Project T01045: The Assessment of Joint Endocrine Effects of Multi-Component Mixtures of Food Contaminants and Additives

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Executive summary

Project T01045, *The Assessment of Joint Endocrine Effects of Multi-Component Mixtures of Food Contaminants and Additives*, investigated whether the combined effects of multiple chemicals could be predicted from the effects of the individual chemicals alone. The chemicals studied in this project were potential endocrine disrupting chemicals (EDCs), a class of chemicals that might affect the endocrine system of exposed humans, for example by mimicking the effect of the female sex hormone estradiol (estrogenicity) or by blocking the effect of the male sex hormone testosterone (anti-androgenicity). This project included the mathematical modeling and experimental testing of over ***50 mixtures*** each containing up to ***31 chemicals.*** The main interest was to explore generic principles of mixture toxicology, using estrogenic and anti-androgenic chemicals as an example, rather than to conduct a risk assessment for endocrine disrupters.

Firstly, mixtures of more than 10 ***active chemicals***, i.e. chemicals that showed activity in the given test system when tested alone, were examined. It was found that the combined effect of mixtures could generally be predicted using a model called *concentration addition*. These mixtures were designed to be balanced, with a fixed mixture ratio in proportion to equi-effective concentrations of each chemical. Each of the chemicals was expected to contribute equally to the effects of the mixture. This experimental design represents an efficient way of examining the usefulness of prediction concepts (*concentration addition*, *independent action*) in approximating a mixture effect by allowing each of the chemicals equal opportunity to exert an effect. *Concentration addition* gave reasonable approximations for the prediction of combination effects. Application of the competing concept of *independent action* led to underestimations of the experimentally observed effects. Deviations from predicted additivity, indicative of synergisms or antagonisms, were only rarely observed, and were relatively small. There is no need for the experimental testing of each and every conceivable mixture, which would indeed make risk assessment unmanageable.

Secondly, the impact of chemicals without estrogenicity or anti-androgenicity, so-called ***modulator chemicals***, was tested to see if they altered the activity of multi-component mixtures of active chemicals. Most chemicals examined in this way did not affect the mixtures they were tested against, however three of the chemicals showed negative modulation in an estrogenicity assay, i.e. they reduced the effect of the mixture they were combined with.

Thirdly, mixtures with a composition approaching the levels of endocrine active chemicals ***found in human tissues*** were tested. These mixtures contained chemicals mixed in the proportions that they may occur at in human tissues, and consequently certain chemicals that are potent and have a high exposure can have a proportionally greater contribution to the mixture effect. The effects of mixtures of more realistic composition, compared to the balanced, equi-effective design described above, remained predictable by the model of *concentration addition*. Furthermore, *concentration addition* was also able to make meaningful estimations of effects for mixtures containing both active and modulator

chemicals, at possible human exposure levels. Our studies have shown that a relatively small number of components explained most of an overall combined effect. If applicable to endpoints of toxicological relevance for risk assessment, the implications of this observation could be very significant: The apparent complexity of real exposure scenarios could be reduced to a manageable group of relevant chemicals, possible health risks assessed more easily and significant risk reductions be achieved through targeted exposure reduction measures. However, for endocrine disrupters the issue cannot be resolved with certainty without information about the range of these chemicals that make up the internal exposure of humans. This information is currently only fragmentary. It remains to be seen whether the phenomenon also appears when a much larger number of chemicals is included for assessment, i.e. whether it is independent of the number of chemicals and thus can be generalized.

In conclusion, this project has produced good evidence that chemicals with common specific modes of action (receptor agonism or antagonism) work together to produce combination effects that are larger than the effects of each mixture component applied singly. Considerations of the joint effects of multi-component mixtures are not only feasible, but also necessary to safeguard against underestimations of risk that might occur with the traditional chemical-by-chemical approach to risk assessment.

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Chapter 1:
Report Introduction

Introduction

This report presents the results and final conclusions from project T01045, The Assessment of Joint Endocrine Effects of Multi-Component Mixtures of Food Contaminants and Additives. This project addressed the predictability of effects of multi-component mixtures of food additives and contaminants. Mixtures were examined in three different *in vitro* assays of relevance to endocrine disruption, and focused on the endpoints of estrogenicity and anti-androgenicity. This project included the experimental testing and mathematical modeling of over 50 mixtures containing up to 31 components.

The project aims were to:

- establish whether multi-component mixtures of food additives and contaminants act together to produce estrogenic and anti-androgenic effects at levels encountered in human tissues.
- explore whether mechanistic information about the mode of action of food contaminants can be used to predict mixture effects.

The main interest was to explore generic principles of mixture toxicology, using estrogenic and anti-androgenic chemicals as an example, rather than to conduct a risk assessment for endocrine disrupters.

Structure of the report

The project progressed through four tasks, each of which is presented in turn in the following chapters of this report:

- Task 1: **Selection of test compounds and compilation of relevant data about internal exposures.** Results from this task were reported during the project as deliverable #1, "*Report on selection of test chemicals and their levels in human tissues*", which is included in this report as chapter 2.
- Task 2: **Evaluation of the estrogenic and anti-androgenic effects of mixtures of food additives/contaminants**. The results from this task were reported as deliverable #5, "*Report on Assessment of Mixtures of Food Contaminants Able To Interact With the Estrogen and Androgen Receptor*", and are included here as chapter 3.
- Task 3: **Assessment of the impact of food additives/contaminants as effect modifiers**. This task was reported in deliverable #7 "*Report on Assessment of the Impact of Effect Modifiers on the Joint Effect of Estrogenic and Anti-androgenic Agents*", which is included here as chapter 4.
- Task 4: **Assessment of mixtures of food additives/contaminants that reflect human tissue levels.** The results from this task have not been reported so far, and are presented in this report in chapter 5.

The report concludes with a discussion of the implications of the project's findings for general mixtures risk assessment (Chapter 6).

The chapters are written as self-contained units, with all relevant details about materials and methods, and assessment models. Consequently, there is some duplication in describing the features of the assays employed for mixture studies, the details of regression modeling and the characteristics of *concentration addition* and *independent action* as assessment concepts.

Published papers arising from project T01045

Inability to confirm estrogenicity of the heterocyclic amine PhIP in two in vitro assays.
Evans RM, Rahte S, Kortenkamp A.
Toxicol In Vitro. 2009. [Epub ahead of print]

The sensitivity of the MDA-kb2 cell in vitro assay in detecting anti-androgenic chemicals - identification of sources of variability and estimation of statistical power.
Ermler S, Scholze M, Kortenkamp A.
Toxicol In Vitro. 2010 May 17. [Epub ahead of print]

Chapter 2:
Selection of Test Chemicals and Their Levels in Human Tissues

Task 1

Deliverable #1

Introduction

The aim of this project task was to select chemicals with relevance to food and with potential concerns as endocrine disrupters for inclusion in multi-component mixtures to be tested as part of project tasks 2 - 4.

To put the selection of test chemicals on a sound footing, it was necessary to conduct a systematic search for information about relevant food contaminants, their documented effect profiles. Of particular importance was to collect data about tissue levels in humans because this information will be used for the preparation of "realistic" mixtures as part of project task 4.

Groups of chemicals of relevance to food and with potential concern as endocrine disruptors were identified and used as the starting point for an extensive literature search. In selecting candidate chemicals, two criteria were of over-riding importance:

- Chemicals had to be present in food
- Data about tissue levels in humans had to be available

In line with the specific aims of the related research programme, pesticides were excluded.

Groups of chemicals selected as the starting point for the literature search are listed below. Because of concerns about their environmental accumulation perfluorinated chemicals (PFCs, including PFOS and PFOA) were included.

Box1: Groups of chemicals identified as being of interest as non-pesticide endocrine disrupters with relevance to food*:*

Food mutagens, including heterocyclic amines such as PhIP
Polychlorinated biphenyls (PCBs) and their hydroxylated metabolites, and polychlorinated dibenzo dioxins (PCDDs) and furans (PCDFs)
Phthalates
Bisphenol A
Anti-oxidants
Phytoestrogens
Polybrominated diphenyl ethers (PBDEs)
Perfluorinated chemicals (PFCs)
Parabens
Polycyclic synthetic musks
UV-filter agents
Heavy metals

Definitions

Endocrine disruptors

In identifying candidate endocrine disrupters for inclusion in multi-component mixtures, the modified Weybridge (EC 1996) definition (IPCS, 2002) was used as a guide:

> "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.
>
> A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations."

Given the aims and objectives of the present project, "endocrine disruption" was limited to estrogenicity and anti-androgenicity.

Estrogenic chemicals

"Estrogenicity" can be defined in various ways, and the use of the term in the scientific literature is not uniform. At the functional, physiological level, the term denotes the ability of a chemical to evoke responses similar to 17β-estradiol (E2), such as cornification of the vaginal epithelium, and uterine cell proliferation. Of toxicological concern is the role of estrogens in breast and ovarian cancer, and 17β-estradiol and synthetic estrogens are recognised human carcinogens. Advances in our understanding of the mode of action of estrogens have led to further definitions which refer to specific steps at various molecular levels. "Estrogenicity" can mean affinity to the estrogen receptor (ERα or β) (although this does not distinguish agonists from antagonists), the ability to activate expression of estrogen-dependent genes, or stimulation of cell proliferation of ER-competent cells (*in vitro* and *in vivo*).

Thus, for the purposes of this report, ***"estrogenic"*** is meant to include chemicals that can mimic the effects of estradiol in the broadest sense of the word. It includes ER agonists, chemicals able to activate expression of estrogen-dependent genes, and substances that induce proliferation of ER-competent cells *in vitro* and *in vivo*.

More specifically, the terms ***"ER binding"*** and ***"ER agonist"*** denote chemicals that can displace estradiol from the ER in binding assays, and agents that bind to, and activate the ER.

Similarly, an ***"ER antagonist"*** is able to block the action of estrogens by occupying the estrogen binding site on the ER, without itself possessing ER agonistic properties.

The term ***"ER modulator"*** includes both ER antagonists and chemicals that can negatively modulate the effects of estrogenic chemicals by diverse, but often unspecified mechanisms.

Anti-androgenic chemicals

As with estrogenic chemicals, the term ***"anti-androgenic"*** is used in differing ways in the literature. In its broadest sense, it describes chemicals able to oppose the action of testosterone. This can include "AR antagonists" and substances that interfere with the synthesis of steroids (e.g. certain phthalates). For the purposes of this deliverable, ***"AR antagonists"*** are defined as substances able to block androgen action by direct interference with the hormones' binding site on the AR.

Effect modulators

Some chemicals can induce modulations of the effects of endocrine active agents by mechanisms other than steroid receptor antagonism. A well-studied example includes co-planar PCBs which are thought to oppose the action of estrogens by interfering with hormone metabolism *via* cytochrome P450 induction, and by modulating the expression of ER.

Modulations of the effects of AR antagonists are less well documented.

It should be noted that "effect modulation" can include both attenuating and diminishing effects.

Literature search strategy

Relevant data was identified in the peer-reviewed literature from PubMed (1985-current) and from the FSA and ATSDR websites. Data on 1) likely endocrine disruptor activity, 2) occurrence in food and 3) occurrence in human tissue was extracted from the literature and tabulated (Appendix 1).

Lists of candidate chemicals

Based on the outcome of the literature searches detailed in Appendix 1, chemicals for inclusion in mixtures (Tasks 2-4) were chosen. This proceeded in a step-wise fashion, as follows:

List 1 includes all chemicals with evidence of estrogenic or AR-antagonist effects and documented occurrence in food and human tissues. At this stage, no attempts for made to assess the strength of evidence for endocrine effects, and chemicals where there are conflicting reports of endocrine activity were included.

List 2 details chemicals from List 1 where conflicting or equivocal evidence of estrogenicity and AR-antagonistic effects was reported in the literature. Some chemicals from this list will be subjected to effect screening using the *in vitro* assays chosen for this project. Chemicals yielding positive effects will be considered for inclusion in mixtures.

List 3 contains chemicals from List 1 with good evidence for endocrine activity and which are therefore considered to be good candidates for inclusion in mixtures.

List 4 shows chemicals chosen from List 3 which will be included in mixtures (Tasks 2-4). In choosing these chemicals, great care was taken to select representatives from as many of the chemical groups listed in Box 1 as possible. List 4 also details substances drawn from List 2 which are to be included after confirmation of positive effects.

List 5 is a selection of chemicals that act as effect modulators of estrogenic agents and AR antagonists. These chemicals will be employed in completion of project tasks 3 and 4.

LIST 1: Candidate chemicals that 1) are likely to occur in food, 2) have been found in human tissues and where tissue levels have been published, and 3) with evidence of either estrogenic or anti-androgenic effects		
3-BC	BHT	Methylparaben
4-MBC	Bisphenol A	*n*-Butylparaben
4-nonylphenol	Cadmium	*n*-Propylparaben
4-tert-octylphenol	Chrysene	o-desmethylangolensin
8-prenylnaringenin	Daidzein	OMC
AHTN	Enterolactone	PCB8
Anthracene	Equol	PCB47
BDE-47	Ethylparaben	PCB52
BDE-99	Fluoranthene	PCB138
BDE-100	Genistein	PFOA
Benz[a]anthracene (BaA)	HHCB	PFOS
Benzo[a]pyrene (BaP)	Isobutylparaben	Phenanthrene
Benzophenone-3	Isoxanthohumol	PhIP
Benzylparaben	Lead	Pyrene
BHA		

LIST 2: chemicals with **conflicting or equivocal** literature reports of estrogenic (2A) or anti-androgenic (2B) effect, and which **will be considered for screening** at SOP	
2A Benzo[a]pyrene (BaP) Benz[a]anthracene (BaA) PCB52 BHA enterolactone BDE-47 BDE-99 BDE-100 PFOS PFOA Cadmium Lead	2B PCB8 BHA Genistein Daidzein 8-prenylnaringenin BHT PFOS PFOA Methylparaben Ethylparaben BDE-99 HHCB AHTN 4-MBC OMC BP-3 3-BC HMS Cadmium Lead

LIST 3: chemicals with **unequivocal** literature reports and which are candidate chemicals for **inclusion in mixtures** of estrogens (3A) and anti-androgens (3B).	
3A PhIP Bisphenol A 4-nonylphenol 4-tert-octylphenol Genistein Daidzein Equol o-desmethylangolensin isoxanthohumol Methylparaben Ethylparaben n-Propylparaben n-Butylparaben Isobutylparaben Benzylparaben HHCB AHTN 4-MBC OMC BP-3 3-BC	3B Benzo[a]pyrene (BaP) Benz[a]anthracene (BaA) Fluoranthene Chrysene Pyrene Phenanthrene Anthracene PCB47 PCB138 Bisphenol A 4-tert-octylphenol BDE-47 BDE-100 n-Propylparaben n-Butylparaben Isobutylparaben Benzylparaben

LIST 4: **proposed mixture compositions** of estrogens (4A) and anti-androgens (4B).	
4A PhIP Bisphenol A Genistein *n*-Propylparaben HHCB AHTN 4-MBC BP-3 *plus one of each group* *(from list 2A after confirmation of positive effects):* *PBDEs* *Metals* *PFOS/PFOA* *BHA* *PAH*	4B Chrysene *plus an additional PAH* PCB138 *plus an additional PCB* Bisphenol A BDE-100 *n*-Propylparaben *plus one of each group* *(from list 2B after confirmation of positive effects):* *PAH* *PCB* *Phytoestrogen* *BHA/BHT* *HMS* *UV-filters* *Musks* *Metals* *PFOS/PFOA*

LIST 5: potential **effect modulators** of estrogenic (5A) and anti-androgenic (5B) chemicals	
5A PCB153 PCB180 Phthalates MeIQx Benzo[a]pyrene (BaP) Naringenin Cadmium Lead	5B PCB153 PCB180 Phthalates MeIQx Naringenin Cadmium Lead

Chapter 3:
Assessment of Mixtures of Food Contaminants Able To Interact With the Estrogen and Androgen Receptor

Task 2

Deliverable #5

Introduction

This report is deliverable number five for the project T01045, and is part of Task 2, "Evaluation of the Estrogenic and Anti-androgenic Effects of Mixtures of Food Additives and Contaminants".

We are able to report complete results from mixture studies carried out in all three of the cell-based assays being used in the project, which comprise two estrogenicity assays, ERLUX (luciferase reporter gene assay using the T47D-KBluc cell line) and ESCREEN (mitogenicity/ proliferation assay using the MCF7-BOS cell line), and an anti-androgenicity assay, ARLUX (luciferase reporter gene assay using the MDA-kb2 cell line).

Firstly, we present data from all three assays on the single agent testing of a panel of substances (the substance selection process was detailed in a previous deliverable (deliverable # 1: "Report on Selection of Test Chemicals and Their Levels in Human Tissues (Task 1)", see chapter 2).

Secondly, we present predictions of the expected effects of three types of mixtures, defined on the basis of the single substance data from each of the assays. The mixture components were 1) substances found to be estrogenic in the ERLUX assay, 2) substances found to be mitogenic in the ESCREEN assay; and 3) substances found to be anti-androgenic in the ARLUX assay. Mixtures were designed using the fixed mixture ratio approach at various equi-effective levels, for example EC_{50}'s, and the concept of concentration addition (CA) was used to combine the single substance concentration-response relationships into predictions of the expected concentration-response relationships for the mixtures.

Finally, we present data for the experimental testing of all three defined mixtures and present a comparison of the observed effects with the expected effects. This comparison allows the mixture effects to be described as "additive" (observed effect similar to expectation), "synergistic" (observed effect greater than expectation) or "antagonistic" (observed effect less than expectation).

The comprehensive results obtained in this task, and reported in this deliverable, provide the baseline for the forthcoming testing of effect modulators (Task 3, see chapter 4) and examination of human exposure scenarios (Task 4, see chapter 5).

In the remainder of this introduction, we present a description of the concepts available for prediction of mixture effects, namely concentration addition (CA) and independent action (IA). The results sections include predictions made using both concepts, however we had a clear expectation that concentration addition would be the more useful concept for the mixtures examined in this phase of the project due to the nature of the endpoints used in the assays, and the nature of the chemicals selected for inclusion in the mixtures. Most importantly, the inclusion of chemicals only if they were proven to be active in the assays provides a good basis for concluding that the chemicals are 'similar' in nature and thus that concentration addition is appropriate.

Concepts available for prediction of joint action of chemicals

When chemicals in a mixture act together to produce an effect, but do not enhance or diminish each others actions, the resulting mixture effect is commonly considered additive. It is important to realize that this particular use of the term "additivity" is specific to mixture toxicology and must not be confused with additivity in the mathematical sense. Sometimes the term "non-interaction" is used synonymously with "additivity".

Various ways of deriving quantitative expectations for mixture additivity have been described. Often, it is implicitly assumed that the anticipated combination effect is accessible by calculating the simple arithmetic sum of the individual effects of all chemicals. However, the fallacy of this expectation becomes obvious when the case of 10 agents is considered that each provoke, say, 15% of a certain response. The expectation that the resulting joint effect should be 10 x 15% = 150% turns out to be biologically impossible, if the maximally inducible effect is only 100%.

Thus, methods are required that allow more reliable calculations of additive mixture effects from information about the responses of individual mixture components. For this purpose, two concepts have emerged, concentration addition (often also called *dose addition* or *Loewe additivity*,) and independent action (sometimes referred to as "*response addition*" or "*Bliss additivity"*)(Greco et al. 1995). These concepts are based on two entirely different ideas about how the joint action of chemicals can be perceived.

Concentration addition (CA)

The concept of concentration addition (CA) looks at mixture effects of chemicals in terms of a "dilution" principle. It assumes that one chemical can be replaced totally or in part by an equal fraction of an equi-effective concentration of another, without diminishing the overall combined effect (Loewe and Muischnek, 1926). If the assumptions of CA hold true, these fractions of equi-effective concentrations, which are also called toxic units, sum up to a value of 1 – therefore the name *concentration* or dose addition. A widely used application of CA is the "toxic equivalence factor" (TEF) concept for the assessment of mixtures of polychlorinated dioxins and furans (PCDD/F) (Van den Berg et al. 2006). Under the additional assumption of parallel dose-response curves, doses of specific PCDD/F isomers are all expressed in terms of the dose of a reference chemical, 2,3,7,8 TCDD, needed to induce the same effect ("equivalent" or "equi-effective" dose), and assessment of the resulting combined effect is obtained simply by adding up all equivalent TCDD doses.

The CA concept implies that every toxicant in any concentration contributes, more or less, to the overall toxicity of a mixture. Whether the individual doses are also effective alone does not matter. Thus, combination effects should also result from toxicants at or below effect thresholds, provided that sufficiently large numbers of components sum up to a sufficiently high total effect dose. It has been proposed that CA is a useful starting point for mixture assessment if the substances in the mixture are 'similar', for example if the substances "act in the same way, by the same mechanism(s), and differ only in their potencies" (COT, 2002).

The mathematical formulae for calculation of mixture effects using the CA concept are provided in the relevant methods section.

Independent action (IA)

Independent action (IA) conceptualises mixture effects in a different way. It assumes that the joint effect of a combination of agents can be calculated from the responses of individual mixture components by adopting the statistical concept of independent events (Bliss, 1939). This means that agents present at doses below effect thresholds (i.e. zero effect levels) will not contribute to the joint effect of the mixture, and if this condition is fulfilled for all components there will be no combination effect. This central tenet of the concept of independent action is commonly taken to mean that exposed subjects are protected from mixture effects as long as the doses of all agents in the combination do not exceed their no-observed-effect-levels (NOEL) (COT, 2002). Unlike CA, use of IA for mixture assessment may be most appropriate when the substances in the mixture should be considered 'dissimilar' to one another and when it is appropriate to assume that no substance in the mixture modulates the activity of any other mixture component (COT, 2002).

The mathematical formulae for calculation of mixture effects according to the IA concept are provided in the relevant methods section.

Methods

Assays employed for testing of estrogenic or anti-androgenic mixtures:

The three assays used for testing of estrogenic or anti-androgenic activity of chemical mixtures are briefly described in the following table (Table 1; for more information on the selection of the assays please refer to the first annual report).

Table 1: *In vitro* assays employed for testing on estrogenic or anti-androgenic mixtures of food additives and contaminants

Purpose:	***Estrogenicity, mitogenic endpoint***	***Estrogenicity, reporter gene endpoint***	***Anti-androgenicity, reporter gene endpoint***
Assay employed:	**ESCREEN**	**ERLUX**	**ARLUX**
Cell line:	MCF-7 BOS	T47D Kbluc	MDA-kb2 cells
Source:	Prof. A. Soto, Boston	ATCC	ATCC
Brief mechanism:	Estrogens produce an increase in cell number; results expressed relative to 17beta estradiol.	Estrogens activate production of luciferase from a stably transfected reporter plasmid; results expressed relative to 17beta estradiol.	Androgens induce the expression of luciferase from a stably transfected AR responsive luciferase reporter-gene construct. Anti-androgens inhibit this androgen induced luciferase induction. The reference androgen is DHT (5α-Dihydrotestosterone).
Reference:	Soto et al. 1995	Wilson et al. 2004	Wilson et al. 2002

Test chemicals

Test chemicals were selected from the list assembled in deliverable #1 (see chapter 2): They comprise members of all major groups of chemicals, which were proposed to be tested (Box 1).

Box 1: Groups of chemicals identified as being of interest as non-pesticide endocrine disrupters with relevance to food:

Food mutagens, including heterocyclic amines such as PhIP
Polychlorinated biphenyls (PCBs) and their hydroxylated metabolites, and polychlorinated dibenzo dioxins (PCDDs) and furans (PCDFs)
Phthalates
Bisphenol A
Anti-oxidants
Phytoestrogens
Polybrominated diphenyl ethers (PBDEs)
Perfluorinated chemicals (PFCs)
Parabens
Polycyclic synthetic musks
UV-filter agents
Heavy metals

A wide variety of chemicals from the above groups were employed in single chemical testing and those regarded suitable were included into mixture testing within the respective assays.

ERLUX

T47D-KBluc cells were obtained from the ATCC and the protocol established by the depositing authors was followed (Wilson et al. 2004). Cells were routinely grown in RPMI (10% FCS). For seven days prior to experiments, cells were maintained in pre-assay media (RPMI, 10% charcoal-dextran stripped FCS (CDFCS)). For experiments, cells were seeded in white plastic 96 well plates at a density of 10,000 cells/well and allowed to attach for 24 hours before removal of media, and application of test chemicals. Test chemicals were dissolved in ethanol to give stock solutions of micromolar concentrations. Test and control solutions were diluted in dosing media (phenol red free RPMI, 5% CDFCS), and in all cases the final concentration of ethanol was 0.5%. Positive controls were 1nM estradiol, and vehicle was 0.5% ethanol. 24 hours after application of test compounds, an equal volume of Steady-Glo assay reagent (Promega) was added and plates were incubated for 10min, with shaking, to allow for cell lysis. Plates were then loaded into a plate reader (FLUOstar Optima, BMG Labtech) and incubated for a further ten minutes in the dark, followed by measurement of luminescence. To reduce variation, the temperature of the plate reader chamber was maintained at 27°C throughout. Controls were run as eight replicates, and compounds were tested in a dilution series comprising eight concentrations tested in triplicate. Results were normalised by subtraction of on-plate vehicle control values and then division by on-plate positive control values.

ESCREEN

MCF7-BOS cells were a kind gift (A. Soto, Boston) and the published ESCREEN method was followed (Soto et al. 1995). Cells were routinely grown in DMEM (5% FCS). For experiments, cells were seeded in clear plastic 96 well plates at a density of 2,500 cell/well and allowed to attach for 24 hours before washing with rinse media (phenol red free DMEM, no supplements). Test chemicals were dissolved in ethanol to give stock solutions of micromolar concentrations. Test and control solutions were diluted prior to application in dosing media (phenol red free DMEM, 10% charcoal-dextran stripped FCS (CDFCS)). The final concentration of ethanol was 0.5% in test and control wells. Positive controls were 25nM estradiol and vehicle control was 0.5% ethanol. At all stages, media removal from cells was carried out gently and in a controlled fashion by use of an electronic multichannel pipette set to the lowest speed possible. The plate layout was designed to reduce variation due to evaporation or spreading of test chemicals, and had been previously optimised in the laboratory. After application of test solutions, plates were incubated for 72 hours before fixation with 10% trichloroacetic acid and sulforhodamine B (SRB) staining to measure protein and allow the indirect quantification of cell number. Controls were run as eight replicates and compounds were tested in a dilution series comprising eight concentrations tested in duplicate. Results were normalised by subtraction of on-plate vehicle control values and then division by on-plate positive control values.

Test strategy (ERLUX and ESCREEN)

For assessment of estrogenicity in either the ERLUX or ESCREEN assays, each chemical was initially tested over a concentration range of 1pM to 10uM; in subsequent repeats the highest concentration tested, and the dilution factor, were chosen to provide coverage of the full concentration-response relationship. The highest concentration tested did not exceed 200µM for any chemical. Each chemical was tested in at least three independent experiments, and further testing was performed if required by the data consistency and quality. All positive chemicals were considered for inclusion in the mixture for each assay, but could be excluded if testing revealed that a candidate produced only a small effect (<20%) at the highest practicable concentration (on the basis of solubility limits or excessive cost). Chemicals could also be excluded if the results from several experiments revealed substantial variability in response. To be described as negative in either assay, a chemical had to show no detectable difference from vehicle controls (when tested at 100µM or the highest practicable concentration) in at least two independent experiments.

Concentration-response data for each chemical was pooled and fitted to an appropriate non-linear regression (NLR) model. Selection of NLR model was by visual inspection of the fit and use of statistical indicators of goodness of fit as appropriate (Scholze et al. 2001). In cases where a decline in response was seen at higher concentrations, presumably due to toxicity or an inactivation of response at high micromolar concentrations, the data points were excluded before performing NLR.

ARLUX

The assay was performed with MDA-kb2 cells (ATCC) using a modified version of the protocol from Wilson et al. (2002). Cells were routinely grown in Leibowitz-15 (L-15) medium, supplemented with 10% FCS in the absence of CO_2. Prior to experiments, cells were kept in L-15 medium containing 10% charcoal dextran stripped FCS (cdFCS) for at least two media changes. For experiments, cells were seeded in assay medium (phenol red free L-15, 10% cdFCS) at a density of 10,000 cells/well in white 96 well plates. Cells were allowed to attach for 24 hours before being treated with the test compounds or mixtures. Chemicals were dissolved in ethanol and diluted in assay medium, the ethanol concentration never exceeding 1%. All test compounds and mixtures were tested in the presence of 0.25 nM DHT to determine their anti-androgenic potential or on their own to identify any androgenic activity. Samples containing the test compound and DHT were tested in quadruplicate and test compounds alone were tested in duplicate. Controls were treated with solvent (ethanol) only (negative control) or with 0.25 nM DHT (positive control). As further positive control a complete DHT concentration-response was recorded in each plate. After 24 h incubation the luciferase activity was determined with the Steady-glow assay reagent (Promega) according to manufacturer's instructions. Luciferase activity was measured in a plate reader (FLUOstar Optima, BMG labtech).

Regression analysis and prediction of confidence intervals

The data was normalised to the positive control, then pooled and subjected to regression analysis. The regression parameters were used to identify the EC50 and EC01 values for each individual compound, which then were used to determine the mixture ratios to be used in the mixture experiments. The parameters also formed the basis for prediction of combination effects for mixtures with fixed mixture ratios. Effect concentrations (ECx) were calculated from the functional inverse F-1 of the best fitting model. These values give concentrations c that are expected to cause a defined effect E. Confidence belts (CI) for effect concentrations were estimated by using the bootstrap approach described in Scholze et al., 2001.

Mean effects and effect concentrations of individual substances are subject to a stochastic variability. Consequently, the calculation of a prediction according to CA has to give a mean that is also affected by statistical uncertainty. This uncertainty was quantified by determining approximately the central 95 % confidence intervals for mean predicted effect concentrations using the bootstrap method. The bootstrap samples were generated on the basis of the effect distributions that were estimated within the fitting process for every individual concentration response function (parametric bootstrap). All computations were programmed using SAS® (1996).

Single chemical testing

For the testing of the single compounds, initial range-finding experiments were conducted. The concentration range was either chosen according to existing literature data or to the potency expected from similar compounds. If none of the above information was available, the highest achievable starting concentration (up to 250 μM), depending on compound solubility, was tested with seven subsequent five fold dilutions. If a compound turned out to act as an anti-androgen, the range finding experiments were followed by two or three further experiments to establish the concentration-response relationship in the relevant concentration range.

Further testing was only performed if deemed necessary because of insufficient data quality.

Mixture testing

10 compound mixture

Chemicals which qualified to be included in the first mixture were chosen based on:

- Quality of data (in terms of limited biological variation) in concentration-response relationships (e.g. high variation could be due to degradation of the tested compound).
- The ability to produce a complete (or sufficiently complete) concentration-response curve, covering an effect range of at least 50%.
- No major androgenicity by the compound in absence of androgen (DHT), because this could interfere with the mixture effect predictions.

All chemicals that did not comply with these criteria were excluded from the mixture experiments.

The chosen chemicals for the first mixture experiment are listed in Table 2.

Reference mixture

To obtain data from a mixture containing only chemically similar compounds, a control mixture of four anti-androgenic parabens was set up (Table 3).

Table 2: Compounds tested in mixtures 1 and 2

Compound	*Abbreviation*	*Chemical Group*
Bisphenol A	BPA	Bisphenol A
n- Propylparaben	n-PP	Paraben
Benzophenone 3 (Eusolex 4360)	BP3	UV-filter
3-Benzylidene Camphor (Unisol S-22)	3-BC	UV-filter
Butylated hydroxy anisole	BHA	Anti-oxidant
Butylated hydroxy toluene	BHT	Anti-oxidant
Tonalide	AHTN	Polycyclic musk
Galaxolide	HHCB	Polycyclic musk
Perfluorooctane sulfonate	PFOS	PFC
Polychlorinated biphenyl 138	PCB138	PCB

Table 3: Parabens tested in mixtures 3 and 4 (reference mixture)

Compound	*Abbreviation*	*Chemical Group*
Methylparaben	MeP	Paraben
Ethylparaben	EtP	Paraben
n- Propylparaben	n-PP	Paraben
n- Butylparaben	n-BP	Paraben

Calculation of mixture effect predictions using concentration addition (CA)

When predicting the effect of combinations of chemicals, specific assumptions are made about the quantitative relationships between the estrogenicity of single substances and those of mixtures. For a multi-component mixture of n chemicals the concept of *CA* states,

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

(Eq. 1)

where c_i are the concentrations of the individual substances present in a mixture with a total effect of X, and ECx_i are the concentrations of the single substances that produce the same effect X on their own. If Equation 1 holds true, a mixture component can be replaced totally or in part by an equal fraction of an equi-effective concentration of another, without altering the overall effect of the mixture.

On the basis of the concentration-response functions of single chemicals, predictions of effect concentrations had to be calculated for mixtures containing all components at defined mixture ratios according to Equation 1. To achieve this, Equation 1 had to be re-arranged, as follows: The concentrations of individual mixture compounds ci were replaced by the relative proportions p_i of the total mixture concentration $c_{mixture}$, i.e. $c_i = p_i \times c_{mixture}$. Assuming that the mixture concentration $c_{mixture}$ produces an effect of X, the corresponding effect concentration $EC_{X(mixture)}$, is given as

$$EC_{X(mixture)} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1}$$

(Eq. 2)

This equation allows an explicit calculation of any effect concentrations of a mixture under the hypothesis of *CA*. The only prerequisites are knowledge about individual effect concentrations ECx_i and the relative concentrations p_i of the mixture components.

Effect concentrations for mixtures ($EC_{X(mixture)}$) denote the mixture concentrations that produce a given quantitative effect X. However, the effect range for X is limited: Equation 2 can only be used when it is possible to determine for each mixture compound a reliable estimate of a concentration that would produce the same effect when applied on its own ($EC_{X(i)}$).

Calculation of mixture effect predictions using independent action (IA)

The model of *independent action* (IA) allows predicted effects of mixtures of known composition to be calculated using the expression;

$$1 - \prod\left[1 - E(c_i)\right]$$

where $E(c_i)$ is the effect E produced by compound i at concentration c. Inherent in this expression is the fact that $E(c_i)$ cannot exceed 1, i.e. $E(c_i)$ is a fraction of a maximal possible effect, making *independent action* a probabilistic model.

