in agreement with the observed effects at low doses. However, dose-additivity underestimated the effects for the high doses of the mixture. The predictions based on the alternative theoretical approach 'independent action' were seen to underestimate the effects for high doses to a greater extent, and overestimate the effect for the low doses. A similar picture was seen for the end-point gestation length. These observations indicate that a synergistic effect had happened. The plasma concentrations of

the pesticides (procymidone and epoxiconazole) indicated that this synergy was due to high pesticide concentrations most likely caused by inhibited metabolism of the active pesticides. To clarify if metabolic overload after mixture exposure causes the higher internal doses, detailed kinetic studies are needed.

In another pesticide research project ('HOPE'), we investigated mixtures of three and five pesticides (propiconazole, cypermethrine and bitertanole, and in the total mixture also terbutylazine and malathione). The *in vivo* study showed that the five pesticides and their metabolites could be dose-dependently detected in the amniotic fluid of rats. Simultaneous exposure to all five pesticides caused markedly lower levels in amniotic fluid of the three individual pesticides compared to when only the three pesticides were given as a mixture. This indicates that the pesticides at these relatively high doses inhibited the internal exposure to one another, possibly by metabolic interference.

As part of the Cocktail Project, we performed an animal study aimed at testing whether toxicokinetic interactions occur at low realistic exposures to chemicals. Such knowledge is important when applying mathematical models for the calculation of cocktail effects. It is important that the combined effect of a mixture can be expected to be additive and that no synergism is taking place; a prerequisite for predictive modelling of mixture effects. The animal study was conducted using three doses of perfluornonanoic acid (PFNA) in the presence or absence of a mixture of 14 chemicals (the 12 endocrine disrupting chemicals ('Contamed' project chemicals) plus two food ingredients). At the toxicokinetic level an interaction on PFNA plasma concentration was found, when animals were given PFNA plus the 14-chemical mixture (Hadrup et al., submitted). The mixture caused a higher PFNA plasma concentration indicating that the mixture affects the kinetics of PFNA. Thus, the presence of some chemicals can affect the internal levels of other chemicals also at low doses.

The conclusion from these studies is that interactions due to toxicokinetics do occur, though usually at higher toxicological dose levels. However, a few observations indicate that these interactions can occur also at lower human relevant exposure levels. Whether this occurs in extraordinary cases only, or is a general phenomenon deserves more attention.

3.3 Low-dose effects and non-monotonic dose-response curves

BPA is a controversial chemical due to the many low-dose effects that have been reported. In spite of this, the regulation of BPA does not take into account these reported low-dose effects. A low-dose BPA developmental toxicity study was performed in order to further clarify this issue. We found that BPA affects AGD at low doses close to human exposure levels. From doses at 250 µg/kg and above, male AGD was significantly decreased, whereas decreased female AGD was seen from 25 µg/kg bw and above (Christiansen et al. 2014). The decreased AGD at birth in both sexes indicates effects on prenatal sexual development and provide new evidence of low-dose adverse effects of BPA in rats in the microgram per kilogram dose-range. Human high-end exposure is estimated by EFSA to be 1.1-1.5 µg/kg. The margin of safety compared with

the effects level of 25 µg/kg in rats is 17-23 and therefore clearly below the safety factor of 100 traditionally applied in risk assessment.

As part of the 'Cocktail project' a PFNA and mixture animal study (mentioned in Section 3.2) was performed at high-end human exposure levels. The lowest PFNA dose applied was estimated to be about 6-15-fold higher than human exposure levels according to the biomonitoring data obtained from the US human population (NHANES: National Health and Nutrition Examination Survey, CDC, USA, 2009), and the mixture was given at a dose close to human exposure levels. We found low-dose effects on androgen plasma concentrations at the high-end human dose levels of the 15-chemical mixture. These effects were found to be non-monotonic, as an increase seen at the low dose was turned into a decrease at high dose (Hadrup et al., submitted).

Another animal study performed under the Cokctail project was using a mixture of 27 chemicals given orally at human relevant dose levels has been conducted. Doses of 1-, 3-, and 10-fold human exposure levels were given to rats for three months. The study aimed at testing the hypothesis that a mixture of chemicals at doses that causes plasma concentrations equal to normal average human exposure does not cause an adverse footprint in the mammalian body. The rats given 10-fold human exposures had to be sacrificed after two months due to severe toxicity. Examples of methods used to monitor biological changes were metabolomics, LC MS/MS steroid measurements, and gene expression analyses. Our data show that even at 1- and 3-fold human exposure levels, biological effects can be observed in the animals. Liver organs were affected at 1-fold human exposure levels and a mechanistic gene expression study indicated that a blocking of the bile ducts has taken place. Regarding metabolomics measurements, clear effects on metabolites were seen at 3- and 10-fold human exposure levels. In the lipids fraction, effects at 1-fold human exposure seem to have occurred as early as after 30 days of administration. These metabolites still need to be identified. Together with colleagues at the Copenhagen University Hospital we are currently analysing the actual serum and urine levels of the animals following exposure, as well as finalizing additional mechanistic studies.

Overall these data show that chemical mixtures given to rats at doses close to human exposure levels can affect hormones and perturb biological pathways in the living organism. Also, there is evidence to conclude that non-monotonic effects can occur. These issues complicate chemical risk assessment and the question is, if we have got the safety margin that we usually think we have for protection of human health.

4. Which mathematical model is best suited for evaluation of mixture effects?

The database generated through the Cocktail Project was used for predictive mathematical modeling (Cocktail III). Various models were tested and compared for their ability to predict mixture effects and included; i) dose addition, ii) independent action, and iii) an extension of the dose addition model, the generalized concentration addition model. The best fit for the actual and the predicted effects were calculated for different end points of varying molecular complexity. This work has given us a better understanding of what types of interactions or lack of interactions are taking place under specific experimental conditions, i.e. whether chemicals act additively, synergistically, antagonistically, or whether there is a potentiating effect. In the current project, we have also investigated whether synergism occurs at human-relevant chemical exposure levels. This is because for mathematical models to be effective, an assumption of additivity and absence of synergism is needed. An evaluation of the models describing mixture effects of chemicals is given and a recommendation of which models to be used as pragmatic tools for risk assessment of chemical mixtures is presented.

4.1 Description of prediction models

The DA model is also known as the concentration addition (CA) model. This model assumes that chemicals behave like 'dilutions' of each other and that they contribute to the joint effect in proportion to their dose. For calculating the additivity expectation from experimental data, one has to add equi-effective doses. This model was previously considered to be applicable only for mixtures of similarly acting chemicals.

The DA concept is included in the generalized concentration addition (GCA) model, which is a special case of the DA, in which the slope of the dose response curves is expected to be 1. The advantage of this model is that it is a simple model to apply for situations in which the maximal effect levels of individual chemicals in the mixture differ.

The IA model is based on stochastic principles. When calculating additivity expectation, one has to apply effect multiplication. When exposure to several chemicals occurs simultaneously, the stochastic principles are only fulfilled when components induce the same effects through different modes of action. It remains to be shown that these requirements can be fulfilled in reality.

Often, DA and IA predictions are identical and the predictions seem not to be dependent on the modes of action. This is surprising because this contradicts the theories underlying these concepts. Thus, there are good reasons to believe that the DA model can be used also to make predictions for dissimilarly acting chemicals (Kortenkamp et al. 2013). This has led to the question if hypotheses about modes of action are a reliable basis for making model choices. The answer is probably no. Thus, the DA model seems so far to be a reasonable and pragmatic choice for predicting mixture effects in most cases (Kortenkamp et al. 2013).

4.2 What is the preferred concept for mixture predictions?

From mathematical modelling based on our *in vivo* database, it is relatively clear that the DA model gives adequate and reliable predictions in most instances. This is also in agreement with other similar studies (Kortenkamp et al. 2013). WHO/IPCS (2009) has recommended using DA as a default, unless there is evidence to the contrary. The DA model has been evaluated as sufficiently conservative.

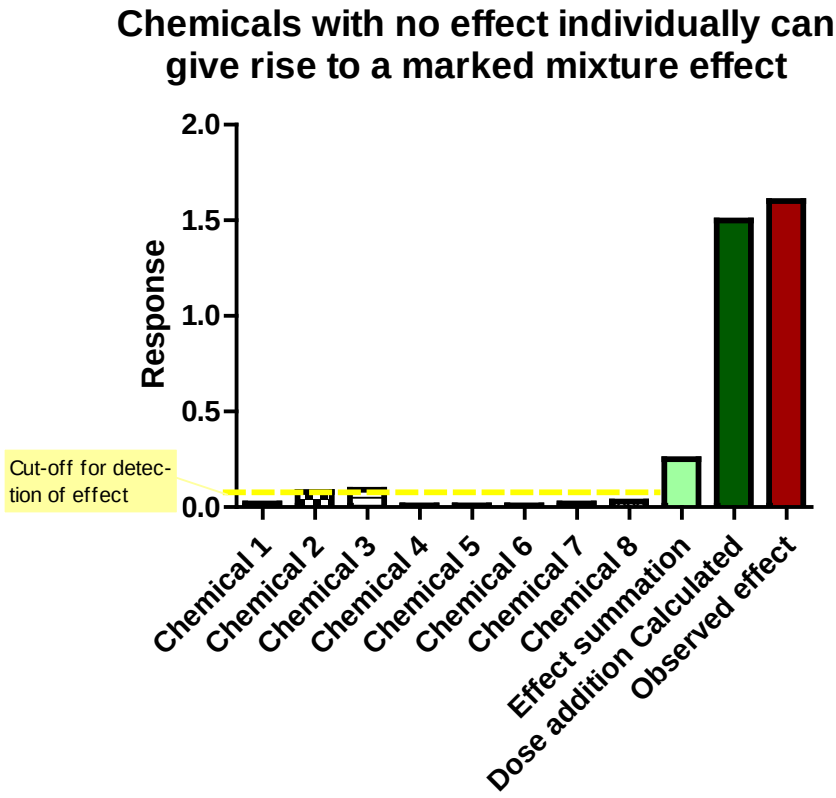


Figure 1. This is an illustration of eight chemicals that individually do not have any harmful effects but which in combination exerts a marked mixture effect. The measured mixture effect can be predicted by dose addition (DA). Simple 'effect summation' is intuitively appealing but underestimates the effect.

We have conducted in vitro experiments to investigate pros and cons of the DA, IA and GCA models. First we measured the effects of single chemicals and mixtures thereof on steroid synthesis in H295R cells (Hadrup et al., 2013). Single chemical data was then applied to the models whereupon predictions of mixture effects were calculated and compared with the experimental mixture data. We investigated two different mixtures. Mixture 1 contained environmental chemicals adjusted in ratio according to human exposure levels. Mixture 2 was a potency-adjusted mixture containing five pesticides. We found that the DA and IA models gave similar predictions, but that the GCA model appeared superior to both for the prediction of effects on testosterone in a situation where individual chemicals did not display equal maximal effect levels. In a special situation with chemicals that exerted opposing effects on a steroid hormone (for instance one chemical that increased and another chemical that inhibited testosterone) none of the models were accurate in their prediction. Therefore, we have identified a need for development of such a model. In addition, we found that once reliable dose-response curves have been obtained for the single chemicals in the

mixture, all three models (IA, DA and GCA) can readily be applied and predictions compared in the process of establishing a toxicological risk evaluation.

There is no documented case in the scientific literature where IA provides more conservative predictions of mixture effects than DA, and where at the same time, IA also produces accurate predictions (Kortenkamp et al. 2012). The use of IA as an assessment concept for mixture effects requires demonstration that modes of action of individual substances in a mixture are strictly independent; a condition that can rarely be met in practice. Therefore, EFSA recommends using cumulative risk assessment methods derived from DA models, including its use for the assessment of mixtures of pesticides with dissimilar modes of action, provided they produce a common adverse outcome. Pesticides that produce common adverse outcomes on the same target organ/system should be grouped together in cumulative assessment groups (CAGs), and their combined effects assessed by using the concept of DA as a pragmatic and conservative default approach for the purpose of assessing cumulative risk in relation to maximum residue level setting or risk assessment of chemical mixtures in practice (EFSA, 2013b)

5. How to group chemicals for evaluation of mixture effects?

It is generally accepted that chemicals should be grouped together according to their adverse outcome (e.g. liver toxicity, neurotoxicity etc) before predicting the mixture effect for each adverse outcome separately. This was done by DTU Food in the comprehensive EFSA CAG project, in which more than 200 pesticides were grouped according to various toxic outcomes (EFSA 2013a). There are several sources that have suggested grouping criteria. The US Environmental Protection Agency (US EPA) has suggested that common mechanisms can be predicted from similar chemical structures. However, the US National Academy of Sciences (2008) stated that similar chemical structures are too narrow and that one should group according to common adverse outcomes.

It is also recognized that other more mechanism-based methods for grouping of chemicals are necessary. The challenge in grouping chemicals however, is that knowledge on mechanisms of action and data on common adverse outcomes often are not available. Also for pesticides, the data requirements for authorization are not geared towards making grouping decisions based on the modes of action. Therefore the Cocktail Project has focused on exploring and finding new ways of grouping chemicals for mixture risk assessment.

5.1 Grouping according to mechanism of action

Under the Cocktail Project, new methods for generating knowledge on the modes of action of chemicals have been applied with the aim of grouping chemicals for predicting their combined effects. In the future we will have much more mechanism-relevant data available primarily due to the ToxCast™ programme performed at the US-EPA, enabling us to group based on mechanism of action.

5.1.1 A metabolomics approach for grouping chemicals

A metabolomics platform has been established to identify changes in the metabolic network caused by EDCs. The goal was to identify biomarkers for specific effects, to elucidate underlying mechanisms of EDCs, and to explore if metabolomics could be applied for grouping of chemicals.

Metabolomics aim to analyze all low-molecular weight metabolites, the metabolome, in a specific biological compartment. Differences in the metabolome between dosed and control animals are detected by multivariate data analysis and will reflect the effects of the dosing on the animals. The platform was established at DTU Food in collaboration with University of Alberta, Canada.

The metabolomics platform consists of a liquid chromatographic system connected to a high-resolution mass spectrometer (HPLC-HRMS) (Skov et al., 2015). The platform has initially been used for analysis of plasma samples from rodents. Plasma samples were subdivided into three fractions in order to obtain good

chromatographic resolution: phospholipids, lipids, and polar metabolites. These three fractions were analyzed by HPLC-HRMS operated in both positive and negative mode followed by multivariate data analysis for detection of affected metabolites.

The platform was used to analyze plasma samples from different mixture studies, where rats had been exposed to various EDC´s. In one study, animals were exposed to a mixture of 14 food-related compounds with or without PFNA (described in section 3.2). The lowest PFNA dose applied was estimated to be about 6-15-fold higher than human exposure levels according to the biomonitoring data obtained from the US human population (NHANES: National Health and Nutrition Examination Survey, CDC, USA, 2009), and the mixture was given at a dose close to human exposure levels. The multivariate data analysis (PLS-DA), expressing the combined effect on the metabolome showed a distinct grouping of animals dosed with different mixtures of EDCs (Figure 2). It can be seen that even after these relatively low dose levels, this technique is relatively good in grouping the chemicals according to the affected plasma metabolome.

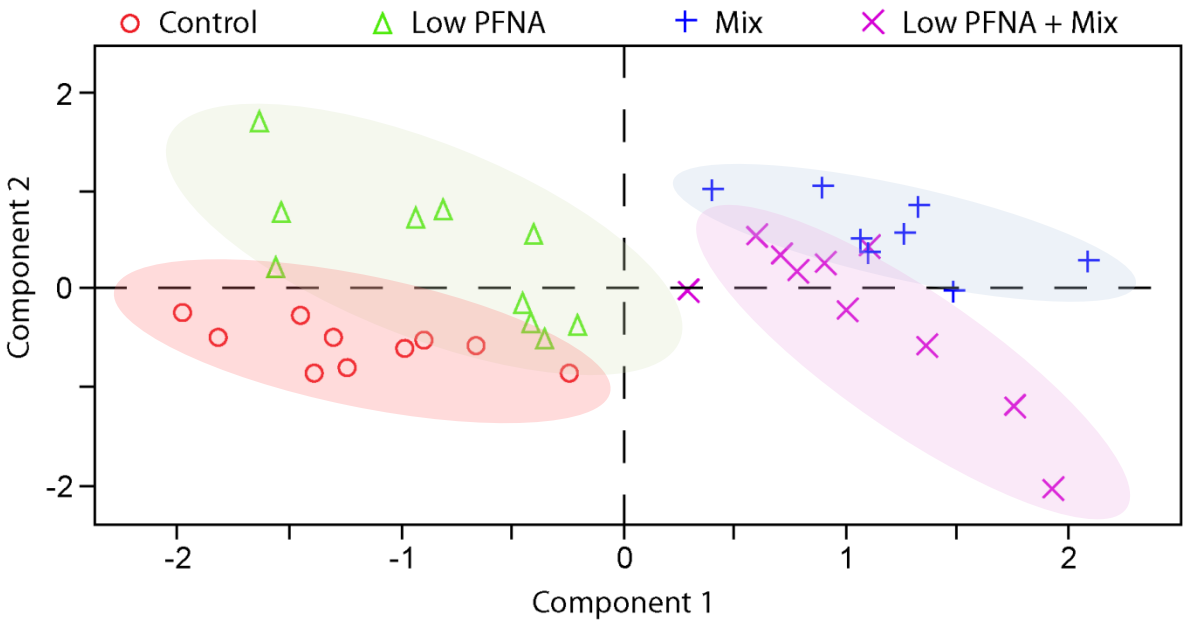


Figure 2. A PLS-DA analysis of endogenous metabolites in control rats, rats treated with a low dose of PFNA, a mixture of 14 chemicals, and a low dose of PFNA plus a mixture of 14 chemicals

In this study 18 metabolites were significantly changed only at the highest dose level, whereas 22 metabolites showed a dose-response pattern. Figure 3 displays six metabolites showing a significant dose-response pattern (figures a-f), whereas figures g-i show a change only at the highest dose level.