Thus, when applying this model to our assay effects $AE(c_i)$, a maximal effect E_{max} has to be defined.

$$E(c_i) = AE(c_i) / E_{max}$$

(Eq. 3)

If the concentration-response relationships of all mixture constituents i are described by an appropriate regression model F_i, the assay effect $AE(c_i)$ can be estimated from the mean effect $F_i(c_i)$ predicted by the regression model. Thus,

$$AE(c_i) = F_i(c_i), \quad \text{and} \quad E(c_i) = F_i(c_i) / E_{max}$$

(Eq. 4)

Substitution of $E(c_i)$ in equation (2) yields

$$e_{mix} = 1 - \Pi\left[1 - F_i(c_i) / E_{max}\right]$$

(Eq. 5)

In order to ensure comparability of the *independent action* predictions with those of *concentration addition* the fractional effects in (3) were rescaled by multiplication with E_{max}, thus:

$$E_{mix} = E_{max} \times e_{mix} \quad , \quad \text{and} \quad E_{mix} = E_{max}\left[1 - \Pi\left[1 - F_i(c_i) / E_{max}\right]\right]$$

(Eq. 6)

Results

A. Results for ERLUX

Single agent testing

Figure A1 presents illustrative experimental results from the ERLUX assay for naringenin, to demonstrate the data quality obtained in the assay. Similar data quality was obtained for all substances. Figure A2 presents non-linear regression relationships for the single substances included in ERLUX mixture experiments. The substances were benzophenone-3, bisphenol A, estradiol, genistein, naringenin, propylparaben and 4-methylbenzylidine-camphor.

Substances that were tested and produced effects of <20% at the highest practicable concentration were fluoranthene and benzo[a]pyrene; the low effect may make them unsuitable for inclusion in mixtures.

Substances that were tested and found to be negative in the ERLUX were: butylated hydroxyanisole (BHA), cadmium chloride, lead nitrate, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP), PCB008, PCB153, PCB180, perfluorooctanoic acid (PFOA), perflurooctane sulfonic acid (PFOS).

Mixture testing

Two mixtures were tested, both containing six food additives/contaminants plus estradiol. MIX1 was formulated using concentrations of each component that were equi-effective to the EC_{01} of estradiol and MIX2 used concentrations of each component that were equi-effective to the EC_{50} of estradiol. Full details of the regression models used to predict mixture effects, and the composition of each mixture, are given in table A1. Predicted mixture effects are presented graphically in Figure A3, alongside experimental data for the tested mixtures.

Since the mixture studies presented in this report commenced, acquisition of single substance data for an additional seven chemicals has been completed and can therefore be considered for inclusion in future mixtures. The additional chemicals are coumestrol, butylparaben, enterodiol, enterolactone, galaxolide, methylparaben and tonalide.

Figure A1. Experimental results from ERLUX for a single substance (naringenin), illustrating the data quality achievable in the assay.

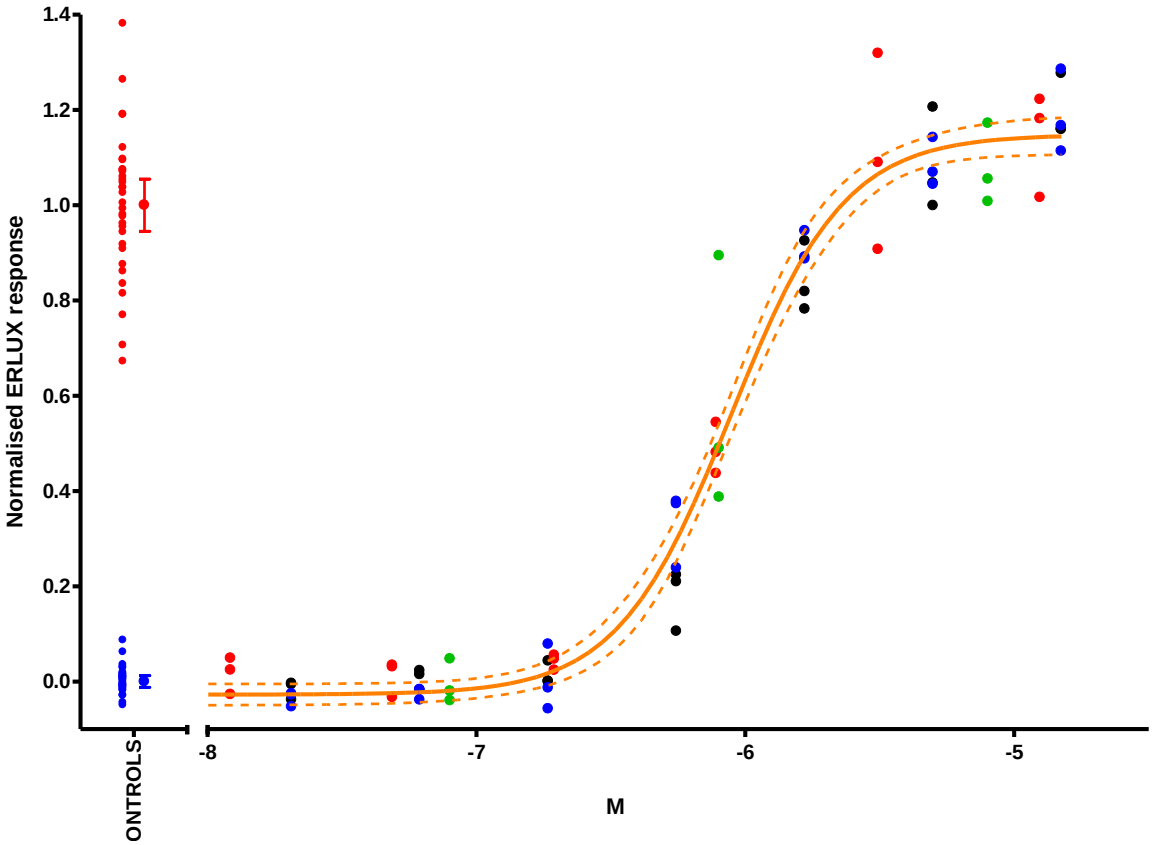


Figure shows experimental data from four experiments (each testing 8 concentrations in triplicate). Filled circles represent individual data points, coloured according to the experiment in which they were obtained. Solid orange line indicates a fitted non-linear regression model (Prism Graphpad, sigmoid) with 95% confidence intervals (dashed lines). Controls are indicated next to the y-axis, red circles indicate individual positive control values (1nM estradiol) and blue circles indicate individual vehicle control values. Single circles with error bars indicate the mean and 95% confidence intervals of the controls (n=32).

Figure A2. Concentration-response relationships from non-linear regression for seven substances to be tested in ERLUX mixtures

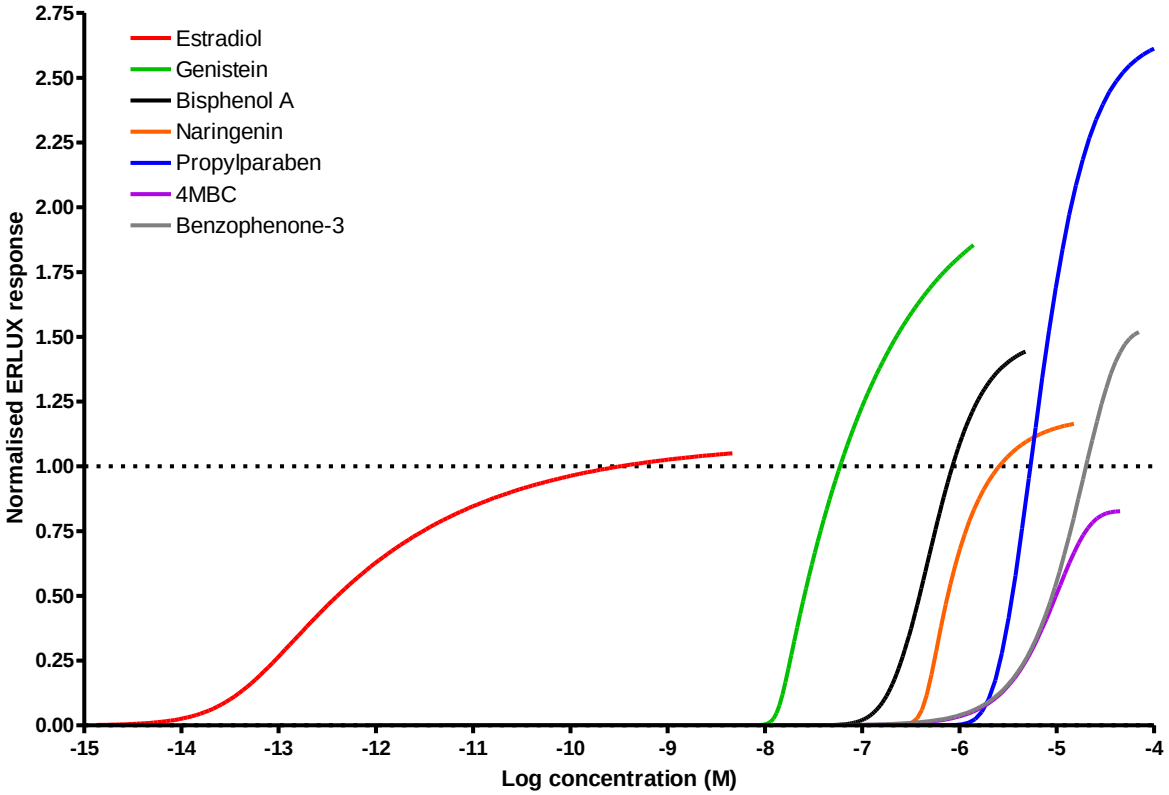


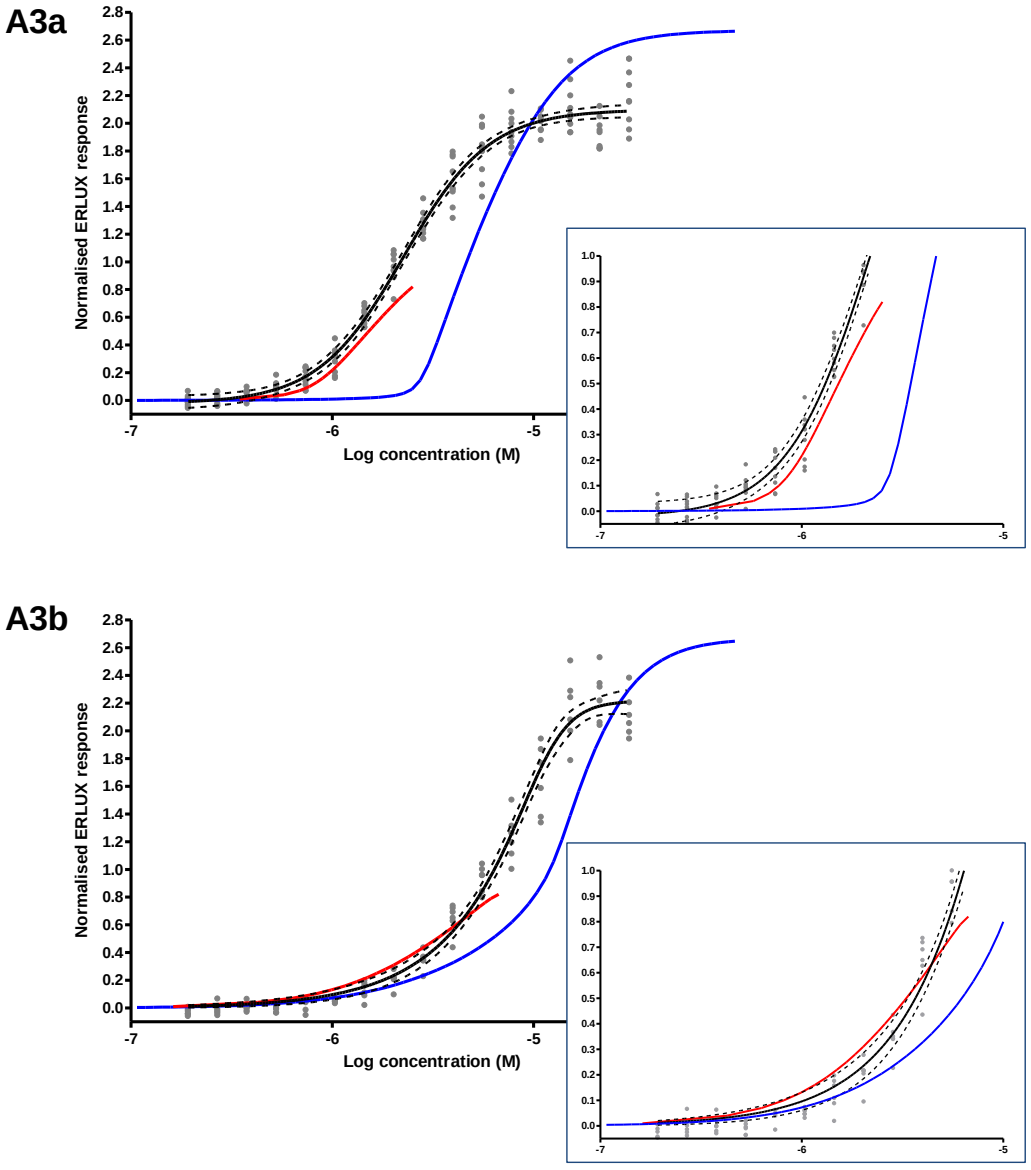
Figure shows non-linear regression models (see table A1) for the concentration-response relationships of the seven substances tested in ERLUX mixtures (see figure A3).

Table A1. Details of single substance regression models and mixture composition for ERLUX studies

Substance name	*Occurrence/ usage*	*n*	*NLR model*	*Regression parameters*				*Mixture 001*		*Mixture 002*	
				T_{max}	*T1*	*T2*	*T3*	EC_{01}	*ratio*	EC_{50}	*ratio*
Estradiol (E2)	endogenous hormone	4	GL2	1.098	42.6	3.19	0.1959	4.90E-15	2.02E-09	4.00E-13	1.73E-08
Genistein (GEN)	phytoestrogen	6	GL2	2.154	183	23.2	0.0423	1.11E-08	0.0046	2.56E-08	0.0011
BisphenolA (BPA)	plasticizer	7	GL1	1.54	22.9	3.46	2.809	8.07E-08	0.0332	3.78E-07	0.0164
Naringenin (NAR)	phytoestrogen	4	GL2	1.201	230	36.2	0.0623	3.82E-07	0.1574	7.49E-07	0.0324
Propylparaben (PropylP)	preservative	3	GL1	2.668	18.3	3.04	9.966	1.20E-06	0.4954	3.50E-06	0.1516
4-methylbenzylidine -camphor (4MBC)	UV filter	3	GL2	0.8221	11.5	3.16	82.36	3.79E-07	0.1564	9.03E-06	0.3906
Benzophenone-3 (BP3)	UV filter	3	GL2	1.72	11.3	2.91	10.31	3.71E-07	0.1530	9.42E-06	0.4079

Notes to table. Substances are listed in order of their EC_{50}. **Abbreviations.** NLR: non-linear regression; T_{max}, T1, T2, T3: derived parameters of the selected regression model; Ratio: ratio in the mixture of the equi-effective concentration of the single substance; GL1: general logit I regression model, y= T_{max} * 1/(1+exp(-T1-T2*log10(x)))^T3, where T_{max}, T1, T2 and t3 are the parameters to be fitted; GL2: general logit II regression model, y= T_{max} * (1-1/(1+exp(T1+T2*log10(x)))^T3) where T_{max}, T1, T2 and t3 are the parameters to be fitted; n: number of independent experiments.

Figure A3. Predicted and observed concentration-response relationships for two ERLUX mixtures.



Graphs show the predicted and observed responses for two mixtures, mixture 001 was based on an equi-effective level of EC_{01} (Fig. A3a) and mixture 002 was based on an equi-effective level of EC_{50} (Fig. A3b). Coloured lines indicate the predicted effect of the mixture based on the single substance data (Fig A2) using the concept of concentration addition (CA, red) or independent action (IA, blue). Filled grey circles indicate individual data points from three (Fig A3a) and two (Fig A3b) independent experiments in which mixtures were tested at 16 concentrations in triplicate. Solid black lines indicate the fitted regression model for the experimental data, with 95% confidence intervals (dashed black lines). Insets show the response range from 0 to 1.0 in more detail.

B. Results for ESCREEN

Single agent testing

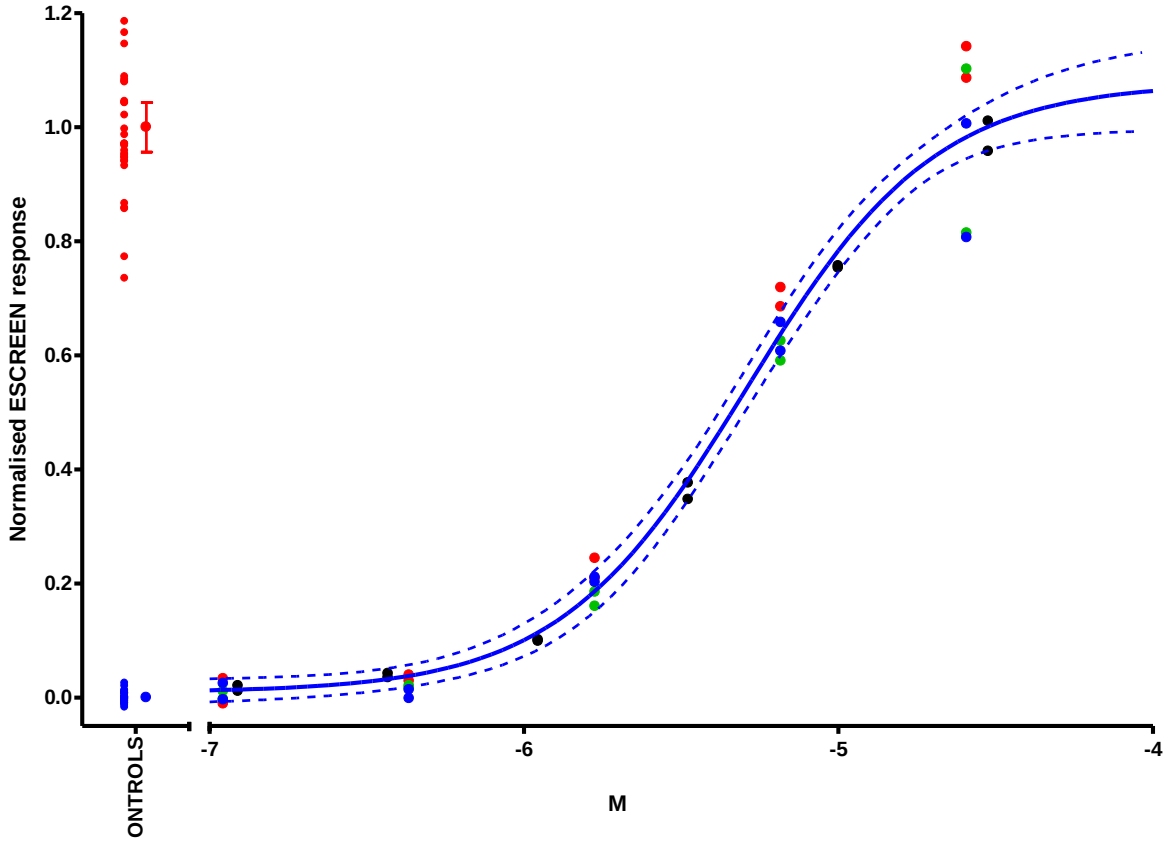
Illustrative data for a single substance (propylparaben) is presented in Figure B1 to demonstrate the data quality obtained in the ESCREEN assay. Similar data quality was obtained for all substances. Figure B2 presents non-linear regression relationships for the single substances included in ESCREEN mixture experiments. The sixteen substances that were investigated are listed in Table B1.

Substances that were tested and found to be negative in the ESCREEN were: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), benzo[a]pyrene, lead nitrate, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP), PCB008, PCB153, PCB180, perfluorooctanoic acid (PFOA) and perflurooctane sulfonic acid (PFOS).

Mixture testing

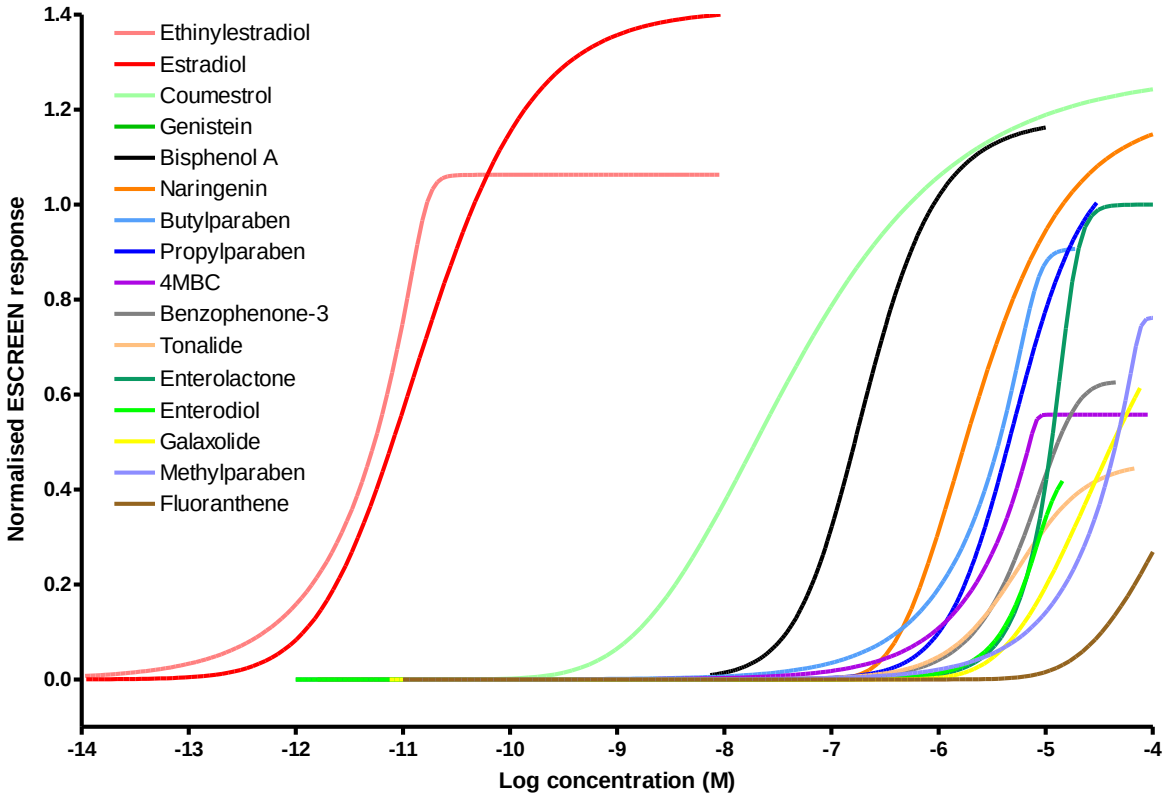
Three mixtures were tested: MIX1 contained 14 food additives/contaminants plus estradiol and ethinylestradiol (16 components); MIX2 omitted ethinylestradiol (15 components) and MIX3 omitted ethinylestradiol and estradiol (14 components). All three mixtures were formulated using concentrations of each component that were equi-effective to the EC_{25} of estradiol. Full details of the regression models used to predict mixture effects and the composition of each mixture are presented in Table B1. Predicted mixture effects are presented graphically in Figure B3, alongside experimental data for the tested mixtures.

Figure B1. Experimental results from ESCREEN for a single substance (propylparaben), illustrating the data quality achievable in the assay.



Graph shows experimental data from four ESCREEN experiments (each testing 8 concentrations in duplicate). Filled circles represent individual data points, coloured according to the experiment in which they were obtained. Solid blue line indicates a fitted non-linear regression model (Prism GraphPad, sigmoid) with 95% confidence intervals (dashed lines). Controls are indicated next to the y-axis, red circles indicate individual positive control values (25nM estradiol) and blue circles indicate individual vehicle control values. Single circles with error bars indicate the mean and 95% confidence intervals of the controls (n=32).

Figure B2. concentration-response relationships from non-linear regression for sixteen substances to be tested in ESCREEN mixtures



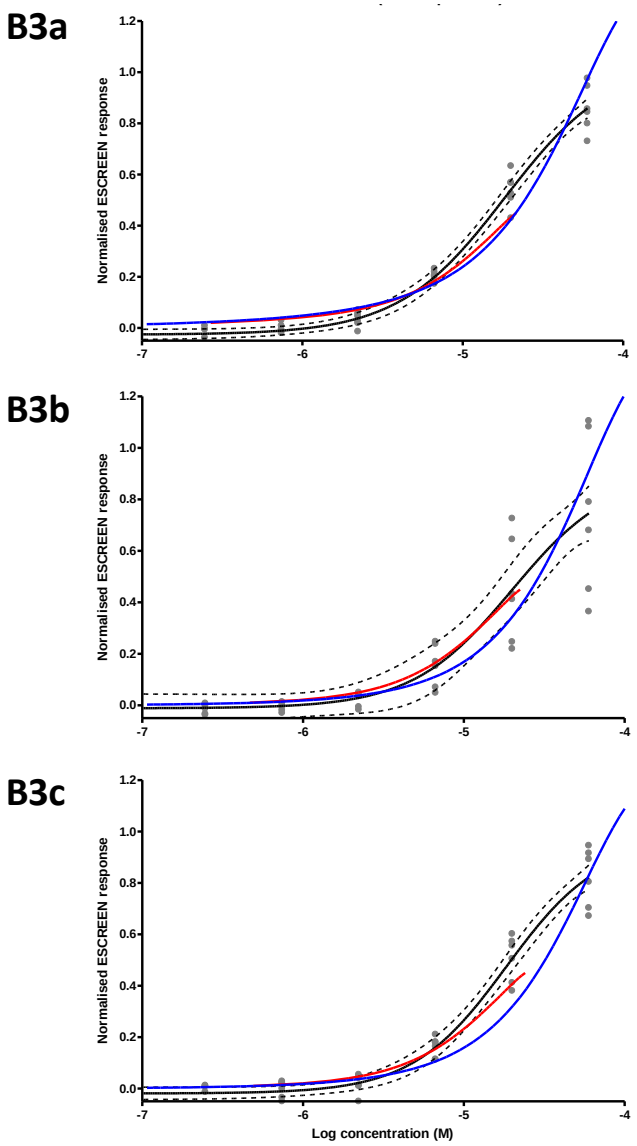
Graph shows non-linear regression models (see table B1) for the concentration-response relationships of the sixteen substances tested in ESCREEN mixtures (see figure B3).

Table B1. Details of single substance regression models and mixture composition for ESCREEN studies

Substance name	*Occurrence/usage*	*n*	*NLR model*	*Regression parameters*				*EC_{25}*	*Mixture 001*	*Mixture 002*	*Mixture 003*
				T_{max}	*T1*	*T2*	*T3*	*(M)*	*ratio*	*ratio*	*ratio*
Ethinylestradiol (EE2)	pharmaceutical	4	GL1	0.995	112	10.29	0.0942	4.59E-13	3.08E-09		
Estradiol (E2)	endogenous hormone	4	GL1	1.453	20.3	1.781	1.897	2.43E-12	1.63E-08	1.63E-08	
Coumestrol (COU)	phytoestrogen	4	GL1	1.278	9.89	0.963	11.6	4.87E-09	3.12E-05	3.12E-05	3.12E-05
Genistein (GEN)	phytoestrogen	4	GL1	0.939	32.6	4.552	0.5557	2.23E-08	0.00015	0.00015	0.00015
Bisphenol A (BPA)	plasticizer	9	GL2	1.181	24.2	3.519	0.6329	7.94E-08	0.00053	0.00053	0.00053
Naringenin (NAR)	phytoestrogen	3	GL1	1.197	14.7	1.745	96.66	8.14E-07	0.00540	0.00540	0.00540
Butylparaben (ButylP)	preservative	3	GL1	0.907	70.3	13.82	0.1233	1.44E-06	0.00967	0.00967	0.00967
Propylparaben (PropylP)	preservative	4	GL1	1.106	15.7	2.93	1.219	2.14E-06	0.01438	0.01438	0.01438
4-methylbenzylidine-camphor (4MBC)	UV filter	3	GL1	0.558	277	54.5	0.0331	2.93E-06	0.01965	0.01965	0.01965
Benzophenone-3 (BP3)	UV filter	3	GL2	0.627	11.8	2.882	17.15	5.10E-06	0.03422	0.03422	0.03422
Tonalide (TON)	musk	3	GL1	0.458	16.8	3.232	0.8499	6.17E-06	0.04138	0.04138	0.04138
Enterolactone (ENL)	phytoestrogen	4	GL1	1	60.7	12.74	0.2928	7.32E-06	0.04811	0.04811	0.04811
Enterodiol (END)	phytoestrogen	3	GL1	0.461	46.2	9.279	0.3756	7.45E-06	0.04999	0.04999	0.04999
Galaxolide (GAL)	musk	4	GL1	0.876	12.4	2.138	6.604	8.65E-06	0.05802	0.05802	0.05802
Methylparaben (MethylP)	preservative	3	GL1	0.762	169	40.98	0.0468	1.99E-05	0.13367	0.13367	0.13367
Fluoranthene (FLUOR)	PAH food contaminant	2	GL1	1	7.339	1.257	13.89	8.99E-05	0.58479	0.58479	0.58479

Notes to table. Substances are listed in order of their EC_{25}(M). **Abbreviations.** See notes to table A1; PAH: polycyclic aromatic hydrocarbon.

Figure B3. Predicted and observed concentration-response relationships for three ESCREEN mixtures



Graphs show the predicted and observed responses for three mixtures in the ESCREEN: mixture 001 contained all sixteen substances shown in Fig B2 (Fig. B3a); mixture 002 omitted ethinylestradiol and so comprised 15 substances (Fig B3b); and mixture 003 omitted ethinylestradiol and estradiol and so comprised 14 substances (Fig, B3c). All three mixtures are based on an equi-effective level of EC_{25}. Coloured lines indicate the predicted effect of the mixture based on the single substance data (Fig B2) using the concept of concentration addition (CA, red) or independent action (IA, blue). Filled grey circles indicate individual data points from three independent experiments for each mixture, in which mixtures were tested at 8 concentrations in duplicate. Solid black lines indicate the fitted regression model for the experimental data, with 95% confidence intervals (dashed black lines).

C. Results for ARLUX

Single compound testing

Figure C1 presents the concentration-response relationship of bisphenol A as a typical example of single chemical testing. The figure shows the data of individual experiments and the regression fit including the 95% confidence interval. The data shown is for treatment with bisphenol A without or in combination with DHT. Similar concentration-response analysis was carried out for each tested compound.

The non-linear regression fits of all compounds which were included in the mixtures 1 and 2 are presented in Figure C2 and the fits of compounds included in mixtures 3 and 4 are shown in Figure C3. The corresponding non-linear regression models used, with their regression parameters, are presented in Table C1. The equations for the used regressions are given in Table C2.

Substances included in the 10 component mixture were bisphenol A; *n*- propylparaben; benzophenone 3; 3-benzylidene camphor; butylated hydroxy anisole; butylated hydroxy toluene; 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline; hexahydrohexamethylcyclopentabenzopyran; perfluorooctane sulfonate and polychlorinated biphenyl 138.

Parabens included in the paraben reference mixture were methylparaben, ethylparaben, *n*-propylparaben and *n*- butylparaben.

Compounds excluded from the mixture because they exhibited insufficient or no anti-androgenicity or were androgenic on their own were: genistein, perfluorooctanic acid, 6:2 fluorotelomere alcohol, 8:2 fluorotelomere alcohol, cadmium chloride, lead nitrate, benzophenone 2, benzo(a)pyrene, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 4-methylenbenzylidene camphor, octyl-methoxycinnamate and polybrominated diphenylether 100.

Mixture testing

Prediction modelling

Based upon the concentration-response relationships for the individual mixture components (Table C1), the expected mixture effects were calculated according to either the model of concentration addition or of independent action. The predictions were based either on mixture ratios that reflect either the EC50 of the individual compounds (mixture 1; Figure C4a) or their EC01 (mixture 2; Figure C4b). The mixture ratios for all mixtures are shown in Table C3.

Testing of 10 component mixture

Next, the mixtures were tested at the same mixture ratios used for prediction modeling and the results were compared to the estimates. Figure C4 shows the results of the testing of both 10 component mixtures (EC50, Figure C4a; EC01, Figure C4b; mixtures 1 and 2, respectively). The figures show, that in both cases a deviation from the concentration addition prediction occurred, especially in the lower dose range. For mixture 1 (EC50), the lower concentration range of the concentration-response curve was well approximated by the IA prediction. It exhibited a very steep slope and at higher doses was in better agreement with the CA prediction. For mixture 2 (EC01) the deviation from CA was less pronounced and at lower doses the data came to lie between both models and again agreed with CA at higher doses.

Testing of a paraben reference mixture

Due to the deviations from additivity observed in the 10 compound mixture of chemically dissimilar compounds, it was decided to seek a reference mixture which was expected to yield additive anti-androgenic effects. To this end, a combination of four parabens (Table 3) was selected. Because of their structural similarity, concentration additive effects were expected. As shown in Figures C5a and b (mixture ratios corresponding to EC50 or EC01 of the individual parabens, mixtures 3 and 4, respectively) these mixtures were well in agreement with CA.

Figure C1: Concentration-response relationship for bisphenol A as an example for single chemical testing.

The graph shows the experimental data from 3 experiments. Cells were either treated with bisphenol A in combination with 0.25 nM DHT (large filled circles) or without DHT (small filled circles). Controls were treated with solvent only (ethanol) are shown next to the y-axis. The red solid line shows the non-linear regression fit, dashed lines indicate the 95% confidence interval.

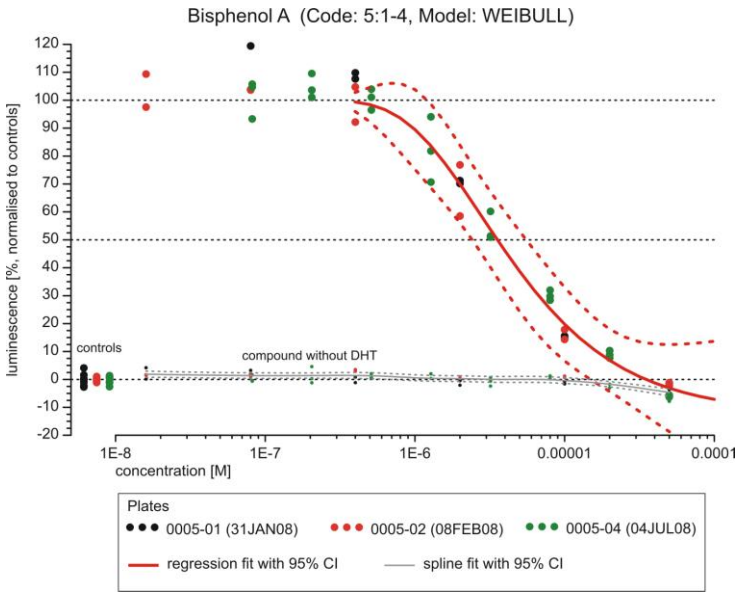


Figure C2: Concentration-response relationships for anti-androgenic compounds included in 10 compound mixture.

Non- linear regression fits for the indicated compounds. More detail on regression models and model parameters is given in Table C1.

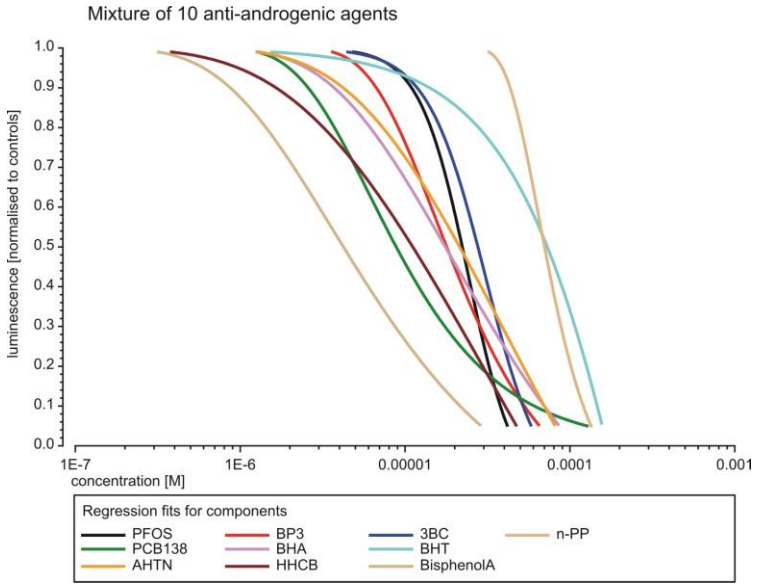


Figure C3: Concentration-response relationships for anti-androgenic parabens included in the paraben reference mixture.

Non- linear regression fits for the indicated compounds. More detail on regression models and model parameters is given in Table C1.

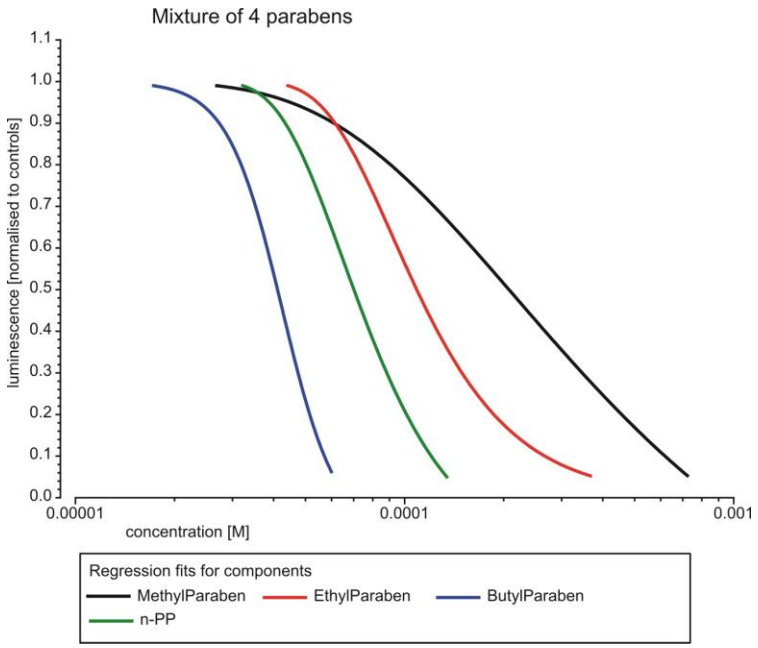


Table C1: Regression modelling data from all compounds included in the tested mixtures. The table contains the respective regression model which fit the data best and the corresponding model parameters. Furthermore, the EC10 and EC50 values for each compound, as derived from the regression analysis are given.

Substance (by order of EC_{50})	*Concentration-Response Function*					*EC_{10}*		*EC_{50}*	
	RM	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_{min}$	$\hat{\theta}_{max}$	*M [CI]*		*M [CI]*	
Ethylparaben	Weibull	-19.54	-4.84	-0.00	1	6.15E-5	[5.22E-5 - 7.24E-5]	1.09E-4	[1.00E-4 - 1.18E-4]
Methylparaben	Weibull	-6.29	-1.71	-0.49	1	5.54E-5	[3.97E-5 - 7.71E-5]	1.88E-4	[1.45E-4 - 2.43E-4]
HHCB	Weibull	-3.88	-0.87	-1.29	1	1.65E-6	[1.12E-6 - 2.43E-6]	1.11E-5	[9.19E-6 - 1.35E-5]
BHA	Weibull	-6.54	-1.38	-0.42	1	3.71E-6	[2.11E-6 - 6.54E-6]	1.75E-5	[1.28E-5 - 2.40E-5]
BP3	Weibull	-11.96	-2.49	-0.17	1	6.69E-6	[4.98E-6 - 8.99E-6]	1.79E-5	[1.51E-5 - 2.11E-5]
AHTN	Weibull	-4.87	-1.11	-0.94	1	4.21E-6	[2.81E-6 - 6.32E-6]	2.14E-5	[1.65E-5 - 2.78E-5]
PFOS	logit	-31.45	-6.81	-0.14	1	1.09E-5	[8.46E-6 - 1.42E-5]	2.22E-5	[2.01E-5 - 2.46E-5]
3-BC	logit	-25.60	-5.67	-0.15	1	1.18E-5	[9.11E-6 - 1.54E-5]	2.77E-5	[2.48E-5 - 3.09E-5]
n-Butylparaben	logit	-52.40	-11.99	-0.09	1	2.73E-5	[2.06E-5 - 3.62E-5]	4.11E-5	[3.59E-5 - 4.71E-5]
n-Propylparaben	Weibull	-21.52	-5.14	-0.16	1	4.34E-5	[3.50E-5 - 5.38E-5]	7.01E-5	[6.02E-5 - 8.18E-5]
BHT	logit	-8.32	-2.44	-2.39	1	1.41E-5	[6.89E-6 - 2.88E-5]	7.29E-5	[6.00E-5 - 8.87E-5]
BPA	Weibull	-8.13	-1.49	-0.23	1	8.70E-7	[5.09E-7 - 1.49E-6]	4.23E-6	[3.25E-6 - 5.51E-6]
PCB 138	Weibull	-11.73	-2.25	0.01	1	2.58E-6	[1.95E-6 - 3.43E-6]	8.83E-6	[7.29E-6 - 1.07E-5]

EC50, EC10: concentration provoking 50% and 10% effect, respectively. Values in brackets denote the upper and lower limits of the approximate 95% confidence interval; the column "RM" indicates the mathematical regression function as defined at Scholze *et al.* (2001): $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}, \hat{\theta}_{max}$ estimated model parameters , given for concentrations expressed in M (rounded values), $\hat{\theta}_{max}$ were set to the fixed value 1 relating to the mean value of the DHT controls;

Table C2: Non-linear regression models (nlRM) used to describe experimental data.

Name	*Function (F)*	*Inverse Function (F^{-1})*
Logit	$E = \theta_{min} + \frac{(\theta_{max} - \theta_{min})}{1 + \exp(-\theta_1 - \theta_2 \log_{10}(c))}$	$c = 10^{(\log_e(k/(1-k)) - \hat{\theta}_1)/\hat{\theta}_2}$
Weibull	$E = \theta_{min} + (\theta_{max} - \theta_{min})\ 1 - \exp\ -\exp(\theta_1 + \theta_2 \log_{10}(c))$	$c = 10^{\log_e(-\log_e(1-k)) - \hat{\theta}_1/\hat{\theta}_2}$
		with $k = \frac{E - \hat{\theta}_{min}}{\hat{\theta}_{max} - \hat{\theta}_{min}}$

E – Effect, expressed as fraction in relation to the mean effect of the positive and the negative controls;

c – Concentration;

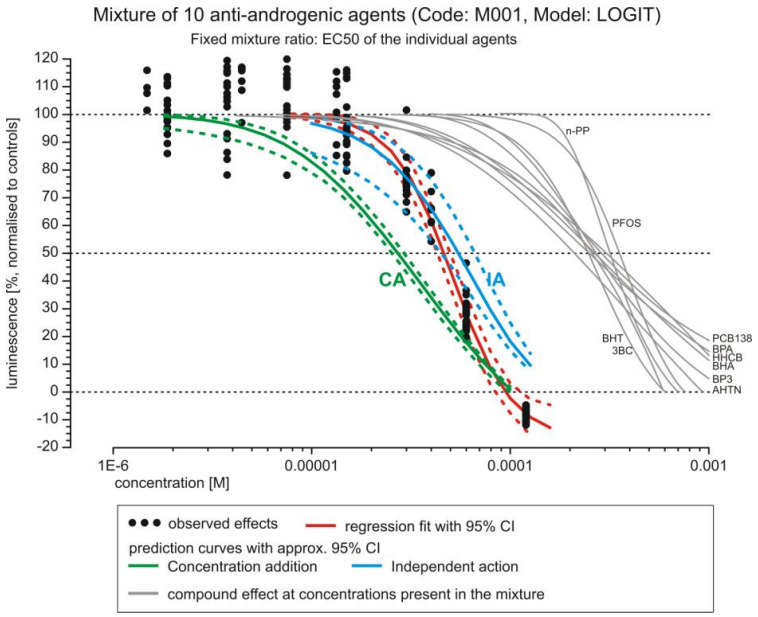
$\theta_1, \theta_2, \theta_3, \theta_{min}, \theta_{max}$ – Model parameters (corresponding statistical estimates marked by ^);

exp(x) = e^x .

Figure C4: Predicted and observed concentration-response relationship for two anti-androgenic 10 component mixtures.

The graphs show the testing of two 10 component mixtures at mixture ratios according A) EC_{50} or B) EC_{01} of the individual mixtures components. Both graphs show the predicted anti-androgenic effects according to the concentration addition (CA) model (green line) and to the model of independent action (blue line). The black filled circles show the data points of 4 independent experiments and the solid red line represents the non-linear regression fit for the data. Dashed lines in the respective colors indicate the 95% confidence interval for the data fit and the predictions. The light grey lines represent the effects of the individual components within the mixture.

A)



B)

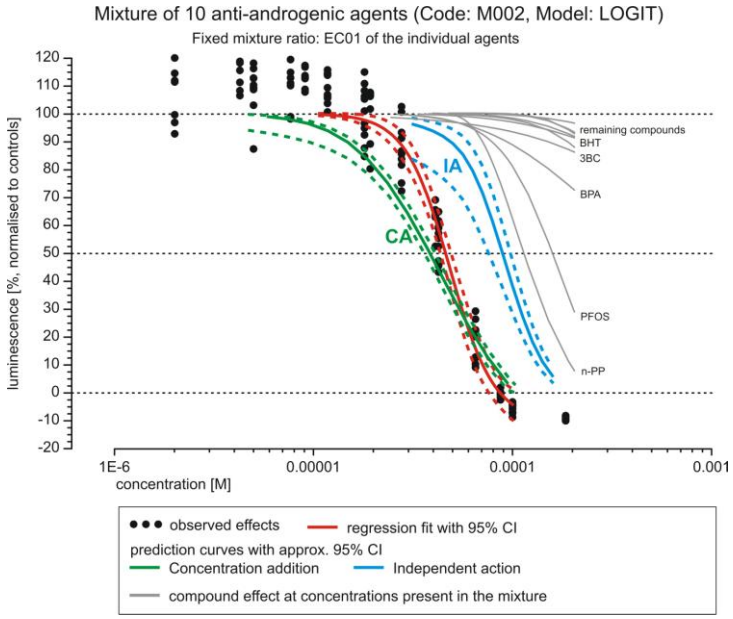
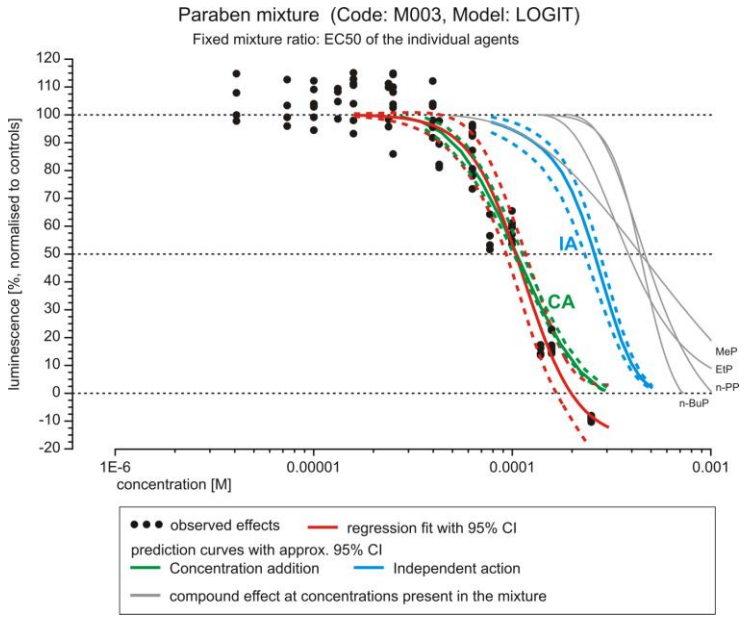


Figure C5: Predicted and observed concentration-response relationship for two anti-androgenic paraben mixtures (reference mixtures).

The graphs show the testing of two paraben mixtures at mixture ratios according A) EC50 or B) EC01 of the individual mixtures components. Both graphs show the predicted anti-androgenic effects according to the concentration addition (CA) model (green line) and to the model of independent action (blue line). The black filled circles show the data points of 4 independent experiments and the solid red line represents the non-linear regression fit for the data. Dashed lines in the respective colors indicate the 95% confidence interval for the data fit and the predictions. The light grey lines represent the effects of the individual components within the mixture.

A)



B)

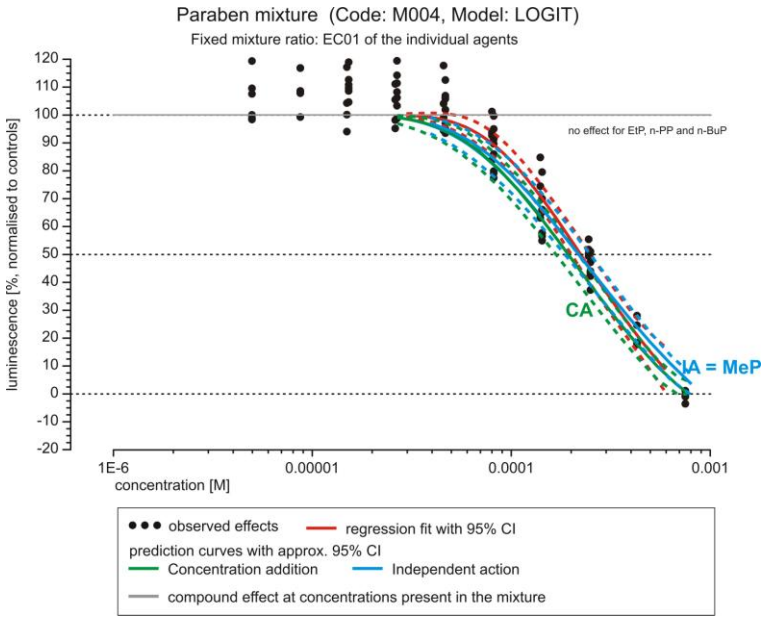


Table C3: Relative proportions of each compound (mixture ratio) for mixtures 1 to 4.

Rounded values are given for the relative proportions

Components	***Relative proportions (percentages)***			
(in order of increasing EC50)	Mixture 1 (10 components)	Mixture 2 (10 components)	Mixture 3 (4 components)	Mixture 4 (4 components)
Ethylparaben	-	-	28.40%	3.04%
Methylparaben	-	-	47.14%	95.13%
HHCB	3.68%	0.73%	-	-
BHA	6.63%	1.62%	-	-
BP3	6.63%	2.93%	-	-
AHTN	10.27%	1.11%	-	-
PFOS	6.28%	14.04%	-	-
3-BC	11.21%	6.32%	-	-
n-Butylparaben	-	-	9.34%	0.47%
n-PP	22.11%	61.88%	15.12%	1.36%
BHT	28.38%	9.55%	-	-
BPA	1.70%	0.94%	-	-
PCB 138	3.09%	1.08%	-	-

Table C4: Statistical uncertainty of predicted and observed effect concentrations for mixtures
CA – concentration addition; IA – independent action, CI – confidence interval.

Effect level (x)	*Effect concentration ECx_{mix} [M]*					
	Observed		Predicted by CA		Predicted by IA	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Mixture 1: ten components (ratio as defined in Table C3)						
10%	2.25E-5	[1.89E-5 - 2.67E-5]	**6.83E-6**	**[4.37E-6 - 8.06E-6]**	1.80E-5	[6.36E-6 - 2.70E-5]
50%	4.63E-5	[4.39E-5 - 4.90E-5]	**2.70E-5**	**[2.52E-5 - 2.90E-5]**	5.46E-5	[4.44E-5 - 6.49E-5]
90%	7.77E-5	[7.47E-5 - 8.15E-5]	7.42E-5	[6.89E-5 - 7.69E-5]	**1.24E-4**	**[1.14E-4 - 1.39E-4]**
Mixture 2: ten components (ratio as defined in Table C3)						
10%	2.68E-5	[2.44E-5 - 2.91E-5]	**1.43E-5**	**[8.95E-6 - 1.61E-5]**	4.85E-5	[1.35E-5 - 5.77E-5]
50%	4.61E-5	[4.35E-5 - 4.86E-5]	**3.86E-5**	**[3.60E-5 - 4.08E-5]**	**8.88E-5**	**[7.59E-5 - 9.63E-5]**
90%	7.09E-5	[6.71E-5 - 7.43E-5]	8.04E-5	[7.41E-5 - 8.46E-5]	**1.44E-4**	**[1.32E-4 - 1.55E-4]**
Mixture 3: four components (ratio as defined in Table C3)						
10%	5.44E-5	[4.06E-5 - 6.51E-5]	5.19E-5	[4.51E-5 - 5.68E-5]	**1.28E-4**	**[9.75E-5 - 1.50E-4]**
50%	1.04E-4	[8.52E-5 - 1.14E-4]	1.07E-4	[1.01E-4 - 1.13E-4]	**2.59E-4**	**[2.29E-4 - 2.76E-4]**
90%	1.66E-4	[1.49E-4 - 1.74E-4]	**2.12E-4**	**[2.02E-4 - 2.23E-4]**	**4.01E-4**	**[3.81E-4 - 4.20E-4]**
Mixture 4: four components (ratio as defined in Table C3)						
10%	7.91E-5	[6.83E-5 - 9.23E-5]	6.03E-5	[4.63E-5 - 7.19E-5]	6.41E-5	[4.85E-5 - 7.75E-5]
50%	2.23E-4	[1.97E-4 - 2.44E-4]	1.93E-4	[1.68E-4 - 2.18E-4]	2.18E-4	[1.86E-4 - 2.49E-4]
90%	5.76E-4	[5.49E-4 - 6.20E-4]	5.41E-4	[4.70E-4 - 5.87E-4]	6.49E-4	[5.50E-4 - 7.18E-4]

Conclusions

ERLUX (estrogenicity assay, reporter-gene endpoint)

For both mixtures tested in the ERLUX assay, the concept of concentration addition (CA) provided a good prediction for the effect of the mixture. The prediction for MIX1 (based on EC_{01}'s, Fig. A3a) showed a slight under-prediction, potentially allowing the experimental results to be described as a very small synergy (observed effect greater than predicted effect). However the difference is extremely small and was not observed in the other mixture tested (based on EC_{50}'s, Fig. A3b). Predictions based on the concept of independent action (IA) under-predicted the effects of both mixtures and also predicted a maximal effect that was greater than the observed maximal effect. For these two reasons, the use of independent action was considered to be unsuitable. Assessment of the ability of CA to accurately predict the observed maximal effect will only be possible after extrapolation of the CA prediction, as described in the results section.

It can be seen from Fig A2 that many, if not most, of the substances tested in ERLUX are able to produce a maximal effect that is greater than that of estradiol, the endogenous hormone and positive control. The occurrence of these supramaximal effects is well known in the ERLUX assay, and in other reporter-gene systems, but a mechanistic explanation is not available. The occurrence of these supramaximal effects is the reason that the CA predictions in Fig. A3 (solid red lines) do not cover the full range of the tested concentrations. The application of CA is correct up to the 100% level, and further effort will be devoted to consideration of whether the concept can be appropriately extended to effects of greater than 100%, for example by effect extrapolation and the use of the concept of toxic units.

In summary, for the ERLUX assay, CA was found to be an acceptable model for prediction of the effect of the mixtures tested. Variation of the mixture composition, by use of mixtures constituted at two different equi-effective levels (EC_{01} and EC_{50}), showed that the applicability of CA was robust since it was acceptably predictive in either case. We did not identify any deviation from additivity (concentration addition) in the ERLUX that requires experimental investigation as part of this task.

ESCREEN (estrogenicity assay, mitogenic endpoint)

For all three mixtures tested in the ESCREEN, predictions according to the use of concentration addition (CA) provided an acceptable estimate of the observed effects. Predictions made using the independent action (IA) concept consistently under-estimated the observed effect, albeit to a smaller extent than was observed for the ERLUX assay (see above); therefore IA is not considered to be an acceptable model in this situation. The mathematical implementation of the CA concept that was used in these studies is limited to prediction of an effect level equal to that of the lowest maximal effect produced by any of the single substances included in the mixture. Consequently, the CA predictions shown in figure B3 extend only as far as the 45% level (the maximal effect of tonalide). Consideration will be given to extending the range of the CA prediction by the use of the toxic unit approach to extrapolate above the current limit.

Unlike the situation observed in the ERLUX assay, supramaximal effects were not observed in the ESCREEN. However, a number of chemicals showed a clear maximal effect that was lower than that of estradiol, the endogenous hormone and positive control.

In summary, for the ESCREEN assay, CA was found to be an acceptable model for prediction of the effect of the mixtures tested. Variation of the mixture composition, by use of mixtures composed of different numbers of components (14, 15 and 16 components), showed that the applicability of CA was robust since it was acceptably predictive in all three cases. We did not identify any deviation from additivity (concentration addition) in the ESCREEN that requires experimental investigation as part of this task.

ARLUX (anti-androgenicity assay, reporter-gene endpoint)

Unexpectedly, the two 10 component mixtures of anti-androgenic compounds both showed a deviation from concentration addition. In both cases, the results suggest an antagonism within the mixture which was more pronounced in mixture 1 (EC50). Due to the assay principle, where anti-androgens are expected to block an androgen (DHT) from activation of the androgen receptor (AR) measured by luciferase activity, it can be argued that concentration addition should be the adequate model for prediction of mixture effects and chemicals are not likely to act according to independent action. Furthermore, the concentration-response curve exhibited a very steep slope in comparison to both prediction models. Such a steep slope was only observed with a minority of compounds during single compound testing and was not expected for the mixture.

An explanation for the deviations could be, that the included chemicals activated signalling pathways within the cells, which might have caused a decrease in anti-androgenic activity of the mixture, e.g. by activation of metabolic enzymes or activation of efflux pumps. These effects could occur on both the anti-androgenic chemicals that were present and on the androgenic hormone DHT.

To test whether the antagonistic effect observed in the 10 component mixtures could be due to the dissimilarities in the chemical structures of the included compounds, two reference mixtures were tested. These two mixtures contained four parabens and thus chemically similar compounds and were in good agreement with concentration addition.

The activation of signalling pathways as explanation for the observed antagonism in anti-androgenicity of the mixtures is more likely than the mixtures acting according to IA. Thus, one possible avenue for future work could be to employ gene expressing profiling and proteomic approaches to characterise the MDA-kb2 cell line with regard to e.g. CYPs, phase II enzymes and efflux pumps and to investigate whether the treatment of the cells with the mixtures causes changes in their gene and protein expression.

General conclusions

This report provides an overview of the data collected from testing of individual mixture components and from mixture assessment of mixtures of food contaminants able to interact with estrogen and androgen receptors (task 2, milestones 02/01 and 02/02).

The next step of the project (task 3) will be the testing of food additives and contaminants which might not act directly on the estrogen or androgen receptor but might act as so-called effect modifiers. The aim of task 3 is to test whether such effect modifiers will produce a deviation of experimental mixture effect from the predicted effect. If deviations are observed, the next step will be to investigate the underlying mechanism by comparison of gene and/or protein expression patterns in response to mixtures that contain effect modifiers and mixtures that act according to CA. Genes of interest will be CYPs, phase II enzymes, efflux pumps and genes involved in DNA repair and cell cycle control.

Chapter 4:
Assessment of the Impact of Effect Modifiers on the Joint Effect of Estrogenic and Anti-androgenic Agents

Task 3

Deliverable #7

Introduction

This report is deliverable number seven for the project T01045, and is part of Task 3, "Assessment of the impact of food additives and contaminants as effect modifiers".

We are able to report the results from testing of food additives and contaminants which might not act directly on the estrogen or androgen receptor but might act as so-called effect modifiers (Task 3). The aim of task 3 is to test whether such effect modifiers will produce a deviation of experimental mixture effect from the predicted effect. If deviations are observed, the next step will be to investigate the underlying mechanism by comparison of gene and/or protein expression patterns in response to mixtures that contain effect modifiers and mixtures that act according to concentration addition (CA). Genes of interest will be CYPs, phase II enzymes, efflux pumps and genes involved in DNA repair and cell cycle control.

The studies of the impact of potential effect modifiers on mixture effects were carried out in all three of the cell-based assays being used in the project, which comprise two estrogenicity assays, ERLUX (luciferase reporter gene assay using the T47D-KBluc cell line) and ESCREEN (mitogenicity/ proliferation assay using the MCF7-BOS cell line), and an antiandrogenicity assay, ARLUX (luciferase reporter gene assay using the MDA-kb2 cell line).

Firstly, we present data from all three assays on the single agent testing of a panel of substances with the potential to act as effect modifiers (the substance selection process was detailed in a previous deliverable (deliverable # 1: "Report on Selection of Test Chemicals and Their Levels in Human Tissues (Task 1)", see chapter 2).

Potential modifiers of estrogenic effects have been studied in both the ERLUX and ESCREEN assays. Potential modulators were examined for their ability to affect the activity of mixtures containing 17 (ERLUX, EC_{10}) and 14 (ESCREEN, EC_{25}) food additives or contaminants that had previously been identified as active in the respective assays, and to have effects predictable by CA. A mixture of 16 modulators (equimolar) was also examined for its ability to affect the activity of a 14 component mixture (ESCREEN, EC_{25}). We also explored the potential for signalling pathways to influence assay outcome by using inhibitors or the MAPK or PI3K pathway (ERLUX).

Testing of potential modifiers on antiandrogenic mixture effects was conducted in the ARLUX assay. There, the ability of the compounds to affect the antiandrogenic activity of chemical mixtures at concentrations reflecting their respective EC_{25} value in the presence of DHT. Two different mixtures, the paraben reference mixture (deliverable #5, see chapter 3) and a 17 component mixture (this chapter) were chosen for modulation studies, because the effects of both mixtures were predictable by CA.

Secondly, we present predictions of the expected effects of additional mixtures of substances found to be antiandrogenic in the ARLUX assay and the data for their experimental testing. These further mixture studies were conducted after a deviation from concentration addition was found in a 10 component

mixture of antiandrogenic chemicals. The mixtures were designed using the fixed mixture ratio approach at various equi-effective levels, for example EC_{50}'s, and the concept of CA was used to combine the single substance concentration-response relationships into predictions of the expected concentration-response relationships for the mixtures. (The concepts for the prediction models of concentration addition (CA) and independent action (IA) have been introduced in detail in deliverable #5, see chapter 3)

Lastly, computational approaches have been used to 1) facilitate exploration of the literature on the wide range of chemicals studied, and 2) to allow analysis of published toxicogenomics profiles with a view to formulating specific hypotheses on the effects of active agents or modulators at the gene or pathway level.

The comprehensive results obtained in this task and reported in this deliverable, together with the results from Task 2 (deliverable #5, see chapter 3), provide the baseline for the forthcoming examination of human exposure scenarios (Task 4, see chapter 5).

Methods

Assays employed for testing of estrogenic or antiandrogenic mixtures and modulators of combined responses:

The three assays used for testing of estrogenic or antiandrogenic activity of chemical mixtures are briefly described in the following table (Table 1; for more information on the selection of the assays please refer to the first annual report).

Table 1: *In vitro* assays employed for testing on estrogenic or antiandrogenic mixtures of food additives and contaminants

Purpose:	***Estrogenicity, mitogenic endpoint***	***Estrogenicity, reporter gene endpoint***	***Anti-androgenicity, reporter gene endpoint***
Assay employed:	**ESCREEN**	**ERLUX**	**ARLUX**
Cell line:	MCF-7 BOS	T47D Kbluc	MDA-kb2 cells
Source:	Prof. A. Soto, Boston	ATCC	ATCC
Brief mechanism:	Estrogens produce an increase in cell number; results expressed relative to 17beta estradiol.	Estrogens activate production of luciferase from a stably transfected reporter plasmid; results expressed relative to 17beta estradiol.	Androgens induce the expression of luciferase from a stably transfected AR responsive luciferase reporter-gene construct. Anti-androgens inhibit this androgen induced luciferase induction. The reference androgen is DHT (5α-Dihydrotestosterone).
Reference:	Soto et al. 1995	Wilson et al. 2004	Wilson et al. 2002

Test chemicals

Test chemicals were selected from the list assembled in deliverable #1 (see chapter 2): They comprise members of all major groups of chemicals, which were proposed to be tested (Box 1).

Box 1: Groups of chemicals identified as being of interest as non-pesticide endocrine disrupters with relevance to food:

Food mutagens, including heterocyclic amines such as PhIP
Polychlorinated biphenyls (PCBs) and their hydroxylated metabolites, and polychlorinated dibenzo dioxins (PCDDs) and furans (PCDFs)
Phthalates
Bisphenol A
Anti-oxidants
Phytoestrogens
Polybrominated diphenyl ethers (PBDEs)
Perfluorinated chemicals (PFCs)
Parabens
Polycyclic synthetic musks
UV-filter agents
Heavy metals

ERLUX and ESCREEN

Note on experimental design and methods

Comprehensive descriptions of the ERLUX and ESCREEN assays, and results from mixture studies, were provided in deliverables #1 and #5, see chapters 2 and 3.

The focus of this report is on modulation studies. In such studies a fixed concentration of a mixture is selected that evokes a response in the range of 40-60 %. The choice of a mid-range effect allows the detection of both positive modulations (effects greater than mixture alone) and negative modulations (effects less than mixture alone). The mixture is composed of chemicals that were active in the respective assay, and is referred to as the reference mixture. In studies of individual modulators the candidate chemical is added to the fixed reference mixture in decreasing concentrations, usually starting in the 1-10 µM range and with 6 (ERLUX) or 7 (ESCREEN) ten-fold dilutions thereof. Mixtures of potential modulators are studied in the same fashion, but using a mixture of candidates constructed from equimolar concentrations.

In either the ERLUX or ESCREEN assays, frank toxicity was considered to be shown as either 1) a reduction below vehicle control values if the chemical was inactive, or 2) a 'tailing off' at the top end of the dose-response curve, if the chemical was active. It should be noted that although 1) can give an indication of toxicity, the measure depends on the small amplitude vehicle signal and is therefore not very sensitive, and 2) can potentially confuse a negative modulation with toxicity.

ARLUX

The assay protocol and details on regression analysis have already been provided in previous reports (deliverable #5, see chapter 3, and 2nd annual report).

This report gives an update on the testing of mixtures of antiandrogenic chemicals and presents the studies on modulation of antiandrogenic mixture responses.

Single chemical testing

For the testing of the single compounds, initial range-finding experiments were conducted. The concentration ranges were either chosen according to existing literature data or to the potency expected from similar compounds. If none of the above information was available, the highest achievable starting concentration (up to 250 μM), depending on compound solubility, was tested with seven subsequent five fold dilutions. If a compound turned out to act as an antiandrogen, the range finding experiments were followed by two or three further experiments to establish the concentration-response relationship in the relevant concentration range.

Candidate chemicals for modulator testing were also subjected to initial range finding experiments. If chemicals showed cytotoxicity at higher concentrations, a concentration range below toxicity was chosen for testing their modulating activity.

Further testing was only performed if deemed necessary because of insufficient data quality.

Mixture testing

10 compound mixture (see also deliverable #5, chapter 3) and 6 and 4 compound sub-mixtures

Chemicals which qualified to be included in the first mixture were chosen based on:

- Quality of data (in terms of limited biological variation) in concentration-response relationships (e.g. high variation could be due to degradation of the tested compound).
- The ability to produce a complete (or sufficiently complete) concentration-response curve, covering an effect range of at least 50%.

- No major androgenicity by the compound in absence of androgen (DHT), because this could interfere with the mixture effect predictions.
- Not more than two chemicals from one chemical group (Box1) to avoid the domination of one group

All chemicals that did not comply with these criteria were excluded from the mixture experiments.

The chosen chemicals for the first mixture experiment are listed in Table 2.

Reference mixture

In this report, the reference mixture of four antiandrogenic parabens (Table 3) was employed to test potential effect modifiers.

17 compound mixture

Chemicals which qualified to be included in the first mixture were chosen based on:

- Quality of data (in terms of limited biological variation) in concentration-response relationships (e.g. high variation could be due to degradation of the tested compound).
- The ability to produce a complete (or sufficiently complete) concentration-response curve, covering an effect range of at least 50%.
- This mixture also included chemicals that exhibited androgenic activity when tested in absence of androgen (DHT).
- More than two chemicals of each group (Box1) were permitted in the mixture.

All chemicals that did not comply with these criteria were excluded from the mixture experiments.

The chosen chemicals for the first mixture experiment are listed in Table 4.

Testing of candidate chemicals as effect modifiers of antiandrogenic mixtures

Potential modulators were tested in the presence of the paraben reference mixture and the 17 component mixture at fixed concentrations reflecting their EC_{25} values. These concentrations were $7.490E^{-05}$ M for the paraben mixture and $2.810E^{-05}$ M for the 17 component mixture. The fixed mixture concentrations were then tested in combination with increasing concentrations of test chemicals to investigate their modulatory effect on the antiandrogenic mixture responses.

The chosen chemicals for the first mixture experiment are listed in Table 5.