Following identification of affected metabolites a mode of action could be suggested. In the lipid fraction a significant decrease in several phospholipids as well as several neutral lipids, such as mono- and diglycerides was observed. For PFNA, the lowered plasma lipid concentrations and the observed fatty liver in the animals were likely biochemically related. Interestingly, the observed changes in the metabolome were detected after only 14 days of dosing, whereas detection of toxic effects may require longer dosing periods.

In the future, identification of affected groups of metabolites following exposure to EDC should make it possible to group EDCs with similar modes of action based on the profiles of the metabolites affected, when a larger database of changes in the metabolome has been collected.

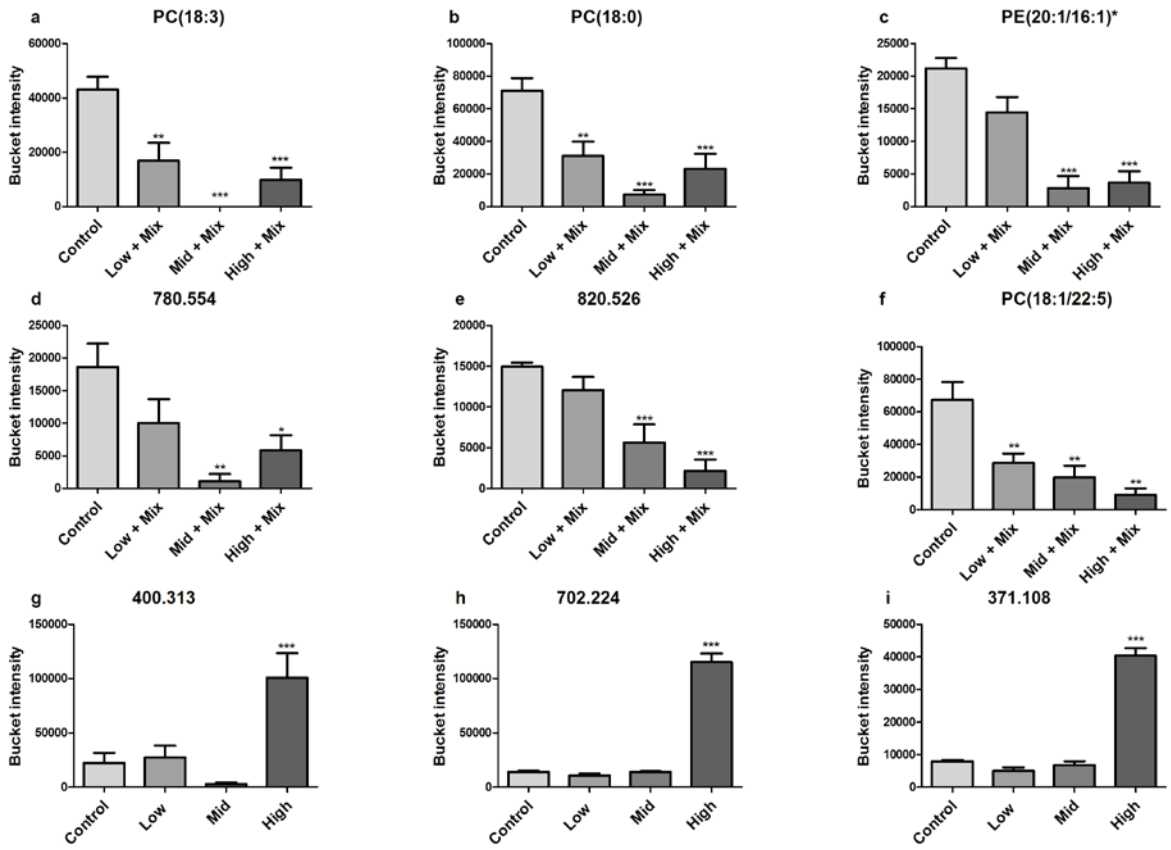


Figure 3. Significantly changed metabolites as a function of three dose levels of PFNA plus the mixture (a-f) or PFNA alone (g-i). In figure a, b and f the plasma concentration of phosphotidylcholine (PC) is shown, in figure c the concentration of phosphotidyleth-anolamine (PE) is shown. In figure d, e, g, h and i masses of unidentified metabolites are shown. This figure represents only part of the changed metabolome, in which 18 out of 40 metabolites have a response similar to that of figure g-i while 22 out of 40 have a response similar to that of figure a-f.

5.1.2 A computational systems biology approach for grouping chemicals

In addition to laboratory tests on cells and animals, advanced bioinformatics tools are used to gain knowledge about the modes of action for chemicals of interest. These computer-generated models constitute additional means of grouping compounds, such that subsequent calculations of mixture effects (e.g. DA calculations) can be obtained.
Bioinformatics analyses were performed in collaboration with Center for Biological Sequence Analysis at

DTU to supplement the traditional toxicological techniques. An examples of a computational approach applied to toxicology was described in two recent articles (Kongsbak et al., 2014a; Kongsbak et al., 2014b). The concept is that predictions of human effects from a set of chemicals are performed solely on the basis of existing literature on associations between human targets and the chemicals in question. We retrieved information about human targets associated to the chemicals of interest (Figure 4) from two databases. This gives an initial overview of known data on human targets for the chemicals. Subsequent steps include 1) enrichment of the initial network with proteins known to interact with the initial human targets, and 2) overrepresentation analyses to determine if the proteins associated with the chemicals are overrepresented in certain diseases or pathways. The final disease-enrichment step has to be carefully evaluated for biological relevance, and existing animal data for the chemicals can serve as evidence for this evaluation. For mixture evaluations, this type of analysis gives an overview of known and potential targets for the investigated chemicals. If chemicals in a mixture target the same molecules and/or are likely to cause the same disease or hit the same pathway, it is likely that these chemicals can be allocated to the same group for mixture risk assessment. For instance, in the example below (Figure 4), mancozeb can be excluded from the cumulative assessment group as it has very different targets related to e.g. inflammatory processes.

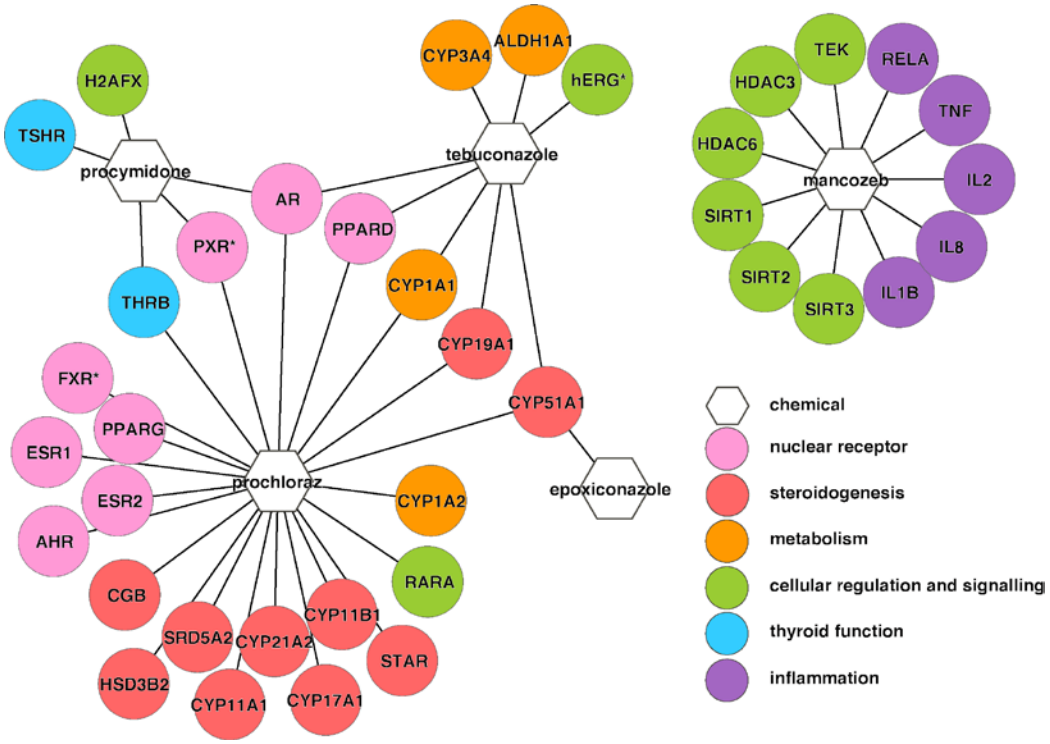


Fig 4: Chemical-protein association network for five pesticides.
Mancozeb (right) shows interactions that do not overlap with those of the other four pesticides. This suggests a different mode of action for mancozeb and therefore it is placed alone in this grouping (Kongsbak *et al.*, 2014b).

5.2 Grouping according to chemical structures

Quantitative structure-activity relationship (QSAR) models can be used to predict the physicochemical and biological (e.g. toxicological) properties of molecules based on their chemical structure. A QSAR is a mathematical model (often a statistical correlation) relating one or more descriptors derived from the chemical structure to a quantitative measure of a property or activity, e.g. a toxicological effect. A set of molecules with experimental test data is used to build the mathematical model (the training set). Thus a QSAR model links information on the chemical structure of compounds to a specific property, which can subsequently be used to predict if other compounds have the same property. Reliable predictions can be made for compounds that are within the domain of the chemical universe of the developed QSAR model, i.e. for compounds that are sufficiently similar in structure to the compounds used to train the model. In this way QSARs can be used to i) evaluate compounds with little or no test data, for example in priority settings, ii) help design testing strategies, iii) further validate test data, and/or iv) devise grouping approaches.

In this project QSAR was used to evaluate five bisphenols (A, B, E, F and S) and the structurally related compound 4-cumylphenol (HPP) with respect to a series of adverse human effects, including endocrine disruption, reproductive toxicity, genotoxicity and cancer. Their metabolism via three key cytochrome P450 (CYP) enzymes and the Pregnane X receptor (PXR) was also evaluated.
All six compounds were predicted or experimentally tested to be positive for estrogenic activity, whereas all but one compound was predicted or experimentally tested positive for anti-androgenic activity. All compounds were predicted to be active in at least one of the models for metabolism (CYP and PXR activity). No strong indications for genotoxicity, cancer and reproductive toxicity were predicted. Overall, the compounds displayed very similar profiles based on the applied QSAR models and these results are described in a recent article (Rosenmai et al, 2014).
In conclusion, QSAR modelling is a valid strategy for grouping of chemicals in cases where data is lacking, provided there is existing data of structurally related chemicals.

6. Exposure of the Danish population to food contaminants

Detailed and consistent data for the presence of chemicals in the diet together with knowledge about Danish food consumption from dietary studies are essential to estimate a realistic human exposure to chemicals. Over the years, numerous chemical analyses have been conducted as part of the food control system or as independent studies. One of the aims of this project was to provide an updated overview of the Danish population's exposure to chemicals including EDCs (Cocktail IV). In 2013, the latest reports were published and included data from the period 2004-2011. The reports comprised estimations of exposure in relation to current toxicological reference values or a margin of exposure (MOE) was calculated and a risk assessment was performed. The two published reports on pesticides and contaminants, respectively, are widely used by the authorities and in teaching.

A challenge with regards to exposure assessment is to align contaminant concentration levels in food and consumption data, and improvements are continuously being made in order to develop more reliable human exposure data. For some of the contaminants, especially trace elements, the chemical analyses have not included all relevant food commodities in the years 2004-2011, thus previous food concentration data were used. Furthermore, only a few food samples were analyzed for some of the chemicals. These limitations introduce uncertainties in the calculations, and therefore it is desirable that more food concentration data for relevant food commodities are generated to improve the exposure assessment.

For the pesticides, a cumulative exposure assessment was performed including all pesticides irrespective of their differential effects and modes of action (Table 1). The hazard index (HI) method was used (explained in Section 10.1.3). Different models for correction of undetected residues as well as the importance of peeling the outer skin/layer of the fruit or vegetable were investigated. The table below shows the results obtained with the chosen model. A HI value above 1 can indicate a risk. It is seen that both values are well below 1. However, pesticides alone may not occupy all of the HI and room should be left for other food contaminants and chemical exposure from other sources. It can be questioned if this 'room' of 56% for other contaminants or chemical exposure from other sources is adequate for the children.

Table 1. Hazard index (HI) for the consumer groups 'Adults' (15-75 years) and 'Children' (4-6 years) using the chosen model for calculation of the exposure (LOR: Limit of reporting)

Reduction for peeling:	½LOR for non-detects; Correction factor limited to 25
Adults	18%
Children	44%

Figure 5 shows which commodities contributed the most to the HI for adults. As can be seen, apples contributed significantly more than other commodities. This is a direct consequence of a high apple consumption of the Danish population, thus the pesticide contamination comes predominantly from this source.

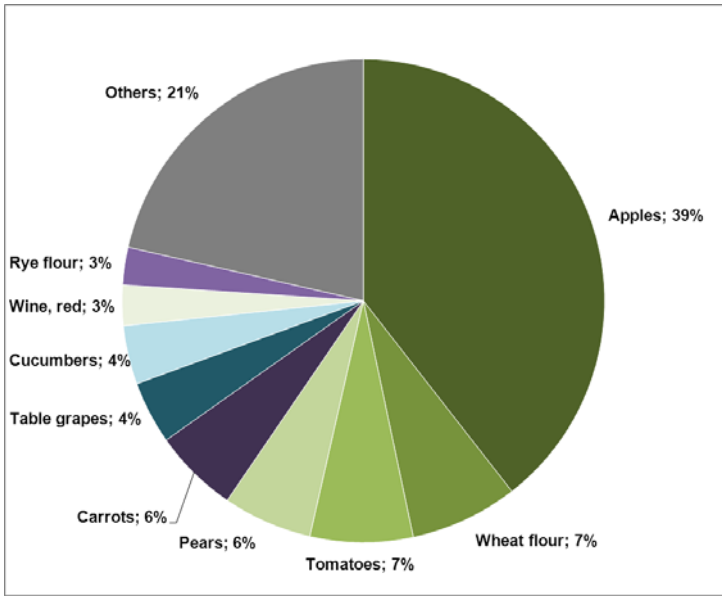


Figure 5: The percentage contribution to the HI for adults caused by various food commodities

In Figure 6, the pesticides that contributed the most to the HI for Danish adults are shown. The phosphor pesticides as a group contributed significantly to the HI. This group includes diazinon, omethoate, pirimiphosmethyl, phosmet, and dimethoate .

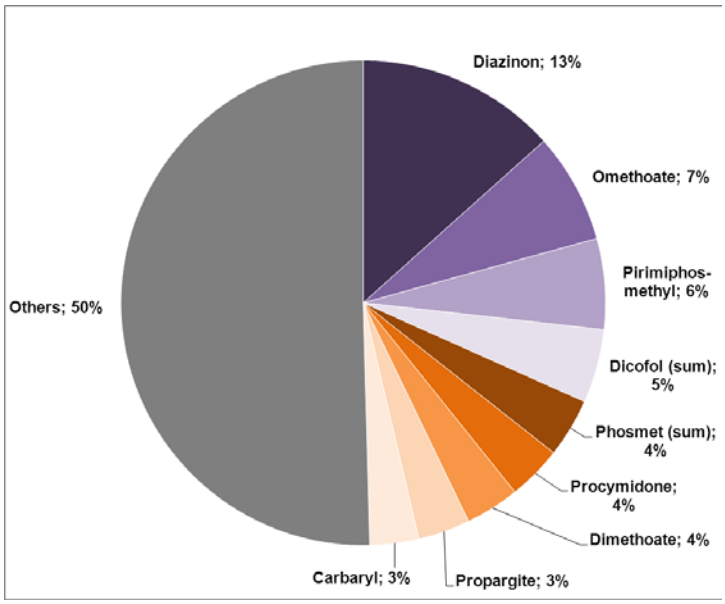


Figure 6: The percentage contribution to the HI for adults from various pesticides

Concerning the other report for contaminants, new substances were included compared with the previous report. These included EDCs such as brominated flame retardants and perfluorinated substances.

For the contaminants, it was concluded that from a health perspective the exposures to lead, cadmium, inorganic arsenic, dioxin and PCB preferably should be lowered, as the exposures to these individual chemicals exceed the toxicological reference values, or the calculated margin of exposure (MOE) is very low. For

certain mycotoxins, brominated flame retardants, organochlorines, nitrate and mercury the calculated exposure from food is below the toxicological reference values. It should be stressed that the above mentioned information on hazards of individual chemicals/chemical classes is based upon the existing regulation of chemicals and do not take cocktail effects into account. For some effects like endocrine disruption, chemicals in general are still not adequately investigated, but in the cases where we have got toxicological information on endocrine disruption, there are indications that the total exposure to these chemicals should be lowered as well.

The objective is to continually include new contemporary data, including data from chemical profiles (Section 8). For pesticides, data are published every year, and for contaminants, the data for 2012 and 2013 will be published in the beginning of 2015. Every year data from the chemical analyses are compiled and reported to EFSA for both contaminants and pesticides.

DTU has been, and will continue to be involved in a EU projects dealing with exposure assessment (respectively 'Acropolis' and 'Euromix'). In Acropolis probabilistic modelling was used to perform cumulative exposure assessments to the triazole pesticides, and in Euromix DTU will be involved in exposure assessments using probabilistic modeling. Probabilistic modelling gives a better estimate of the exposure. It is the aim to expand the use of probabilistic modelling to other chemicals such as cadmium and lead. To perform cumulative exposure modeling it is also preferable to use probabilistic modeling. If possible cumulative modeling will also be performed for other substances than pesticides in the future.

7. Existing and emerging chemicals in food contact materials

In the food contact materials (FCMs) industry, new innovative packaging and articles are constantly being developed and introduced. New chemicals are being applied in order to improve their performance or to enhance their properties. More traditional FCMs are produced from natural materials or from recycled materials where knowledge about chemical constituents is limited. In all cases, only a minority of the chemicals, including unknown impurities and reaction products, has been satisfactorily assessed for their potential health risks.

Traditionally, chemicals migrating from FCMs to foods are determined only on a one-by-one basis using validated analytical methods developed by the industry. However, the number of substances that can potentially contaminate foods is high, and the number of validated detection-methods is very limited. This is an unsustainable situation for regulatory authorities that aim at protecting human health, thus additional methods must be developed.