Table 2: Compounds tested in mixtures 1 and 2 (10 component mixture) and the two sub mixtures 5 (red) and 6 (blue)

Compound	*Abbreviation*	*Chemical Group*
Bisphenol A	BPA	Bisphenol A
n- Propylparaben	n-PP	Paraben
Benzophenone 3 (Eusolex 4360)	BP3	UV-filter
3-Benzylidene Camphor (Unisol S-22)	3-BC	UV-filter
Butylated hydroxy anisole	BHA	Anti-oxidant
Butylated hydroxy toluene	BHT	Anti-oxidant
Tonalide	AHTN	Polycyclic musk
Galaxolide	HHCB	Polycyclic musk
Perfluorooctane sulfonate	PFOS	PFC
Polychlorinated biphenyl 138	PCB138	PCB

Table 3: Parabens tested in mixtures 3 and 4 (reference mixture)

Compound	*Abbreviation*	*Chemical Group*
Methylparaben	MeP	Paraben
Ethylparaben	EtP	Paraben
n- Propylparaben	n-PP	Paraben
n- Butylparaben	n-BP	Paraben

Table4: Compounds tested in mixtures 7 and 8 (17 component mixture)

Compound	*Abbreviation*	*Chemical Group*
Bisphenol A	BPA	Bisphenol A
Methylparaben	MeP	Paraben
Ethylparaben	EtP	Paraben
n- Propylparaben	n-PP	Paraben
n- Butylparaben	n-BP	Paraben
Benzophenone 2	BP2	UV-filter
Benzophenone 3 (Eusolex 4360)	BP3	UV-filter
3-Benzylidene Camphor (Unisol S-22)	3-BC	UV-filter
4-Methylbenzylidene Camphor	4-MBC	UV-filter
Butylated hydroxy anisole	BHA	Anti-oxidant
Butylated hydroxy toluene	BHT	Anti-oxidant
Tonalide	AHTN	Polycyclic musk
Galaxolide	HHCB	Polycyclic musk
Perfluorooctane sulfonate	PFOS	PFC
Polychlorinated biphenyl 138	PCB138	PCB
Polybrominated diphenyl ether 100	BDE100	PBDE
Benzo(α)pyrene	BaP	Mutagen

Table5: Compounds tested as potential effect modifiers

Compound	*Abbreviation*	*Chemical Group*
Mercury Chloride	$HgCl_2$	Heavy metal
Cadmium Chloride hemi(pentahydrate)	$CdCl_2$	Heavy metal
Lead (II) nitrate	Pb	Heavy metal
Polychlorinated biphenyl 118	PCB118	PCB
Bis(2-ethylhexyl)phthalate	DEHP	Phthalate
Dibutylphthalate	DBP	Phthalate
Perfluorooctanoic acid	PFOA	PFC

Calculation of mixture effect predictions using concentration addition (CA) and independent action (IA)

Details on the calculation of mixture effect predictions have been described in detail in deliverable #5, see chapter 3.

Results

A. Studies of effect modifiers (ERLUX)

Modulation studies examining mercury and DEHP

In the ERLUX, we examined the ability of 1) mercury chloride and 2) di-ethyl-hexyl-phthalate (DEHP) to modulate the effects of a 17 component reference mixture of food additives and contaminants. The mixture was previously shown to conform to CA and the potential modulators were screened against a concentration of the mixture that evoked a response of approximately 40%. The composition of the reference mixture is shown in Table A1.

Neither mercury chloride (5 nM to 0.5 µM, Figure A1) nor DEHP (0.5 nM to 50 µM, Figure A2) showed any evidence for modulation. Both DEHP and mercury chloride were not estrogenic when tested alone (Insets to Figures A1 and A2).

Activity of a set of phthalates in ERLUX

To consider the activity of phthalates more generally, we examined the estrogenicity of three further phthalates: BBP, DBP and DEP. Interestingly we found that DBP showed no estrogenicity, similar to DEHP, whilst both BBP and DEP were estrogenic (activity seen at concentrations of 0.1-1 µM and above) (Figure A3). In the case of BBP there was evidence for toxicity within the dose-response analysis, and this appeared to occur close to the concentration at which estrogenicity was observed. Because of the different patterns of activity and toxicity seen, the group of phthalates was not considered further for examination as modulators in the ERLUX. The same panel of phthalates was examined in the ESCREEN (see next section).

Modulators found to be unsuitable for examination due to estrogenicity when tested alone

Prior to testing a chemical as a potential effect modulator, we screened for activity of the chemical when tested alone. Benzo[a]pyrene (BaP) was examined alone in the ERLUX and found to be active. This was unexpected, based on the literature, and meant that BaP could not be considered as an effect modulator. It was however included in the 17 component mixture of active chemicals, against which modulators were tested. Interestingly BaP was not active when tested alone in the ESCREEN, and so we were able to test it as an effect modifier in the ESCREEN (see next section).

Ability of MAPK and PI3K pathway inhibitors to inhibit the ERLUX response

The initial strategy for our modulator studies was to use ERLUX and ESCREEN in a complementary fashion since it was expected the ERLUX would function as a simple reporter-gene assay whilst ESCREEN would serve as a much more complex assay for estrogenicity.

Data from our group had indicated that inhibition of the MAPK pathway can reduce the effect of estrogens in the ESCREEN, consequently we examined the effects of MAPK pathway inhibition in the ERLUX, and also studied the effects of PI3K pathway inhibition. These pathways are of relevance because both the MAPK and PI3K pathway could be affected by chemicals considered as active estrogens, especially the phytoestrogens, or by modulators, in which case they can be considered as potential targets for modulations to occur at.

We found that inhibition of both the MAPK (with PD98059) and PI3K (with LY294002) pathway reduced the effects of estradiol, bisphenol A or genistein (Figure A4A, B). We examined a further three compounds, naringenin, fluoranthene and benzo[a]pyrene, for the effects of MAPK pathway inhibition and found that, in all cases, MAPK pathway inhibition reduced the effects of the active compounds (Figure A4A, right).

These results may suggest that the ERLUX assay is not as simple as previously considered, however the ESCREEN assay should still be considered the more complex assay because of the use of cell proliferation as an endpoint, allowing for many further options for the integration and influence of modulators with the effects of active estrogens.

Table A1: ERLUX reference mixture

Composition	**Components**
Components: **17**	Estradiol (E2)
Design: **fixed ratio**	Ethinyl estradiol (EE2)
Mixture ratio: **equieffective at EC_{10}**	Genistein (GEN)
	Naringenin (NAR)
	Enterolactone (ENL)
	Coumestrol (COU)
	Bisphenol A (BPA)
	Benzo[a]pyrene (BaP)
	Fluoranthene (FLUOR)
	Brominated diphenyl ether-100 (BDE100)
	Propylparaben (PropylP)
	Methylparaben (MethylP)
	Butylparaben (ButylP)
	4-methylbenzylidene camphor (4MBC)
	Benzophenone-3 (BP3)
	Galaxolide (GAL)
	Tonalide (TON)

Figure A1: results of modulations study (ERLUX) for mercury chloride

Graph shows the effect of increasing concentrations of mercury chloride (indicated on the axis) on the ERLUX effect evoked by a fixed concentration of a 16 component reference ERLUX mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (estradiol, pale red) and vehicle controls (pale blue) are shown. Figure shows individual data values for three (concentration response) or eight (controls) replicates per plate, solid line indicates a linear regression model (dashed lines denote 95% confidence intervals). Inset shows the effect of mercury chloride when tested alone.

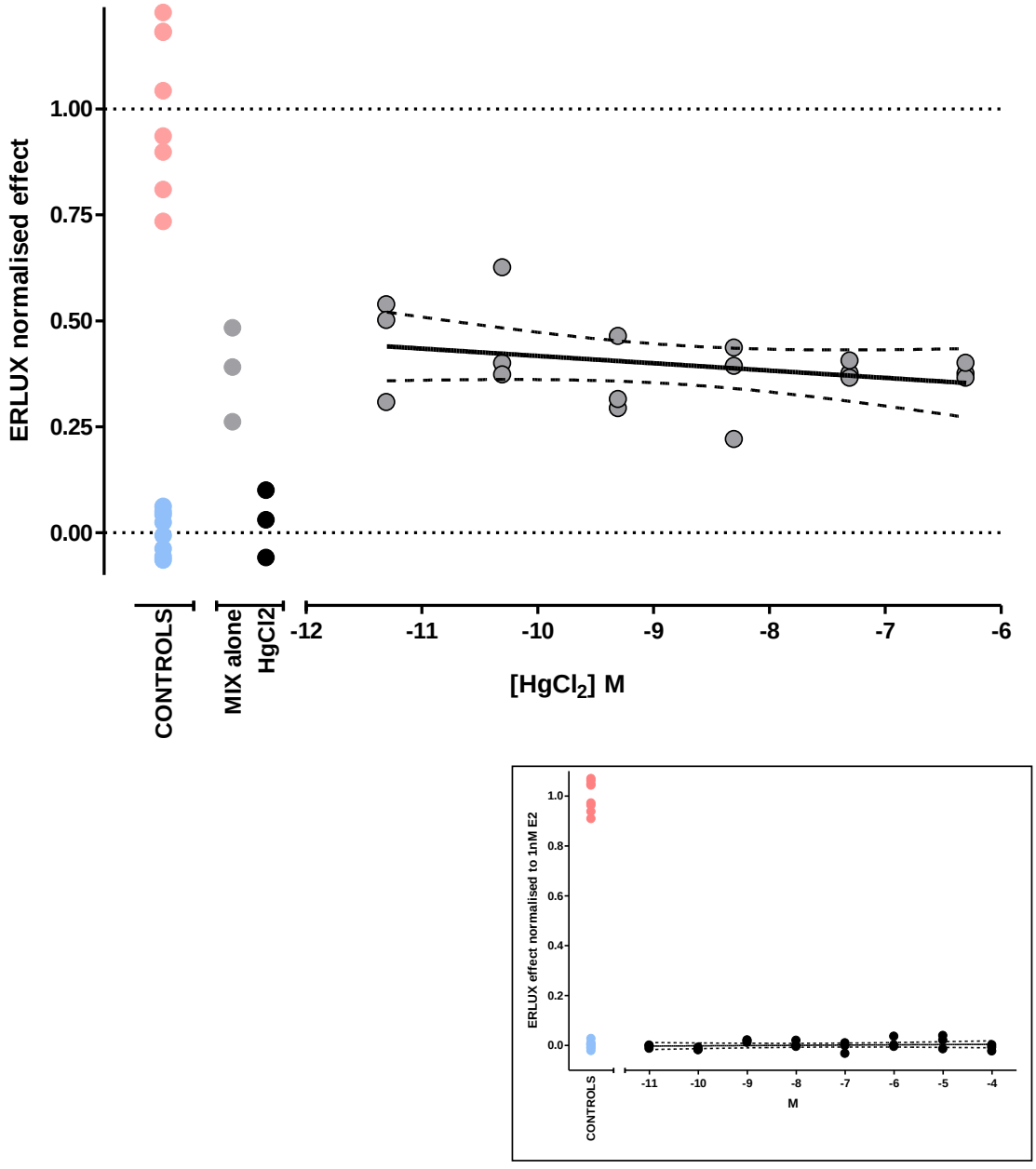


Figure A2: results of modulations study (ERLUX) for DEHP

Graph shows the effect of increasing concentrations of DEHP (indicated on the axis) on the ERLUX effect evoked by a fixed concentration of a 16 component reference ERLUX mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (pale red) and vehicle controls (pale blue) are shown. Figure shows individual data values for three (concentration response) or eight (controls) replicates per plate, solid line indicates a linear regression model (dashed lines denote 95% confidence intervals). Inset shows the effect of DEHP when tested alone.

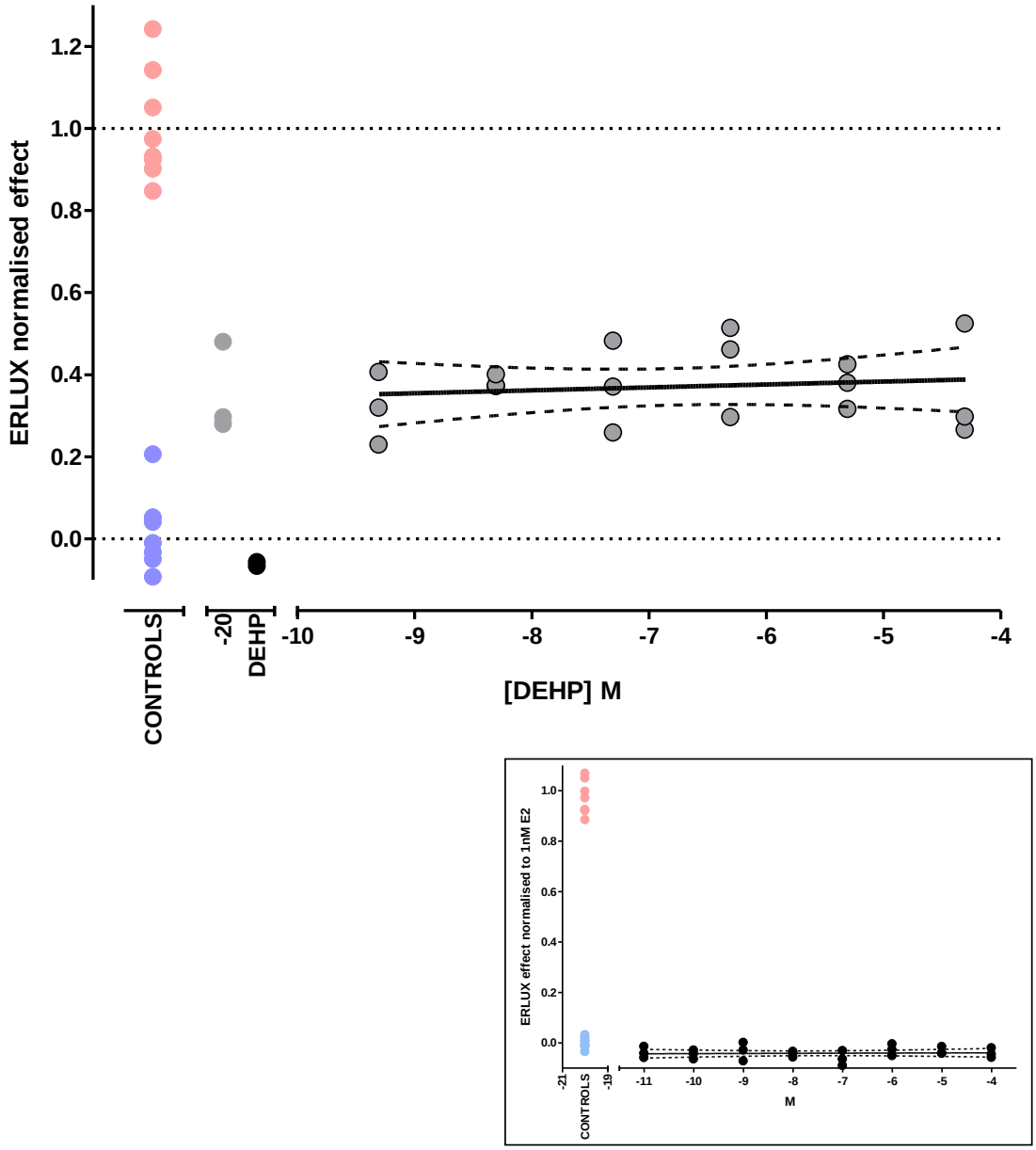


Figure A3: ERLUX results for four phthalates

Graph shows ERLUX results for 4 phthalates: DEHP (stars), BBP (diamonds), DBP (squares), DEP (circles).

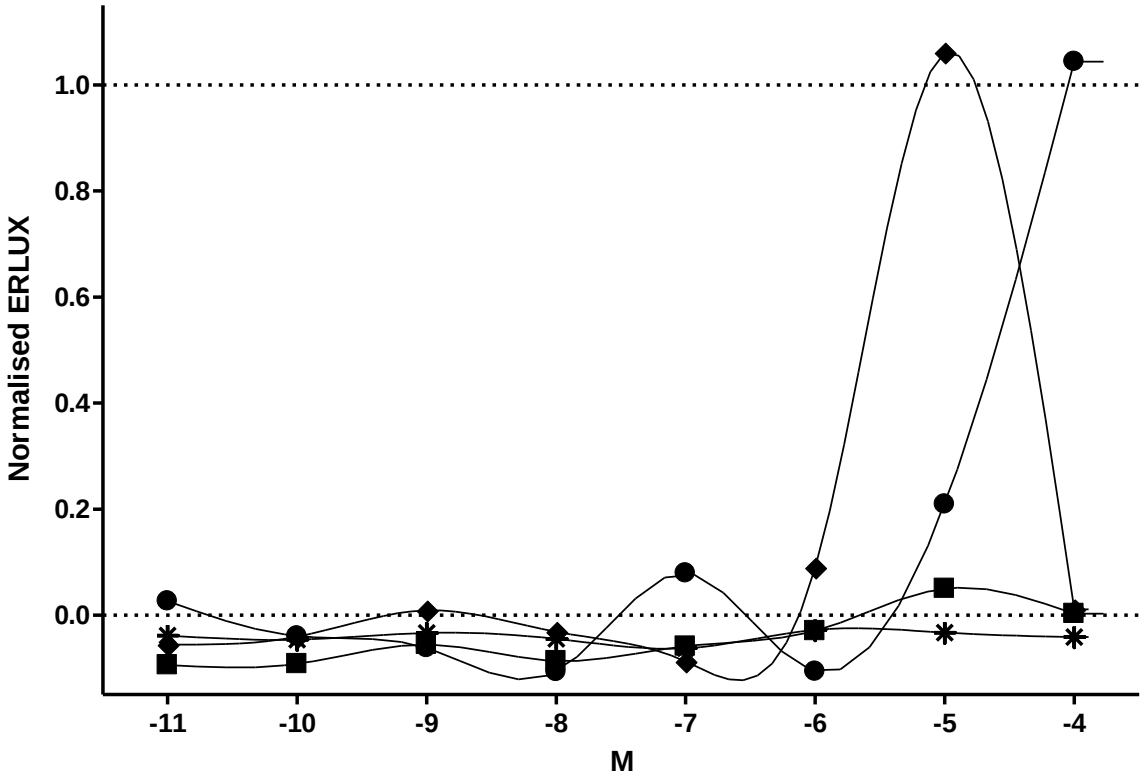
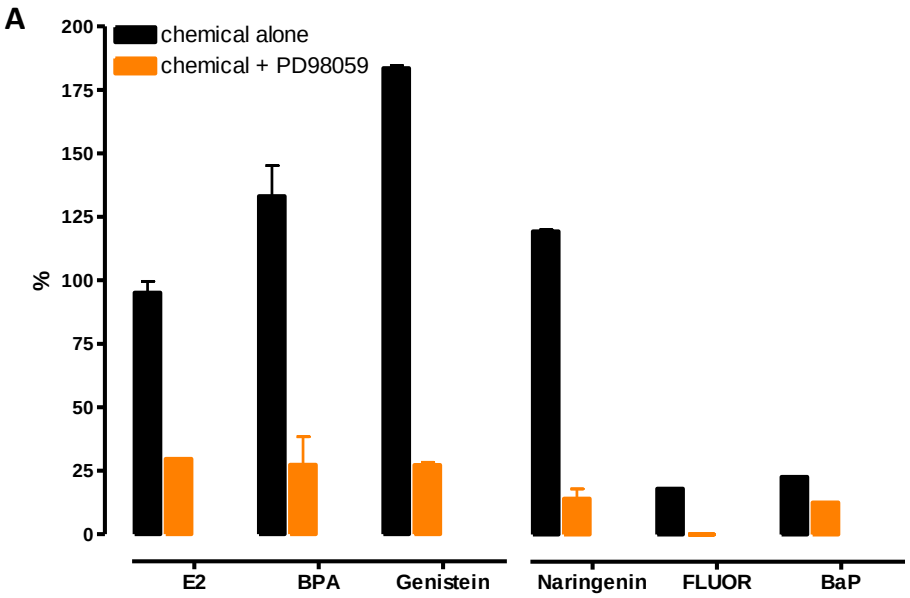
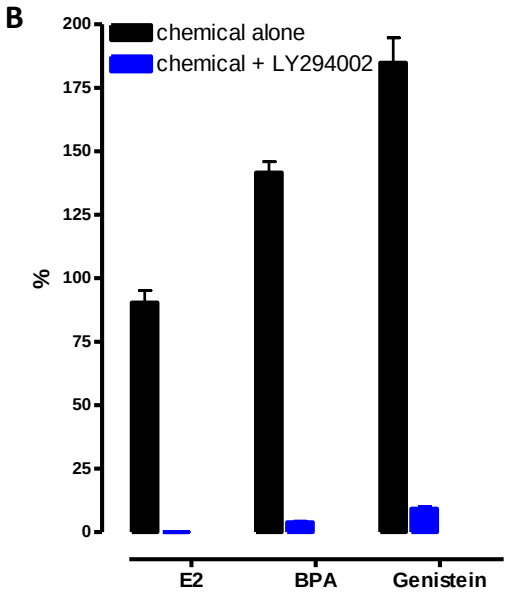


Figure A4: effects of MAPK or PI3K pathway inhibition on ERLUX results

Graphs show the effect of inclusion of A) a MAPK pathway inhibitor (PD98059, 50uM) and B) a PI3K pathway inhibitor (LY294002, 50uM) on the effects of estrogenic chemicals in the ERLUX. The concentrations of the chemicals tested were estradiol (E2, 0.4 nM), bisphenol A (BPA, 5 µM), genistein (1.5 µM), naringenin (15 µM), fluoranthene (FLUOR, 50 µM) and benzo[a]pyrene (BaP, 5 µM).





B. Studies of effect modifiers (ESCREEN)

Modulation studies examining mercury and DEHP

In the ESCREEN, we examined the ability of 1) mercury chloride and 2) di-ethyl-hexyl-phthalate (DEHP) to modulate the effects of a 14 component reference mixture of food additives and contaminants. The mixture was previously shown to conform to CA and the potential modulators were screened against a concentration of the mixture that evoked a response of approximately 50-60%. The composition of the reference mixture is shown in Table B1.

Mercury chloride (5 nM to 5 µM) showed no evidence for modulation (Figures B1). DEHP (5 nM to 5 µM showed no clear evidence for modulation although the data showed greater variability than usual (Figure B2) and further testing would be required for a firm conclusion to be drawn.

When tested alone, mercury chloride showed no indication of estrogenicity up to 1 µM but appeared to be toxic at concentrations of 10 µM or higher (Figure B2). The modulator testing was restricted to the range below which toxicity was seen. DEHP was found to be estrogenic when tested alone, in contrast to the data obtained in the ERLUX (see previous section). The estrogenicity of DEHP occurred at concentration of greater than 1 µM, and toxicity was seen before a full estrogenic response was reached (toxicity began at 5 µM). Data for the estrogenicity of DEHP is compared to that of three other phthalates in the following paragraph.

Activity of a set of phthalates in ESCREEN

For comparison to the data collected in the ERLUX on a set of four phthalates, we tested the same set in the ESCREEN (DEHP, BBP, DBP, DEP). In contrast to the ERLUX, where two of the phthalates were inactive, all four phthalates showed activity in the ESCREEN (Figure B3). At concentrations above the highest concentrations shown in Figure B3, both DEHP and BBP showed toxicity.

Modulation studies using a mixture of 16 potential modulators

In addition to the single modulator studies described above, we also examined the ability of a large multi-component mixture to modulate the activity of the reference 14 component mixture. The composition of the reference mixture is shown in Table B1 and the composition of the mixture of modulators is shown in Table B2.

Potential modulators were first screened individually for modulatory activity and then combined and tested as a mixture.

Screening of potential modulators

Initially we screened 14 potential modulators, in addition to mercury and DEHP, individually for modulation. Data for the 16 potential modulators that were studied in total is presented in Figure B4. Data on estrogenicity and toxicity for these chemicals is listed in Table B3.

Considering the 16 potential modulators studied individually:

- Three chemicals showed a **clear negative modulation**: PCB126, benzo[a]pyrene and cadmium chloride.
- Five chemicals showed a **possible negative modulation**: lead nitrate, PhIP, DBP, BHA and BHT.
- Eight chemicals showed **no indication of modulation** in either direction: DEHP, mercury chloride, PCB008, PCB153, PCB180, MeIQx, BBP and DEP.
- None of the potential modulators showed any indication of a positive modulation.

Mixture of modulators study

In the mixture study, a fixed concentration of the reference mixture was added to increasing concentrations of the mixture of modulators. The mixture of modulators was designed using an equimolar ratio, rather than a ratio of equieffective levels because it is not clear what the appropriate effect actually is for a modulator. The effect that is important for the modulation may not itself be eloquent in the assay used, consequently equieffect levels cannot be set. Substances showing weak estrogenicity at micromolar concentrations were not excluded from the mixture of modulators.

The mixture of modulators study showed that there was a clear negative modulation at a mixture concentration of 15.2 µM (i.e. contains 16 modulators each present at 0.95 µM) and 1.6 µM but not at 0.16 µM or lower (Figure B5). Similarly to the individual modulators, there was no indication of any positive modulation by the mixture of modulators.

A major difficulty in interpreting negative modulations is the potential confounding of toxicity. Because of the complexity of the ESCREEN and the occurrence of cell proliferation within the assay, a simple cell viability assay cannot be used. One approach is to model any toxicity observed for the individual modulators and then to formulate a mixture prediction for toxicity. We are currently exploring this approach. Where modulators cause a negative modulation that plateaus at a level greater than zero, then this is evidence for a negative effect that is very likely not due to toxicity (which would eventually produce a complete abolition of effect, if the appropriate concentration range was covered).

Table B1: ESCREEN reference mixture

Composition	**Components**
Components: **14**	Estradiol (E2)
Design: **fixed ratio**	Ethinyl estradiol (EE2)
Mixture ratio: **equieffective at EC_{25}**	Genistein (GEN)
	Naringenin (NAR)
	Enterolactone (ENL)
	Coumestrol (COU)
	Bisphenol A (BPA)
	Fluoranthene (FLUOR)
	Brominated diphenyl ether-100 (BDE100)
	Propylparaben (PropylP)
	Methylparaben (MethylP)
	Butylparaben (ButylP)
	4-methylbenzylidene camphor (4MBC)
	Benzophenone-3 (BP3)

Table B2: mixture of potential modulators

Composition	**Components**
Components: **16**	Benzo[a]yrene (BaP)
Design: **fixed ratio**	Butylated hydroxyl anisole (BHA)
Mixture ratio: **equimolar**	Butylated hydroxytoluene (BHT)
	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)
	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)
	PCB #8
	PCB #126
	PCB #153
	PCB #180
	Cadmium chloride ($CdCl_2$)
	Lead nitrate ($Pb(NO_3)_2$)
	Mercury chloride ($HgCl_2$)
	Butyl benzyl phthalate (BBP)
	Di butyl phthalate (DBP)
	Di ethyl hexyl phthalate (DEHP)
	Di ethyl phthalate (DEP)

Table B3: observations of estrogenicity or toxicity for chemicals screened as potential modulators

Name of potential modulator	Observed modulation	Signs of estrogenicity*	Signs of toxicity**
Benzo[a]yrene (BaP)	Clear negative	None	Possible toxicity at 3uM and greater
Butylated hydroxyl anisole (BHA)	Possible negative	None	None
Butylated hydroxytoluene (BHT)	Possible negative	None	None
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)	None	None	None
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	Possible negative	None	None
Cadmium chloride ($CdCl_2$)	Clear negative	None	
Lead nitrate ($Pb(NO_3)_2$)	Possible negative	None	None
Mercury chloride ($HgCl_2$)	None	None	Possible toxicity at 10uM and greater
PCB #8	None	None	None
PCB #126	Clear negative	Active at 10uM or higher	None
PCB #153	None	None	None
PCB #180	None	None	None
Butyl benzyl phthalate (BBP)	None	Active at 1uM or higher	Toxic at 100uM
Di butyl phthalate (DBP)	Possible negative	Active at 10uM or higher	None
Di ethyl hexyl phthalate (DEHP)	None	Active at 1uM or higher	Toxic at 50uM and greater
Di ethyl phthalate (DEP)	None	Active at 10uM or higher	None

**Active'* indicates a positive signal in ESCREEN (estrogenicity), clearly distinguishable from assay variability and noise and usually supported by a dose-response (i.e. not reliant on data from only a single concentration).

**'Possible toxicity'* indicates a decrease in value for treated wells below that of vehicle controls, this signal is small so the assignment of toxicity is not certain; '*Toxic'* indicates a reduction in signal evoked by increasing concentrations above those at which a chemical showed activity (estrogenicity).

Figure B1: results of modulation study (ESCREEN) for mercury chloride

Graph shows the effect of an increasing concentration of mercury chloride (indicated on the axis) on the ESCREEN effect evoked by a fixed concentration of a 14 component reference ESCREEN mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (estradiol, pale red) and vehicle controls (pale blue) are shown. Individual data points are shown for two (concentration response) and eight (controls) replicates. Inset shows the effect of mercury chloride when tested alone.

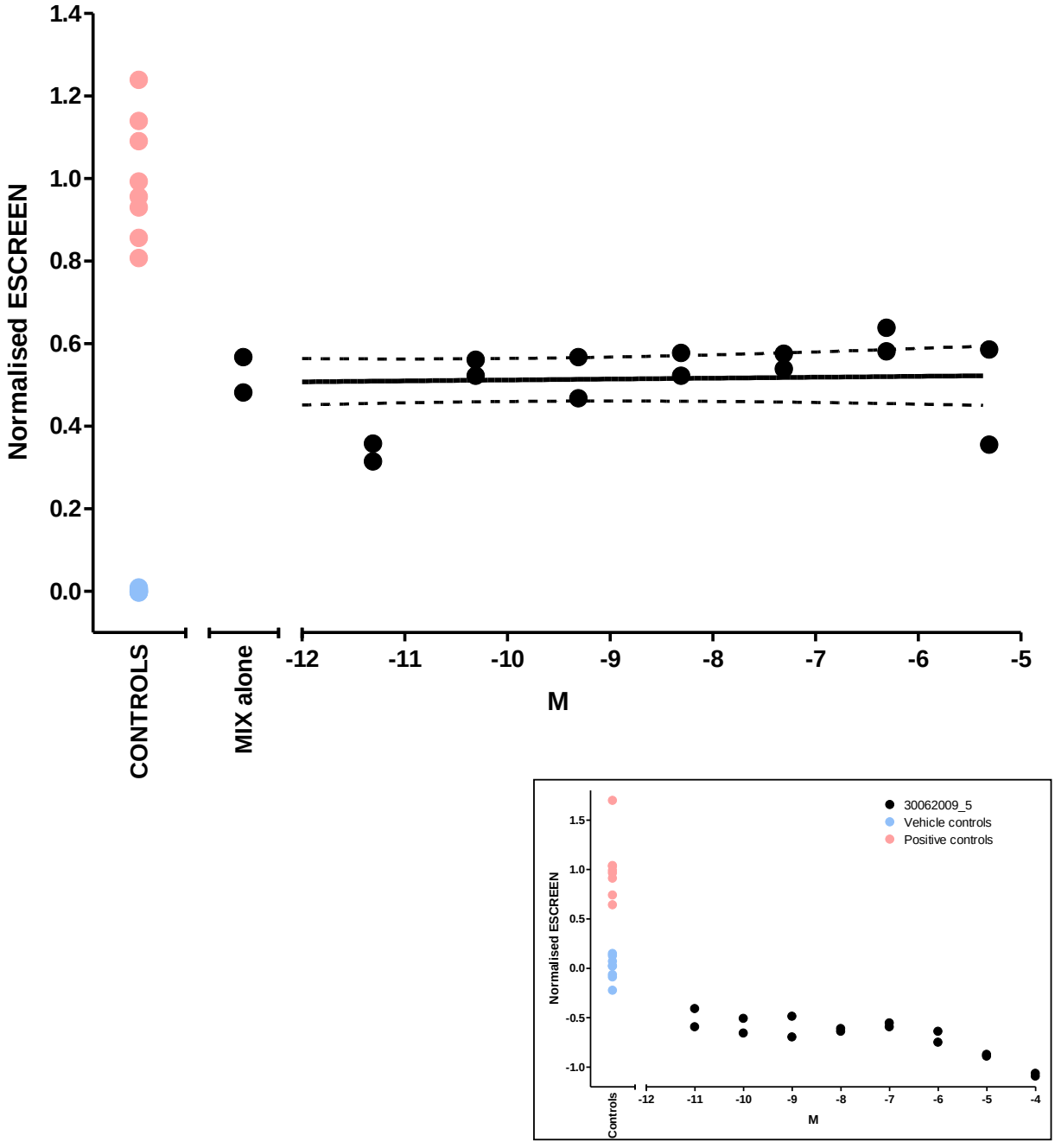


Figure B2: results of modulation study (ESCREEN) for DEHP

Graph shows the effect of an increasing concentration of DEHP (indicated on the axis) on the ESCREEN effect evoked by a fixed concentration of a 14 component reference ESCREEN mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (pale red) and vehicle controls (pale blue) are shown. Inset shows the effect of DEHP when tested alone. Note: on this plate, the reference mixture alone had a lower effect than was observed on three other plates run in the same experiment. Individual data points are shown for two (concentration response) and eight (controls) replicates. Data from the parallel plates is therefore also shown (grey circles) for comparison.

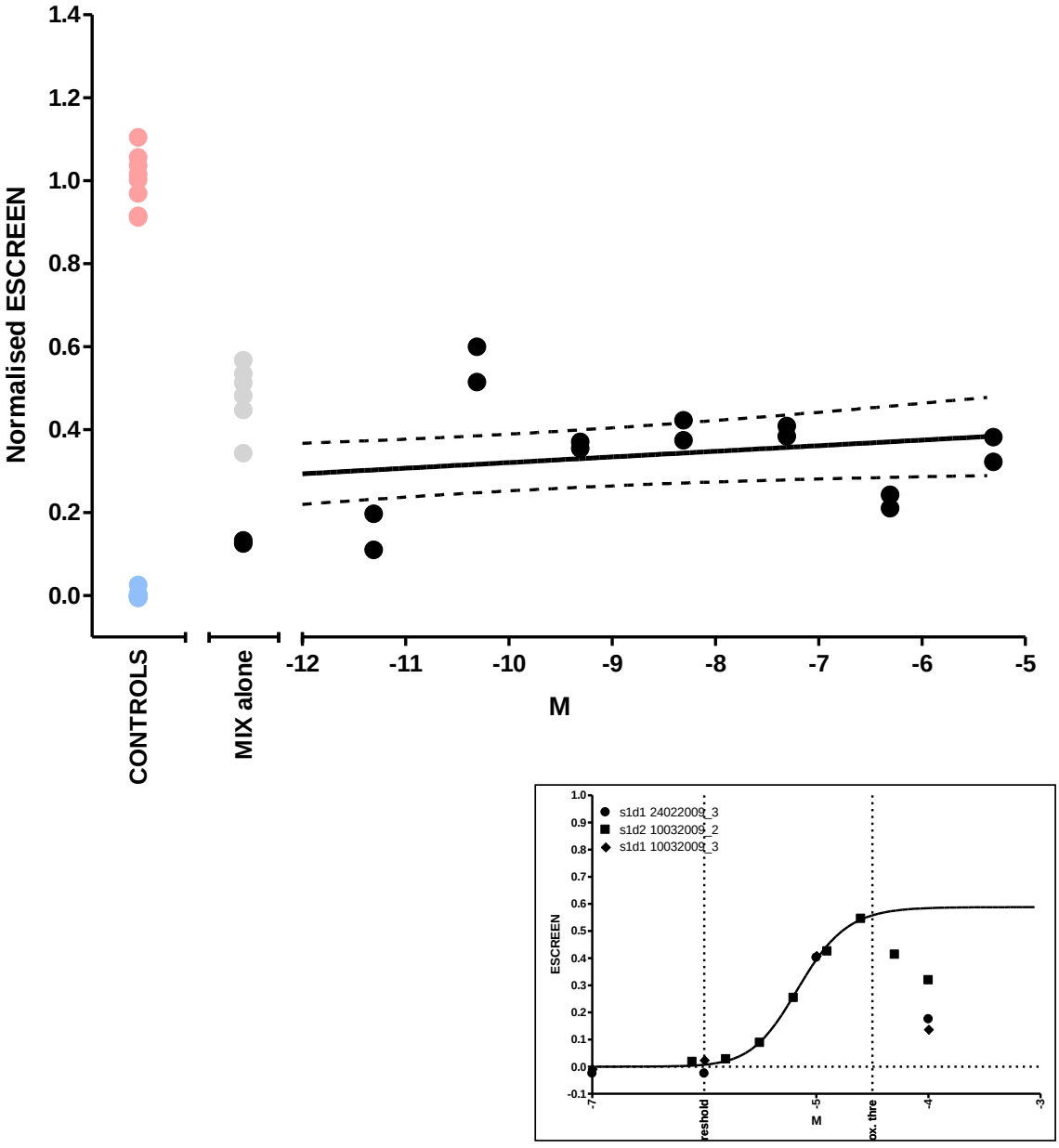


Figure B3: ESCREEN results for four phthalates

Graph shows ESCREEN results for 4 phthalates: DEHP (circles), BBP (diamonds), DBP (squares), DEP (stars).