The aim of our studies was to investigate whether more efficient alternative techniques to traditional chemical analyses could be employed when predicting the safety of FCMs (Cocktail V). Tools such as in vitro toxicological testing for various end points in combination with preparative liquid chromatography and advanced high-resolution mass spectrometry (MS) methods were used in order to develop a strategy for handling mixtures of known and unknown FCM substances.

The wider perspective of this strategy was being able to solve the emerging task of evaluating new and existing FCMs more efficiently using an interactive analytical chemical/toxicological process.

Another aim was to analyze further the presence and activity of certain bisphenols and fluorinated substances, which are known or suspected to be used in FCM. Bisphenol A has been banned from some FCM applications in recent years and a dedicated analytical method for the determination of bisphenols potentially used as alternatives to bisphenol A was developed and applied.

7.1 Strategy for screening of chemicals in food packaging

The strategy was developed for investigation of a variety of samples of paper and board from either recycled or new cellulose fibers and included the chemicals coming from additives, inks, coatings etc. (Figure 7). Paper and board packaging rarely contain a functional barrier that can efficiently prevent migration from these materials.

In the first place a range of important in vitro assays were selected for inclusion in the strategy. The assays are predicting genotoxic effects, endocrine disrupting effects and some general cytotoxic effects like oxidative stress. To focus upon the most critical samples, it was important first to prioritize the FCMs for further analysis based on the in vitro results on the raw extracts. Secondly, to facilitate identification, it was important to isolate (by fractionation) the bioactive substances from the bulk of extracted materials and substances. The fraction(s) were tested again in the relevant bioassay(s). Fractions showing bioactivity underwent chemical analyses to produce a list of tentatively identified substances. By expert judgment many

compounds could be eliminated from the list, with the remaining pure substances procured if commercially available. The individual chemicals were tested in the bioassay, and it was calculated if the amount present in the extract could explain the bioactivity.

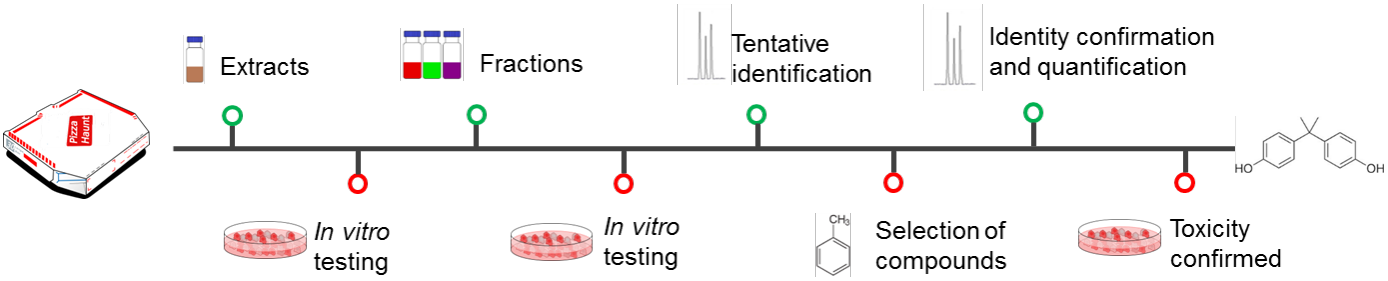


Figure 7: Flowchart illustrating the applied strategy for assessment of paper and board packaging.

7.1.1 Test strategy – tools for in vitro analysis and analytical chemistry

An overview of the individual tools developed and/or used in the strategy is listed here:

- Laboratory equipment suitable for collection of volatiles and extracting, fractionating and concentrating substances present in huge samples of paper and board.
- A high-resolution MS-based analytical platform for improved detection and identification of migrants. A dedicated database containing around 2300 FCM-substances for use in LC-HRMS identification was built.
- A panel of bioassays for toxicological testing of raw extracts and their fractions. Cell culture (in vitro*)* assays covering end points related to endocrine disruption, metabolism, gene toxicity/mutagenicity, oxidative stress and teratogenicity were included.
- QSAR models: computer-based tools for predicting the toxicity of substances was used for initial assessment of observed toxicity. QSAR was used both to predict the effects of specific chemicals and also to guide chemists in the identification of migrating chemical structures.

7.1.2 Results

Twenty packaging materials made from paper and board for various food products were selected for initial in vitro testing of the highly concentrated crude extracts. One sample had a significant mutagenic effect in a bacterial mutagenicity test, the Ames test. Some FCMs showed significant effects on estrogen receptor (ER) activation or blocking of androgen receptor (AR) activity. All extracts exhibited some effect on aryl hydrocarbon receptor activity (AhR that is involved in metabolism) indicating the presence of some active, possibly inherent, substances in the cellulose fibers. However, large differences between the samples with respect to response in the various assays were evident. FCMs with a marked toxicological profile according to this in vitro profiling were selected for further testing.

In vitro testing of the 22 fractions obtained from the raw extracts proved fruitful, and activity was measured in a few fractions from each FCM. In these active fractions chemicals were identified by analytical chemistry. It was not possible to determine directly all of the substances present in the fraction using high-resolution MS, even with the newly developed database, but numerous tentatively identified substances suspected to show activity were identified and compiled. Information about these substances was then listed together with data about chemical structure, known use in FCM, and specific toxicity on the endpoint in question (when available). Knowledge on biophores that are involved in e.g. ER activation or AR antagonism was a valuable contribution in this step. In one case, the number of substances on the initial lists could be reduced from around 70 to a handful of commercially available substances. The complexity of this phase of the strategy depended upon the toxicological endpoint in question, as our knowledge on the chemical structures that are important for activation of the various receptors differ to a large extent.

In these cases the substance(s) responsible for assay activity were attempted being identified:

- A pizza box was shown to have estrogenic activity. The responsible chemicals were BPA, di-*n*-butylphthalate (DBP), and butylbenzylphthalate (BBP). BPA was the primary responsible chemical (Rosenmai et al., in preparation).
- A sandwich wrapper was shown to have marked antiandrogenic activity. Chemical analysis showed that the responsible chemicals were dehydroabietic acid (DHAA) and abietic acid, the latter being the main cause of the effect. This is to our knowledge the first reporting of antiandrogenicity of DHAA (Rosenmai et al., in preparation).
- A pizza box exhibited high aryl hydrocarbon receptor (AhR) activity. The major contributor(s) to the observed activity remains to be identified, but a minor part could be attributed to two pigments found in printing inks. Analyses are ongoing. (Bengtström et al., in preparaton).
- A popcorn bag showed genotoxic activity in the Ames test. Current activity testing is ongoing (Bengtström et al., in preparation).

7.1.3 QSAR analysis of chemicals in non-plastic food contact materials

To further develop abilities to identify hazards of FCM, QSAR models were used to screen approximately 2,000 substances used in FCMs for their potential to be carcinogenic, mutagenic or toxic to reproduction (CMR) (Dybdahl et al.). The in vitro estrogenic and anti-androgenic activities were also predicted. Predictions from a number of relevant models were combined to reach overall calls for CMR effects. More than 25% of the FCM substances were predicted as being positive for at least one of the end points (Figure 8).

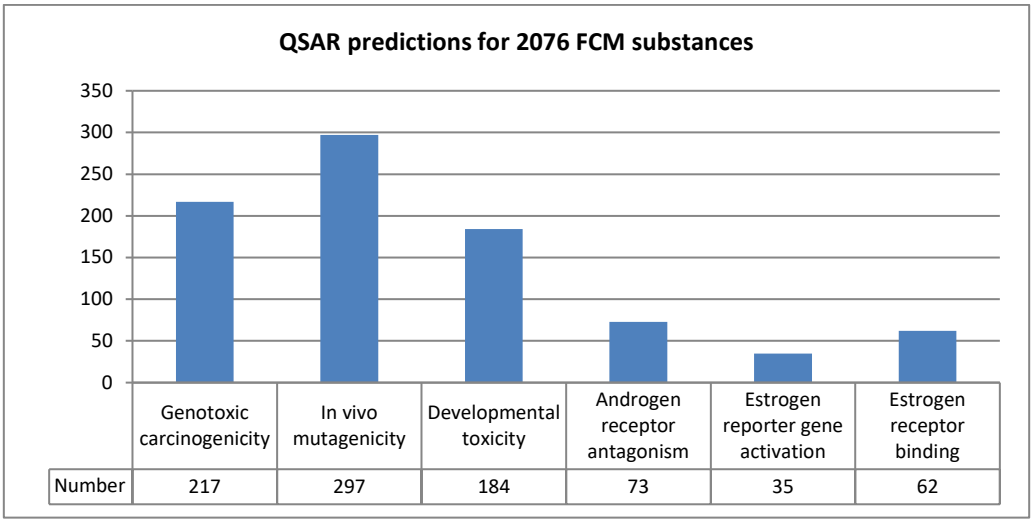


Figure 8. QSAR predictions for 2076 chemicals reported being used in FCMs.

The resulting list of FCM substances with potential harmful effects to human health may be used to prioritize substances for further evaluation. While plastics are covered by a specific regulation comprising positive lists of substances, non-plastic FCMs, e.g. coatings, paper and board, adhesives, printing inks and rubber are not covered by a specific regulation. As a result, thousands of substances used to manufacture non-plastic FCMs have not been evaluated at the EU level for their safety. The present work may also be of value to the non-plastic FCM activities ongoing in EFSA.

7.1.4 Evaluation of the strategy

The strategy proved to be a useful tool to find and assess potential hazards posed by chemicals present in FCM made from paper and board and in identifying the problematic substances in the selected samples. This multidisciplinary approach - using in vitro testing in combination with advanced chemical analyses - set new standards for risk assessment of FCM.

However, it is important to acknowledge certain limitations of the current approach: Already when selecting the solvent for the initial extraction, it is known that not all substances are completely soluble in said solvent, and will thus not be 100% extracted. Other substances are potentially toxic to the cells used in the bioassays and can mask for a substance having other adverse effects. Also, the crude procedures used when producing the highly concentrated extract can lead to at least partial loss of some substances.

A modified approach that involves a refined fractionation procedure generating more fractions from one extract would be beneficial, thereby limiting the number of chemicals in each fraction. This would ultimately make the task of identifying the active chemicals more easy.

The conclusion this far is that problematic substances present in FCMs made from paper and board can in many cases be identified by applying the described procedure. These problematic chemicals can be present as contaminants in the recycled fibers or as constituents in printing inks and coatings applied to the FCMs.

7.2 Adverse effect of fluorinated chemicals

In recent years, DTU Food has highlighted the issue of the presence, and potential harmful effects, of fluorinated chemicals like PAPS molecules in FCMs. For most fluorinated substances little is known about their toxicity and use. Therefore we selected 19 fluorinated chemicals suspected to be used in FCM and tested them in vitro. The chemicals (and three commercially available technical mixtures) are either used as coating on food packaging materials or may appear as impurities or metabolic products. A common effect of many fluorinated chemicals is their peroxisome proliferator-activated receptors (PPARα) activating properties that can be linked to their liver toxic effects in vivo. From our results it appears that some of these compounds (e.g. PAPS molecules) can decrease the synthesis of the male sex hormones and increase the synthesis of female sex hormones. The longer-chained perfluorinated chemicals also displayed an ability to activate estrogen receptors (Rosenmai *et al.*, 2013; Rosenmai et al., in preparation).

7.3 Chemical analysis of alternative bisphenols in food packaging

A literature survey of the use of BPA, its substitutes, and their use in different types of FCMs was conducted and revealed that BPA, BPB and BPS can be found in human tissues and that several BPs have been found in canned food and drinks, as well as in thermal paper or receipts (BPS). We are lacking information to what extent the alternative BPs are used and in which foods they occur. However, a rough estimate of the human exposure to BPA from FCMs based on published data was presented (Pedersen, 2013).

BPA substitutes (bisphenol B, E, F, S and 4-cumylphenol) were successfully incorporated into our existing mass spectrometric method for detection of BPA. A specific analytical method for determination of BPA and its substitutes in raw paper and board extracts was validated; using FCM extracts from the aforementioned project, BPA was found in seven out of the 20 samples. Only one BPA substitute (BPS) was found at low levels in a cardboard tray for tomatoes. Further, a selection of 18 lacquered cans and lids for food contact were analyzed for the content of BPA substitutes. Cumylphenol was found in three of the samples and BPF in one sample, both at low concentrations.

7.4 Toxicological profiling of alternative bisphenols

BPA is a compound known for its potential to cause endocrine disruption and its use has been prohibited in some instances. In recent years, substitutes such as BPA analogues have replaced BPA in food packaging. Some analogues have been measured in food packaging materials and in human blood, suggesting that these are already in use and that humans may be exposed. We have investigated five analogues to BPA to assess whether these have a similar toxicological profile to BPA in vitro and in silico (QSAR models). The in vitro tests comprised reporter gene assays for the estrogen, the androgen, the Ah receptor, and p53, oxidative stress, as well as effects on sex steroid synthesis.

Results showed that the six compounds generally had similar toxicological profiles and thus had qualitatively almost same effects. The main effects were endocrine disruption and the compounds tended to target both the sex hormone receptors and sex hormone synthesis. One exception was BPS which wasn't very potent as an antiandrogen, but showed a marked effect on progesterone synthesis. In general we found slight differences in the quantitative effects (Rosenmai *et al.*, 2014). Based on these findings, it is advised that substituting BPA with these analogues should be carried out with caution before more knowledge is available.

8. Screening of food for mycotoxins and pesticides

We know about the presence of a significant number of contaminants in foods, but also believe that there are many more contaminants not yet detected. This represents a 'black box' in food safety and the main reason for this lack of information is likely because the presence of many chemicals in foods have not yet been analyzed. It could also be that we do not know about their existence and/or that these chemicals have not yet caught the attention of the researchers or authorities. An analytical chemical profiling strategy for foods has been developed to supplement traditional analytical techniques, and was designed to measure one or more specifically selected chemicals (Cocktail VI). By adapting principles from metabolomics, the focus was on creating coherent, broad chemical profiles in order to obtain a basis for more detailed chemical risk profiling of food. A spin-off would be a strengthening of the contingency plans, as it enables the subsequent use of data mining to search for unexpected relationships in data. In this way, we aimed to obtain an overview on the extent environmental contaminants in foods pose potential problems into the future. With this knowledge, strategic decisions in relation to chemical food safety will be simpler in the future. The strategy was developed in collaboration with DTU Systems Biology.

8.1 Glycosylated mycotoxins - occurrence and formation

We carried out investigations into new glucosylated fusarium toxins (HT-2 and T-2 toxin) in cereal grain. In addition to the glucosylated forms, a further 10 forms of A-trichothecenes were identified: acetyl-T-2 toxin, neosolaniol, diacetoxyscirpinol, buturyl-NEO, hydroxy HT-2, Hydroxy-T-2, dehydroxy-HT-2, dehydroxy-T-2, 3-aldo-methylbutyryl-T-2, and NT-1 toxin. These new fusarium toxins are expected to add another 20%-30% to the total trichothecene contents in foods. These toxins (except neosolaniol) have previously not been described in small cereal grains. Furthermore, the alkaloid chrysogine was found in most samples and correlated with the T-2 content. Glucosides of HT-2 are - as other masked mycotoxins - assumed to be formed in the plants in response to fungal contamination. We have recently found that HT-2 glucosides can be formed at significant levels by a specific fungus (*F. langsethiae)*. This shows that glycosylation of HT-2, and maybe also other mycotoxins, can be carried out by the fungus itself and does not only occur in plants as previously thought.

8.2 Multi-method for screening of mycotoxins and pesticides

We developed and implemented a multi-method analysis capable of evaluating the presence of mycotoxins and pesticides in cereals. A LC-QTOF approach obtained a very good confirmation of the chemical structure with respect to accurate mass, isotopic pattern, retention time and fragment ions. A library was built, now containing around 600 pesticides and 40 mycotoxins.

In selecting the best method for sample preparation, compromises must be made. And thus, this study used the QuEChERS method as a starting point for optimization; a simple extraction and cleanup strategy used for pesticides. Recovery and matrix effects were investigated for 25 mycotoxins and around 150 pesticides at three different steps of the QuEChERS method, as specified in Figure 6. Most compounds benefitted from a freeze-out step to remove lipids and sugars (except for fumonisin B1 and B2). The last step in the QuEChERS method is the cleanup, which removes acid components in the matrix, and the analysis of many compounds was improved using this step due to a pronounced reduction of matrix effect. However, compounds containing carboxylic acids were also removed including ochratoxin, fumonisins and pesticides such as 2,4-D. Therefore, a compromise was taken skipping the cleanup step, thereby accepting matrix effect for many compounds, but enabling us to analyze for mycotoxins and pesticides containing carboxylic acids. However, this had a consequence for several pesticides as it resulted in lower detection limits.

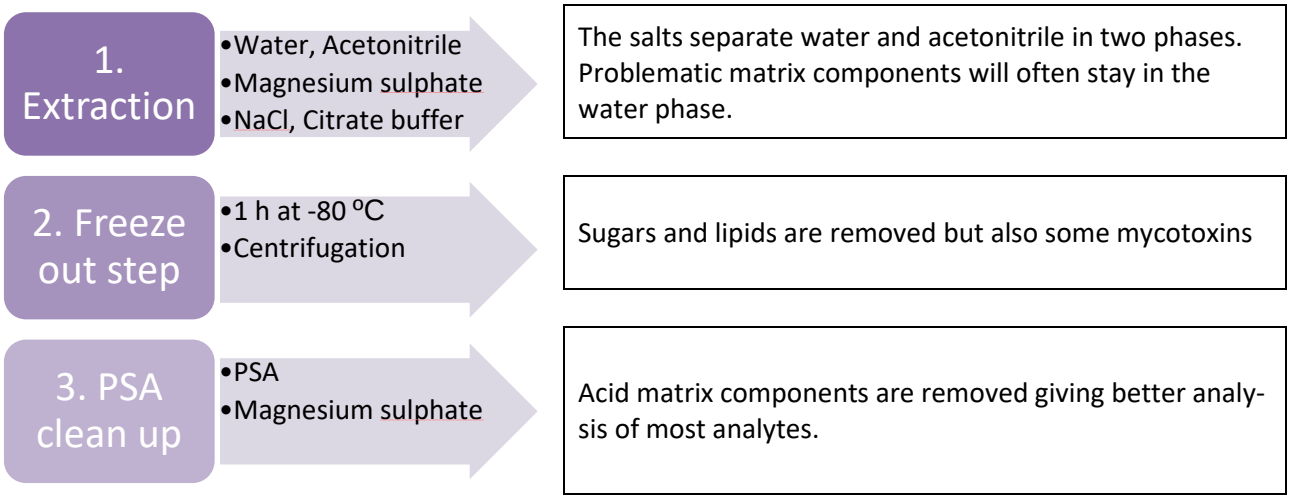


Figure 9. Sample preparation steps used for optimizing the method. Step 3 was skipped in the final method to be able to analyse for acid analytes.