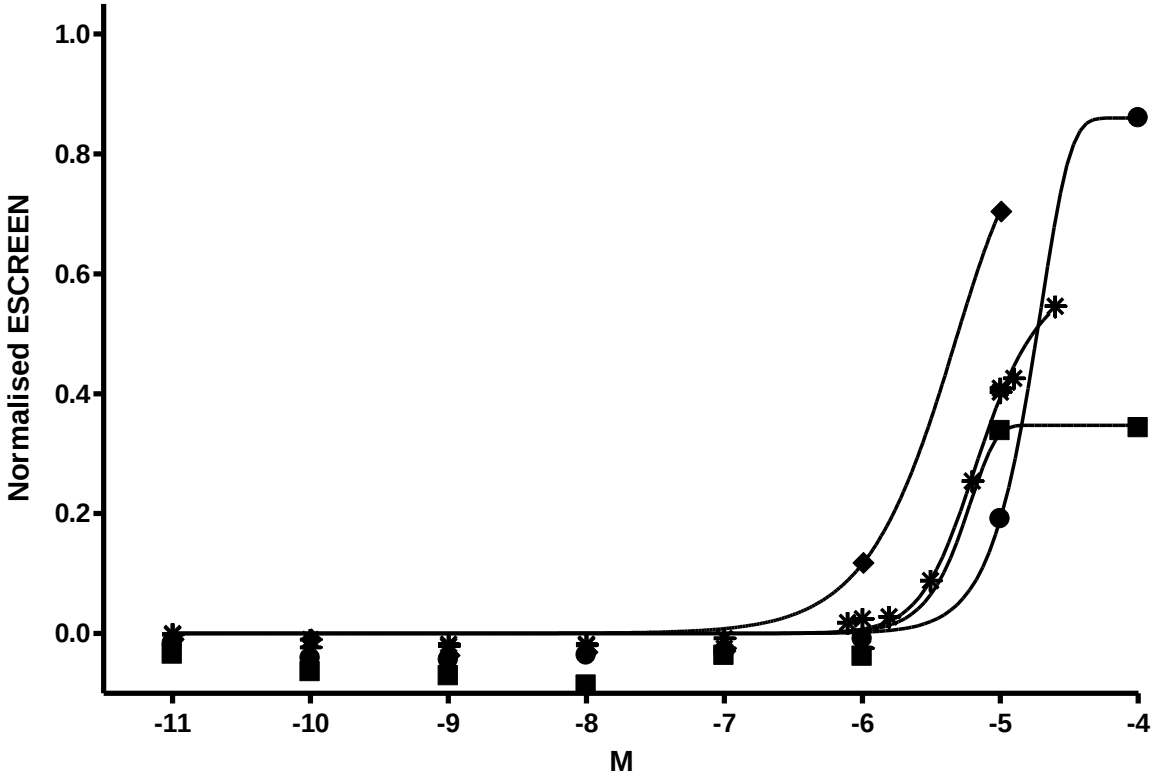


Figure B4: results of modulation studies for 16 potential modulators

Each graph shows the effect of an increasing concentration (indicated on the axis) of a potential modulator on the ESCREEN effect evoked by a fixed concentration of a 14 component reference mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (pale red) and vehicle controls (pale blue) are shown. Each modulator was studied on one multiwell plate, and data points are shown as mean +/- range for two (concentration response) or eight (controls) replicates. Data were fitted with a linear or sigmoid model as appropriate (solid line; with +/- 95% confidence interval, dashed line).

A vertical dotted line is drawn at 0.1 μM, for comparison to the following figure, Figure B5, where the vertical dotted line indicates a mixture concentration of 1.6 μM (contains each potential modulator at 0.1 μM).

See figure on following page.

Figure B4

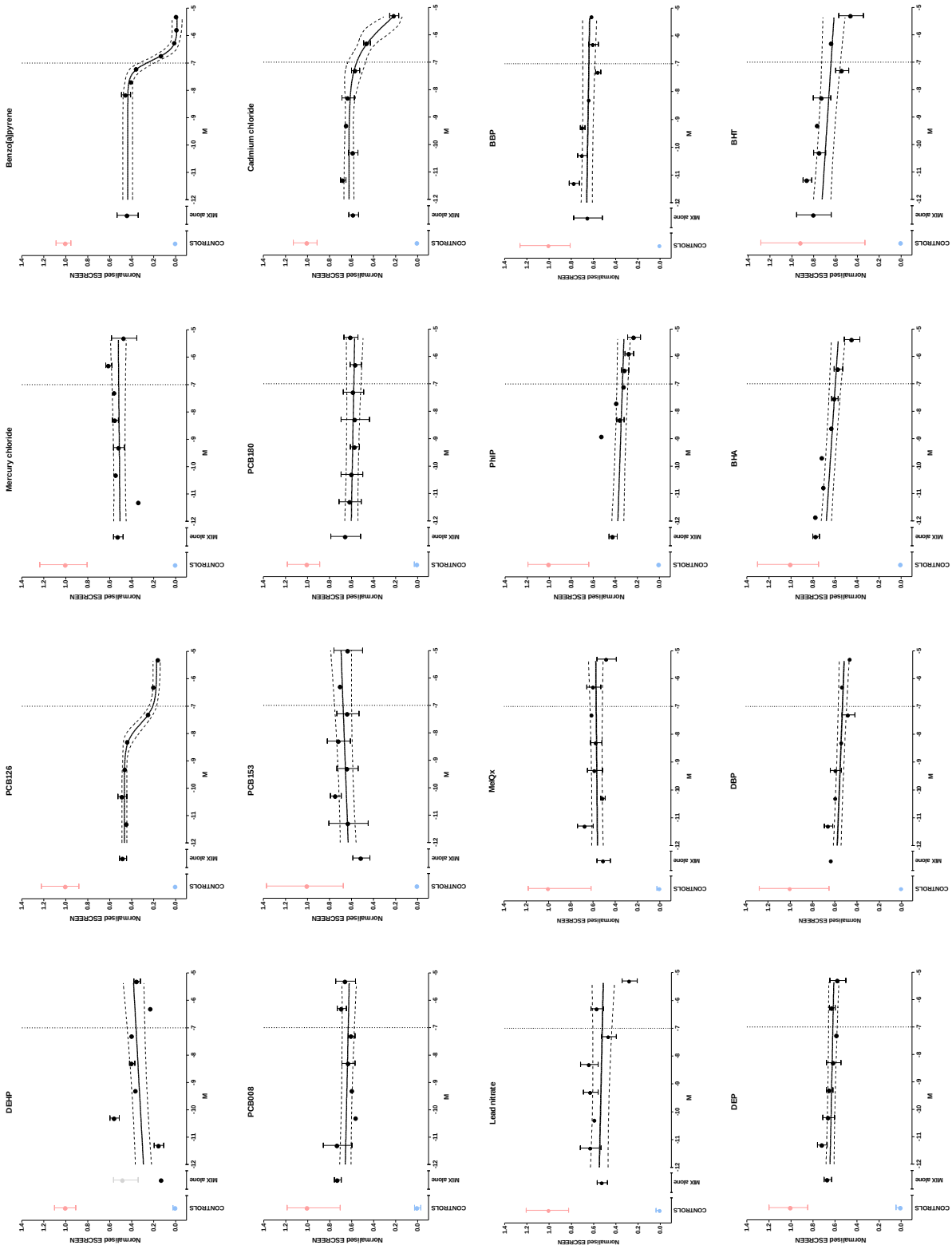
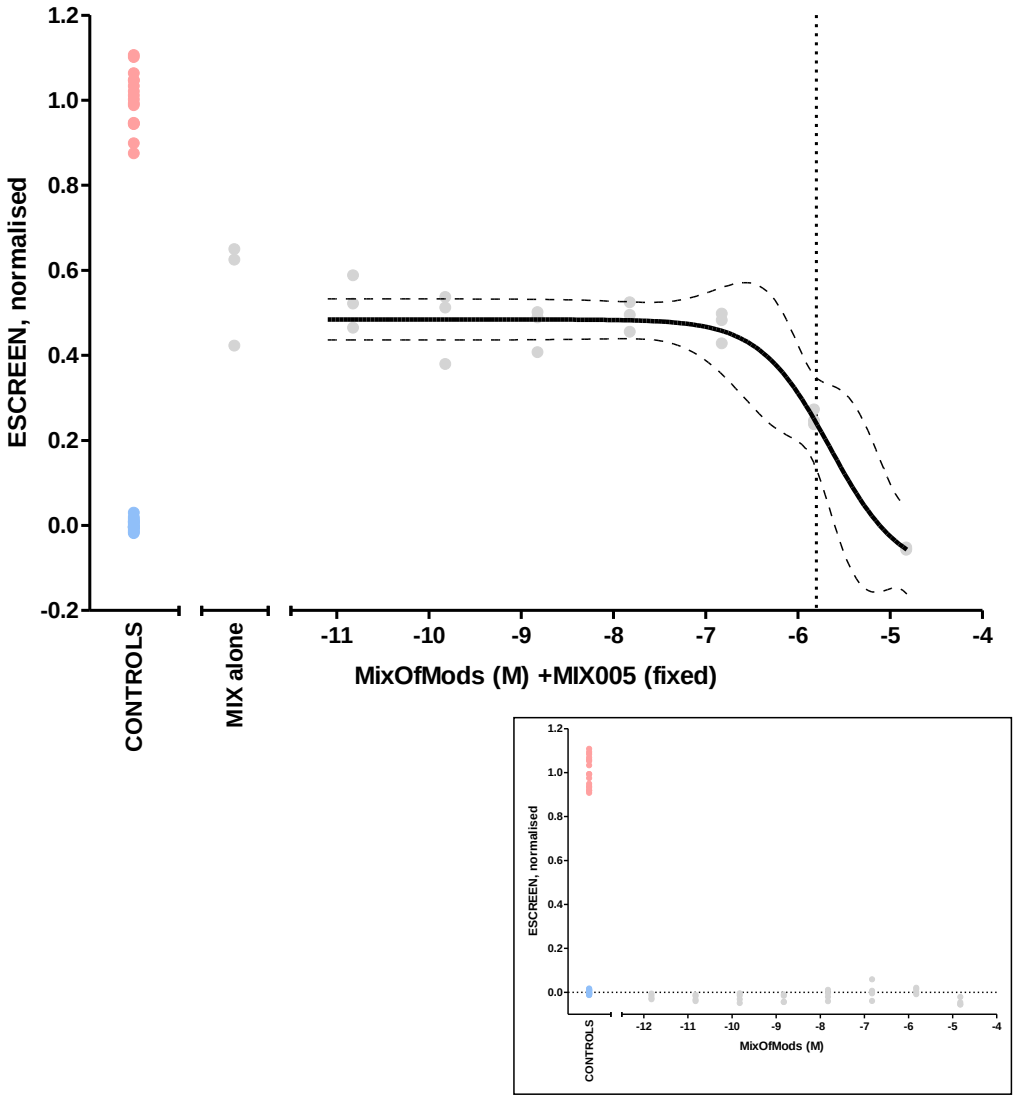

Benzo[a]pyrene
Cadmium chloride
BBP
BHT
Mercury chloride
PCB180
PhIP
BHA
PCB126
PCB153
MeIQx
DBP
DEHP
PCB008
Lead nitrate
DEP
Normalised ESCREEN
CONTROLS
MIX alone
M

Figure B5: results of modulation study for a 16 component mixture of potential modulators

Graph shows the effect of an increasing concentration (indicated on the axis) of an equimolar mixture of 16 potential modulators (see composition in table B2) on the ESCREEN effect evoked by a fixed concentration of a 14 component reference ESCREEN mixture (effect of the reference mixture alone is indicated separately). Values for positive controls (estradiol, pale red) and vehicle controls (pale blue) are shown. Individual data points are shown for two (concentration response) and eight (controls) replicates. Inset shows the effect of the mixture of modulators when tested alone. A vertical dotted line is drawn at a mixture concentration of 1.6 µM (contains each potential modulator at 0.1 µM), for comparison to the preceding figure, Figure B4, where vertical dotted line is drawn at 0.1 µM for each individual modulator. It appears that the degree of downward modulation seen with the mixture of 16 modulators is due to the downward modulation observed with benzo-[a]-pyrene, cadmium chloride and PCB 126.



C. Results for ARLUX

Mixture testing (update)

The testing of the ten component mixture and the paraben reference mixture has been described in deliverable #5 (chapter 3). Here, we present an update on further mixture studies that have been conducted since deliverable #5 and the 2nd annual report.

Prediction modelling

Based upon the concentration-response relationships for the individual mixture components (Table C1), the expected mixture effects were calculated according to either the model of concentration addition (CA) or of independent action (IA). The predictions were based on mixture ratios that reflect either the EC_{50} or EC_{20} values of the individual compounds. The mixture ratios for all mixtures are shown in Table C2.

Testing of two mixtures containing compounds tested in the 10 component mixture (split mixture)

The 10 component mixture (Figure C1 and deliverable #5, see chapter 3) was found to exhibit a deviation from expected concentration addition, suggesting an antagonism within the mixture. Substances included in the 10 component mixture were bisphenol A (BPA), *n*- propylparaben (n-PP), benzophenone 3 (BP3), 3-benzylidene camphor (3BC), butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN), hexahydrohexamethylcyclopentabenzopyran (HHCB), perfluorooctane sulfonate (PFOS) and polychlorinated biphenyl 138 (PCB138). Due to the assay principle, where antiandrogens are expected to block an androgen (DHT) from activating the androgen receptor (AR) measured by luciferase activity, it can be argued that CA should be the adequate model for predicting mixture effects and chemicals are not likely to act according to IA. Furthermore, the concentration-response curve of the 10 component mixture exhibited a very steep slope in comparison to both prediction models. Such a steep slope was only observed with a minority of compounds during single compound testing and was not expected for the mixture.

To investigate the cause for this deviation, the 10 components were split into two mixtures. This was based on the slopes of the curves of the effect of the individual compounds within the mixture (Figure C1, grey curves). The 6 component mixture comprised the compounds with shallower individual effects (BPA, BP3, BHA, AHTN, HHCB, PCB138), while the remaining four compounds had steeper responses (PFOS, n-PP, 3BC, BHT).

First, the mixture effects of these two mixtures were predicted according to the models of CA and IA. Next, they were tested at the same mixture ratios used for the prediction and the results were compared to the estimates. Figure C2 shows the results of testing of both mixtures at a fixed mixture ratio according to their EC_{50} values (Figure C2a: 6 component mixture, Figure C2b: 4 component mixture). Both mixtures agreed rather well with the CA prediction.

Testing of 17 component mixture

The 17 component mixture included chemicals that showed an antiandrogenic response when tested in the presence of 0.25 nM DHT. This mixture also incorporated compounds that were androgenic when tested without DHT. Substances included in the 17 component mixture were BPA, n-PP, BP3, 3BC, BHA, BHT, AHTN, HHCB, PFOS, PCB138, methyl paraben (MeP), ethyl paraben (EtP), *n*- butylparaben (n-BP), benzophenone 2 (BP2), 4-methylbenzylidene camphor (4MBC), polybrominated diphenyl ether 100 (BDE100) and benzo(α)pyrene (BaP).

The non-linear regression fits of all compounds which were included in the mixture are presented in Figure C3. The corresponding non-linear regression models used, with their regression parameters, are presented in Table C1.

In Figure C4 the three components that also showed androgenic activity (BDE100, BaP, BP2) are presented. BDE100 showed an almost complete concentration response for antiandrogenicity up to the concentration where it exhibited androgenic when tested in the absence of DHT. Testing of BaP did not deliver a full concentration response for antiandrogenicity up to the highest concentration tested. BP2 was antiandrogenic in presence of DHT up to a concentration of 20 µM. Above this concentration it showed an androgenic response with and without DHT. Concentrations above 200 µM were cytotoxic.

This mixture was first tested at a fixed mixture ratio reflecting the EC_{50} values for the individual chemicals (FigureC5a: mixture 7). The tested mixture effect of this mixture agreed well with the effect predicted according to CA. However, due to the incomplete concentration response of BaP, this compound dominated the mixture effect. Thus, the same compounds were combined at concentrations reflecting their EC_{20}, where each compound contributed equally to the mixture effect (Figure C5b: mixture 8). This mixture also acted according to CA.

Testing of potential effect modulators of antiandrogenic mixtures

To test potential modulators of antiandrogenic effects, several compounds were tested against the reference mixture consisting of 4 parabens (deliverable #5, see chapter 3) and the 17 component mixture (above). These two mixtures were chosen, as they acted according to concentration addition. Thus, any deviation from the expected effect can be regarded as effect

modulation. The 10 component mixture was not included in modulator testing, as it exhibited a deviation from CA itself.

The chemicals tested for their modulating effects were di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), cadmium chloride, mercury chloride, lead nitrate and perfluorooctanoic acid (PFOA) (Figures C6 to C11). Other candidate modulator compounds were PBDEs and PCBs. However, during earlier testing, BDE100 and PDC138 showed antiandrogenic activity and therefore were included in the mixture of antiandrogens. Another, co-planar PCB that was tested later, PCB118, also acted as an antiandrogen in combination with 0.25 nM DHT and also showed weak androgenicity when tested on its own (Figure C12) and was thus rejected from modulator testing.

For modulator testing, chemicals were first tested alone to determine a concentration range below cytotoxicity for testing their modulatory effect on antiandrogenic mixtures. Next, a suitable concentration range was chosen to test the modulators in combination with the paraben mixture and the 17 component mixture. Both mixtures were kept at a constant concentration reflecting their individual EC_{25} values (paraben mixture: $7.490E^{-05}$ M; 17 component mixture: $2.810E^{-05}$ M).

DEHP showed no AR antagonistic activity when tested in combination with DHT, however cytotoxicity occurred at the highest test concentration (500 µM, Figure C6a). This was also apparent from the decrease in luciferase activity when DEHP was tested without DHT. After testing of DEHP at lower concentrations (2.7 nM – 6 µM) in combination with both, the paraben and the 17 component mixture, no effect on the antiandrogenic activity of the mixtures could be observed (Figures C4b and c).

DBP, when tested in presence or absence of DHT showed cytotoxicity beginning at concentrations exceeding 5 µM (Figure C7a). Cytotoxicity for DBP was not as marked as for DEHP but was observed over a concentration range from 5 to 100 µM. In this case, it was not possible to clearly differentiate between a potential antiandrogenic response, which might be masked cytotoxicity at higher concentration and mere cytotoxicity. Therefore, DBP concentrations up to 100 µM were also included in the testing of the modulatory effect of DBP on the paraben and the 17 component mixture (Figures C7b and c). In both mixtures DBP did not have an effect on the antiandrogenic activity of the mixture at lower concentrations. Cytotoxicity was observed in the same concentration ranges as in the testing of DBP alone. If DBP exhibits some antiandrogenic activity at concentrations below the cytotoxic concentrations it cannot be detected within this assay because of the overlap of the two concentration ranges.

Cadmium chloride and mercury chloride had no antiandrogenic effect but showed some cytotoxicity at higher concentrations (100 µM, Figures C8a and C9a). When tested in combination with the paraben and 17 component mixtures no modulating effects were observed except the cytotoxicity at the higher concentrations (Figures C8b and c and C9b and c).

Lead nitrate did not show any antiandrogenicity or cytotoxicity over the entire concentration range tested (1 nM to 10 µM, Figure C9a). The same concentration range was then tested in combination with the paraben and 17 component mixture (Figure C9b and c). No modulation of the antiandrogenic effect of either mixture by lead nitrate was observed.

PFOA did not show any antiandrogenic response when tested in the presence of DHT and no cytotoxicity was observed (Figure C11). However, in combination with the paraben mixture, higher concentrations of PFOA seemed to enhance the antiandrogenic effect of the mixture (>10 µM, Figure C11b). The same effect was seen in combination with the 17 component mixture at 100 µM PFOA (Figure C11c).

Figure C1: Predicted and observed concentration-response relationship for an antiandrogenic 10 component mixtures (as in deliverable #5, see chapter 3).

The graph shows the testing of a 10 component mixture at mixture ratios according the EC_{50} of the individual mixtures components. The predicted antiandrogenic effects are shown according to the CA concept (green line) and the IA concept (blue line). The black filled circles show the data points of 4 independent experiments and the solid red line represents the non-linear regression fit for the data. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The light grey lines represent the effects of the individual components within the mixture.

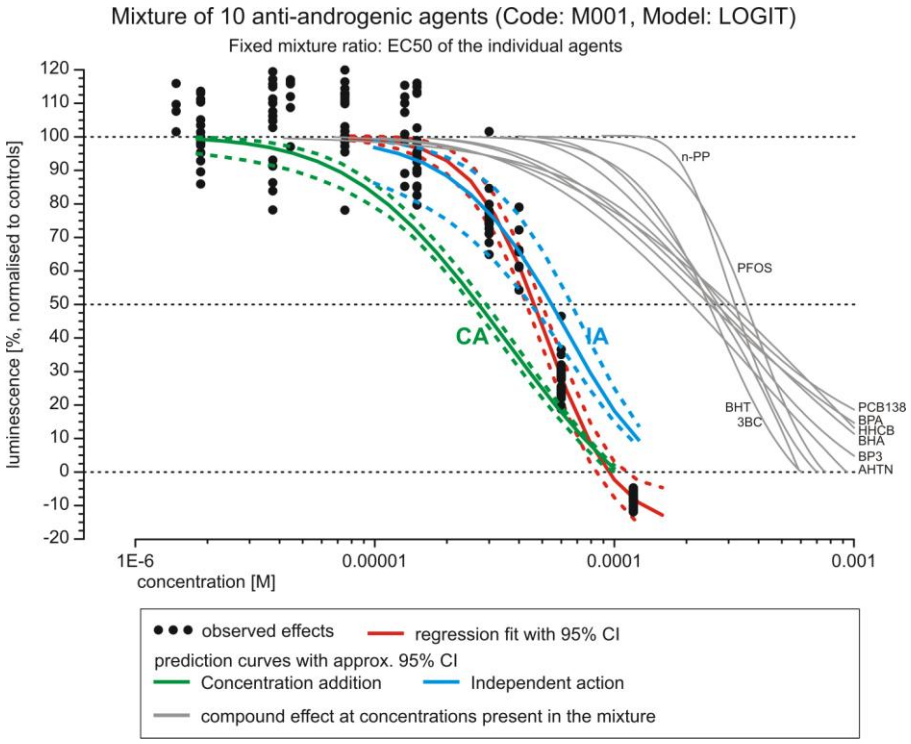
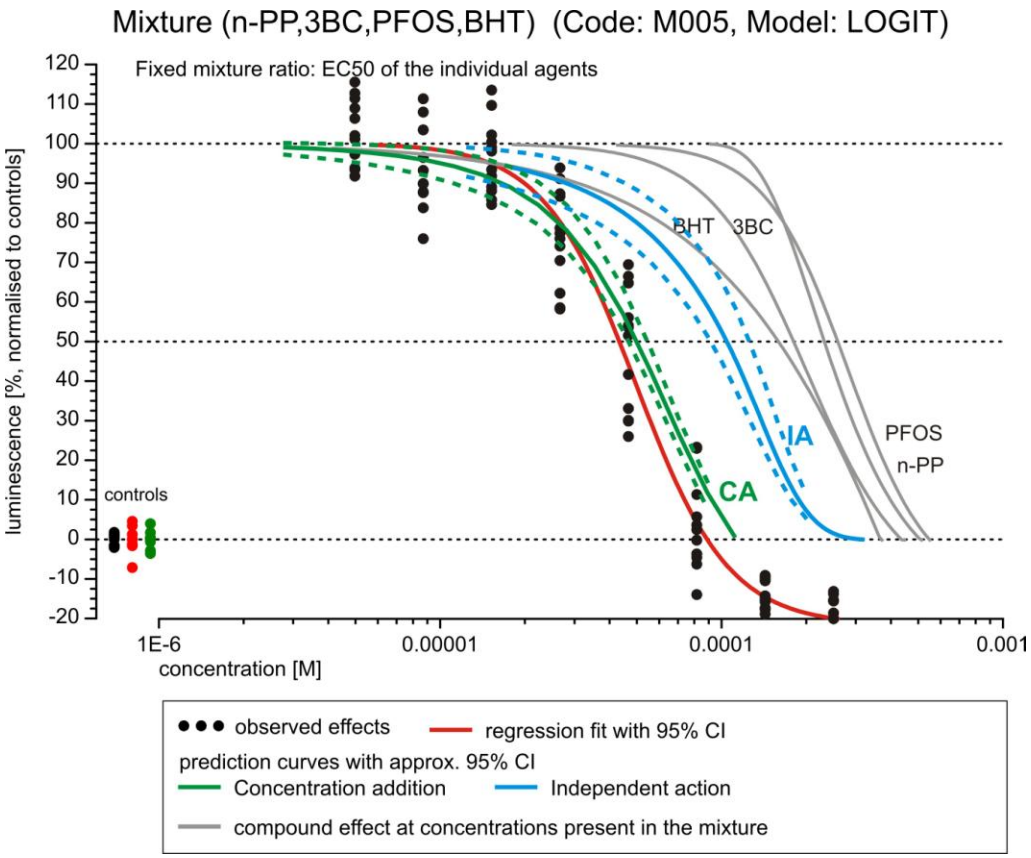


Figure C2: Concentration-response relationships for antiandrogenic compounds included in 2 sub-mixtures of the 10 compound mixture (Figure C1 and deliverable #5, see chapter 3).

The graph shows the testing of two sub-mixtures of the 10 component mixture (Figure C1) at mixture ratios according the EC_{50} of the individual mixtures components. The 4 component mixture (A) was composed of the compounds that exhibit the steeper curves for their individual effects within the mixture (as shown in Figure C1), whereas the 6 component mixture (B) consisted of the 6 compounds with shallower individual effect curves. The predicted antiandrogenic effects are shown according to the CA concept (green line) and to the IA concept (blue line). The black filled circles show the data points of 4 independent experiments and the solid red line represents the non-linear regression fit for the data. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The light grey lines represent the effects of the individual components within the mixture.

A)



B)

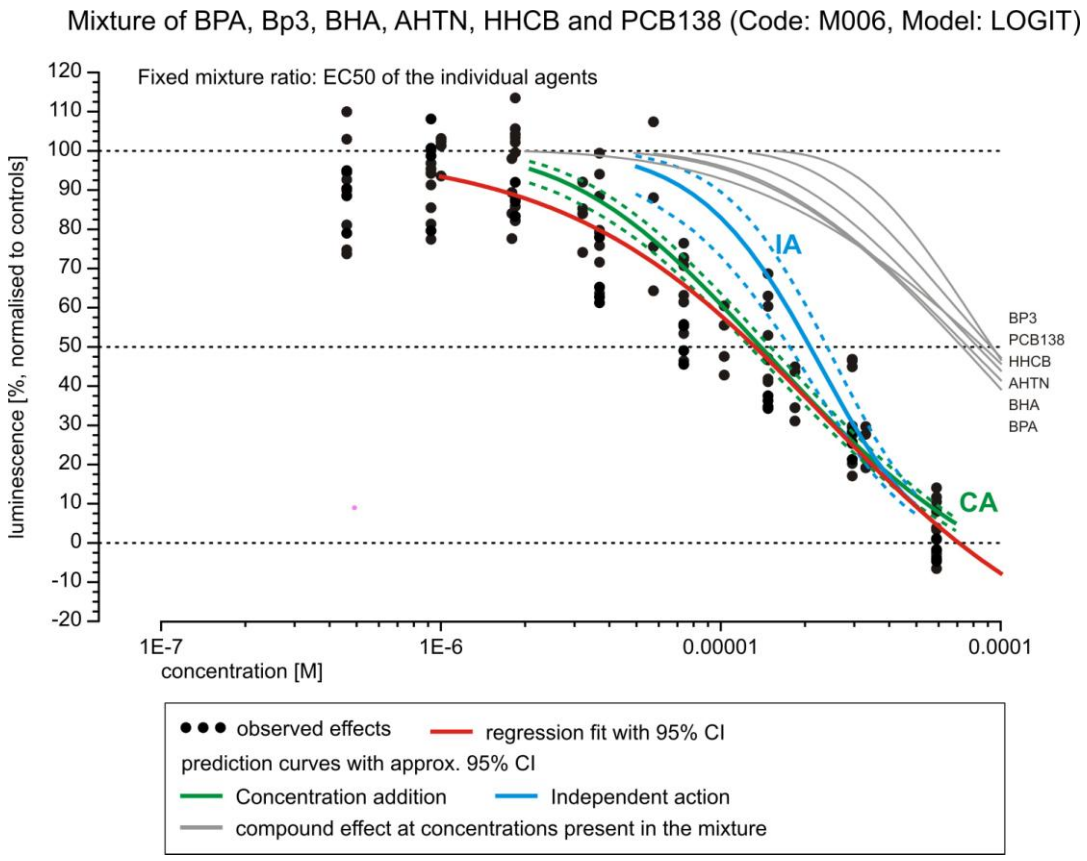

Mixture of BPA, Bp3, BHA, AHTN, HHCB and PCB138 (Code: M006, Model: LOGIT)
Fixed mixture ratio: EC50 of the individual agents
luminescence [%, normalised to controls]
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
1E-7
1E-6
0.00001
0.0001
concentration [M]
IA
CA
BP3
PCB138
HHCB
AHTN
BHA
BPA
observed effects
regression fit with 95% CI
prediction curves with approx. 95% CI
Concentration addition
Independent action
compound effect at concentrations present in the mixture

Figure C3: Concentration-response relationships for antiandrogenic compounds included in the 17 compound mixture.

Non-linear regression fits for the indicated compounds. More detail on regression models and model parameters is given in Table C1.

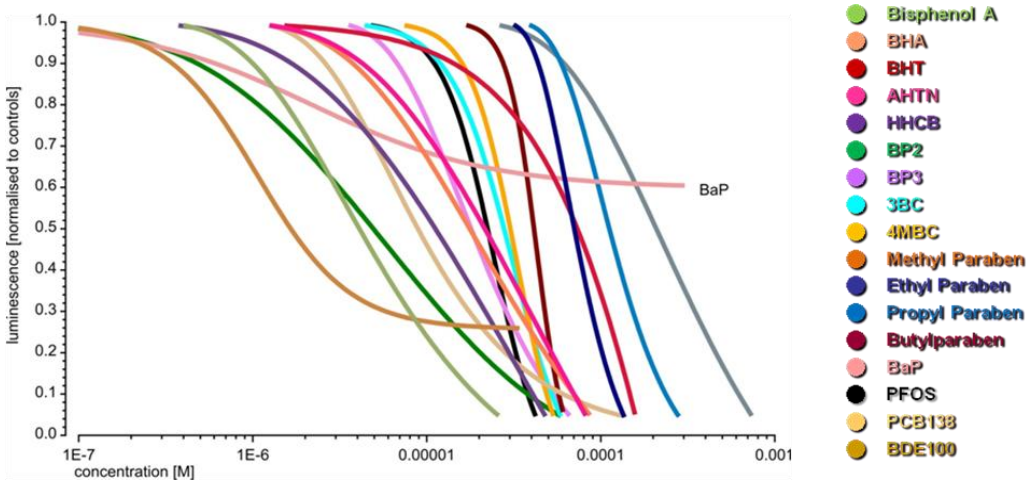
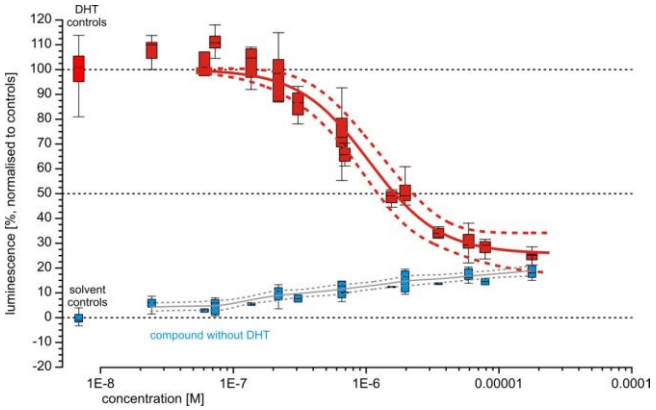


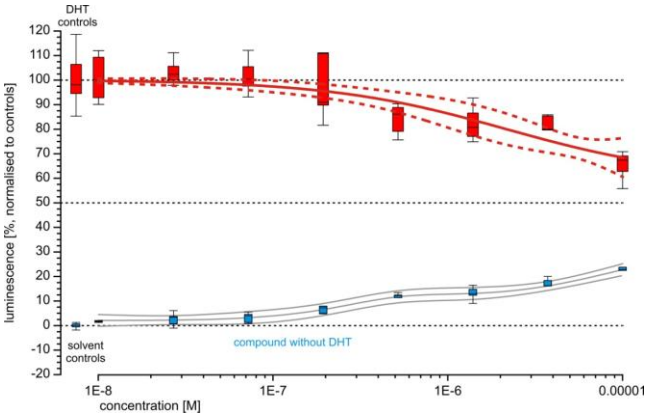
Figure C4: Concentration-response relationships for compounds with androgenic and antiandrogenic activity included in the 17 compound mixture.

BDE 100 (A), BaP (B) and BP2 (C) were investigated in the presence of 0.25 nM DHT to test their antiandrogenic activity (red). All three compounds also showed androgenic activity when tested in the absence of DHT (blue).

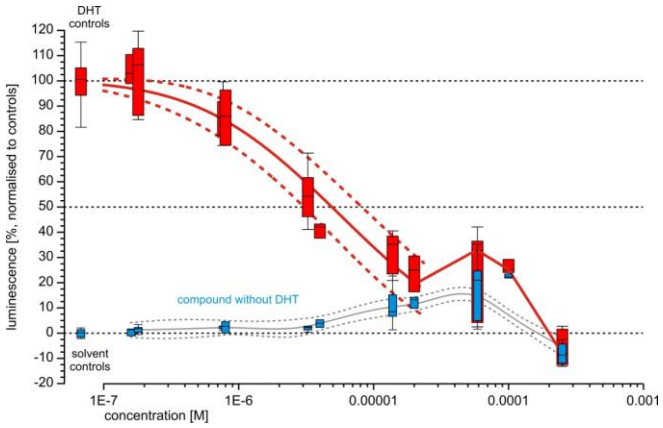
A)



B)



C)



box and whisker plot for pooled data from at least three independent plates:
- bottom and top edges of the box are located at the sample 25th and 75th percentiles
- center horizontal line is drawn at the 50th percentile (median)
- whiskers are drawn from the box to the most extreme point within 1.5 interquartile ranges

regression fit with 95% CI spline fit with 95% CI

Table C1: Regression modelling data from all compounds included in the tested mixtures. The table contains the respective regression model which fit the data best and the corresponding model parameters. Furthermore, the EC_{10} and EC_{50} values for each compound, as derived from the regression analysis are given.

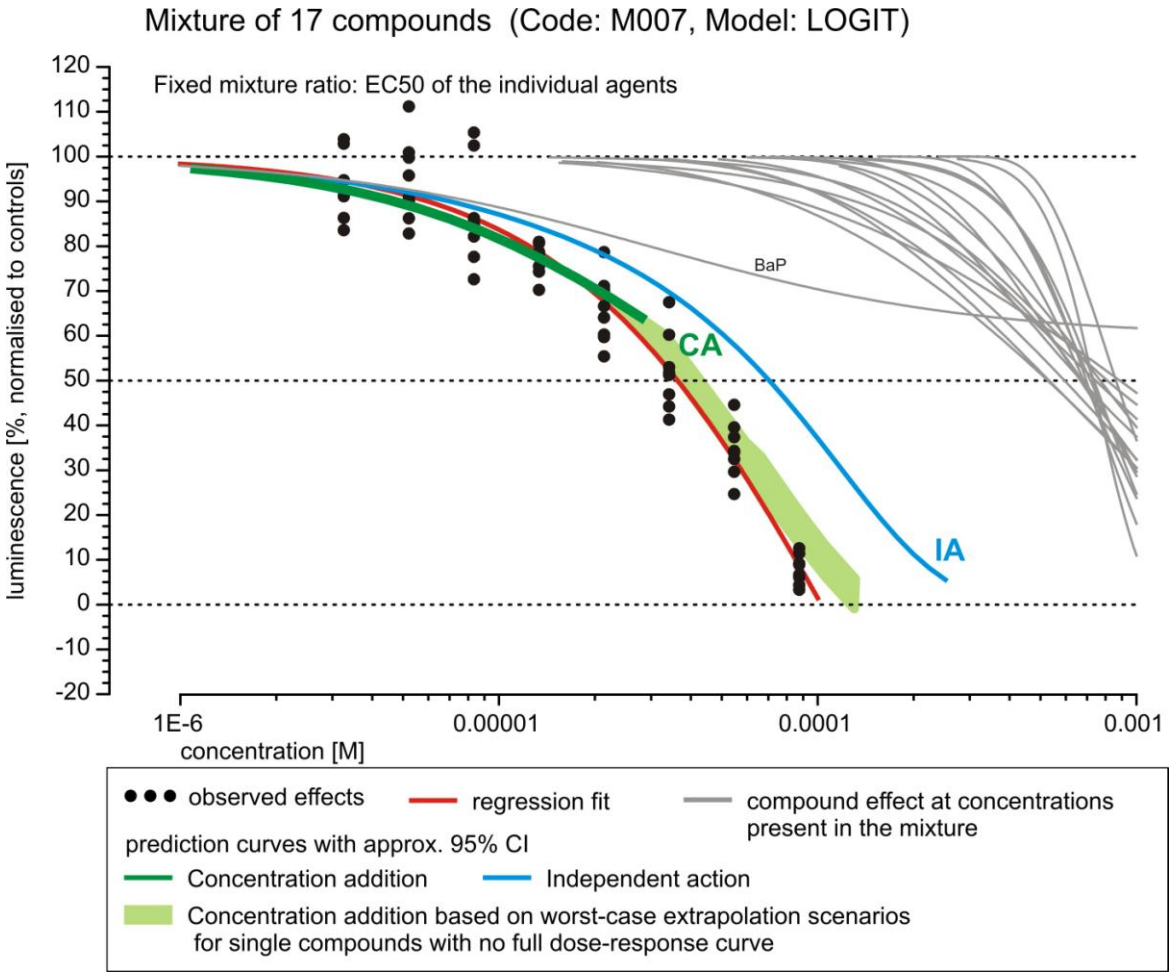
Substance (by order of EC_{50})	*Concentration-Response Function*					*EC_{10}*		*EC_{50}*	
	RM	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_{min}$	$\hat{\theta}_{max}$	*M [CI]*		*M [CI]*	
Ethylparaben	Weibull	-19.54	-4.84	-0.00	1	6.15E-5	[5.22E-5 - 7.24E-5]	1.09E-4	[1.00E-4 - 1.18E-4]
Methylparaben	Weibull	-6.29	-1.71	-0.49	1	5.54E-5	[3.97E-5 - 7.71E-5]	1.88E-4	[1.45E-4 - 2.43E-4]
HHCB	Weibull	-3.88	-0.87	-1.29	1	1.65E-6	[1.12E-6 - 2.43E-6]	1.11E-5	[9.19E-6 - 1.35E-5]
BHA	Weibull	-6.54	-1.38	-0.42	1	3.71E-6	[2.11E-6 - 6.54E-6]	1.75E-5	[1.28E-5 - 2.40E-5]
BP2	probit	-6.83	-1.22	-0.07	1	4.71E-7	[2.34E-7 – 9.49E-7]	4.90E-6	[2.99E-6 – 8.01E-6]
BP3	Weibull	-11.96	-2.49	-0.17	1	6.69E-6	[4.98E-6 - 8.99E-6]	1.79E-5	[1.51E-5 - 2.11E-5]
AHTN	Weibull	-4.87	-1.11	-0.94	1	4.21E-6	[2.81E-6 - 6.32E-6]	2.14E-5	[1.65E-5 - 2.78E-5]
PFOS	logit	-31.45	-6.81	-0.14	1	1.09E-5	[8.46E-6 - 1.42E-5]	2.22E-5	[2.01E-5 - 2.46E-5]
3-BC	logit	-25.60	-5.67	-0.15	1	1.18E-5	[9.11E-6 - 1.54E-5]	2.77E-5	[2.48E-5 - 3.09E-5]
4-MBC	logit	-33.79	-7.53	-0.14	1	1.58E-5	[1.10E-5 – 2.27E-5]	3.00E-5	[2.51E-5 – 3.60E-5]
n-Butylparaben	logit	-52.40	-11.99	-0.09	1	2.73E-5	[2.06E-5 - 3.62E-5]	4.11E-5	[3.59E-5 - 4.71E-5]
n-Propylparaben	Weibull	-21.52	-5.14	-0.16	1	4.34E-5	[3.50E-5 - 5.38E-5]	7.01E-5	[6.02E-5 - 8.18E-5]
BHT	logit	-8.32	-2.44	-2.39	1	1.41E-5	[6.89E-6 - 2.88E-5]	7.29E-5	[6.00E-5 - 8.87E-5]
BPA	Weibull	-8.13	-1.49	-0.23	1	8.70E-7	[5.09E-7 - 1.49E-6]	4.23E-6	[3.25E-6 - 5.51E-6]
PCB 138	Weibull	-11.73	-2.25	0.01	1	2.58E-6	[1.95E-6 - 3.43E-6]	8.83E-6	[7.29E-6 - 1.07E-5]
BDE 100	logit	-22.58	-3.78	0.26	1	3.40E-7	[2.32E-7 - 4.99E-7]	1.64E-6	1.20[E-6 - 2.25E-6]
BaP	logit	-11.11	-1.96	0.60	1	5.94E-7	[2.97E-7 - 1.19E-6]	n.d.	n.d.

EC50, EC10: concentration provoking 50% and 10% effect (antiandrogenicity), respectively. Values in brackets denote the upper and lower limits of the approximate 95% confidence interval; the column "RM" indicates the mathematical regression function as defined at Scholze *et al.* (2001): $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}, \hat{\theta}_{max}$ estimated model parameters , given for concentrations expressed in M (rounded values), $\hat{\theta}_{max}$ were set to the fixed value 1 relating to the mean value of the DHT controls; n.d. = not determined.

Figure C5: Predicted and observed concentration-response relationship for two antiandrogenic 17 component mixtures.

The graphs show the testing of two 17 component mixtures at mixture ratios according A) EC_{50} or B) EC_{20} of the individual mixtures components. Both graphs show the predicted antiandrogenic effects according to CA (green line) and IA (blue line). The light green area is an extrapolation of the CA model beyond the predictability due to the lack of full concentration response data for BaP. The black filled circles show the data points of 4 independent experiments and the solid red line represents the non-linear regression fit for the data. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The light grey lines represent the effects of the individual components within the mixture.

A)



B)

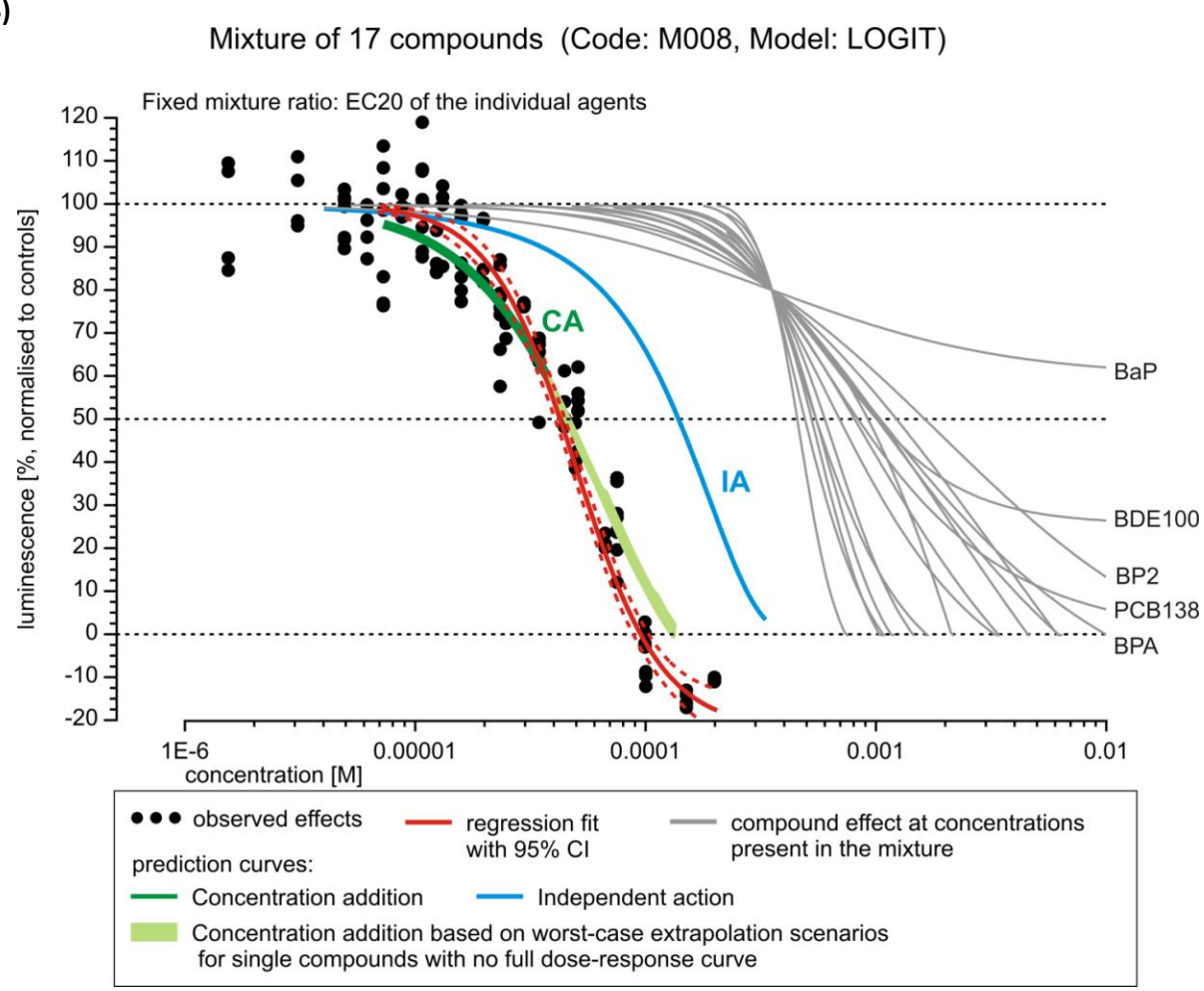

Mixture of 17 compounds (Code: M008, Model: LOGIT)
Fixed mixture ratio: EC20 of the individual agents
luminescence [%, normalised to controls]
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
CA
IA
BaP
BDE100
BP2
PCB138
BPA
1E-6
0.00001
0.0001
0.001
0.01
concentration [M]
observed effects
regression fit with 95% CI
compound effect at concentrations present in the mixture
prediction curves:
Concentration addition
Independent action
Concentration addition based on worst-case extrapolation scenarios for single compounds with no full dose-response curve

Table C2: Relative proportions of each compound (mixture ratio) for the examined mixtures.

Rounded values are given for the relative proportions

Components	***Relative proportions (percentages)***				
(in order of increasing EC50)	Mixture of 10 components	Mixture of 4 components	Mixture of 6 components	Mixture of 17 components (EC50 ratio)	Mixture of 17 components (EC20 ratio)
Ethylparaben	-	-	-	15.55%	20.02%
Methylparaben	-	-	-	26.95%	25.33%
HHCB	3.68%	-	12.99%	2.11%	0.89%
BHA	6.63%	-	23.20%	3.29%	1.71%
BP3	6.63%	-	19.30%	2.68%	2.54%
AHTN	10.27%	-	29.88%	3.60%	2.05%
PFOS	6.28%	8.60%	-	3.02%	3.99%
3-BC	11.21%	15.35%	-	4.05%	4.55%
n-Butylparaben	-	-	-	5.63%	8.91%
n- Propylparaben	22.11%	30.25%	-	8.13%	14.07%
BHT	28.38%	45.80%	-	10.77%	7.79%
BPA	1.70%	-	4.96%	0.57%	0.40%
PCB 138	3.09%	-	9.66%	1.31%	1.04%
BP2	-	-	-	0.55%	0.30%
4-MBC	-	-	-	4.31%	5.64%
BDE 100	-	-	-	0.2%	0.16%
BaP	-	-	-	7.28%	0.60%

Table C3: Statistical uncertainty of predicted and observed effect concentrations for mixtures

CA – concentration addition; IA – independent action; CI – confidence interval; * CA prediction is based on worst-case extrapolation scenarios for single compounds with no full dose-response curve

Effect level (x)	*Effect concentration ECx_{mix} [M]*					
	Observed		Predicted by CA		Predicted by IA	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Mixture of ten components (ratio as defined in Table C2)						
10%	1.61E-5	[1.23E-5 - 2.04E-5]	7.16E-6	[5.56E-6 - 7.89E-6]	1.99E-5	[1.01E-5 - 2.46E-5]
50%	3.47E-5	[2.97E-5 - 4.07E-5]	2.69E-5	[2.52E-5 - 2.90E-5]	5.74E-5	[4.68E-5 - 6.48E-5]
Mixture of four components (ratio as defined in Table C2)						
10%	1.85E-5	[1.26E-5 - 2.58E-5]	1.77E-5	[1.10E-5 - 2.01E-5]	3.11E-5	[1.52E-5 - 3.72E-4]
50%	4.09E-4	[3.30E-5 - 5.01E-4]	4.99E-4	[4.63E-4 - 5.33E-4]	2.59E-4	[8.88E-5 - 1.20E-4]
Mixture of six components (ratio as defined in Table C2)						
10%	1.59E-6	[8.66E-7 - 2.59E-6]	3.10E-6	[2.35E-6 - 3.66E-6]	7.43E-6	[4.37E-6 - 9.79E-6]
50%	1.321E-5	[9.77E-6 - 1.79E-5]	1.38E-5	[1.27E-5 - 1.50E-5]	2.07E-5	[1.70E-5 - 2.45E-5]
Mixture of 17 components (EC20 ratio as defined in Table C2)						
10%	1.59E-5	[1.30E-5 - 1.95E-5]	1.29E-5	[1.03E-5 - 1.41E-5]	3.74E-5	[1.68E-5 - 4.59E-5]
50%	4.56E-5	[4.23E-5 - 4.92E-5]		4.63E-5 - 4.65E-5*	1.46E-4	[1.17E-4 - 1.60E-4]

Figure C6: Testing of modulators of antiandrogenic mixture responses: DEHP.

Luciferase activity of DEHP in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

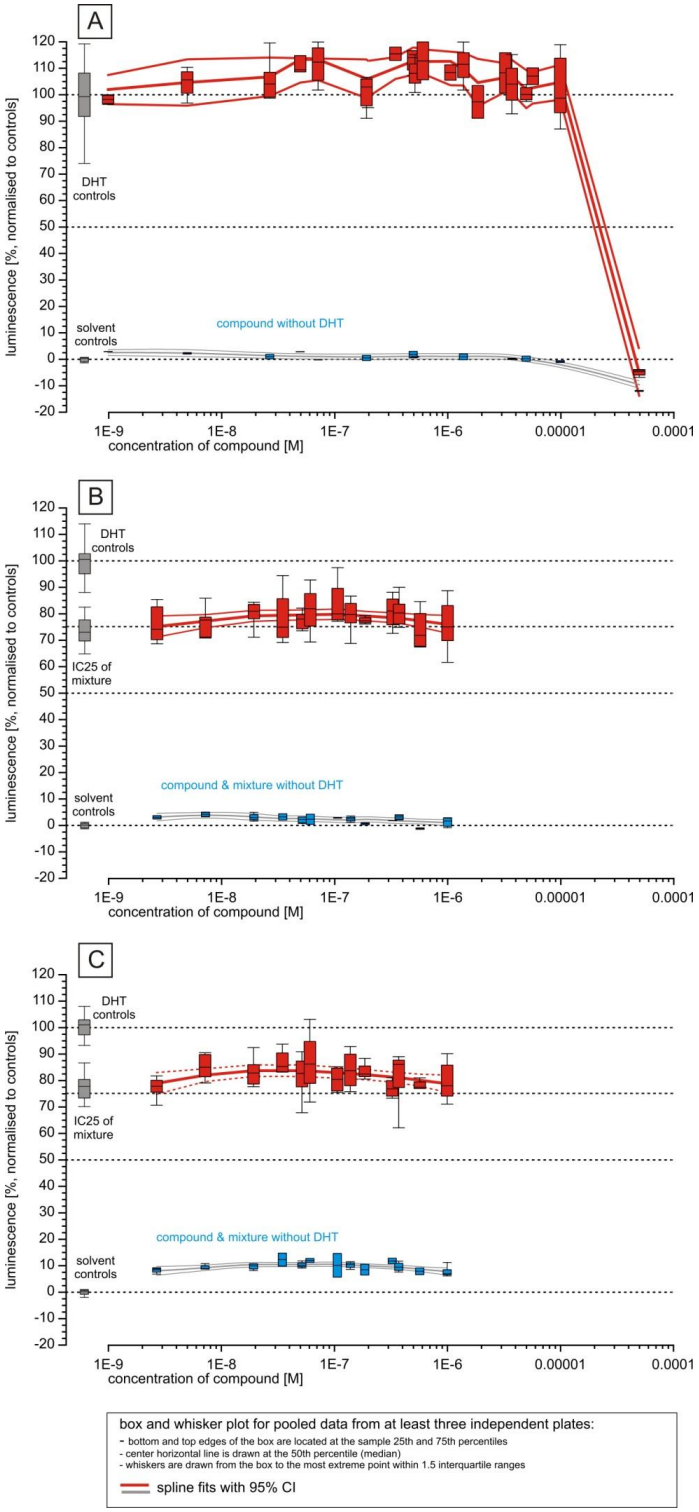


Figure C7: Testing of modulators of antiandrogenic mixture responses: DBP.
Luciferase activity of DBP in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

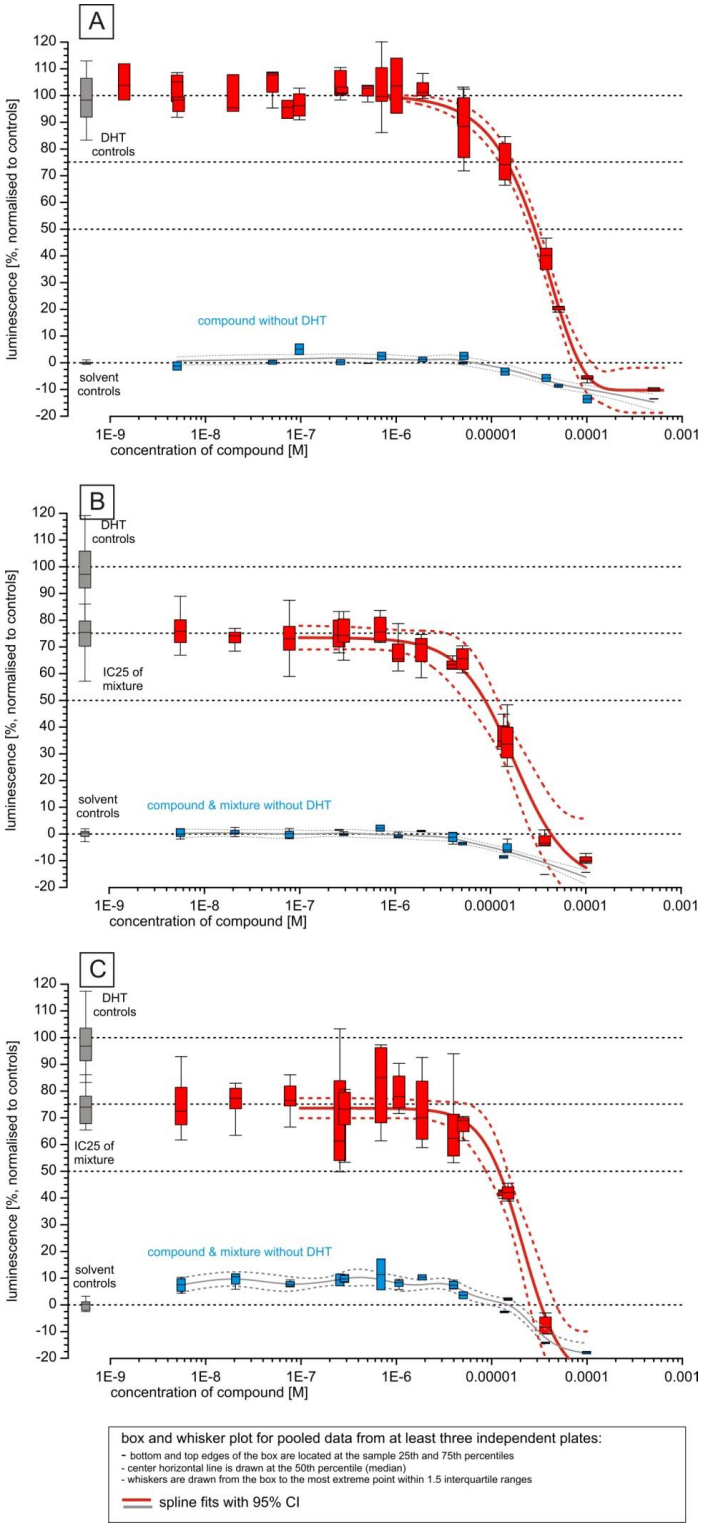


Figure C8: Testing of modulators of antiandrogenic mixture responses: Cadmium chloride.
Luciferase activity of cadmium chloride in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

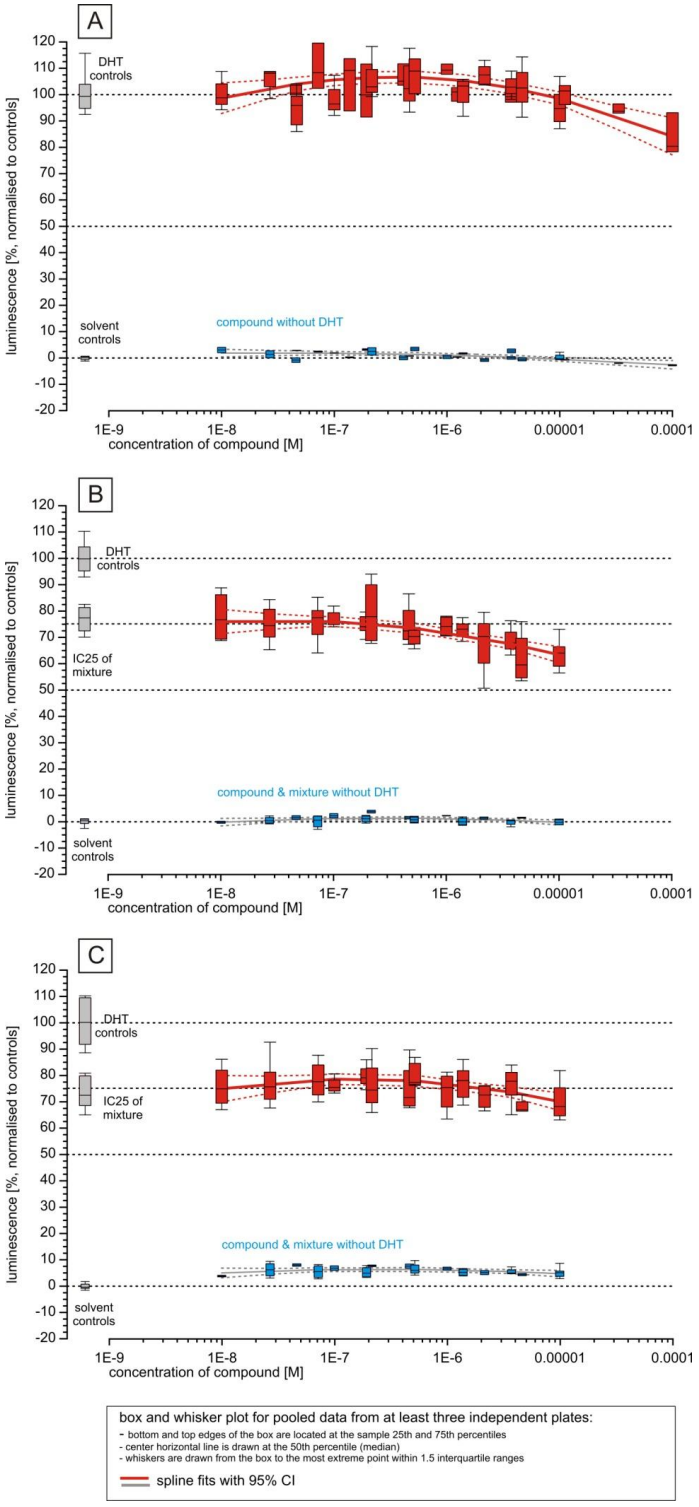


Figure C9: Testing of modulators of antiandrogenic mixture responses: Mercury chloride.

Luciferase activity of mercury chloride in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

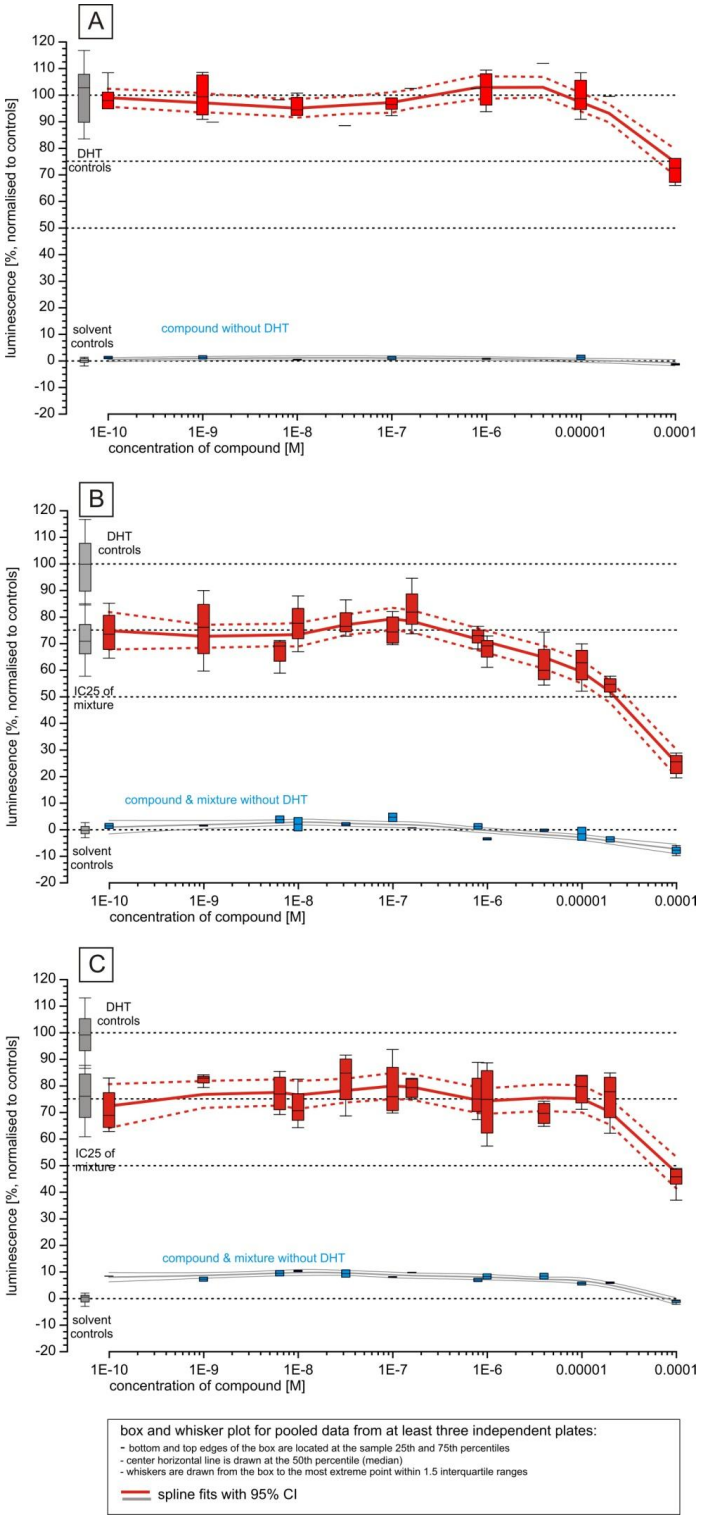


Figure C10: Testing of modulators of antiandrogenic mixture responses: Lead nitrate.

Luciferase activity of lead nitrate in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

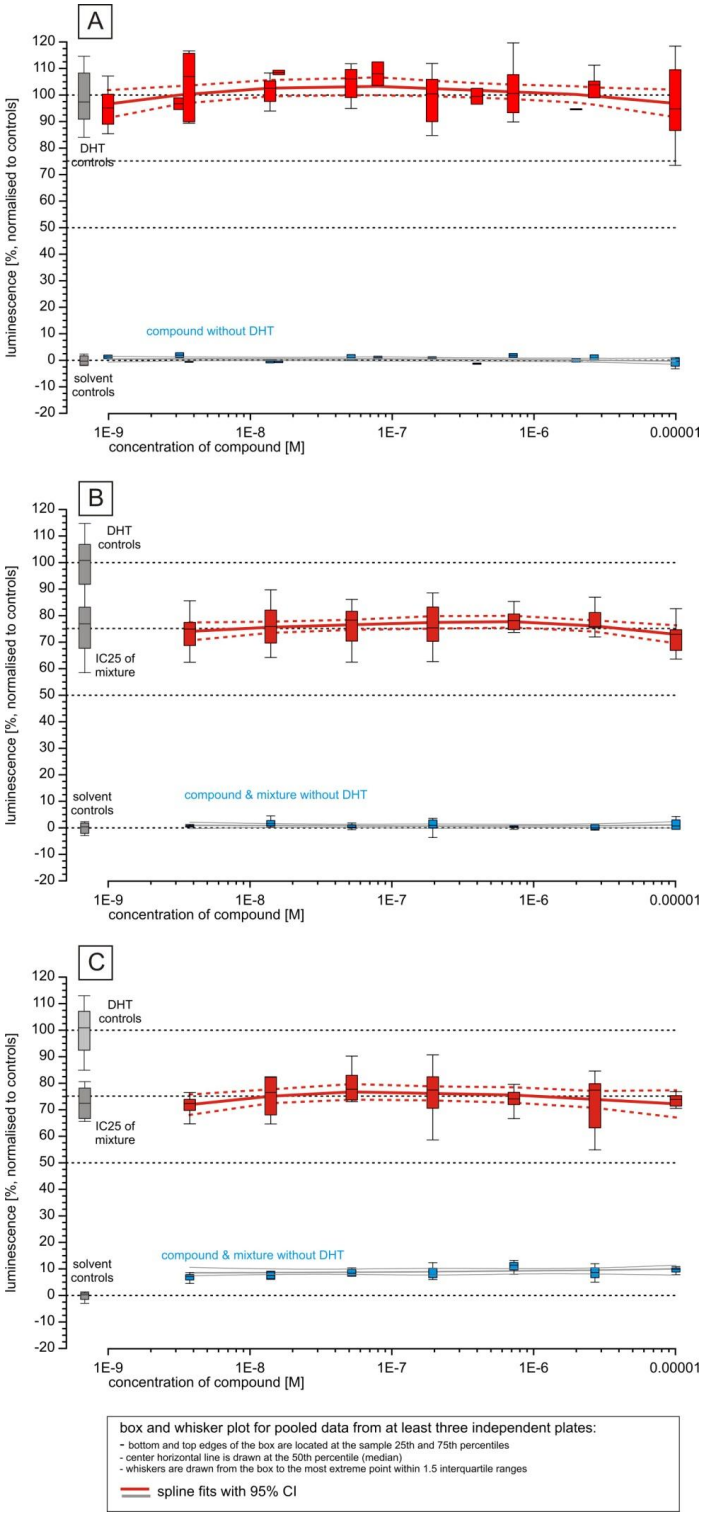


Figure C11: Testing of modulators of antiandrogenic mixture responses: PFOA.

Luciferase activity of PFOA in the presence of 0.25 nM DHT (A), of 0.25 nM DHT and the EC_{25} produced by four parabens (B), and of 0.25 nM DHT and the EC_{25} produced by 17 antiandrogens (C).