The developed library was used on data files of rice samples screened for pesticides as part of the monitoring program for the Danish Veterinary and Food Administration. These samples were cleaned up using the QuEChERS method, which prevented analysis of mycotoxins and pesticides containing carboxylic acids. Traces of ergot-alkaloids were found in three rice samples. This is an example of how these multi methods can give more information than initially expected.
Recently we have developed a new method for determination of different mycotoxins in coffee based on the QuEChERS extraction (Rasmussen et al. 2014).

8.3 Toxicological effects of mycotoxins

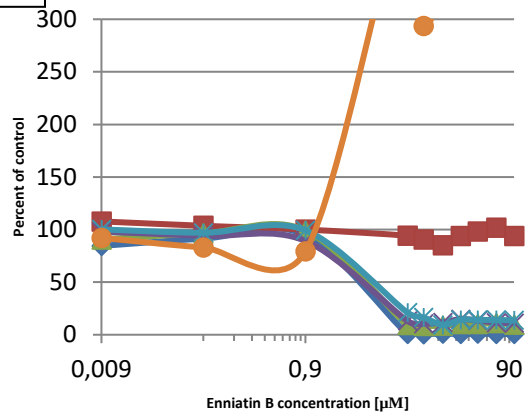
Toxicological information is lacking for many mycotoxins and therefore we aimed at investigating the toxicological characteristics of newly identified mycotoxins (From Section 8.2). Initially, the focus was on substances that may be produced by new mold species that appear because of changes to the climate and

therefore could be expected to occur in our foods. These substances include both known and emerging mycotoxins, as well as other fungal metabolites. The toxicological effects of most of these metabolites and their impact on humans are largely unknown.

As part of this project we developed a Comet assay for detecting genotoxicity in human sperm cells. This assay was developed to investigate potential genotoxic effects for new mycotoxins. None of the investigated mycotoxins revealed genotoxicity by this assay when tested as single compounds or in mixtures. However, it was found that enniatin B was much more cytotoxic in this assay than DON, in the order of 15-fold. A real world mixture of infected barley grains containing high concentrations of HT-2 and T-2 toxin, the glycosylated forms of the two toxins and different enniatins (A, A1, B, B1, B2) was investigated. This extract induced genotoxicity in a concentration-related manner. However, which compound(s) in the extract that induced DNA damage remains to be shown.

One critical effect of known mycotoxins is their severe cytotoxicity. We wanted to establish a method for toxicological profiling of new mycotoxins and selected a liver toxicity assay that performs well in predicting human liver toxicity. The assay is based on a cytotoxicity test in HepG2 cells where multiple end points (membrane integrity, oxidative stress, lysosomal and mitochondria toxicity etc) are detected by 'high content imaging'. This High Content Imaging (HCI) screening method is based of fluorescence imaging of single cells by fully automated image capturing and conversion into phenotypic analyses by statistical algorithms. We applied this strategy to characterize the effects of new mycotoxins and extracts of grain. The following six parameters indicating various kinds of cytotoxicity were investigated: Nuclei count, nuclear area, lysosome activity (LA), mitochondrial membrane potential (MMP), mitochondrial area (MA) and the plasma membrane integrity (PMI) (Figure 10A). The mycotoxins that have been analyzed so far make it clear that most of them are very potent (Figure 10B). Especially, enniatin B and beauvericin appear to be more potent than the positive controls. These results are very promising and further studies designed to elucidate these effects are in progress.

A



B

	Nuclei count		Nuclear area		LA		MMP		MA		PMI	
Hours of exposure	24	72	24	72	24	72	24	72	24	72	24	72
Positive control												
Disulfiram	67	67	100			100	44			100		
Chlorpromazine	20	20	67	44		20	13	30	44	44	44	20
Mycotoxins												
Deoxynivalenol		0,9		9		0,9	67		67			
Enniatin B	9	9			9	9	9	9	9	9	9	9
Citrinin				100		67		67				100
Penicillic acid				67						100		100
Beauvericin	9	9		100		9	9	9	9	9		9
Alternariol*	20	-	20	-	100	-	100	-	20	-		-
Aflatoxin B1*	20	-	20	-		-	100	-		-	100	-

Figure 10A: Enniatin B exposure causes a decrease in: nuclei count (dark blue), lysosomal activity (LA; Green), mitochondrial membrane potential (MMP; purple) and mitochondrial activity (MA; teal), as well as an increase for plasma membrane integrity (PMI; orange), but no change for nuclear area (red).
Figure 10B: Seven mycotoxins and two positive controls were tested for liver toxicity on the parameters nuclei count, nuclear area, LA, MMP, MA and PMI. Each number indicates the lowest observed effect concentration (μM).

9. How to predict toxicity of emerging chemicals?

One of the obstacles for predicting mixture effects is the lack of data for toxicity of chemicals. Therefore we here focus on various methods for predicting toxicity of individual chemicals in cases where there are data gaps.

9.1 A computational systems biology approach for predicting human hazards

Computational systems biology methods can be applied to predict potential human hazards of chemicals. This method was also described in section 5.1.2 where it was used for grouping chemicals, exemplified by a potential workflow in figure 8. This approach relies on existing knowledge about the chemical of interest, as well as mechanisms of actions (or targets) involved in the diseases. Therefore, animal data are not required for such predictions of human hazards.

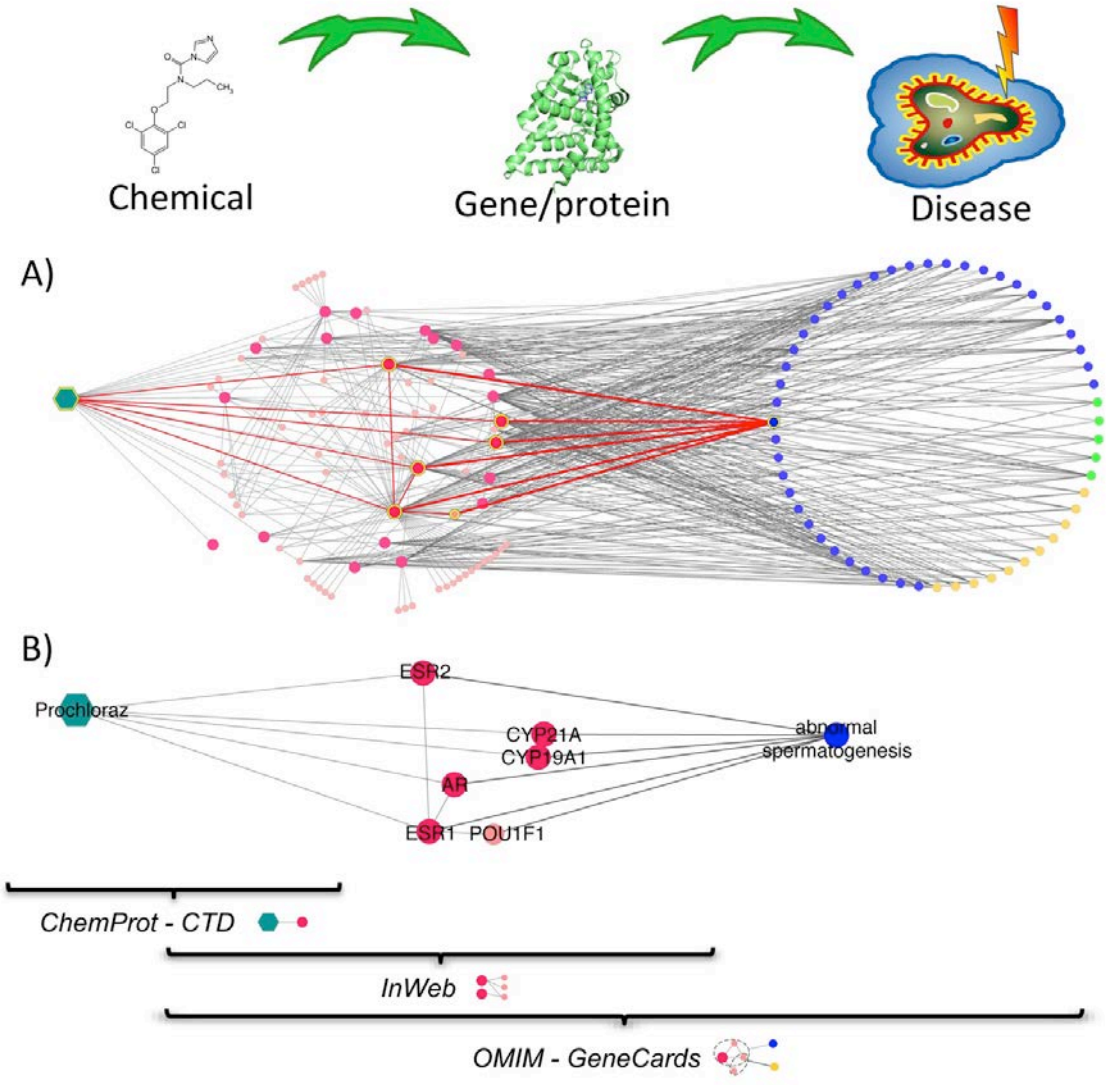


Figure 11. Workflow of a computational systems biology approach for predicting potential human hazards of the pesticide, prochloraz (Kongsbak et al., 2014a). Human targets (magenta circles) for the study chemical (dark cyan hexagon) are retrieved from data-

bases, the network of targets is enriched with interacting proteins (pink circles) and the total list of targets and interacting proteins is tested for overrepresentation in diseases (blue: reproductive disorders, yellow: adrenal disorders and green: other disorders) or pathways (not shown).

9.2 QSAR models for predicting hazards of chemicals

QSAR can be used for predicting health effects of chemicals where experimental data are lacking. The QSAR method is described in section 5.2. QSAR can be used to predict in vitro and in vivo effects including human effects, depending on the experimental data available for building the predictive models.

9.3 Cell-based assays for predicting human hazards

Cell-based assays can be very useful for screening and predicting toxicity of chemicals. We have a portfolio of assays available that together can give an indication whether this chemical has endocrine disrupting activity, liver toxicity and/or genotoxity. These assays are especially valuable when no animal data - or no data at all - exist for the chemicals.

Our battery of cell assays was successfully applied for:

- Investigation of mixture effects of EDC (section 3.1)
- Toxicological profiling of extracts of 20 food packaging materials (section 7.1)
- Toxicological profiling of bisphenol A alternatives (section 7.4)
- Investigation of mycotoxins for liver toxicity (section 8.3)
- Investigation of mycotoxins for genotoxicity on human sperm (section 8.3)
- Investigation of embryotoxicity of pesticides (Dreisig *et al.*, 2013)

Apart from our own assays, we have been using assays generated under the Toxicity Forecaster (ToxCast™) project, which is conducted at the National Center for Computational Toxicology (NCCT), US EPA. The philosophy behind ToxCast™ is that large collections of high-throughput in vitro assay data can be used to predict adverse effects of chemicals provided there is sufficient in vitro data available and models to predict exposure. In ToxCast™, more than 2000 chemicals have been screened across more than 700 high-throughput assays, covering approximately 300 signaling pathways and more is underway. By using these high-throughput data together with in vivo data for the same chemicals, statistical analyses allows for development of predictive signatures or models for in vivo effects of the chemicals based on in vitro data. We have established collaboration with the US EPA and have received access to data for modeling purposes like a comprehensive ER data set. We're in the process of modelling these effects.

10. How to assess the risk of chemical mixtures

Chemical risk assessment provides threshold doses or concentrations of regulatory concern such as acceptable daily intakes (ADI) or predicted no effect concentrations (PNECs) for individual chemicals based on points of departure (no observed adverse effect levels, NOAELs, no observed effect concentrations, NOECs, or benchmark doses). Exposures below these levels are usually considered safe. The experimental evidence on mixture effects provokes the question as to whether there is also sufficient protection against combined exposures, if each component is present below their individual threshold doses (concentrations). That conjecture has been tested experimentally by combining chemicals at levels commonly used to derive estimates of safe exposures (Cocktail I & II and by others). Taken together, these studies have produced **strong evidence that mixture effects may arise when several chemicals are combined at doses or concentrations around, or below, points of departure.**

10.1 Pragmatic evaluation of cocktail effects

One aim of the Cocktail Project was to deliver a toolbox and recommendations for its use in risk assessment of chemical mixtures (Cocktail VII). This is a complex challenge that remains critically dependent on high quality data on everything from chemical structures to in vitro and in vivo effects of single chemicals. Thus, as long as this information remains lacking the complete toolbox will also remain lacking in its predictive powers. Therefore, we suggest taking a pragmatic approach for assessing risks in relation to food contaminants.

10.1.1 Suggested approach for evaluation of mixture effects

Several proposals for handling risk assessments of chemical mixtures have been put forward and been discussed (NRC, 2008; Kortenkamp and Hass, 2009; WHO/IPCS, 2009). On the basis of this knowledge, and the results generated in the Cocktail Project, we have compiled a flowchart that suggests a step-by-step procedure for mixture risk assessment (Figure 12).

- Initially the problem with the specific mixture is defined: e.g. do we know the exact composition? Are combined exposures to humans in fact likely? Do the chemicals occur concomitantly?
- Is there relevant toxicity information on the individual chemicals? If NOAELs from in vivo experiments exist, these are used.

If no in vivo toxicity data exists, it may be possible to estimate toxicity. If e.g. in vitro data for a key initiating event exists, it can be combined with a human exposure level to calculate hazard quotients. If no data exists, read-across data (or quantitative QSAR data) may be applied and toxicity values for similar chemicals can be used as a surrogate. Alternatively, the threshold of toxicological concern (TTC) approach can be applied although it may be less reliable. In this case an uncertainty factor should be de-

fined in order to reach an 'acceptable level' (AL) and this uncertainty factor is typically set to 100 based on knowledge of uncertainty within and between individuals.

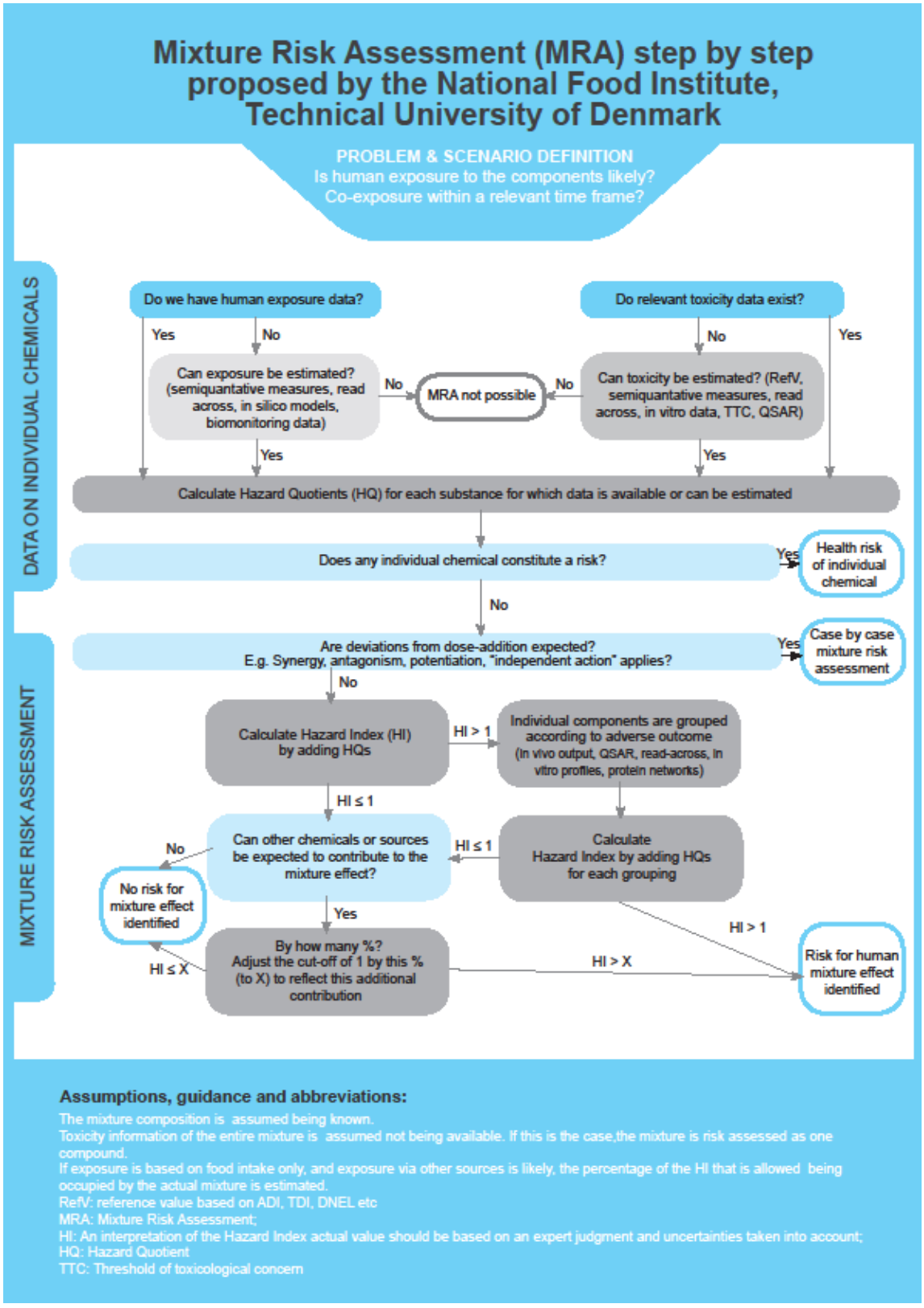


Figure 12. Flow chart describing a step-by-step procedure on how to handle mixture risk assessment.

- Next, do human exposure data exist? Can exposure via the food be estimated from contaminant data for specific food items? Does exposure between consumer groups vary? Should mean exposure values or 95% percentiles be applied to protect the more sensitive people as well? Is anything about exposure from sources other than food known?
- In cases where toxicity and exposure information is available for only a subset of the chemicals in the mixture, a risk assessment can still be performed on this subset to give an idea of the risk.
- Hazard quotients are calculated and it is evaluated if there is a problem with any of the individual chemicals (HQ is above or close to 1), regulatory action should take place.
- It is evaluated if it can be expected that deviations from additivity are likely. Are there reasons to believe that interaction between the chemicals will occur either due to toxicodynamic or toxicokinetic interactions? Are CYP450 interactions likely?
- If not, a rough calculation of the hazard index (HI) is performed. At this step, all chemicals are added in the same formula, irrespective on which toxicity it is based.
- In cases where the **HI becomes less than 1**, it should be evaluated if chemicals from other sources might contribute to the HI.
- If other sources of the same chemicals are identified or if other chemicals may contribute to the mixture effect, the cut-off of 1 should be adjusted to X to take this extra exposure into account.
- If the **HI exceeds 1,** it will be necessary to group the chemicals (tier 2) according to their specific toxicities. This is done according to in vivo outcomes or alternatively grouping can be performed according to mechanisms of actions, QSARs etc. as explained in section 5.
- For each group of chemicals, new HI's are calculated.
- If any of these HI's exceed 1, regulatory action should be decided upon by the authorities.
- For those HI's that are less than 1, are there other sources of the same chemicals that can be identified, or may other chemicals contribute to the mixture effect. If so, the cut-off of 1 should be adjusted to take this extra exposure into account. This should be done by calculating the percentage allocated to contributions from other sources and the percentage allocated to contributions from foods. If any of these HI's exceeds 1, the regulatory authorities should be contacted.

Hazard Index:

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

EL - exposure level, e.g. intake in mg/kg/d
AL - acceptable level, e.g. ADI (acceptable daily intake) or TDI (tolerable daily intake)
EL and AL must be expressed in the same unit

Simplifications:

- AL not necessarily in relation to same end point
- Different uncertainty factors may have been used to define AL
- AL represents effect doses associated with the same (small or zero) effect

Numerous mixture risk assessment methods are available, including the hazard index (HI), point of departure index (PODI), relative potency factors (RPF) and toxicity equivalency factors (TEF). Calculation of HI, point of departure (PODI) or relative potency factors (RPFs) for dioxins and PCBs is in all cases simplifications of the DA method.

We have developed a user interface that should make the process of mixture risk assessment more feasible, and this is described in Section 10.1.2.

Tiered approaches as suggested by WHO

The approach described in section 10.1.1 can be performed at various levels also called various 'tiers'. Tiered methods have been proposed, when the quality of the data that is available for risk assessments are less than optimal (data poor versus data rich situations). This approach may be useful to initially explore the problem and utilize more sophisticated models and associated supporting data when needed. An assumption of the tiered approach is that chemicals act additively and that effects can be predicted by using DA or IA. Exposure assessment, hazard assessment and risk characterization of multiple chemicals (whole mixture approach/component-based approaches) follow a tiered approach and ranges from predictive methodologies and conservative assumptions in early tiers (tier 0 and 1 = data poor situations), to more refined approaches based on increased data information and probabilities (tiers 2 and 3 = data rich situations).

A more detailed description of the tiered approach is presented below:

Tier 0

At this lowest tier, all chemicals that occur together in the exposure setting are considered irrespective of the effects they elicit. At this stage, it is suggested that an HI is constructed commensurate with the (low) quality of data that are included in the analysis. ***Crude and semi-quantitative exposure estimates*** may be used for the development of an HI. Similarly, ***quite basic potency estimates*** can be entered into the calculation. Thresholds of toxicological concern (TTC) or read-across may be used, with the aim of bridging data gaps. If available, ADIs may be entered into the calculation of an HI, but there is no need to enter ADIs consistently for all chemicals considered at this stage. The ADI values may be derived from a variety of different end points and species, and may include different uncertainty factors (UFs). During the risk characterization step, the margin between estimated exposure and hazard is considered as the decision basis for determining whether a more refined analysis is required. If the HI exceeds 1, the analysis should proceed to Tier 1.

Tier 1

All chemicals relevant to the exposure scenario are considered in Tier 1, irrespective of the effects they produce and without consideration to the modes of action involved. The assumptions on exposures may still be deterministic and may reflect **worst-case assumptions**, but they should rely on **measured values** as much as possible. For hazard assessments, estimates of potency for each chemical are incorporated, such as ADIs or benchmark doses. Simplifying assumptions, such as TTC or potencies similar to the most toxic known substance present in the mixture should be abandoned. The potency estimates may be for a variety of different end points and can be derived from studies with a variety of different test species. Alternatively, and if possible, hazard assessments may be based on point of departures (PODs) for all the chemicals considered, with application of the point of departure index (PODI) in the risk characterization step. The risk characterization step determines whether further refinements of analysis should be conducted. This should be pursued if the HI is larger than 1, or if the margin of safety is judged to be inappropriate.

Tier 2

In the preceding tiers, no chemical present in the mixture was excluded from the analysis. In Tier 2, this restriction may be relaxed, by considering the effect profile of chemicals, with the intention of **creating assessment groups of chemicals**. It is suggested to exclude those chemicals that are known not to produce a chosen common adverse outcome. Consequently, the assessment group may include chemicals where there is a degree of uncertainty as to whether they can contribute to a common effect. This is done in order to avoid a situation where the analysis will underestimate the mixture effect by defining too narrow common assessment groups on the basis of positive effect criteria. As before, the exposure assessment should rely on measured data. The hazard assessment may utilize ADIs (or, alternatively PODs) that were derived for common end points, irrespective of any consideration of mode of action. If the risk estimates exceed acceptable levels, the analysis may be refined and carried forward to Tier 3.

Tier 3

At this stage, the analysis may adopt **more restrictive criteria about common adverse outcomes**, and may define groupings of chemicals for assessment on the basis of phenomenological effect criteria. The exposure assessment element may utilize **probabilistic data**, if available. Tier 3 assessments for hazards may incorporate increasingly refined potency estimates for the specific end points that form the basis for defining groups of chemicals for assessment. At this stage, PODs may be used, with the aim of constructing a PODI. In the interest of consistency of analysis, the PODs should derive from the same species. In this way, the analysis approaches a level of detail, where all single chemicals and the mixture were studied under similar conditions. Nevertheless, the PODs may still reflect some differences in terms of data quality and experimental standards.

In Tier 3, construction of a **PODI**, rather than an HI, may be regarded as more appropriate for the following reasons: It is assumed that the PODs that form the basis of the analysis are of similar quality. In this case, it is appropriate that the aggregation for mixture effects should be conducted at the level of experimental data by calculating a PODI. This practice achieves a high level of consistency that comes nearer to the application of DA as a mixture assessment concept in experimental mixture studies. It realizes a high level of transparency by avoiding the introduction of too many assumptions (e.g. use of different uncertainty factors in inconsistent ways).

10.1.2 A user interface for finding information on toxicity and exposure data

A pragmatic tool for the risk assessment of mixture effects has been developed. This tool collects the available toxicity and exposure information for a broad range of chemicals, which is presently spread over various databases, reports, etc. The toxicity information is limited to the critical toxicity on which the present EU regulation is based. However, links to reports that describes risk assessments of and exposure information on the most important chemical classes are given. The collected information can be used for the calculation of the HI for the actual mixture by using the calculation module. The user interface is expected to make the task of assessing mixture effects more feasible. The first version of this tool is currently available (Figure 13 & 14), but further development is recommended, and when this is done it will be made publicly available.

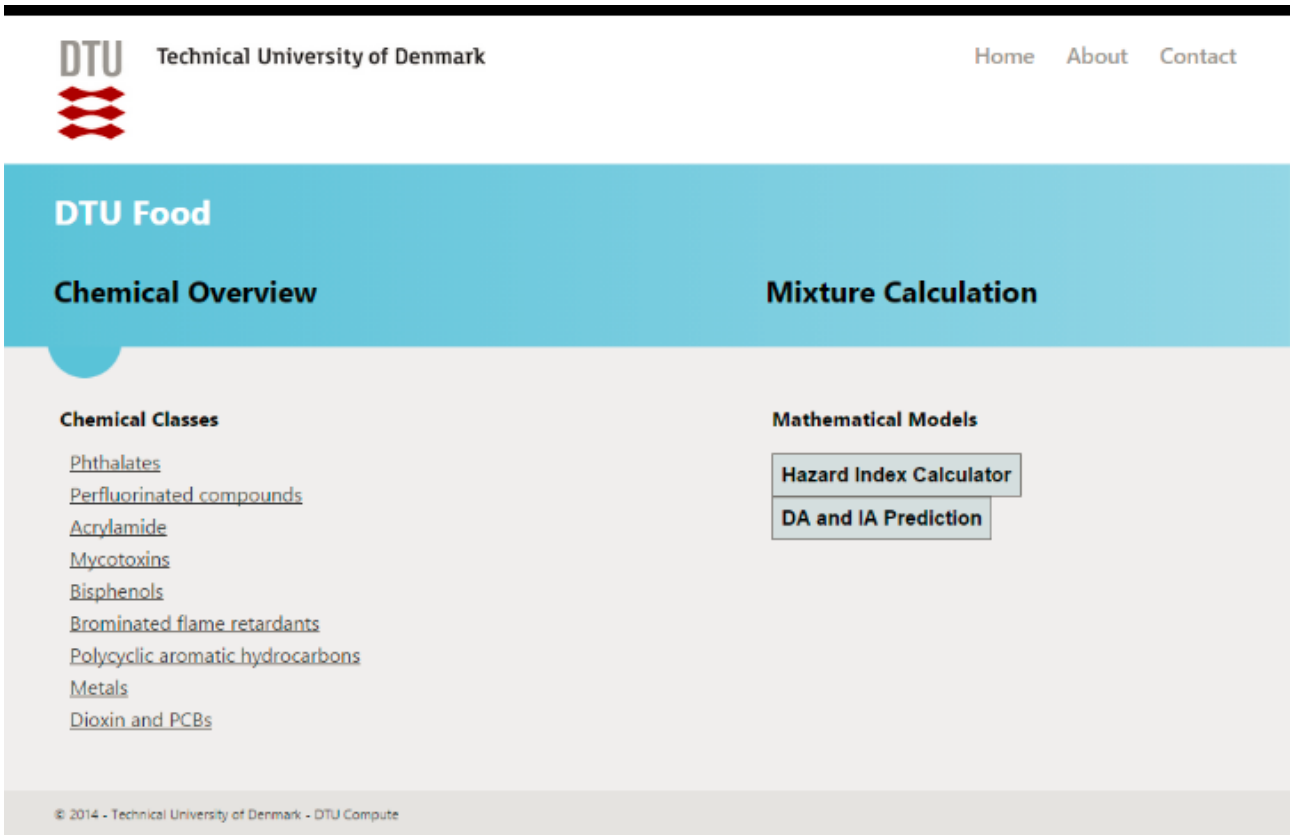


Figure 13. Start page of the user interface showing entries to toxicity and exposure information for a range of chemical classes and to the calculation modules.

The user interface consists of three modules: 1) a toxicity/exposure information module, 2) an HI calculation module, and 3) a research-oriented module for calculation of mixture effects.

Module 1 collects the information on chemicals from selected chemical groups including phthalates, perfluorinated compounds, mycotoxins, brominated flame retardants, polycyclic aromatic hydrocarbons, metals, pesticides, dioxins and polychlorinated biphenyls. The toxicity information includes values for POD and reference dose, as well as the critical effect for the POD. The exposure information includes intake values for defined consumer groups. Specifically, the collected exposure information concerns the exposure via food and does not cover the exposure from other sources. Hazard quotients for the individual chemicals are, if possible, calculated based on the collected information. The module also contains links to reports containing the collected toxicity and exposure information.

Module 2 calculates the HI. In the HI calculation module, the HI for a mixture of selected chemicals can be calculated based on the hazard quotients (HQs) provided in the information module (Figure 13). These calculations also give the user the option to apply other relevant HQ values (for example based on intake values for other consumer groups).

Module 3 is included in the user interface as a research-oriented calculation tool. The module can be used for calculation of mixture effects using DA or IA.

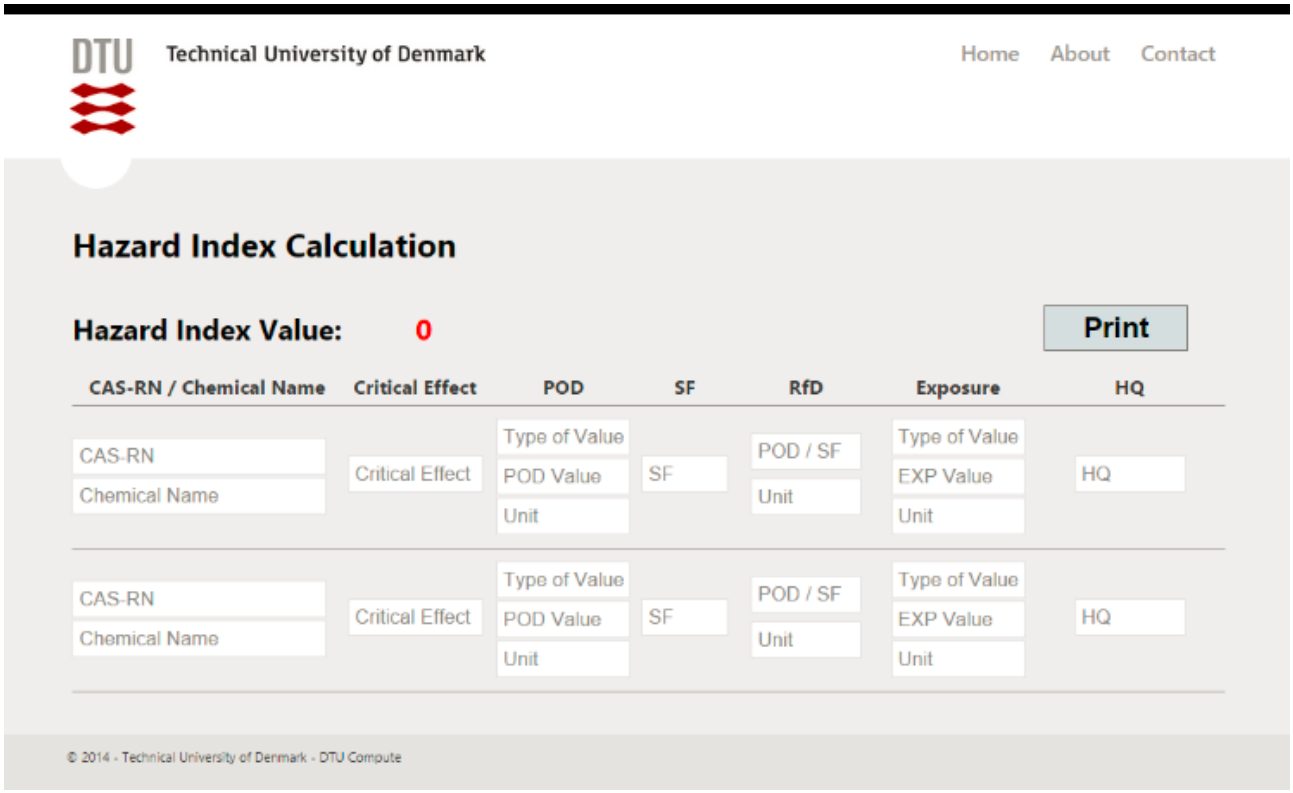


Figure 14. The HI calculation page where the HI is automatically calculated based on the HQs of the selected chemicals in the mixture.

10.1.3 A preliminary evaluation of human hazards caused by food contaminants

In the Cocktail Project we asked ourselves if we could make some preliminary calculations to give us an idea how large a problem we have got in relation to human health effects caused by chemical mixtures from food. In order to reduce the complexity, we made preliminary calculations of human health risk to chemical exposures via the food by including several assumptions and/or exclusion criteria. Although this approach has obvious limitations, it may offer valuable insights into otherwise unknown risk parameters, and will improve as new data becomes available. The assumptions and exclusions were:

- Only chemical exposure from foods is considered.
- Only substances for which we have information about toxicology or exposure via the food are included.
- Only the critical toxicological effect on which the acceptable or tolerable daily intake (ADI/TDI) is based has been taken into account.
- All types of adverse effects on the living organism are grouped together. It is anticipated that adverse effects are linked to each other and can affect the outcome of one another. This is expected to overestimate the risk.
- In general the mean exposure levels have been used in the calculations.
- If data are available, also high-end human exposures, e.g. the 95th percentile, have been used.

- The calculations are based on available data and the existing regulations. This means that for some groups of chemicals, e.g. fluorinated chemicals and endocrine disrupting chemicals, the risk is likely underestimated. Furthermore, humans are exposed to many chemicals for which we have no risk assessments and this too will lead to an underestimation.
- Toxicity and exposure data from EFSA and Danish reports are primarily used for the calculations. In some cases other data such as Joint FAO/WHO Expert Committee on Food Additives (JECFA) or European Chemical Agency (ECHA) data have been used.
- Where no reference value such as TDI or ADI is established, a reference dose was calculated using the formula: *point of departure / safety factor*.

This rough calculation resulted in the following ranking of HQs for the various chemicals/chemical classes: lead, inorganic arsenic, cadmium, aluminium, dioxins, dioxin-like PCBs, non-dioxin like PCBs, acrylamide, deoxynivalenol, phthalates, zearalenone, organic mercury. For lead and arsenic the HQ exceeded 1, indicating a human health challenge with these metals. For the remaining chemicals, the HQ ranged from 0.1 to 0.8. Based on existing regulations, the aforementioned chemicals are problematic. However, it should be stressed that certain endpoints like endocrine disruption and neurotoxicity has not been dealt with adequately in the present risk assessments of most chemicals due to lack of data. **This means that for some chemicals these HQs do not represent the actual human risk.** Chemicals, like bisphenols and fluorochemicals, are expected to get a higher ranking when these new endpoints in the future will be required in the legislation.