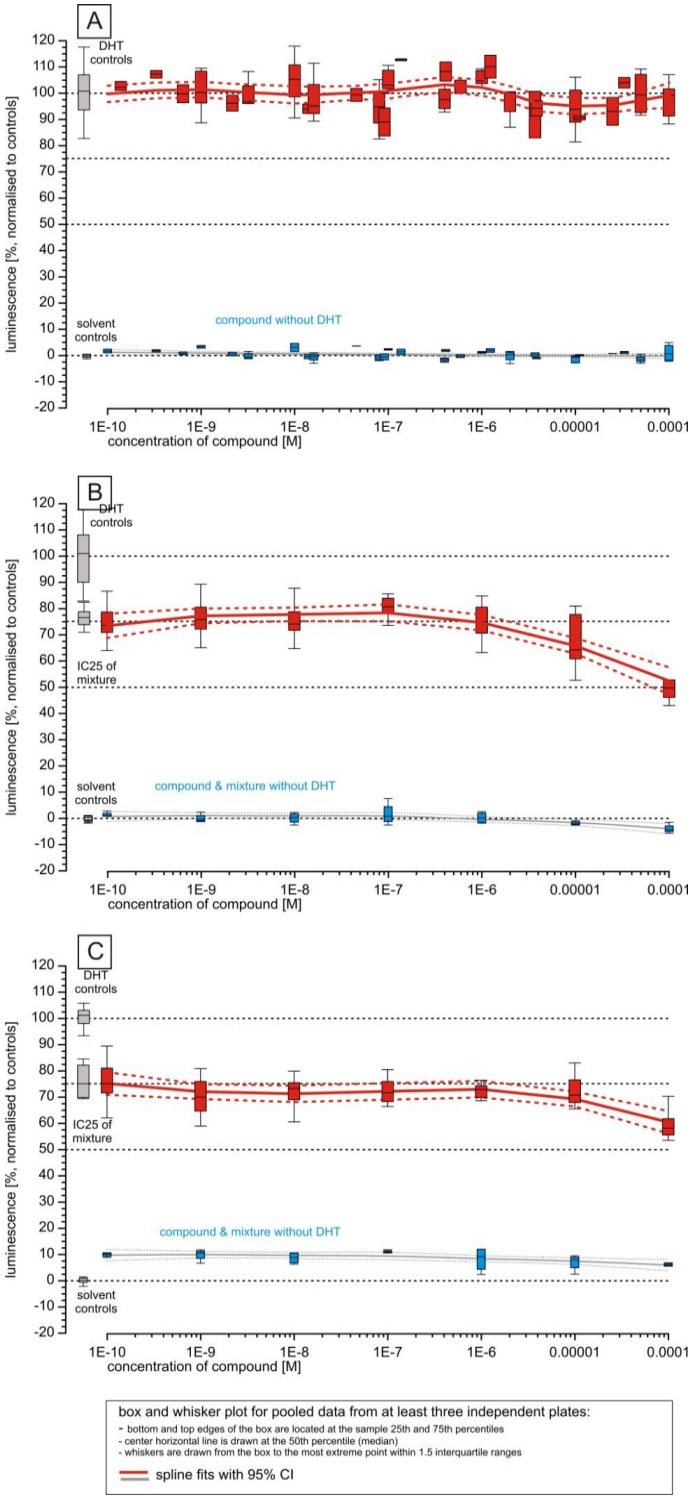
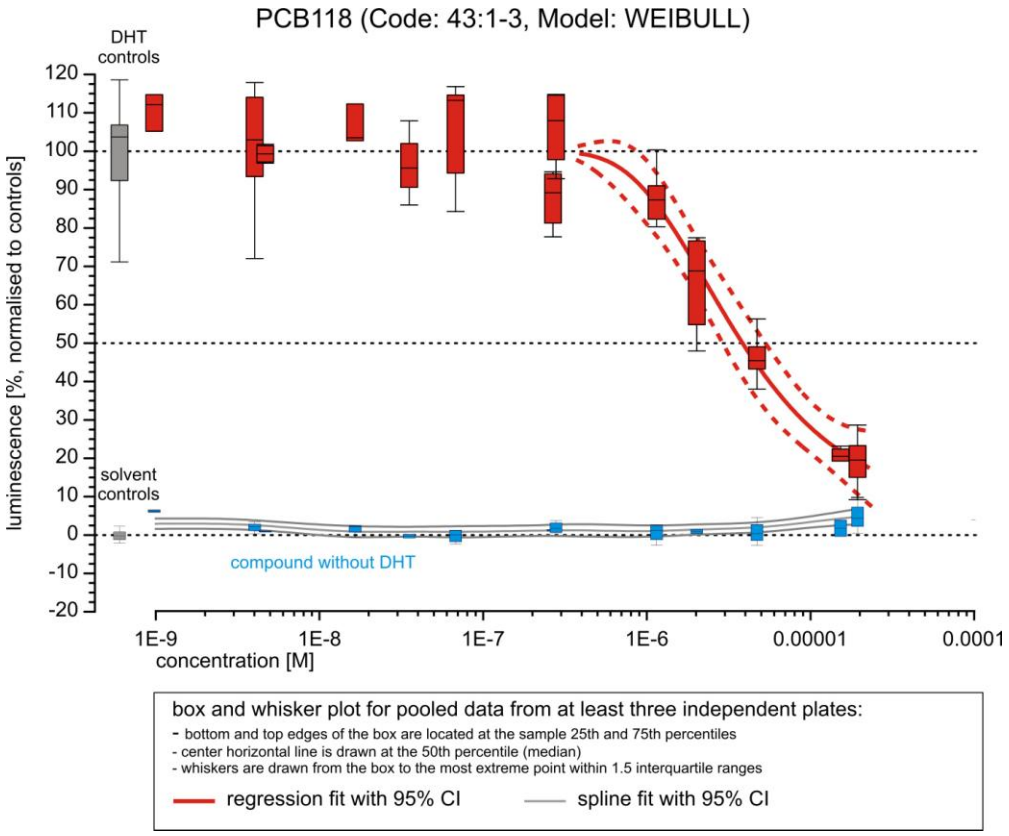


Figure C12: Testing of modulators of antiandrogenic mixture responses. PCB 118
Luciferase activity of PCB 118 in the presence of 0.25 nM DHT (red) and without DHT (blue). PCB 118 turned out to act as an antiandrogen and was thus not tested in the presence of the mixtures.



D. Computational approaches to studying effect modifiers

Within this task, computational approaches have been used with two main aims: firstly, to assist with the design of experimental studies (for example selection of modulators, formulation of expectation of modulations (occurrence and direction), prediction of affected genes/proteins/pathways); and secondly, to interpret experimental results (for example are observed changes supported by the literature, by data in repositories or by previous studies, can the results of modulation studies be reliably predicted).

The reason for developing and using computational approaches was to consider their utility for the purposes of studying mixtures of estrogenic chemicals and the potential for modulation of mixture effects. The aim is not to use these approaches immediately in a formal risk assessment.

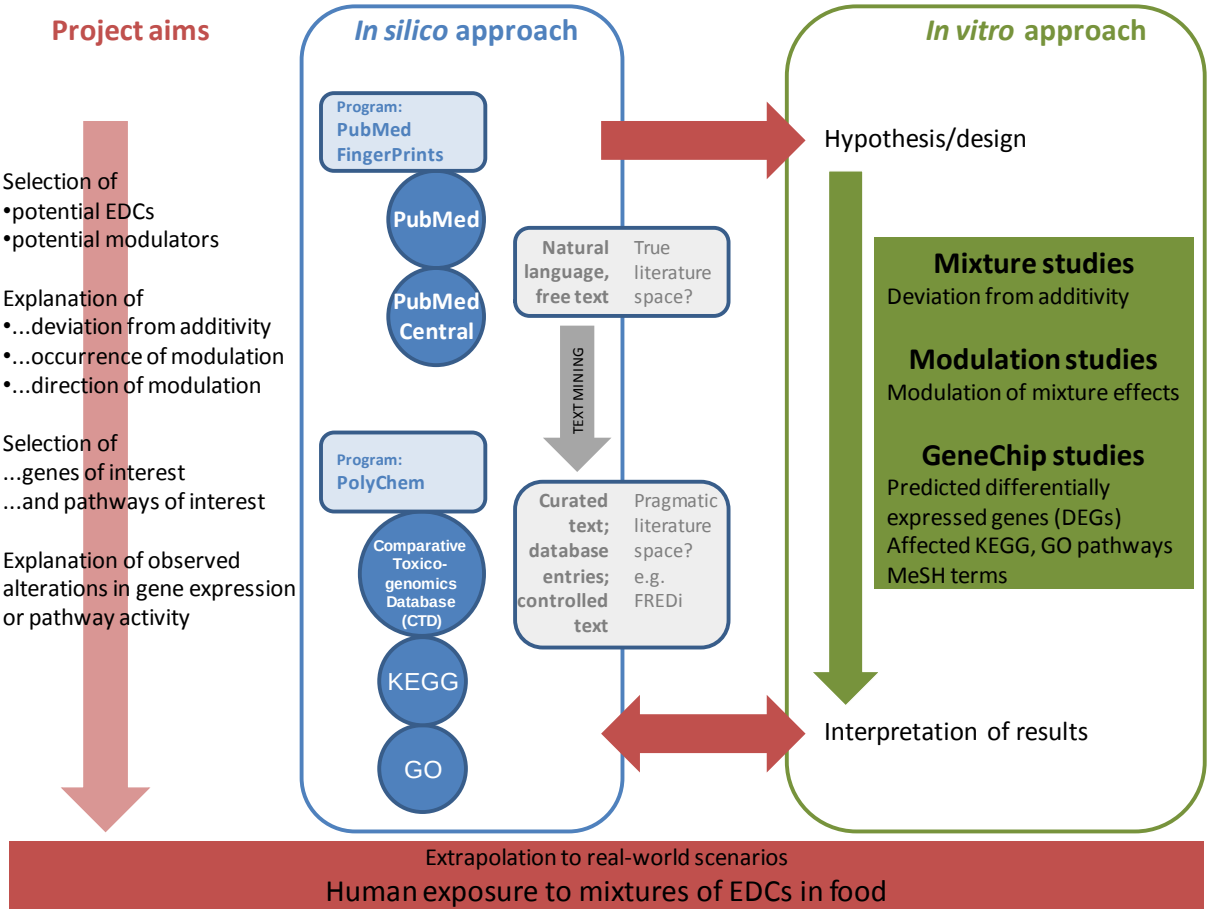
Two programs have been written, *PubMed FingerPrints* and *PolyChem (for CTD).* Both utilise public resources (PubMed and PubMedCentral for *PubMed FingerPrints*, and the Comparative Toxicogenomics Database for *PolyChem*) and provide an interface and analysis options that make these resources suitable for mixture studies. Mixture studies have specific needs because the usual dimensions of a search or analysis are increased, for example there are many chemicals instead of one or two, and there are many relevant endpoints.

Both programs are stand-alone executable files that can be run on any Windows computer. The user interfaces are simple and tools are provided to help the user with the input of lists of chemicals, search terms, identifiers etc. Both programs were written in Visual Basic using Visual Basic 2005 Express Edition (Microsoft) and the .NET framework (Microsoft, version 2.0).

Both programs make heavy use of external Web Services which are published as WSDL (Web Services Description Language) files and which allow computer programs to use SOAP (Simple Object Application Protocol) to access functions residing on remote web servers. *PubMed FingerPrints* uses the SOAP interface to the eUtils service provided by the NCBI (National Center for Biotechnology Information) to access PubMed and PubMed Central. *PolyChem* uses the SOAP API for KEGG (Kyoto Encyclopedia of Genes and Genomes) and local copies of the Comparative toxicogenomics Database (CTD), Gene ontology (GO) and GO annotations (GOA) which are downloaded and directly accessed by the program.

Each program is now briefly described and their functions illustrated in the accompanying diagrams. Figure D1 provides a schematic to illustrate how the various *in silico* and *in vitro* approaches relate to the overall aims of the project.

Figure D1: Schematic illustrating the position of computational (*in silico*) and experimental (*in vitro*) approaches in the project



Abbreviations: **EDCs**, endocrine disruption chemicals; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **GO**, Gene Ontology; **MeSH**, Medical Subject Headings; **FREDi**, Food-Related Endocrine Disruptor information (Microsoft Access database compiled as part of deliverable #1, see chapter 2).

PubMed FingerPrints program; defining a literature 'space'

PubMed FingerPrints allows the user to enter a list of chemicals of interest and a list of search terms of interest. The program then performs pair-wise searches between each chemical and each search term, searches are carried out in PubMed (for abstracts) and PubMedCentral (for full text). The results are displayed as a 'heatmap', which is a colour-shaded grid of chemical name versus search terms in which intensely coloured cells indicate searches with a large number of results. Clicking on a cell in the heatmap takes the user directly to the PubMed or PubMedCentral results; this 'click-through' approach allows the user to go rapidly from an overview of an entire area of the literature down to a single, relevant full-text paper. An example of a *PubMed FingerPrints* heatmap is shown in figure D2. The user can change the threshold that is used to colour the heatmap, and this provides different levels of information, a low threshold shows all the searches with **any** results and thus allows easy identification of data gaps; conversely a higher threshold is more stringent and gives prominence to searches with many results, allowing the user to focus on the data rich searches which are likely to contain useful information. The usefulness of different threshold values is illustrated in figure D3.

We are using *PubMed FingerPrints* to identify the literature on mixture components and potential modulators. The heatmap can be viewed as a crude analysis of the potential for interactions; if certain search terms are intensely coloured for most of the components in a mixture then that search term may well represent an important mechanism or attribute by which the effects of the mixture occur. Furthermore, if modulators are also intensely coloured for the same search terms, then the search term may indicate an area that could give rise to modulations.

PubMed FingerPrints is also able to perform a very basic level of Text Mining, a computational technique that is part of information extraction (IE). The aim of IE is to provide a user with information extracted from text sources, such as the abstracts in PubMed, rather than a list of potentially relevant sources from which the user must then manually extract information; for example *PubMed FingerPrints* can perform a PubMed search and then present the user with a list of all the sentences within the relevant abstracts that contain both the chemical and search term of interest, rather than a list of full abstracts which will contain many irrelevant sentences.

These Text Mining functions are not currently very developed but the intention is to link the users' list of search terms and chemicals, which represent 'domain-specific' information, i.e. information that an expert has manually identified as relevant, to text mining pipelines that are publicly available online. One example of these services is the WhatIzIt suite of tools provided by the EBI. In this way the current functionality of *PubMed FingerPrints* may be usefully enhanced, but without requiring a significant programming effort on our part, which would be outside the scope of this project.

Figure D2: Example of a *PubMed FingerPrints* heatmap

Figure shows part of the heatmap generated by *PubMed FingerPrints* from a list of the 14 ESCREEN mixture components and 16 potential modulators tested in the modulator studies described in this report, and a list of search terms comprised of all 51 human cytochrome P450 isoforms. Numbers shaded in green indicate the number of search results for the chemical or search term alone, numbers shaded red indicate the results for pair-wise searches of each chemical with each search term.

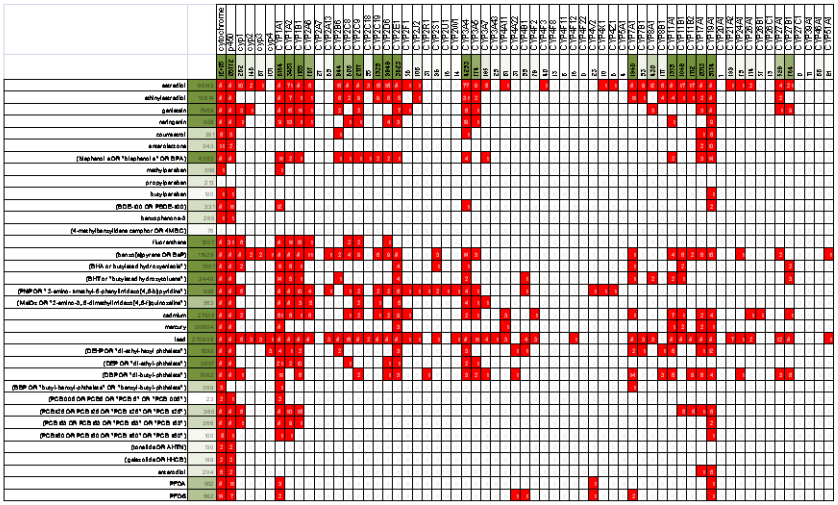
		cytochrome	p450	cyp1	cyp2	cyp3	cyp4	CYP1A1	CYP1A2	CYP1B1	CYP2A6	CYP2A7	CYP2A13	CYP2B6	CYP2C8	CYP2C9	CYP2C18	CYP2C19	CYP2D6	CYP2E1	CYP2F1	CYP2J2	CYP2R1	CYP2S1	CYP2U1	CYP2W1	CYP3A4	CYP3A5	CYP3A7	CYP3A43	CYP4A11
		1E+05	26772	282	146	87	101	6164	3461	1165	767	27	63	844	608	2177	35	1323	3348	3623	56	108	31	36	16	14	4293	174	185	29	81
estradiol	95143	#	#	10	2	1	0	#	71	#	6	0	0	15	32	40	2	15	18	20	1	1	0	0	0	0	77	9	1	0	1
ethinylestradiol	10614	#	#	0	0	0	0	24	7	1	1	0	0	8	2	9	0	9	8	5	0	1	0	0	0	0	31	2	0	0	0
genistein	7969	#	74	2	1	0	0	34	15	11	1	0	0	2	0	2	0	0	0	7	1	0	0	0	0	0	8	1	0	0	0
naringenin	958	94	59	1	0	0	0	9	13	1	1	0	0	0	1	1	0	0	2	2	0	0	0	0	0	0	19	1	0	0	0
coumestrol	351	8	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
enterolactone	343	11	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
bisphenol A	6382	60	24	0	0	0	0	11	2	1	0	0	0	1	1	1	1	2	2	1	0	0	0	0	0	0	2	0	1	0	0
methylparaben	355	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
propylparaben	213	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
butylparaben	100	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BDE100	331	24	11	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
benzophenone-3	265	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-methylbenzylidene camphor	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
fluoranthene	1207	85	31	8	0	0	0	52	11	13	1	0	0	0	2	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
(benzo[a]pyrene OR BaP)	11928	#	#	33	2	2	1	#	#	#	11	0	1	2	4	9	0	6	9	49	0	0	0	3	0	0	14	3	0	0	0
(BHA or butylated hydroxyanisole")	1801	#	38	2	0	0	0	6	5	1	0	0	0	0	0	0	0	0	0	4	0	0	0	1	0	0	1	0	0	0	0
(BHT or "butylated hydroxytoluene")	3440	#	49	0	0	0	0	14	5	1	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	2	0	0	0	0
PhIP	958	#	79	3	0	0	0	50	99	10	3	0	1	0	1	2	0	2	2	8	1	1	1	2	1	1	3	1	0	0	0
MeIQx	563	115	48	0	0	0	0	22	65	3	5	0	0	0	0	2	0	1	0	5	0	0	0	0	0	0	2	1	0	0	0
cadmium	27535	#	90	2	0	0	0	51	8	1	6	0	0	0	1	2	0	1	2	9	1	0	0	0	0	0	2	1	0	0	3
mercury	30604	#	26	0	0	0	0	23	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
lead	270938	#	#	3	2	2	1	#	92	28	20	0	3	24	18	66	2	50	#	88	1	1	0	1	0	1	#	11	3	1	3
DEHP	1899	95	28	0	0	0	2	4	1	2	0	0	0	2	0	0	0	0	0	3	0	0	0	0	0	0	2	0	0	0	0
DEP	3657	53	24	0	0	0	0	21	2	10	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	2	1	0	0	0
DBP	7062	#	46	1	0	0	0	11	0	8	0	0	0	0	0	2	0	0	1	3	0	0	1	0	0	0	3	2	1	0	0
BBP	369	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PCB 008	23	1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PCB 126	365	#	43	8	0	0	0	97	10	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PCB 153	366	56	26	1	0	0	0	29	9	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PCB 180	100	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tonalide	130	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Galaxolide	149	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
enterodiol	204	8	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PFOA	552	20	11	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PFOS	562	14	7	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure D3: Use of different threshold values to interpret heatmaps

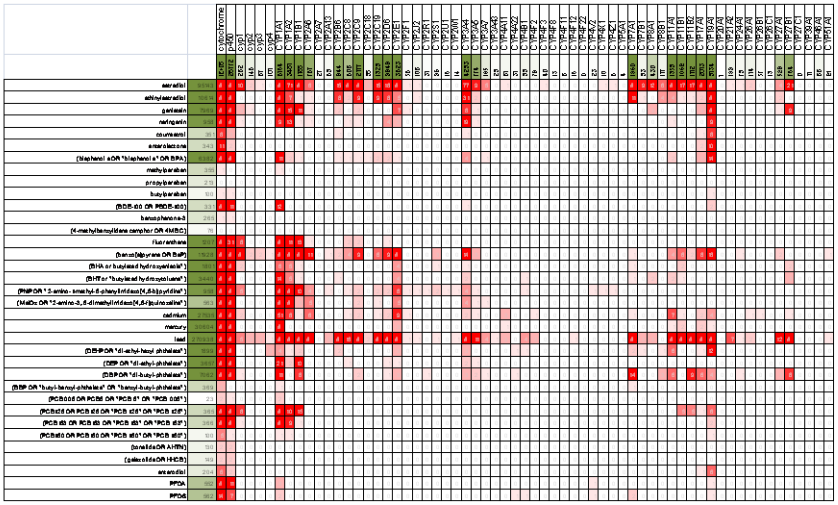
Figure shows three versions of the full heatmap shown in Figure D2. Each heatmap was drawn using a different colour threshold, which can be used to aid the interpretation of the heatmap, as indicated in the figure.

Threshold **1**

Identification of data gaps

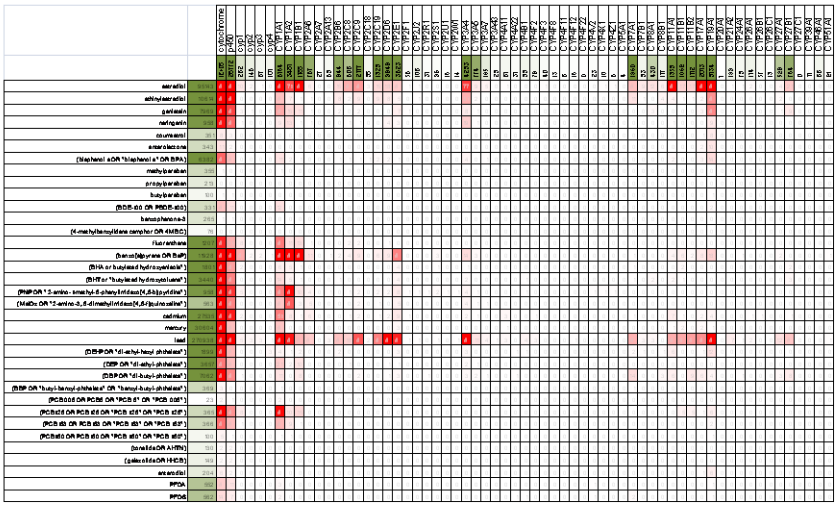


Threshold **10**



Threshold **100**

Identification of data-rich search areas



PolyChem (for CTD) program; toxicogenomics profile of EDCs

PolyChem allows the user to query the comparative toxicogenomics database (CTD, http://ctd.mdibl.org/) with a list of chemicals of interest. The CTD is a publicly-available database of toxicogenomics information compiled from the literature by curators at CTD; however the tools provided by CTD for querying the database are limited to a maximum of three chemicals and to one gene at a time (or to many genes but only one chemical). The *PolyChem* program allows mixtures to be profiled by aggregating the results for individual chemicals. There is no limit to the number of chemicals or genes that can be studied, and the only real limit is the availability of data within the CTD.

PolyChem provides the following analyses of data in the CTD:

1. An assessment of data availability, *PolyChem* presents a bar graph on which the amount of information in the CTD for each chemical of interest is shown. This allows the user to check that all of the chemicals are covered in sufficient detail, and to temper their interpretation of the results if coverage is low. See Figure D4.

2. An assessment of species contribution to the data, each data piece in the CTD is linked to a species and this information is collected by *PolyChem* and shown in a pie chart, allowing the user to check that the data is not biased to a species that might not be relevant. The results obtained to date have predominantly come from human, rat and mouse studies. Integration of results from multiple species is useful for studying potential modulations because the pathways from which modulation may arise may not have been clearly elucidated in every species. See Figure D4.

3. A profile of genes/proteins known to interact with chemicals of interest (chemical-gene interactions). The basic piece of information in CTD is a curated statement that a given chemical has an interaction with a given gene/protein. *PolyChem* aggregates this information and presents a table listing chemicals and the genes with which they are known to interact. The table is colour-coded to indicate the density of information and the list of genes can be sorted on the basis of how many of the chemicals of interest have known interactions with each gene. Genes with interactions with most of the active components of a mixture are likely to be important to the mixture effect, for example, estrogenicity, whilst genes that interact with potential modulators may be targets whereby for modulation can occur, if the gene is also important to the mixture effect. The user can click on a chemical-gene interaction to see the exact entry in CTD and can follow links to the original cited paper.

4. A comparison of the gene interaction profile of each chemical with each other chemical to give a 'comparison matrix' indicating the extent to which the profiles of each chemical overlap. The user can then interpret this depending on whether they have entered chemicals that have additive mixture effects, or chemicals that are potential modulators. See Figure D5

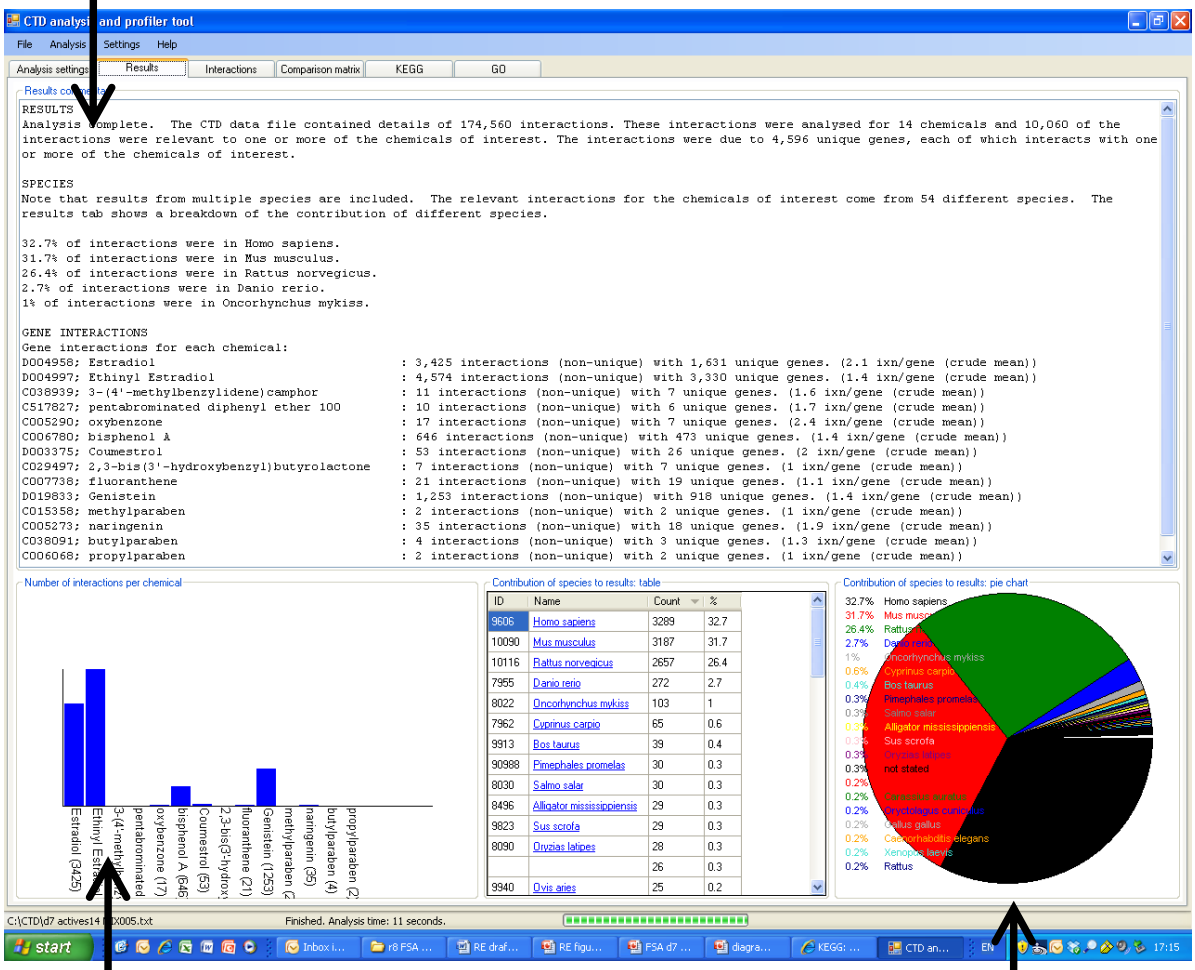
5. An analysis of KEGG pathways associated with chemicals of interest, providing one approach to the problem of interpreting lists of gene interactions. Long list of hundreds of genes can be very hard to comprehend, and *PolyChem* allows the user to reduce the dimensionality of this problem by

showing the KEGG pathways that are affected rather than the individual genes. The number of affected pathways is usually less than the number of gene interactions, and the results are given context by the KEGG annotation which can be readily linked to. The KEGG results can be shown graphically as a Circle Plot (see Figure D6) and as a more information-dense Pathway Tree (see Figure D7). In the very near future, *PolyChem* will also perform an analysis of associated GO (Gene Ontology) pathways, in a manner similar to the current KEGG analysis.

Figure D4: Screenshot showing the basic analysis provided by the *PolyChem* program

Figure shows the first results page from the *PolyChem* program, which indicates the coverage of CTD for the chemicals of interest (bar chart), and the contribution of different species to the dataset (pie chart: black, Homo sapiens; red, Mus musculus; green, Rattus rattus).

Text commentary containing details of analysis performed



Bar chart indicating data coverage Note that in this graph the first two bars, indicating estradiol and ethinylestradiol, cause the amount of data available on the other chemicals to look less than it is.

Pie chart showing the contribution of different species to the data set

Figure D5: A chemical-chemical comparison matrix from *PolyChem*

Figure shows a table in which each chemical is listed as both a row and a column, and the values in each cell indicate the overlap of the genes that the pair of chemicals is known to interact with.

Matrix shows percentage or fraction of genes known to interact with a chemical (column, x) that are also known to interact with each other chemical (row, y)

	butylbenzyl phth	Dibutyl Phthalate	Diethylhexyl Pht	diethyl phthalate	PCB 180	2,4'-dichlorobiphe	Cadmium	Cadmium Chlorid	Lead	lead chloride	Mercury	2-amino-1-meth	2-amino-3,8-dim	Butylated Hydrox	Butylated Hydrox	Benzo(a)pyrene
butylbenzyl phthalate	100	16	14	50	0	100	2	2	2	0	1	0	0	8	9	2
Dibutyl Phthalate	81	100	36	70	38	100	4	6	2	14	3	10	0	8	4	4
Diethylhexyl Phthalate	86	43	100	70	25	100	9	9	9	29	3	5	0	28	11	8
diethyl phthalate	24	6	5	100	12	100	1	2	7	0	1	0	0	8	2	1
PCB 180	0	3	2	10	100	0	1	1	0	0	0	14	25	0	0	2
2,4'-dichlorobiphenyl	5	1	1	10	0	100	0	0	0	0	0	0	0	2	2	0
Cadmium	24	10	20	40	38	100	100	67	41	43	22	29	0	40	20	17
Cadmium Chloride	24	16	22	60	50	100	74	100	30	43	21	38	25	45	20	18
Lead	5	1	3	30	0	0	7	5	100	14	2	0	0	5	2	3
lead chloride	0	1	2	0	0	0	1	1	2	100	1	0	0	0	0	1
Mercury	14	6	6	20	12	0	22	18	11	29	100	14	0	12	7	6
2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine	0	2	1	0	38	0	2	3	0	0	1	100	75	8	2	5
2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline	0	0	0	0	12	0	0	0	0	0	0	14	100	0	0	0
Butylated Hydroxyanisole	14	3	9	30	0	100	6	6	4	0	2	14	0	100	20	6
Butylated Hydroxytoluene	19	2	4	10	0	100	3	3	2	0	1	5	0	22	100	4
Benzo(a)pyrene	24	12	20	40	62	100	20	18	17	43	8	67	25	50	29	100

Figure D6: Example of a Circle Plot for KEGG analysis in *PolyChem*

Figure shows a Circle Plot (upper, box) and an expanded view of one area of the plot (lower). A Circle Plot indicates the KEGG pathways that chemicals may interact with, and is based on the genes that each chemical interacts with. The inner, concentric circle of dots (black, grey fill) shows all of the KEGG pathways affected by any of the chemicals of interest. Working outwards, each concentric circle (coloured dots, see legend) then shows whether a specific chemical affects the specific pathway. The lower expanded view of part of the Circle Plot illustrates the interpretation of the plot: common pathways that may be affected by many or all of the chemicals become very apparent visually. Conversely pathways that owe their presence in the plot to one or two chemicals are also clearly seen and could represent important areas for considering the differences between chemicals.

Note that when the Circle Plot is viewed inside the PolyChem program moving the mouse over each dot shows information about the KEGG pathways, and dynamic information is presented to the user in a manner that cannot be appreciated from the static view shown in the figure.

See figure on following page.