The table is shown in Appendix C. However due to space limitations, the links to toxicity and exposure information is not given in the table but will be made publicly available.
The hazard quotients for all chemicals/chemical classes were added for adult exposures. The mean exposures (instead of 95th percentile exposures) were selected in order not to overestimate this point. For the 189 chemicals, a total HI of 25 was calculated. Not surprisingly the HI exceeded 1, which is the cut-off for indication of a human risk. This is a further indication that the combined exposure of humans to chemical cocktails may be problematic and there is an urgent need to perform mixture risk assessments.

10.1.4 Case studies on pragmatic mixture risk assessment

CASE: Exposure of toddlers to the four phthalates DEHP, DBP, DiBP and BBP

In 2012, the Danish Environmental Protection Agency proposed a ban of the four phthalates DEHP, DBP, DiBP and BBP in articles that are used indoors or may come in contact with the skin. This ban was based on a mixture risk assessment using dose addition to predict the mixture effects (ECHA 2012).

A HI approach was applied, in which exposure from different sources was calculated for each of the four phthalates and compared with derived no-effect levels (DNELs, corresponding to AL as defined in section 10.1.1) for each substance. The individual DNELs for each phthalate were used to calculate HQs (exposure divided by DNEL) that were summarized to a total HI for all four phthalates and for all exposure routes. When this cumulative HI exceeds 1, the risk is considered not to be controlled for the chemicals (ECHA part

E, 2008). This approach is described as a useful approach by the Scientific Committees in their joint opinion on 'Toxicity and assessment of chemical mixtures' (SCCS, SCHER, SCENIHR 2011).

The formula used is: $HI = HQ1 + HQ2 + HQ3 + HQ4 = \frac{exposure1}{DNEL1} + \frac{exposure2}{DNEL2} + \frac{exposure3}{DNEL3} + \frac{exposure4}{DNEL4}$

The DNELs were based on no- or lowest-observed adverse effect levels (NOAELs or LOAELs) for effects on reproductive toxicity (anti-androgenic effects) for each phthalate. Thus, the specific effects for setting the DNELs were different, but the mode of action inducing these effects was similar.

In the cumulative risk assessment of the four phthalates, HIs were calculated for different age groups (2-year olds, 6/7-year olds and adults). The results with regards to the estimated exposure from food for 2-year olds, i.e. toddler are shown in Table 2.

Table 2. HQs and HI for toddlers based on estimated exposure to DEHP, DBP, DiBP and BBP from food

Phthalate	Estimated exposure from food (97.5th percentile, µg/kg bw/day)	DNEL, µg/kg bw/day	HQ based on 97.5th percentiles
DEHP	9.9	35	0.28
DBP	1	6.7	0.15
DiBP	2.7	420	0.006
BBP	1.3	500	0.003
Total HI			**0.44**

As seen from Table 2, the HI for exposure of toddlers from food was 0.44; thus did not exceed 1. This value was added to the HIs calculated for exposure to DEHP, DBP, DiBP and BBP from other sources (articles, dust and indoor air) using a realistic worst case exposure scenario (average of 95th percentiles), see Table 3. For the group of toddlers, the total HI was 1.36, i.e. exceeded 1, and therefore indicating a risk for the total exposure.

Table 3. HI for toddlers based on estimated exposure to phthalates from food, articles, dust and indoor air

DEHP, DBP, DiBP and BBP from	HI
Food	0.44
Articles	0.14
Dust	0.72
Indoor air	0.06
Total HI (food, articles, dust and indoor air)	**1.36**

If using the approach described in section 10.1.1, the percentage contribution from "other sources" would be 0.14+0.72+0.06 = 0.92 = 92%. This would allow only 8% contribution from foods (X=8%). With an HI of 0.44 from foods, it is clear that HI>X, leading to the conclusion that a possible risk of mixture effects is identified.

This cumulative risk assessment of the phthalates DEHP, DBP, DiBP and BBP included only these four substances. However, it is well known that several other substances can induce similar reproductive toxicity

effects (anti-androgenicity) and therefore would be relevant to include in a cumulative risk assessment for such effects. These substances include other phthalates and pesticides that humans may also be exposed to. Thus, the total HI could be larger than 1.36.

This highlights an issue related to exposure to EDCs and is a further indication that mixture risk assessment is urgently needed.

11. An international perspective on mixture effects

SCHER has stated:

'… at present, risk assessment on the combined effects of chemicals in a mixture is not commonly carried out, nor required by most EU regulations.' (SCHER *et al.*, 2011).

Currently, mixture effects of pesticides are taken into account by the Danish authorities when maximum residue limits are discussed in the EU; however this is an exception rather than a rule.

Some of the key events with regards to regulatory considerations for mixture risk assessment are listed below. The list illustrates the long road to implementation of mixture risk assessment, which has yet to be finalized.

- 1996, US Food Quality Protection Act calls for mixture risk assessment of pesticides with a common mode of action
- 2002, US EPA guidance on mixture risk assessment of pesticide chemicals that have a common MoA
- 2002–2007, US EPA mixture risk assessments for organophosphates, triazines, chloroacaetanilides, carbamates
- 2005, EU Pesticide Maximum Residue Limit regulation calls for the development and use of methodologies for mixture risk assessment of pesticide residues
- 2005–2008, EFSA working groups on mixture risk assessment established, Scientific Colloquium on mixture risk assessment, evaluation of existing methodologies, recommendation for a tiered approach.
- 2009, Test results of a suggested methodology for the case of triazole fungicides. Conclusions: appropriate but not yet applicable on a routine basis.
- 2009, Requirement for mixture risk assessments established under the new EU pesticide regulation:
- 'Active substances and plant protection products shall not have any harmful effects on human health, taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available,… '
- 2013, Scientific opinion on relevance of dissimilar mode of action and its appropriate application for cumulative risk assessment of pesticide residues in food
- 2014, Scientific report on regulatory requirements and guidance published by the European Commission

The European Parliament has passed several EU food-related regulations (European Parliament, 2005; European Parliament, 2009) that call for a consideration of 'cocktail effects' of chemical residues. However, due to the complexity of the issue, the implementation of practicable testing and risk assessment methods for chemical cocktails remains a challenge.

In 2009, the European Council of Ministers called on the European Commission to assess how and whether relevant existing legislation adequately addresses risks from exposure to multiple chemicals (UNION, 2009).

In response, the European Commission prepared a communication based on an opinion of the Scientific Committee on Health and Environmental Risks (SCHER et al., 2011). The communication emphasized that while the scientific basis for conducting mixture risk assessments is established, significant knowledge and data gaps exist that act as barriers to applying these methods.

From 2005 onwards, EFSA has been engaged in various activities concerning cocktail effects of pesticides (EFSA, 2013a; EFSA, 2013b). With this work, several challenges have come to light that hamper the implementation of mixture risk assessment methods for pesticide residues, many of them echoing the generic gaps identified in the European Commission communication:

- Mechanistic data to support the grouping of pesticides into assessment groups for mixture risk assessment are often not accessible or missing altogether and therefore grouping has to rely on proxy measures, such as common target organ toxicity.
- Data about non-critical toxicities (not relevant to ADIs, but potentially relevant for mixture toxicity) are often sparse.
- Mixture risk assessments have to rely on data about the co-occurrence of several pesticides and contaminants in food, but such data are often not available.

These difficulties exist not only in relation to pesticide residues, but also apply to food contaminants, and are even more acute in relation to less investigated substances, such as feed and food additives and chemicals released from food packaging (i.e. FCMs).

Significant contributions to the fields of human mixture risk assessment have come from Kortenkamp and his collaborators. In 2009 the report 'State of the Art Report on Mixture Toxicity' was published (Kortenkamp et al, 2009) and generated attention, as it provided the first thoughts on how to handle the issue of mixture risk assessment. Later in 2013 the report 'Investigation of the state of the science on combined actions of chemicals in food through dissimilar modes of action and proposal for science-based approach for performing related cumulative risk assessment' was published for EFSA (Kortenkamp et al., 2013). Here it was evaluated that dissimilar acting compounds with common adverse outcome could be considered as the similar acting chemicals and that dose addition could be used for MIXTURE RISK ASSESSMENT.

In 2014, the Joint Research Centre (European Commission) published a report 'Assessment of mixtures – review of regulatory requirements and guidance' (Kienzler et al., 2014). Here the current legislation relating to mixture effects and various guidance for human toxicology as well as ecotoxicology is gathered. They distinguish between intentional (e.g. products) and unintentional mixtures (often complex).

For ***intentional mixtures*** the following parts of EU legislation have been identified:

- industrial chemicals under REACH (Regulation No 1272/2008).
- Plant Protection Products (Regulation No 1107/2009, 283/2013 and 284/2013) and biocides (Regulation No 528/2012).
- Human and veterinary pharmaceuticals (Directive 2001/83/EC).
- Veterinary products (Directive 2001/82/EC)
- Cosmetic products (Regulation 1223/2009)
- Food and feed stuff can be regarded as complex mixtures, and additives (Regulation No 1333/2008) are assessed for toxicity.

Unintentional mixtures (contaminants, by-products and environmental pollutants) are much more challenging to assess, because they are of varying and often complex composition, and many of the substances present are unidentified and toxicity data are lacking. The following pieces of (mostly environmental) legislation have been identified that address the toxicological risk of unintentional mixtures: a) Regulations on contaminants in food (Regulation No 315/93/EEC and follow-up regulations) generally do not take mixture toxicity into account, with the exception of dioxins and dioxin-like compounds. b) Food contact materials are addressed separately (Regulation No 1935/2004) and considers cumulative effects (undefined) but not mixture toxicity, c) Pesticide residues in food and feed are also regulated separately (Regulation No 396/2005), which acknowledges the need for cumulative and mixture toxicity assessment, d) Water contaminants (Water Framework Directive 2006/60/EC), with the related Groundwater Directive (Directive 2006/118/EC) do not specifically address mixtures or aggregated exposure. However, reference is made to Environmental Quality Standards (Directive 2013/39/EU), which do address mixture toxicity, e) The marine environment is covered separately by the Marine Strategy Framework Directive (2008/56/EC), which emphasizes that risk assessment should also consider cumulative and mixture effects but without further specification, f) The Waste Framework Directive is linked to the Directive on Integrated Pollution Prevention and Control (Directive 2010/75/EU), which addresses (waste) emissions to the air, including waste incineration. This directive does not address mixtures with the exception of dioxins and furans, g) To ensure the safety of workers against chemical agents at work, Directive 98/24/EC specifies maximum levels for individual substances. It also refers explicitly to chemical agents in combination, covering both intentional and coincidental mixtures, h) A separate directive (Directive 2011/92/EU) is in place for the environmental impact assessment of large scale public and private projects (e.g. motorways, airports) that are likely to have significant effects on the environment. This includes estimations of emissions of pollutants, including cumulative effects, but mixture toxicity is not specifically addressed.

Overall, it seems that several EU regulations address the need for mixture risk assessment, and some guidance have been developed, but presently mixture risk assessment is not commonly carried out.

12. Future perspectives

One of the aims of the Cocktail project was to give recommendations to the authorities on how to handle mixture risk assessment. In section 10, tools were presented that have been developed on the basis of our current knowledge. These are pragmatic and easy to use tools. Nevertheless, the tools represent a significant improvement to existing chemical risk assessment procedures with regards to mixture risk assessment and should be relatively feasible to apply.

In order to refine and improve the tools, more work is needed, including the points listed below:

- One of the present limitations of mixture risk assessment is lack of data on single chemicals. Approximately 800 chemicals have been risk assessed and been allocated an ADI (TDI) so far, but several thousand additional chemicals are in use (10-30,000) that may have food relevance. Such data gaps need to be reduced regarding toxic effects of single chemicals. This can be done by developing alternative ways of risk assessing chemicals based on e.g. batteries of in vitro models, computational tools like (Q)SARs and physiologically-based kinetic modelling.
- For specific chemical classes like alternative chemicals that are substituting BPA, fluorochemicals, and EDCs we need more knowledge on hazards and exposure.
- Better human exposure data and methods to calculate exposure in general is required.
- Further development and refinement of the tools for mixture risk assessment. For instance to further develop the software 'Cocktail Effect Calculator' to be applied for mixture risk assessment. More detailed information could be included in the software.
- More knowledge on the effects caused by 'real-world' mixtures. What happens when humans are exposed to complex relevant mixtures at low, realistic dose levels? Is synergy an important phenomenon at realistic low exposure levels?
- Concerning FCMs of paper and board a refinement of the strategy for evaluation of FCM is needed, e.g. an improved methods for fractionation of extracts and for investigating volatile substances.
- Many chemicals are toxic to the liver and are regulated based on that endpoint. Our new established method is promising and could be applied for screening larger numbers of mycotoxins, botanicals and other chemicals. The future perspective is that this method can become validated as a test guideline to partly replace this endpoint in the rodent model.

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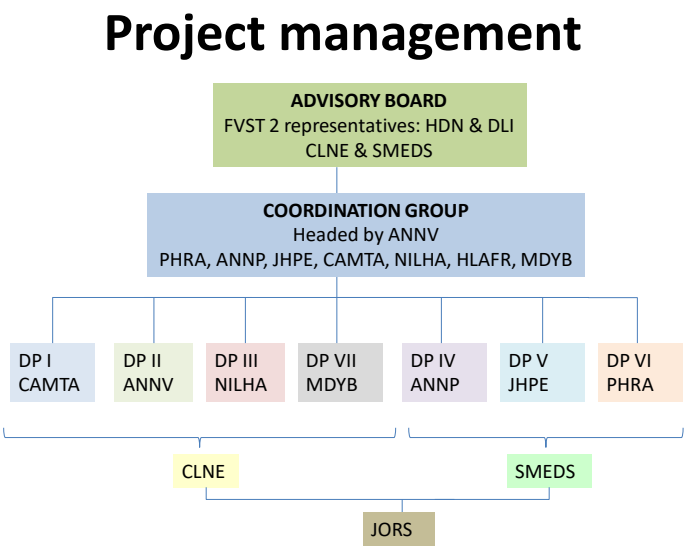
Appendix B: Management of Cocktail Project and people involved

Organization of the project as of Aug 2014



DTU persons involved in Cocktail

Academics:

Anne Marie Vinggaard (ANNV) - project coordinator and Cocktail 2 leader
Camilla Taxvig (CAMTA) - Cocktail 1 leader
Niels Hadrup (NILHA) – Cocktail 3 leader
Annette Petersen (ANNP) – Cocktail 4 leader
Jens Højslev Petersen (JHPE) – Cocktail 5 leader
Peter Have Rasmussen (PHRA) – Cocktail 6 leader
Marianne Dybdahl (MDYB) – Cocktail 7 leader

Ulla Hass
Julie Boberg
Karen Mandrup
Anne Lykkeberg
Xenia Trier
Lisbeth Krüger Jensen
Gitte Alsing Pedersen
Kit Granby
Mona-Lise Binderup
Anoop Kumar Sharma

Terje Svingen
Sofie Christiansen
Marta Axelstad Petersen

PhD students
Kristine Kongsbak
Linda Bengtström
Anna Kjerstine Rosenmai
Kasper Skov
Hanna Johansson

Technicians
Annie Foverskov
Anni Helleskov
Vivian Jørgensen
Birgitte Møller Plesning
Heidi Letting
Lene Sofie Svensson
Dorte Lykkegaard
Lillian Sztuk
Sarah Grundt Simonsen
Ulla Baroudy
Vibeke Kjær

The animal facility at DTU Food

Activities performed under Cocktail

- Half-year reporting to the steering committee for the entire Cocktail project
- Internal Cocktail Project seminars every half year
- Conference March 2015 to mark the finalization of the Cocktail project
- Richard Judson from US-EPA visited DTU Food and gave a talk entitled " Improving the Human Hazard Characterization of Chemicals: A Tox21 Update", June 2013
- Seminar on cumulative risk assessment of chemicals having dissimilar modes of action. Susanne Hovgaard from Danish EPA presented a new EFSA report in January 2014.
- ANSES/BfR/DTU workshop December 2013 where Anne Marie Vinggaard and Ulla Hass gave talks on mixture effects

Appendix C: Overview of Hazard Quotients for various chemical classes based on exposure from foods

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Phthalates	DEHP	117-81-7	Testes (rat)	NOAEL: 5	TDI: 0.05	Toddlers (2.5-3.5 y). UK 95% perc., LB	6.9	0,138
Phthalates	DEHP	117-81-7	Testes (rat)	NOAEL: 5	TDI: 0.05	Children (4-6 y), UK, 95% perc., LB	5.5	0,11
Phthalates	DEHP	117-81-7	Testes (rat)	NOAEL: 5	TDI: 0.05	Adults (18-65 y), UK, 95% perc. LB	3.4	0,068
Phthalates	DEHP	117-81-7	Testes (rat)	NOAEL: 5	TDI: 0.05			
Phthalates	DEHP	117-81-7	Testes (rat)	NOAEL: 5				
Phthalates	BBP	85-68-7	Developmental (rat)	NOAEL: 50	TDI: 0.5	Toddlers (2.5-3.5 y). UK 95% perc., LB	0.07	0,00014
Phthalates	BBP	85-68-7	Developmental (rat)	NOAEL: 50	TDI: 0.5	Children (4-6 y), UK, 95% perc., LB	0.06	0,00012
Phthalates	BBP	85-68-7	Developmental (rat)	NOAEL: 50	TDI: 0.5	Adults (18-65 y), UK, 95% perc. LB	0.04	0,00008
Phthalates	BBP	85-68-7	Developmental (rat)	NOAEL: 50	TDI: 0.5			
Phthalates	BBP	85-68-7	Developmental (rat)	NOAEL: 50				
Phthalates	DBP	84-74-2	Testes (rat)	LOAEL: 2	TDI: 0.01	Toddlers (2.5-3.5 y). UK 95% perc., LB	0.4	0,04
Phthalates	DBP	84-74-2	Testes (rat)	LOAEL: 2	TDI: 0.01	Children (4-6 y), UK, 95% perc., LB	0.4	0,04
Phthalates	DBP	84-74-2	Testes (rat)	LOAEL: 2	TDI: 0.01	Adults (18-65 y), UK, 95% perc. LB	0.2	0,02
Phthalates	DBP	84-74-2	Testes (rat)	LOAEL: 2	TDI: 0.01			
Phthalates	DINP	68515-48-0	Liver (rat)	NOAEL: 15	TDI: 0.15	Adults	1	0,0067
Phthalates	DINP	68515-48-0	Liver (rat)	NOAEL: 15	TDI: 0.15	Children	6.5	0,0433
Phthalates	DINP	68515-48-0	Liver (rat)	NOAEL: 15				
Phthalates	DIDP	68515-49-1	Liver (rat)	NOAEL: 15	TDI: 0.15	Adults	2	0,013
Phthalates	DIDP	68515-49-1	Liver (rat)	NOAEL: 15	TDI: 0.15	Children	13	0,087
Phthalates	DIDP	68515-49-1	Liver (rat)	NOAEL: 15				
Perfluorinated compounds	PFOA	335-67-1	Liver (rat)	$BMDL_{10}$: 0.3	TDI: 0.0015	All (4-75 y), DK	0.00045	0,0003
Perfluorinated compounds	PFOS	2795-39-3	Lipids, thyroid hormones (monkey)	NOAEL: 0.03	TDI: 0.00015			
Acrylamide	Acrylamide	79-06-01	Tumours (mouse)	$BMDL_{10}$: 0.18	RfD: 0.0018	Adults (15-75 y), DK, mean	0.19	0,106
Acrylamide	Acrylamide	79-06-01	Tumours (mouse)	$BMDL_{10}$: 0.18	RfD: 0.0018	Children (4-14 y) DK, mean	0.33	0,183
Acrylamide	Acrylamide	79-06-01	Tumours (mouse)	$BMDL_{10}$: 0.18	RfD: 0.0018	Adults (15-75 y), DK, 95% percentile	0.46	0,256
Acrylamide	Acrylamide	79-06-01	Tumours (mouse)	$BMDL_{10}$: 0.18	RfD: 0.0018	Children (4-14 y), DK, 95% percentile	0.89	0,494
Mycotoxins	Deoxynivalenol	51481-10-8	Body weight (mouse)	NOAEL: 0.1	TDI: 0.001	All (4-75 y), DK	0.215	0,215
Mycotoxins	Deoxynivalenol	51481-10-8	Body weight (mouse)	NOEL: 0.1	PMTDI: 0.001			
Mycotoxins	T-2 & HT-2		Immunotox (pig)	$BMDL_{05}$: 0.01	TDI: 0.0001	All (4-75 y), DK. Sum for the two substances	0.0008	0,008

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Mycotoxins	Ochratoxin A	303-47-9	Kidney (pig)	LOAEL: 0.008	TWI: 0.00012 mg/kg/week	All (4-75 y), DK	0.0004	0,00048
Mycotoxins	Ochratoxin A	303-47-9	Kidney (pig)	LOAEL: 0.008	TWI: 0.00012 mg/kg/week			
Mycotoxins	Ochratoxin A	303-47-9	Kidney (pig)	NOEL: 0.021	PTWI: 0.0001 mg/kg/week			
Mycotoxins	Patulin	149-29-1	Body weight (rat)	NOEL: 0.043	PMTDI: 0.0004	Adults (≥ 18 y), FR, mean, LB	0.00063	0,0016
Mycotoxins	Patulin	149-29-1	Body weight (rat)	NOEL: 0.043	PMTDI: 0.0004	Children (3-17 y), FR, mean, LB	0.00121	0,003
Mycotoxins	Patulin	149-29-1	Body weight (rat)	NOEL: 0.043	PMTDI: 0.0004	Adults (≥ 18 y), FR, 95% percentile, LB	0.00321	0,008
Mycotoxins	Patulin	149-29-1	Body weight (rat)	NOEL: 0.043	PMTDI: 0.0004	Children (3-17 y), FR,)%5 percentile LB	0.00686	0,0172
Mycotoxins	Ergot alkaloids		Muscular atrophy (rat)	$BMDL_{10}$: 0.33	TDI: 0.0006	Children (1-2 y), mean, LB	0.056	0,0933
Mycotoxins	Ergot alkaloids		Muscular atrophy (rat)	$BMDL_{10}$: 0.33	TDI: 0.0006	Adults (18-64 y), mean, LB	0.011	0,018
Mycotoxins	Ergot alkaloids		Muscular atrophy (rat)	$BMDL_{10}$: 0.33	TDI: 0.0006	Children (1-2 y), 95% percentile, LB	0.155	0,258
Mycotoxins	Ergot alkaloids		Muscular atrophy (rat)	$BMDL_{10}$: 0.33	TDI: 0.0006	Adults (18-64 y), 95% percentile, LB	0.030	0,05
Mycotoxins	Zearalenone	17924-92-4	Oestrogenic (pig)	NOEL: 0.01	TDI: 0.00025	Children (1-2 y), mean, LB	0.013	0,052
Mycotoxins	Zearalenone	17924-92-4	Oestrogenic (pig)	NOEL: 0.01	TDI: 0.00025	Adults (18-64 y), mean, LB	0.0043	0,017
Mycotoxins	Zearalenone	17924-92-4	Oestrogenic (pig)	NOEL: 0.01	TDI: 0.00025	Children (1-2 y), 95% percentile, LB	0.104	0,416
Mycotoxins	Zearalenone	17924-92-4	Oestrogenic (pig)	NOEL: 0.01	TDI: 0.00025	Adults (18-64 y), 95% percentile, LB	0.028	0,112
Mycotoxins	Citrinin	518-75-2	Kidney (rat)	NOAEL: 0.02	RfD: 0.0002	No data		
Mycotoxins	Fumonisins		Kidney (rat)	NOAEL B1: 0.2	TDI: 0.002	Adults (≥ 18 y), FR, mean, LB	0.0099	0,005
Mycotoxins	Fumonisins		Kidney (rat)	NOAEL B1: 0.2	TDI: 0.002	Children (3-17 y), FR, mean, LB	0.0219	0,011
Mycotoxins	Fumonisins		Kidney (rat)	NOAEL B1: 0.2	TDI: 0.002	Adults (≥ 18 y), FR, 95% percentile, LB	0.0325	0,0165
Mycotoxins	Fumonisins		Kidney (rat)	NOAEL B1: 0.2	TDI: 0.002	Children (3-17 y), FR,)%5 percentile LB	0.0737	0,0369
Mycotoxins	Fumonisins		Kidney (rat)	NOEL, Fumonisin B1: 0.2	PMTDI: 0.002			
Mycotoxins	Nivalenol	23282-20-4	Immuno/haematotox (rat)	$BMDL_{05}$: 0.35	TDI: 0.0012	Children (1-2 y), mean, LB	0.0063	0,0053
Mycotoxins	Nivalenol	23282-20-4	Immuno/haematotox (rat)	$BMDL_{05}$: 0.35	TDI: 0.0012	Adults (18-64 y), mean, LB	0.0016	0,0013
Mycotoxins	Nivalenol	23282-20-4	Immuno/haematotox (rat)	$BMDL_{05}$: 0.35	TDI: 0.0012	Children (1-2 y), 95% percentile, LB	0.015	0,0125
Mycotoxins	Nivalenol	23282-20-4	Immuno/haematotox (rat)	$BMDL_{05}$: 0.35	TDI: 0.0012	Adults (18-64 y), 95% percentile, LB	0.004	0,0033
Bisphenols	BPA	80-05-7		NOAEL: 5	TDI: 0.05	Children, (3-9 y), DK, mean, MB	0.102	0,002
Bisphenols	BPA	80-05-7		NOAEL: 5	TDI: 0.05	Men (18-45 y), DK, mean, MB	0.051	0,001
Bisphenols	BPA	80-05-7		NOAEL: 5	TDI: 0.05	Children, (3-9 y), DK, 95% percentile, MB	0.165	0,0033
Bisphenols	BPA	80-05-7		NOAEL: 5	TDI: 0.05	Men (18-45 y), DK, 95% percentile, MB	0.080	0,0016

Chemical class	Chemical name	CAS RN	Critical effect	Point of departure mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Brominated flame retardants	Polybrominated diphenyl ether (BDE-47)	5436-43-1	Neurotox (mouse)	$BMDL_{10}$: 0.309	RfD: 0.00309	Children (1-3 y), EU, mean, LB,	0.00103	0,00033
Brominated flame retardants	Polybrominated diphenyl ether (BDE-47)	5436-43-1	Neurotox (mouse)	$BMDL_{10}$: 0.309	RfD: 0.00309	Children (1-3 y), EU high consumers, LB	0.0044	0,00129
Brominated flame retardants	Polybrominated diphenyl ether (BDE-47)	5436-43-1	Neurotox (mouse)	$BMDL_{10}$: 0.309	RfD: 0.00309	Adults, EU, mean, LB,	0.00029	0,00009
Brominated flame retardants	Polybrominated diphenyl ether (BDE-47)	5436-43-1	Neurotox (mouse)	$BMDL_{10}$: 0.309	RfD: 0.00309	Adults, EU, highe consumers, LB	0.0011	0,00036
Brominated flame retardants	Polybrominated diphenyl ether (BDE-99)	60348-60-9	Neurotox (mouse)	$BMDL_{10}$: 0.012	RfD: 0.00012	Children (1-3 y), EU, mean, LB,	0.00058	0,0048
Brominated flame retardants	Polybrominated diphenyl ether (BDE-99)	60348-60-9	Neurotox (mouse)	$BMDL_{10}$: 0.012	RfD: 0.00012	Children (1-3 y), EU high consumers, LB	0.00136	0,0113
Brominated flame retardants	Polybrominated diphenyl ether (BDE-99)	60348-60-9	Neurotox (mouse)	$BMDL_{10}$: 0.012	RfD: 0.00012	Adults, EU, mean, LB,	0.00011	0,00092
Brominated flame retardants	Polybrominated diphenyl ether (BDE-99)	60348-60-9	Neurotox (mouse)	$BMDL_{10}$: 0.012	RfD: 0.00012	Adults, EU, highe consumers, LB	0.00030	0,0025
Brominated flame retardants	Polybrominated diphenyl ether (BDE-153)	68631-49-2	Neurotox (mouse)	$BMDL_{10}$: 0.083	RfD: 0.00083	Children (1-3 y), EU, mean, LB,	0.00009	0,00011
Brominated flame retardants	Polybrominated diphenyl ether (BDE-153)	68631-49-2	Neurotox (mouse)	$BMDL_{10}$: 0.083	RfD: 0.00083	Children (1-3 y), EU high consumers, LB	0.00020	0,00241
Brominated flame retardants	Polybrominated diphenyl ether (BDE-153)	68631-49-2	Neurotox (mouse)	$BMDL_{10}$: 0.083	RfD: 0.00083	Adults, EU, mean, LB,	0.00003	0,00004
Brominated flame retardants	Polybrominated diphenyl ether (BDE-153)	68631-49-2	Neurotox (mouse)	$BMDL_{10}$: 0.083	RfD: 0.00083	Adults, EU, highe consumers, LB	0.00007	0,00008
Brominated flame retardants	Polybrominated diphenyl ether (BDE-209)	1163-19-5	Neurotox (mouse)	$BMDL_{10}$: 1.7	RfD: 0.017	Children (1-3 y), EU, mean, LB,	0.00155	0,00009
Brominated flame retardants	Polybrominated diphenyl ether (BDE-209)	1163-19-5	Neurotox (mouse)	$BMDL_{10}$: 1.7	RfD: 0.017	Children (1-3 y), EU high consumers, LB	0.00086	0,00051
Brominated flame retardants	Polybrominated diphenyl ether (BDE-209)	1163-19-5	Neurotox (mouse)	$BMDL_{10}$: 1.7	RfD: 0.017	Adults, EU, mean, LB,	0.00035	0,00002
Brominated flame retardants	Polybrominated diphenyl ether (BDE-209)	1163-19-5	Neurotox (mouse)	$BMDL_{10}$: 1.7	RfD: 0.017	Adults, EU, highe consumers, LB	0.00070	0,00004
Brominated flame retardants	Hexabromocyclododecane		Neurotox (mouse)	$BMDL_{10}$: 0.79	RfD: 0.0079	Adults (15-75 y), DK, mean	0.00019	0,00002
Brominated flame retardants	Hexabromocyclododecane		Neurotox (mouse)	$BMDL_{10}$: 0.79	RfD: 0.0079	Children (4-14 y) DK, mean	0.00023	0,00003
Brominated flame retardants	Hexabromocyclododecane		Neurotox (mouse)	$BMDL_{10}$: 0.79	RfD: 0.0079	Adults (15-75 y), DK, 95% percentile	0.00075	0,00009
Brominated flame retardants	Hexabromocyclododecane		Neurotox (mouse)	$BMDL_{10}$: 0.79	RfD: 0.0079	Children (4-14 y), DK, 95% percentile	0.00128	0,00016
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene	50-32-8	Genotox	$BMDL_{10}$: 0.07	RfD: 0.0007	All (4-75 y), DK, mean	0.0045	0,00006

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Polycyclic aromatic hydro-carbons	Benzo[a]pyrene	50-32-8	Genotox	$BMDL_{10}$: 0.07	RfD: 0.0007	Children (4-14 y), DK, mean	0.0069	0,0001
Polycyclic aromatic hydro-carbons	Benzo[a]pyrene	50-32-8	Genotox	$BMDL_{10}$: 0.07	RfD: 0.0007	All (4-75 y), DK, 95% percentile	0.0093	0,00013
Polycyclic aromatic hydro-carbons	Benzo[a]pyrene	50-32-8	Genotox	$BMDL_{10}$: 0.07	RfD: 0.0007	Children (4-14 y), DK, 95% percentile	0.013	0,00019
Polycyclic aromatic hydro-carbons	PAH4		Genotox	$BMDL_{10}$: 0.34	RfD: 0.0034	All (4-75 y), DK, mean	0.019	0,00006
Polycyclic aromatic hydro-carbons	PAH4		Genotox	$BMDL_{10}$: 0.34	RfD: 0.0034	Children (4-14 y), DK, mean	0.031	0,00009
Polycyclic aromatic hydro-carbons	PAH4		Genotox	$BMDL_{10}$: 0.34	RfD: 0.0034	All (4-75 y), DK, 95% percentile	0.039	0,00011
Polycyclic aromatic hydro-carbons	PAH4		Genotox	$BMDL_{10}$: 0.34	RfD: 0.0034	Children (4-14 y), DK, 95% percentile	0.056	0,00016
Metals	Mercury, organic (MeHg)	22967-92-6	Neurotox (human)		TWI: 0.0013 mg/kg/week	All (4-75 y), DK, mean	0.018	0,1
Metals	Mercury, organic (MeHg)	22967-92-6	Neurotox (human)		TWI: 0.0013 mg/kg/week	All (4-75 y), DK, 95% percentile	0.051	0,27
Metals	Mercury, organic (MeHg)	22967-92-6	Neurotox (human)		PTWI: 0.0016 mg/kg/week			
Metals	Mercury, inorganic		Kidney (rat)	$BMDL_{10}$: 0.06	TWI: 0.004 mg/kg/week	All (4-75 y), DK, mean	0.012	0,022
Metals	Mercury, inorganic		Kidney (rat)	$BMDL_{10}$: 0.06	TWI: 0.004 mg/kg/week	All (4-75 y), DK, 95% percentile	0.034	0,06
Metals	Mercury, inorganic				PTWI: 0.004 mg/kg/week			
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	Adults (15-75 y), DK, mean	0.23	4,6
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	Children (4-14 y) DK, mean	0.30	6
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	Adults (15-75 y), DK, 95% percentile	0.41	8,2
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	Children (4-14 y), DK, 95% percentile	0.56	11,2
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	All (4-75 y), DK, mean	0.25	5
Metals	Lead	7439-92-1	Neurotox (children)	$BMDL_{01}$: 0.0005	RfD: 0.00005	All (4-75 y), DK, 95% percentile	0.46	9,2
Metals	Cadmium	7440-43-9	Kidney (human)	$BMDL_{05}$: 4 µg Ca/g crea	TWI: 0.0025 mg/kg/week	All (4-75 y), DK, mean	0.18	0,47
Metals	Cadmium	7440-43-9	Kidney (human)	$BMDL_{05}$: 4 µg Ca/g crea	TWI: 0.0025 mg/kg/week	All (4-75 y), DK, 95% percentile	0.38	0,99
Metals	Cadmium	7440-43-9	Kidney (human)	$BMDL_{05}$: 4 µg Ca/g crea	TWI: 0.0025 mg/kg/week	Adults (15-75 y), DK, mean	0.15	0,39
Metals	Cadmium	7440-43-9	Kidney (human)	$BMDL_{05}$: 4 µg Ca/g crea	TWI: 0.0025 mg/kg/week	Children (4-14 y), DK, 95% percentile	0.31	0,8
Metals	Cadmium	7440-43-9	Kidney (human)	$BMDL_{05}$: 4 µg Ca/g crea	TWI: 0.0025 mg/kg/week			

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Metals	Cadmium	7440-43-9	Kidney (human)		PTMI: 0.025 mg/kg/month			
Metals	Arsen, inorganic	7440-38-2	Lung cancer (human)	$BMDL_{01}$: 0.0003	RfD: 0.00003	All (4-75 y), DK, mean	0.12	4
Metals	Arsen, inorganic	7440-38-2	Lung cancer (human)	$BMDL_{01}$: 0.0003	RfD: 0.00003	All (4-75 y), DK, 95% percentile	0.21	7
Metals	Arsen, inorganic	7440-38-2	Lung cancer (human)	$BMDL_{01}$: 0.0003	RfD: 0.00003	All (4-75 y), DK, 99% percentile	0.30	10
Metals	Arsen, inorganic	7440-38-2	Lung cancer (human)	$BMDL_{0.5}$: 0.002	RfD: 0.0002			
Metals	Aluminium	7429-90-5	Neurotox (rat)	NOAEL: 30	PTWI: 1 mg/kg/week	Adults (≥ 18 y), FR, mean	40.3	0,28
Metals	Aluminium	7429-90-5	Neurotox (rat)	NOAEL: 30	PTWI: 1 mg/kg/week	Children (4-17 y), FR, mean	62.2	0,44
Metals	Aluminium	7429-90-5	Neurotox (rat)	NOAEL: 30	PTWI: 1 mg/kg/week	Adults (≥ 18 y), FR, 95% percentile	69.7	0,49
Metals	Aluminium	7429-90-5	Neurotox (rat)	NOAEL: 30	PTWI: 1 mg/kg/week	Children (4-17 y), FR, 95% percentile	118.8	0,83
Metals	Aluminium	7429-90-5		LOEL: 50	PTWI: 1 mg/kg/week			
Metals	Nickel	7440-02-0	Reprotox (rat)	NOAEL: 2.2	TDI: 0.022	All (4-75 y), DK, mean	1.5	0,068
Metals	Nickel	7440-02-0	Reprotox (rat)	NOAEL: 2.2	TDI: 0.022	All (4-75 y), DK, 95% percentile	2.7	0,122
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	All (4-75 y), DK, mean	0.55 pg/kg bw/day	0,275
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	Children (4-14 y), DK, mean	0.87 pg/kg bw/day	0,435
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	All (4-75 y), DK, 95% percentile	1.2 pg/kg bw/day	0,6
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	Children (4-14 y), DK, 95% percentile	1.9 pgkg bw/day	0,95
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	All (4-75 y), 99% percentile	2 pg/kg bw/day	1
Dioxin and PCBs	Dioxins and dioxin-like PCBs		Reprotox	LOAEL: 25 ng/kg/day	TWI: 14 pg TEQ/kg/week	Children (4-14 y), DK, 99% percentile	2.4 pg/kg bw/day	1,2
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	All (4-75 y), DK, mean	1.8 ng/kg bw/day	0,18
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	Children (4-14 y), DK, mean	2.7 ng/kg bw/day	0,27
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	All (4-75 y), DK, 95% percentile	4.3 ng/kg bw/day	0,43
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	Children (4-14 y), DK, 95% percentile	6.3 ng/kg bw/day	0,63
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	All (4-75 y), 99% percentile	7.1 ng/kg bw/day	0,71
Dioxin and PCBs	Non-dioxin like PCBs		Neurotox	NOAEL: 0.093 µg/kg/day	TDI: 10 ng/kg/day	Children (4-14 y), DK, 99% percentile	9.4 ng/kg bw/day	0,94
Pesticides	2,4-D (sum)				0.05	Adults (15-75 y), DK, mean	0.0023	0,000046
Pesticides	Acephate				0.03	Adults (15-75 y), DK, mean	0.0012	0,000039
Pesticides	Acetamiprid				0.07	Adults (15-75 y), DK, mean	0.011	0,00015
Pesticides	Aclonifen				0.07	Adults (15-75 y), DK, mean	0.00003	0,0000004

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Pesticides	Aldicarb (sum)				0.003	Adults (15-75 y), DK, mean	0.0013	0,00043
Pesticides	Aldrin and Dieldrin				0.0001	Adults (15-75 y), DK, mean	0.00023	0,0023
Pesticides	Atrazine				0.02	Adults (15-75 y), DK, mean	0.000041	0,000002
Pesticides	Azinphos-methyl				0.005	Adults (15-75 y), DK, mean	0.022	0,0044
Pesticides	Azoxystrobin				0.2	Adults (15-75 y), DK, mean	0.021	0,00011
Pesticides	Benalaxyl (sum)				0.04	Adults (15-75 y), DK, mean	0.00014	0,0000036
Pesticides	Benfuracarb				0.01	Adults (15-75 y), DK, mean	0.0017	0,00017
Pesticides	Bifenthrin				0.015	Adults (15-75 y), DK, mean	0.0064	0,00043
Pesticides	Biphenyl				0.125	Adults (15-75 y), DK, mean	0.0011	0,0000089
Pesticides	Bitertanol				0.003	Adults (15-75 y), DK, mean	0.009	0,003
Pesticides	Bromopropylate				0.03	Adults (15-75 y), DK, mean	0.011	0,00037
Pesticides	Bupirimate				0.05	Adults (15-75 y), DK, mean	0.012	0,00024
Pesticides	Buprofezin				0.01	Adults (15-75 y), DK, mean	0.0022	0,00022
Pesticides	Captan/Folpet (sum)				0.1	Adults (15-75 y), DK, mean	0.053	0,00053
Pesticides	Carbaryl				0.0075	Adults (15-75 y), DK, mean	0.043	0,0057
Pesticides	Carbendazim and benomyl				0.02	Adults (15-75 y), DK, mean	0.087	0,0043
Pesticides	Carbofuran (sum)				0.00015	Adults (15-75 y), DK, mean	0.00017	0,0011
Pesticides	Carbosulfan				0.005	Adults (15-75 y), DK, mean	0.0048	0,00097
Pesticides	Chlorfenvinphos				0.0005	Adults (15-75 y), DK, mean	0.0028	0,0057
Pesticides	Chlormequat				0.04	Adults (15-75 y), DK, mean	0.076	0,0019
Pesticides	Chlorothalonil				0.015	Adults (15-75 y), DK, mean	0.012	0,00082
Pesticides	Chlorpropham				0.05	Adults (15-75 y), DK, mean	0.031	0,00063
Pesticides	Chlorpropham (sum)				0.05	Adults (15-75 y), DK, mean	0.0051	0,0001
Pesticides	Chlorpyrifos				0.01	Adults (15-75 y), DK, mean	0.023	0,0023
Pesticides	Chlorpyrifos-methyl				0.01	Adults (15-75 y), DK, mean	0.02	0,002
Pesticides	Chlorthal-dimethyl				0.01	Adults (15-75 y), DK, mean	0.00031	0,000031
Pesticides	Clofentezine				0.02	Adults (15-75 y), DK, mean	0.00096	0,000048
Pesticides	Cyfluthrin (sum)				0.003	Adults (15-75 y), DK, mean	0.0029	0,00095
Pesticides	Cyhalothrin, lambda-				0.005	Adults (15-75 y), DK, mean	0.0087	0,0017
Pesticides	Cypermethrin (sum)				0.05	Adults (15-75 y), DK, mean	0.01	0,00021
Pesticides	Cyprodinil				0.03	Adults (15-75 y), DK, mean	0.019	0,00062
Pesticides	Cyromazine				0.06	Adults (15-75 y), DK, mean	0.0084	0,00014
Pesticides	DDT (sum)				0.01	Adults (15-75 y), DK, mean	0.0017	0,00017
Pesticides	Deltamethrin				0.01	Adults (15-75 y), DK, mean	0.0091	0,00091
Pesticides	Diazinon				0.0002	Adults (15-75 y), DK, mean	0.0047	0,024
Pesticides	Dichlofluanid				0.3	Adults (15-75 y), DK, mean	0.0018	0,0000061
Pesticides	Dichlorprop				0.06	Adults (15-75 y), DK, mean	0.00014	0,0000024
Pesticides	Dichlorvos				0.00008	Adults (15-75 y), DK, mean	0.000096	0,0012
Pesticides	Dicloran				0.005	Adults (15-75 y), DK, mean	0.0027	0,00055
Pesticides	Dicofol (sum)				0.002	Adults (15-75 y), DK, mean	0.017	0,0086
Pesticides	Diethofencarb				0.43	Adults (15-75 y), DK, mean	0.0035	0,0000082

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Pesticides	Difenoconazole				0.01	Adults (15-75 y), DK, mean	0.0066	0,00066
Pesticides	Diflufenican				0.2	Adults (15-75 y), DK, mean	0.0012	0,0000061
Pesticides	Dimethoate				0.001	Adults (15-75 y), DK, mean	0.0063	0,0063
Pesticides	Dimethomorph				0.05	Adults (15-75 y), DK, mean	0.014	0,00028
Pesticides	Diniconazole				0.02	Adults (15-75 y), DK, mean	0.0009	0,000045
Pesticides	Diphenylamine				0.075	Adults (15-75 y), DK, mean	0.065	0,00086
Pesticides	Dithiocarbamates				0.05	Adults (15-75 y), DK, mean	0.21	0,0042
Pesticides	Endosulfan (sum)				0.006	Adults (15-75 y), DK, mean	0.0085	0,0014
Pesticides	Epoxiconazole				0.008	Adults (15-75 y), DK, mean	0.000077	0,0000097
Pesticides	Ethion				0.002	Adults (15-75 y), DK, mean	0.00083	0,00041
Pesticides	Ethoxyquin				0.005	Adults (15-75 y), DK, mean	0.0057	0,0011
Pesticides	Famoxadone				0.012	Adults (15-75 y), DK, mean	0.0031	0,00026
Pesticides	Fenarimol				0.01	Adults (15-75 y), DK, mean	0.0021	0,00021
Pesticides	Fenazaquin				0.005	Adults (15-75 y), DK, mean	0.0045	0,00091
Pesticides	Fenbuconazole				0.006	Adults (15-75 y), DK, mean	0.00036	0,000059
Pesticides	Fenhexamid				0.2	Adults (15-75 y), DK, mean	0.072	0,00036
Pesticides	Fenitrothion				0.005	Adults (15-75 y), DK, mean	0.0054	0,0011
Pesticides	Fenoxaprop-P-Ethyl				0.01	Adults (15-75 y), DK, mean	0.000077	0,0000077
Pesticides	Fenpropathrin				0.03	Adults (15-75 y), DK, mean	0.001	0,000034
Pesticides	Fenpropimorph				0.003	Adults (15-75 y), DK, mean	0.0011	0,00035
Pesticides	Fenthion (sum)				0.007	Adults (15-75 y), DK, mean	0.00074	0,00011
Pesticides	Fenvalerat, esfenvalerat, RR- and SS-				0.02	Adults (15-75 y), DK, mean	0.0044	0,00022
Pesticides	Fenvalerat, esfenvalerat, RS- and SR-				0.02	Adults (15-75 y), DK, mean	0.0007	0,000035
Pesticides	Flucythrinate				0.02	Adults (15-75 y), DK, mean	0.00026	0,000013
Pesticides	Fludioxonil				0.37	Adults (15-75 y), DK, mean	0.025	0,000069
Pesticides	Fluoxastrobin				0.015	Adults (15-75 y), DK, mean	0.000051	0,0000034
Pesticides	Fluroxypyr (sum)				0.8	Adults (15-75 y), DK, mean	0.0013	0,0000016
Pesticides	Flusilazole				0.002	Adults (15-75 y), DK, mean	0.00039	0,0002
Pesticides	Flutriafol				0.01	Adults (15-75 y), DK, mean	0.001	0,0001
Pesticides	Fluvalinate, tau-				0.005	Adults (15-75 y), DK, mean	0.0038	0,00076
Pesticides	Glyphosate				0.3	Adults (15-75 y), DK, mean	0.11	0,00037
Pesticides	Hexaconazole				0.005	Adults (15-75 y), DK, mean	0.00037	0,000075
Pesticides	Hexythiazox				0.03	Adults (15-75 y), DK, mean	0.0022	0,000074
Pesticides	Imazalil				0.025	Adults (15-75 y), DK, mean	0.072	0,0029
Pesticides	Iprodione				0.06	Adults (15-75 y), DK, mean	0.099	0,0016
Pesticides	Iprovalicarb				0.015	Adults (15-75 y), DK, mean	0.0086	0,00057
Pesticides	Kresoxim-methyl				0.4	Adults (15-75 y), DK, mean	0.0029	0,0000071
Pesticides	Lindane				0.005	Adults (15-75 y), DK, mean	0.00000066	0,0000001

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Pesticides	Linuron				0.003	Adults (15-75 y), DK, mean	0.01	0,0034
Pesticides	Malathion (sum)				0.03	Adults (15-75 y), DK, mean	0.019	0,00064
Pesticides	Mecoprop (sum)				0.01	Adults (15-75 y), DK, mean	0.00085	0,000085
Pesticides	Mepiquat				0.2	Adults (15-75 y), DK, mean	0.0054	0,000027
Pesticides	Metalaxyl (sum)				0.08	Adults (15-75 y), DK, mean	0.012	0,00015
Pesticides	Methacrifos				0.006	Adults (15-75 y), DK, mean	0.000032	0,0000053
Pesticides	Methamidophos				0.001	Adults (15-75 y), DK, mean	0.0029	0,0029
Pesticides	Methidathion				0.001	Adults (15-75 y), DK, mean	0.00097	0,00097
Pesticides	Methiocarb (sum)				0.013	Adults (15-75 y), DK, mean	0.0016	0,00012
Pesticides	Methomyl and Thiodicarb				0.0025	Adults (15-75 y), DK, mean	0.0083	0,0033
Pesticides	Metribuzin				0.013	Adults (15-75 y), DK, mean	0.005	0,00038
Pesticides	Mevinphos				0.0008	Adults (15-75 y), DK, mean	0.00019	0,00023
Pesticides	Monocrotophos				0.0006	Adults (15-75 y), DK, mean	0.0000052	0,0000087
Pesticides	Myclobutanil				0.025	Adults (15-75 y), DK, mean	0.006	0,00024
Pesticides	Omethoate				0.0003	Adults (15-75 y), DK, mean	0.0039	0,013
Pesticides	Orthophenylphenol				0.4	Adults (15-75 y), DK, mean	0.011	0,000028
Pesticides	Oxamyl				0.001	Adults (15-75 y), DK, mean	0.0026	0,0026
Pesticides	Oxydemeton-methyl (sum)				0.0003	Adults (15-75 y), DK, mean	0.00074	0,0025
Pesticides	Parathion-methyl (sum)				0.003	Adults (15-75 y), DK, mean	0.0016	0,00053
Pesticides	Penconazole				0.03	Adults (15-75 y), DK, mean	0.0019	0,000064
Pesticides	Pendimethalin				0.125	Adults (15-75 y), DK, mean	0.0011	0,0000088
Pesticides	Permethrin (sum)				0.05	Adults (15-75 y), DK, mean	0.0017	0,000033
Pesticides	Phenthoate				0.003	Adults (15-75 y), DK, mean	0.000086	0,000029
Pesticides	Phorate (sum)				0.0007	Adults (15-75 y), DK, mean	0.00016	0,00023
Pesticides	Phosalone				0.01	Adults (15-75 y), DK, mean	0.019	0,0019
Pesticides	Phosmet (sum)				0.003	Adults (15-75 y), DK, mean	0.021	0,0069
Pesticides	Pirimicarb (sum)				0.035	Adults (15-75 y), DK, mean	0.0082	0,00023
Pesticides	Pirimiphos-methyl				0.004	Adults (15-75 y), DK, mean	0.043	0,011
Pesticides	Prochloraz (sum)				0.01	Adults (15-75 y), DK, mean	0.0064	0,00064
Pesticides	Procymidone				0.0028	Adults (15-75 y), DK, mean	0.018	0,0064
Pesticides	Profenofos				0.03	Adults (15-75 y), DK, mean	0.00046	0,000015
Pesticides	Propamocarb (sum)				0.29	Adults (15-75 y), DK, mean	0.032	0,00011
Pesticides	Propargite				0.01	Adults (15-75 y), DK, mean	0.061	0,0061
Pesticides	Propiconazole				0.04	Adults (15-75 y), DK, mean	0.00035	0,0000087
Pesticides	Propoxur				0.02	Adults (15-75 y), DK, mean	0.00000039	
Pesticides	Propyzamide				0.02	Adults (15-75 y), DK, mean	0.0006	0,00003
Pesticides	Pymetrozine				0.03	Adults (15-75 y), DK, mean	0.0057	0,00019
Pesticides	Pyraclostrobin				0.03	Adults (15-75 y), DK, mean	0.023	0,00076
Pesticides	Pyrazophos				0.004	Adults (15-75 y), DK, mean	0.001	0,00025
Pesticides	Pyridaben				0.01	Adults (15-75 y), DK, mean	0.0027	0,00027

Chemical class	Chemical name	CAS RN	Critical effect	Point of de-parture mg/kg/day	Reference dose mg/kg/day	Consumer group	Intake µg/kg/day	Hazard quotient
Pesticides	Pyridate (sum)				0.036	Adults (15-75 y), DK, mean	0.000056	0,0000015
Pesticides	Pyrimethanil				0.17	Adults (15-75 y), DK, mean	0.043	0,00025
Pesticides	Pyriproxyfen				0.1	Adults (15-75 y), DK, mean	0.003	0,00003
Pesticides	Quinoxyfen				0.2	Adults (15-75 y), DK, mean	0.000037	0,0000002
Pesticides	Quintozene (sum)				0.01	Adults (15-75 y), DK, mean	0.0061	0,00061
Pesticides	Spiroxamine				0.025	Adults (15-75 y), DK, mean	0.0014	0,000056
Pesticides	Tebuconazole				0.03	Adults (15-75 y), DK, mean	0.013	0,00042
Pesticides	Tebufenozide				0.02	Adults (15-75 y), DK, mean	0.01	0,0005
Pesticides	Tebufenpyrad				0.01	Adults (15-75 y), DK, mean	0.0064	0,00064
Pesticides	Tecnazene				0.02	Adults (15-75 y), DK, mean	0.000095	0,0000048
Pesticides	Tetraconazole				0.004	Adults (15-75 y), DK, mean	0.00037	0,000092
Pesticides	Tetradifon				0.015	Adults (15-75 y), DK, mean	0.00038	0,000026
Pesticides	Thiabendazole				0.1	Adults (15-75 y), DK, mean	0.11	0,0011
Pesticides	Thiophanate-methyl				0.08	Adults (15-75 y), DK, mean	0.031	0,00039
Pesticides	Tolclofos-methyl				0.064	Adults (15-75 y), DK, mean	0.0018	0,000028
Pesticides	Tolylfluanid (sum)				0.1	Adults (15-75 y), DK, mean	0.019	0,00019
Pesticides	Triadimefon (sum)				0.05	Adults (15-75 y), DK, mean	0.028	0,00055
Pesticides	Triallate				0.025	Adults (15-75 y), DK, mean	0.00008	0,0000032
Pesticides	Triazophos				0.001	Adults (15-75 y), DK, mean	0.000082	0,000082
Pesticides	Trichlorfon				0.045	Adults (15-75 y), DK, mean	0.00065	0,000014
Pesticides	Trifloxystrobin				0.1	Adults (15-75 y), DK, mean	0.0095	0,000095
Pesticides	Triflumuron				0.014	Adults (15-75 y), DK, mean	0.007	0,0005
Pesticides	Vinclozolin (sum)				0.005	Adults (15-75 y), DK, mean	0.009	0,0018