FigureD6

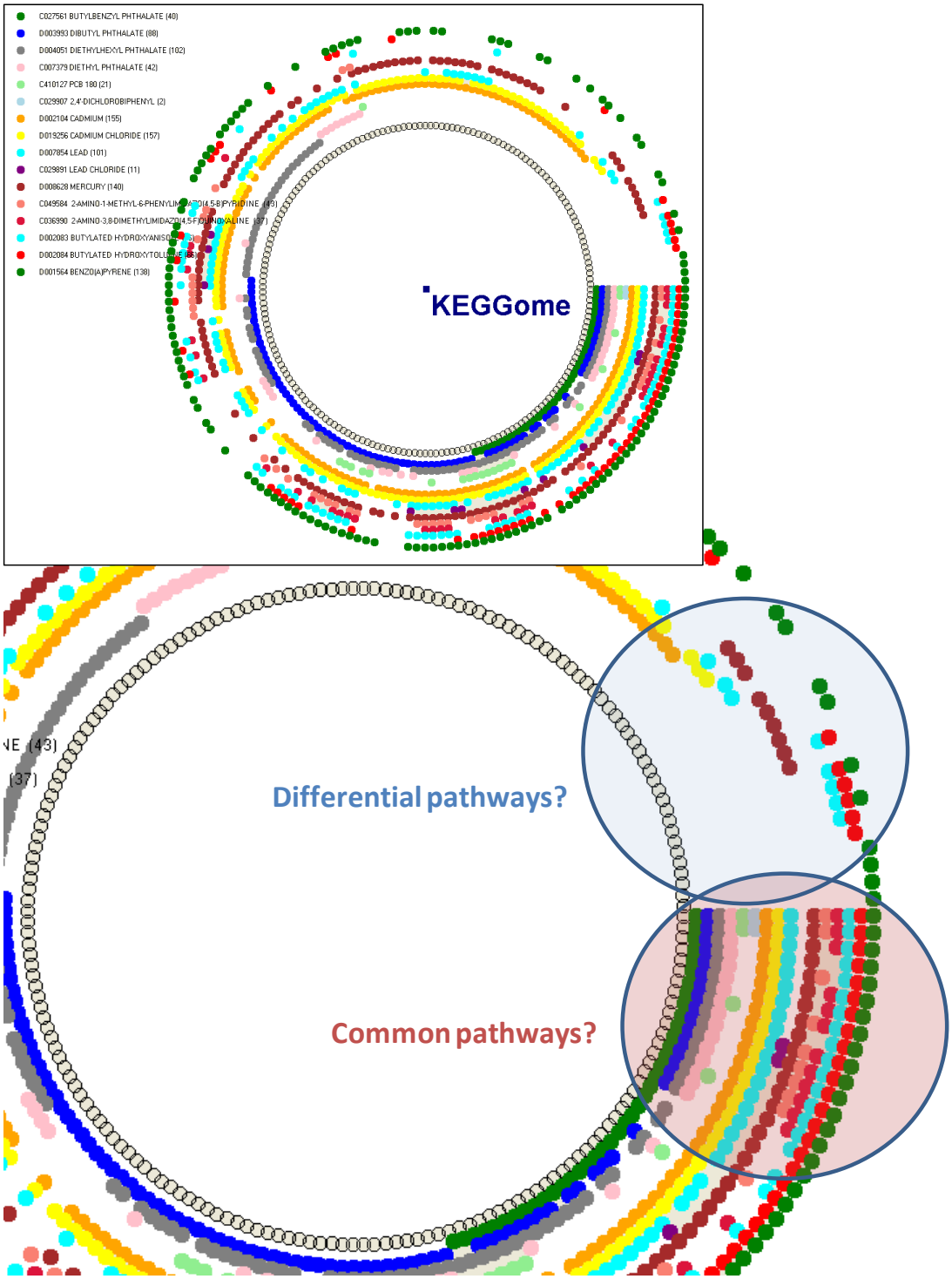

C027561 BUTYLBENZYL PHTHALATE (40)
D003993 DIBUTYL PHTHALATE (88)
D004051 DIETHYLHEXYL PHTHALATE (102)
C007379 DIETHYL PHTHALATE (42)
C410127 PCB 180 (21)
C029907 2,4'-DICHLOROBIPHENYL (2)
D002104 CADMIUM (155)
D019256 CADMIUM CHLORIDE (157)
D007854 LEAD (101)
C029891 LEAD CHLORIDE (11)
D008628 MERCURY (140)
C049584 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-B]PYRIDINE (43)
C036990 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-F]QUINOXALINE (37)
D002083 BUTYLATED HYDROXYANISOLE (3)
D002084 BUTYLATED HYDROXYTOLUENE (88)
D001564 BENZO(A)PYRENE (138)
KEGGome
Differential pathways?
Common pathways?

Figure D7: Example of a Pathway Tree for KEGG analysis in *PolyChem*

Figure shows a Pathway Tree in two versions, A) collapsed and B) with one section fully expanded. The Pathway Tree shows similar information to a Circle Plot, namely the KEGG pathways affected by chemicals of interest, but is more information dense.

KEGG pathways are organised into three levels, the top level contains very general umbrella terms, such as “Human Diseases”, the second level contains sub headings such as “Cancers”, whilst the third level is the most specific and conatins terms such as “Pathways in Cancer”, “Prostate Cancer” etc. The third level contains the actual KEGG pathways, which are expert-curated, manual drawings of pathways. In a Pathway Tree colour shading is used to indicate the relevance of the second (blue) and third (red) level terms to the group of chemicals being considered. Each third level KEGG term is also accompanied by a ‘BarDash’ plot (see b) showing which chemicals affect the individual pathway, in this way the user can immediately see how relevant the pathway is and can begin to discern patterns in the BarDash plots that relate to sub-groups of chemical with common KEGG effect profiles.

See figure on following pages

Figure D7

A) collapsed view

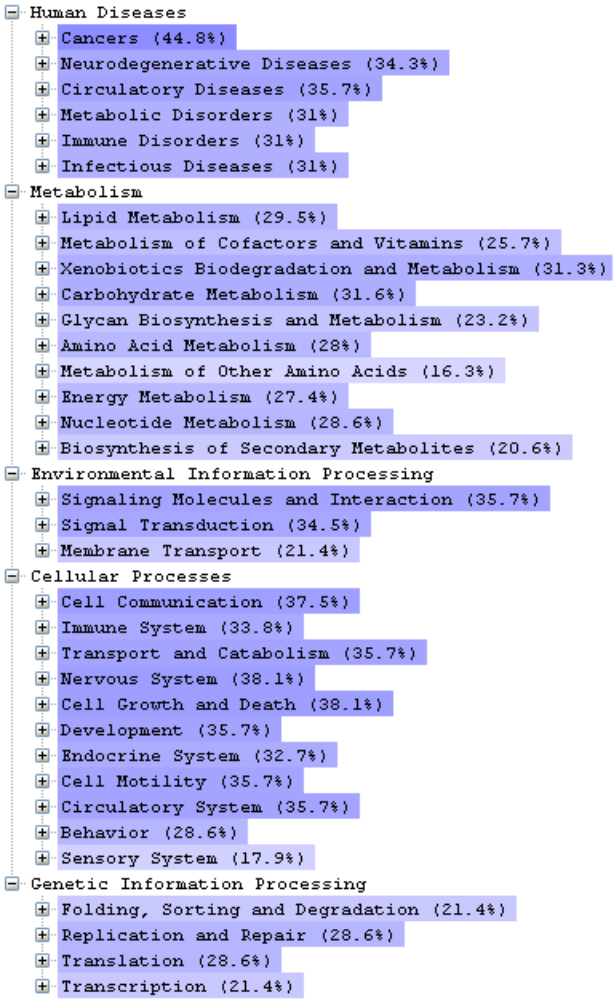
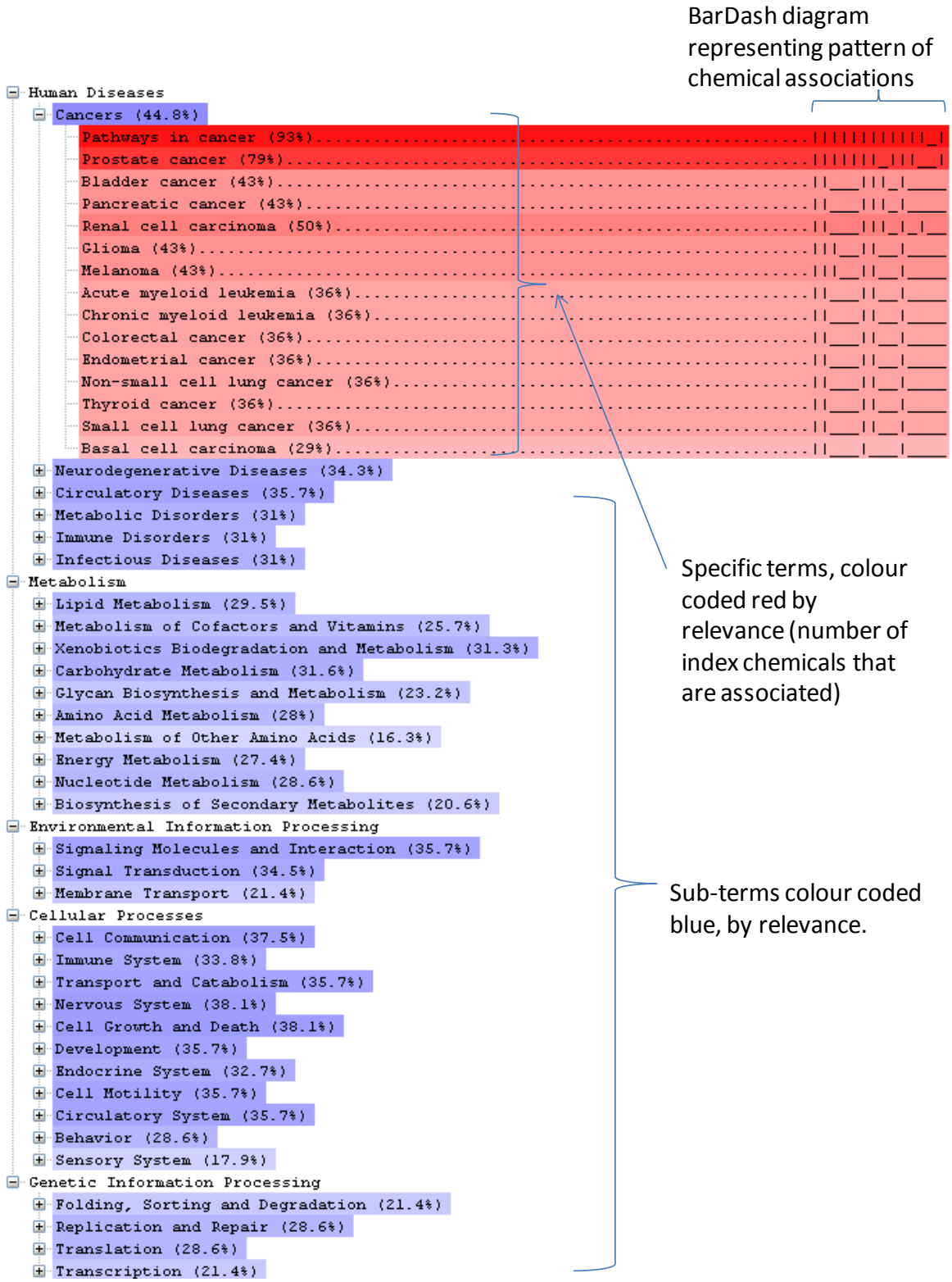


Figure D7

B) expanded view



GeneChip hypotheses derived from *PolyChem* analyses

14 component mixture of estrogenic food additive and contaminants

Table D1 lists, in alphabetical order, 150 genes that were found by *PolyChem* to have an interaction with 4 or more of the chemicals in the reference mixture. Six of the genes were found to have an interaction with 7 or more (>50%) of the mixture chemicals. 17 genes were found that interact with 4 or more of the potential modulators, see also following paragraph and Table D2.

We hypothesise that these 150 genes will be differentially expressed genes (DEGs) for the reference mixture in a GeneChip study.

16 component mixture of potential modulators

Table D2 lists, in alphabetical order, 53 genes that were found by *PolyChem* to have an interaction with 4 or more of the potential modulators. 17 of these genes were also found to have an interaction with 4 or more components of the reference mixture, see Table D1.

We hypothesise that these 53 genes will be differentially expressed genes (DEGs) for the mixture of modulators in a GeneChip study, and that the 17 genes that interact with chemicals in both the reference and modulator mixtures, will be lead targets to explain the effect of the mixture of modulators on the reference mixture.

Table D1: 150 genes found by *PolyChem* to interact with four or more of the chemicals in the reference mixture

ABCC1	CDKN3	FEN1	MYB	SERPINB9
ABCC2**	CEACAM1	FKBP4	MYBL2	SHBG
ACTA2	CHEK1	**FOS****	**MYC****	SLC2A1
AGR2	CKB	FSHB	MYH6	SLC6A14
AHNAK	CKS1B	**GADD45A****	NCAPD2	SLCO1A1
AHR* **	CLU	GJA1	NCOA2	SOD2
ALAS1	COL12A1	GMNN	NDRG2	SOX4
ALPL	COL1A1	GSTO1	**NFKB1****	SQSTM1
APOB	CPE	H2AFX	NR1I2	STAR
AR* **	CRISPLD2	H2AFZ	OLFM1	STAT3
ARL3	CTPS	HMGB2	OLFML3	STMN1
ATF3	CTSC	HSD11B2	OXT	SULT1A1
ATP5O	CXCL10	HSD17B1	PBK	SULT1E1
AURKB	CYP17A1	HSP90AA1	PCNA	TACC3
BCL2	CYP19A1	**HSPA1A****	PCP4	TFF1*
BGLAP	CYP19A1A	HSPCB	PGR*	TFF3
BHLHB2	CYP19A1B	ID3	PIAS3	TIMP3
BIRC5	**CYP1A****	IGF1	PLK4	TK1
BTG1	**CYP1A1****	IGF1R	PLOD2	TMEM97
BUB1B	**CYP1B1****	IGFBP4	POLD4	TOP2A
C3	EBP	IL2	PPAP2B	TPM3
CAMK2N1	EDG2	KIF20A	PPAP2C	UBE2C
CASP3**	EGR1	KIF23	PPID	UGT1A1
CCNB2	EMP2	LDHA	PRL	UGT1A10
CCND1	ENO1	**MAPK1****	RACGAP1	UGT1A7
CDC20	ENPP2	**MAPK3****	RAD51AP1	UGT1A8
CDC6	ERBB3	MCM2	RBP4	UGT1A9
CDK2AP2	**ESR1*** **	MCM6	RRM2	VEGFA
CDKN1A**	**ESR2*** **	MELK	S100G	WNT5A
CDKN2C	ETS2	MGST1	SELENBP1	ZFP36L1

* one of six genes found to interact with seven or more (>50%) of the chemicals in the reference mixture

** one of 17 genes found to interact with four or more of the chemicals in the mixture of modulators

Table D2: 53 genes found by *PolyChem* to interact with four or more of the chemicals in the mixture of modulators

ABCC2**	EPHX1	GSTP1	LHCGR	NFKBIA
ABCG2	**ESR1****	HBB	**MAPK1****	NQO1
ADRA1A	**ESR2****	HMOX1	**MAPK3****	PPARG
AHR**	**FOS****	HNF1A	MAPK8	PTGS2
AR**	**GADD45A****	HSP70	MT1	RELA
CASP3**	GCLC	**HSPA1A****	MT1A	TNF
CAT	GPER	HSPA5	MT2	TP53
CDKN1A**	GPT	IFNB1	MT2A	TRP53
CYP1A**	GPX2	IFNG	**MYC****	TXNRD1
CYP1A1**	GSTA1	IL10	NFE2L2	
CYP1B1**	GSTA3	JUN	**NFKB1****	

** one of 17 genes found to interact with four or more of the chemicals in the reference mixture

Conclusions

ERLUX and ESCREEN

Studies of individual potential modulators of estrogenic effects have been performed in ERLUX and ESCREEN assays. Individual modulators showed either no modulation or a negative modulation, whilst positive modulations were not seen. A mixture of 16 potential modulators was studied and was found to negatively modulate a 14 component mixture of estrogenic substances. The possibility that this negative modulation is due to toxicity may need to be examined. A GeneChip study is proposed and offered for discussion with FSA. Two computer programs were written to assist with the design and interpretation of these studies, and allowed us to formulate specific hypotheses in terms of predictions of differentially expressed genes.

Plan for ongoing experiments

According to the original grant proposal, the negative modulation shown by the mixture of modulators against a 14 component mixture of active chemicals in the ESCREEN (Figure B5), could now be explored in a genomic study.

However, due to the large cost of such a study, and the need for careful design prior to execution, we would like to discuss this plan with FSA before continuing.

The proposed study would utilise the Affymetrix GeneChip platform (available through the Institute of Child Health, UCL), and test the specific hypotheses set out in section C (Tables C1, C2, and accompanying text). The genomic profile of MCF7-BUS cells would be compared under the following treatments:

1. Vehicle (0.5% ethanol).
2. Reference mixture (14 components) at a concentration evoking a 50% effect.
3. Estradiol at a concentration evoking a 50% effect.
4. Reference mixture (50% conc.) plus the mixture of modulators (16 components) at a concentration that reduces the effect by around 50% (50% inhib. conc.).
5. Mixture of modulators (50% inhib. conc.) plus an increased concentration of the reference mixture such that the evoked effect is returned to 50%.

Treatments 2, 3 and 5 allow important comparison of the genomic profile under different conditions but with the same integrated, functional endpoint (a 50% proliferative effect).

Alternatives to an immediate GeneChip study could include:

- Further examination of potential for toxicity to explain the negative modulation, prior to a GeneChip study.
- Examination, in a GeneChip study, of the effects of a single modulator for which toxicity appears less likely an explanation of the negative modulation, on the basis of the available data. A possible candidate would be PCB126 which showed a negative modulation that reached a plateau above zero, indicating that any toxicity that is present is incomplete.

ARLUX (antiandrogenicity assay, reporter-gene endpoint)

Mixture testing

The 10 component mixture, which exhibited a deviation from concentration addition was further analysed by splitting it into two sub-mixtures. The components for these mixtures were chosen according to the shape of the response curves of the individual compounds within the mixture. Four of the compounds showed a steeper individual response as compared to the other six. The deviation of the 10 component mixture was more pronounced at the lower mixture concentration, where the compounds with the steeper individual effect curves contributed less to the mixture effect than at higher concentrations. Furthermore, the slope of the 10 component mixture concentration response curve was similar to the steeper slope of the four compounds. Thus, the subdivision according to slope could might give some information about the chemicals responsible for the deviation. Both of the tested mixtures acted according to concentration addition, so it was not possible to narrow the number of chemicals down, which might contain the compound, causing the deviation. Currently, both mixtures, together with the 10 component mixtures are investigated by quantitative RT-PCR, to find out, whether the regulation of certain CYP enzymes differ between the mixtures and, thus, could be responsible for the deviation from concentration addition seen in the 10 component mixture.

Furthermore, we tested a mixture of 17 compounds, to examine, whether the deviation persists with an increasing number of chemicals. This mixture also included compounds, which were not only antiandrogenic but also showed an androgenic response when tested without DHT. Although this mixture contained all the chemicals which were also present in the 10 component mixture, it acted according to concentration addition. This can be explained by the fact, that with increasing numbers of chemicals, the contribution of each individual chemical to the mixture effect is less pronounced. Thus, if the deviation seen in the 10 component mixture was caused by one specific compound e.g. by induction of cytochrome P450 emzymes (CYPs) or ABC efflux pumps, thus decreasing the concentration of one active compound, this effect may not be detected in a mixture composed of a higher number of chemicals.

Effect modulators of antiandrogenic mixtures

Candidate chemicals for potential effect modulators of antiandrogenic combination effects were heavy metals, mutagens such as benzo(α)pyrene, PBDEs and PCBs. The perfluorinated compound PFOA did not exhibit any antiandrogenic activity, but was recently described to have an effect on certain CYP isoforms in the rare minnow (Liu et al. 2008). During early testing PCB138, BDE100 and benzo(α)pyrene exhibited antiandrogenic activity and, thus, were included in the mixtures of antiandrogens. The co-planar PCB118 tested later as another candidate compound to for modulation, but was also found to be antiandrogenic.

The majority of tested potential modulators did not show any modulation of mixture effects. The only effect observed was cytotoxicity at higher test concentrations. For DBP it was not clear, whether cytotoxicity is masking a potential antiandrogenic effect which might add to the antiandrogenicity of the mixture compound and effect which can be observed if the concentration range of antiandrogenic activity overlaps with the concentration for cytotoxicity (Frische et al. 2009). The only compound that showed a potential modulatory effect was PFOA. Whereas PFOA in its own did not show any effect or toxicity by itself, it increased the antiandrogenicity of both mixtures at higher concentrations. Another explanation for the decrease in luciferase signal could be toxicity that occurs when PFOA is combined with the mixture. Generally, any observed effects where at rather high concentrations which are probably of now relevance with regard to human exposure. To investigate this further, a selection of these chemicals will also be included in the mixtures that will be designed according to human exposure to see if any effects can be observed in these more realistic scenarios.

General conclusions

In summary, the general aims of the modulation studies proposed as task 3 of the project could be realised. It is now possible to begin work on the final part of the project, the testing and evaluation of mixtures composed according to information about the tissue levels of the investigated endocrine active chemicals.

Chapter 5:
Assessment of Mixtures of Food Contaminants and Additives That Reflect Human Tissue Levels

Task 4

Introduction

This chapter describes task 4 of the project T01045, “Assessment of mixtures of food contaminants and additives that reflect human tissue levels”.

The work on the previous tasks of this project has shown that the prediction and assessment of mixtures composed of more than 15 components is conceptually and technically feasible. It is clear that inclusion of more and more agents in a mixture will diminish the concentrations of each individual component necessary to produce an overall mixture effect. The question that informed work on task 4 was: Are there combined effects of the selected estrogenic or anti-androgenic chemicals at levels approaching those reported for human tissues for each individual chemical? Are these combination effects predictable on the basis of single chemical concentration-response relationships? Can such effects be demonstrated with the *in vitro* assays for estrogenicity and androgen receptor antagonism utilized in this project?

Here, we report on experiments with estrogenic and anti-androgenic chemicals that were designed to address this question.

As the dose metric for these studies we chose molar concentrations of estrogenic and anti-androgenic chemicals in human tissues. Because we utilized *in vitro* assays, human intake could not be used as the dose metric. In many cases, the concentrations measured in serum were used to guide decision making about the composition of the tested mixtures. Where necessary, adipose tissue levels were used, but adjusted to serum levels, assuming average lipid contents of serum. The data in the literature reflect internal exposure to the chosen chemicals, after uptake, absorption and metabolism.

We have examined mixtures containing both active estrogens or anti-androgenic agents and potential modulator compounds with no or weak activity and which were designed using a fixed ratio based on measured human tissue levels, so-called ‘real-world’ mixtures. In contrast to the mixtures tested in previous tasks, which had a fixed ratio based on equi-effective levels, this ‘real-world’ design means that the components of the mixture are present in similar proportions to a human internal exposure scenario. Mixtures of up to 29 components were tested in both the ESCREEN and ERLUX assays, and the utility of mixture effect predictions using the concentration addition model was evaluated.

Mixtures containing anti-androgenic as well as inactive chemicals were investigated using different exposure scenarios (average and high). Anti-androgenic mixtures of up to 31 compounds were tested in the MDA-kb2 assay to assess whether they acted according to the mixture effect predicted by the concentration addition model.

Materials and methods

ERLUX and ESCREEN

Detailed descriptions of the assays and mathematical models used were presented in previous deliverables, see chapter 3, and will not be reiterated here.

Mixture formulation

The mixtures used in this task were all designed using a fixed mixture ratio proportional to the chemical concentrations found in human tissues, mostly human serum. We refer to these mixtures as 'real-world' (RW) mixtures. The chemicals included in the mixtures in this section of the report are listed in Table 1.

To select RW mixture ratios, tissue concentrations that were identified during Task 1, see chapter 2 and appendix 1, were entered into a database; concentrations were then converted into a common unit, lipid adjusted if required (value in ng/g lipid multiplied by 6 to give ng/L serum), and serum levels from normal adults were selected if available. If serum levels were not available, levels in human milk or from breast tissue biopsy were used as an interim measure; concentrations were used directly without compensation for conversion between tissues, representing a worse case scenario. Analysis of mixture effects through the toxic unit approach, see below, was used to examine the consequence of these assumptions on the overall credibility of the resulting mixture design. If data from different geographical areas was available, data from Europe or the UK were selected but in many cases a full range of data was not available and data from other geographical areas was used. Finally, the available data was averaged over different studies.

Purpose written software (db96, RE) was used to make CA predictions and plot toxic unit distributions for the RW case, then the following adjustments were made:

- The estradiol level was reduced to avoid dominance of the mixtures by a single component, note that selecting a single concentration as the relevant human exposure to estradiol is not trivial since there is a wide physiological range with variation due at least to gender, age and menstrual status.
- The contribution of other components was inspected by TU analysis and reduced if the component would dominate the mixture; levels remained within the realistic human range.

The aim of adjustment was not to produce a balanced mixture, since this was achieved in earlier tasks using equi-effective mixture designs (see chapters 3 and 4), but to produce a mixture more relevant to a human exposure scenario. However, it was still necessary to ensure that the final mixture was not so biased towards one or two components that it became trivial i.e. the mixture effect is almost entirely due to one or two components and the test becomes not a mixture test but a test of the few dominant components.

Human tissue concentrations of phthalates

It has been proposed that some of the historical published data on phthalate levels in human tissues is erroneously high due to laboratory contamination by the parent phthalate compounds (diesters). More recent studies report levels of phthalate metabolites, such as monoesters, which arise from metabolic conversion, rather than levels of parent phthalate compounds which can be due to contamination (Barr *et al*. 2003). The available data on phthalate metabolites could not be used directly in our mixture designs since we used the parent phthalate compounds for single chemical testing. Consequently we addressed this data gap by performing two mixtures, see following results section on modulator mixture testing; one mixture contained phthalates at the reported human exposure concentrations and which may be artificially high, and a second mixture containing phthalates at reduced concentrations which may be closer to the real human exposure scenario.

Testing strategy

Mixtures of actives or modulators were tested at eight concentrations spanning the defined "RW" level. Mixtures were formulated so that the highest concentration tested was 10-times the 'real-world' level. Use of a dilution series with a dilution factor of 2.15 meant that the fourth dilution was equivalent to "1X", and might be considered to reflect the human exposure situation for active chemicals. Combination experiments were performed using the fourth dilution of the actives mixture and a dilution series of the modulator mixtures, consequently the combination of the fourth dilution of both actives and modulators can be considered to represent a 'real-world' scenario for both active and modulator chemicals.

Toxic units

Toxic unit plots were used to show the contribution of each component to the mixture effect, assuming concentration addition, and are derived during the calculation process for CA predictions. For a multi-component mixture of n chemicals the concept of *CA* states,

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

For a given chemical and a given effect concentration, the TU is calculated by dividing the concentration (c_i) of the chemical in the mixture (at a mixture concentration that has the given effect), by the concentration (ECx_i) of the chemical that has the given effect when applied alone. The toxic units add up to 1 if a mixture follows concentration addition. In this sense TUs provide a breakdown of the concentration addition prediction.

Default additivity expectation

In previous tasks we have evaluated two additivity models: concentration addition (CA) and independent action (IA). We have routinely found that CA is the more useful model and therefore in this task we have considered CA to be the default additivity model and have not considered IA any further.

Table 1: List of chemicals tested in mixtures designed in proportion to human tissue concentrations

*Compound**	*Abbreviation*	*Chemical Group*
Bisphenol A	BPA	Bisphenol A
Methylparaben	MethylP	Paraben
Propylparaben	PropylP	Paraben
Butylparaben	ButylP	Paraben
Benzophenone 3 (Eusolex 4360)	BP3	UV-filter
Genistein	GEN	Phytoestrogen
Estradiol	E2	Endogenous hormone
4-Methylbenzylidene Camphor	4MBC	UV-filter
Fluoranthene	FLUOR	PAH
Naringenin	NAR	Phytoestrogen
Enterolactone	ENL	Phytoestrogen
Coumestrol	COU	Phytoestrogen
Polybrominated diphenyl ether 100	BDE100	PBDE
Benzo[a]pyrene**	BaP	Mutagen
Polychlorinated biphenyl 008	PCB008	PCB
Polychlorinated biphenyl 126	PCB126	PCB
Polychlorinated biphenyl 153	PCB153	PCB
Polychlorinated biphenyl 180	PCB180	PCB
Butylated hydroxy anisole	BHA	Anti-oxidant
Butylated hydroxy toluene	BHT	Anti-oxidant
Mercury Chloride	Hg	Heavy metal
Cadmium Chloride hemi(pentahydrate)	Cd	Heavy metal
Lead (II) nitrate	Pb	Heavy metal
MeIQx	MeIQx	Heterocyclic amine
PhIP	PhIP	Heterocyclic amine
Bis(2-ethylhexyl)phthalate	DEHP	Phthalate
Dibutylphthalate	DBP	Phthalate
Benzybutylphthalate	BBP	Phthalate
Diethylphthalate	DEP	Phthalate

*Active estrogens are shown in blue, potential modulator compounds are shown in red.

**BaP was inactive in the ERSCREEN assay but showed activity in the ERLUX assay, consequently it was tested as a potential modulator in ESCREEN mixtures but as an active chemical in ERLUX mixtures.

MDA-kb2 assay (ARLUX)

The assay protocol and details on regression analysis have already been provided in task 2 ("Evaluation of estrogenic and anti-androgenic effects of mixtures of food contaminants", see chapter 3).

Mixture designs

The anti-androgenic mixtures in this task have been designed at mixture ratios reflecting human tissue levels. For this purpose, the tissue levels of food contaminants and additives that were compiled in task 1 ("Selection of test compounds and compilation of relevant data about internal exposures", see chapter 2) were used to formulate the mixtures. Where no data for the same tissue (serum levels) was available, the data was adjusted to human serum levels (see below). The adjusted human tissue level data was used to design mixtures at fixed mixture ratios reflecting the tissue levels, and the mixture effects were predicted according to the models of concentration addition (CA) and independent action (IA). Details about the calculation of mixture effect predictions have been described in task 2 (see chapter 3). The data from task 1 was used to test average and high exposure settings. Only if the predictions showed that the mixture would have been dominated by one or too few chemicals, the assumed tissue level was reduced accordingly to allow for investigation of a true mixture effect.

Next, the mixtures were tested over their whole predicted active range to test, whether they acted according to the prediction models. The hypothesised appropriate model was CA. We also ensured that the actual exposure concentration of the mixture, i.e. the sum of the actual tissue concentrations of all agents present in the mixture, was included in the test range to identify whether this concentration was in the effective range of the mixture effects.

Four scenarios were modelled and tested. Scenario I used average human tissue levels of 21 antiandrogenic compounds to calculate the mixture ratios. For the second scenario, 10 non-anti-androgenic compounds reflecting average tissue levels were added to the mixture of 21 anti-androgenic compounds. These compounds comprised the chemicals tested as effect modulators (task 3, "assessment of the impact of food additives and contaminants as effect modifiers", see chapter 4) plus further inactive compounds. Scenarios III and IV were high exposure scenarios, containing the same compounds as one and two, respectively, but at a mixture ratio reflecting maximal tissue levels.

All compounds, anti-androgenic and inactives, employed in mixture studies reflecting human tissue levels are shown in table 2.

Adjustments and conversions of human tissue level data

Human tissue levels were extracted from the data gathered in task 1, see chapter 2 and appendix 1. If possible, human serum levels were used directly. If no serum levels were available, we used the approach described for *p,p'*-DDE by López-Cervantes et al. (2004). To convert data to serum chemical levels in lipid bases (ng/g) the concentrations found in wet bases (ng/ml) were multiplied by a factor of 129.8 to convert them to serum levels in lipid bases. If only tissue levels from adipose tissue were available, they were divided by a factor of 4.2 to obtain an estimate of the concentration in lipid bases. Although these factors have been specifically determined for *p,p'*-DDE, we used them for the compounds employed in the mixture studies reflecting human tissue levels, as no similar data is available for other compounds. However, where no data for the same tissue matrix was available, this was a practical approach to homogenise the data.

Point of departure index (PODI)

To evaluate, which chemicals contribute most to the mixture effect, we ranked them in an approach similar to the "point of departure index" (PODI) for cumulative risk assessment (Wilkinson et al. 2000). As point of departure, we chose the EC_{10} of the respective compound in the MDA-kb2 assay. The EC_{10} was chosen as a low effect concentration, where effects can be distinguished readily from the response seen with the androgen DHT on its own. The tissue levels [M] were divided by the EC_{10} [M], resulting in the so called risk unit (RU) for each compound. The RUs indicate the contribution of each compound to the overall mixture concentration equaling the point of departure, in our case the EC_{10} of the mixture designed at a fixed mixture ratio according to tissue levels. Whereas for equi-effective mixtures these values are the same for each compound, the RUs for mixtures reflecting tissue levels are different for the individual compounds. This allows ranking of the chemicals according to their effect within the mixture. The PODI for the mixture is the sum of the individual RUs. For mixture summation plots first the RU of the most effective compound is shown and, subsequently the additional effect of the following, ranked chemicals are added to the overall mixture effect (cumulative plot). For a PODI above unity, a mixture effect at actual exposure levels can be expected (i.e. greater or equal 10%), whereas below unity, no measurable effect is to be expected. For example, a PODI of 0.5 means that a measurable effect is expected to occur at a mixture concentration twice as high as the one leading to a PODI of 1 (assuming that the mixture composition remains unchanged).

Table 2: Compounds tested in mixtures reflecting human tissue levels

Active anti-androgens are shown in blue, inactive compounds are shown in red.
(Note that "inactive" in this context stands for non-anti-androgenic. Some chemicals within this group exhibited cytotoxicity at higher concentrations. Genistein acted as an androgen when tested in combination with 0.25 nM DHT).

Compound	*Abbreviation*	*Chemical Group*
Bisphenol A	BPA	Bisphenol A
Methylparaben	MeP	Paraben
Ethylparaben	EtP	Paraben
n- Propylparaben	n-PP	Paraben
n- Butylparaben	n-BP	Paraben
Benzophenone 2	BP2	UV-filter
Benzophenone 3 (Eusolex 4360)	BP3	UV-filter
3-Benzylidene Camphor (Unisol S-22)	3-BC	UV-filter
4-Methylbenzylidene Camphor	4-MBC	UV-filter
Butylated hydroxy anisole	BHA	Anti-oxidant
Butylated hydroxy toluene	BHT	Anti-oxidant
Tonalide	AHTN	Polycyclic musk
Galaxolide	HHCB	Polycyclic musk
Perfluorooctane sulfonate	PFOS	PFC
Polychlorinated biphenyl 138	PCB138	PCB
Polybrominated diphenyl ether 100	BDE100	PBDE
Benzo(α)pyrene	BaP	Mutagen
Polychlorinated biphenyl 118	PCB118	PCB
Polychlorinated biphenyl 126	PCB126	PCB
Polychlorinated biphenyl 153	PCB153	PCB
Polychlorinated biphenyl 180	PCB180	PCB
Mercury Chloride	$HgCl_2$	Heavy metal
Cadmium Chloride hemi(pentahydrate)	$CdCl_2$	Heavy metal
Lead (II) nitrate	$PbNO_3$	Heavy metal
Bis(2-ethylhexyl)phthalate	DEHP	Phthalate
Dibutylphthalate	DBP	Phthalate
Octyl-methoxycinnamate (Eusolex 2292)	OMC	UV-filter
Perfluorooctanoic acid	PFOA	PFC
6:2 Fluorotelomer alcohol	6:2 FTOH	PFC
8:2 Fluorotelomer alcohol	8:2 FTOH	PFC
4',5,7-Trihydroxyisoflavone	Genistein	Phytoestrogen

Results

A. ESCREEN

The mixture experiments in this section tested 13 chemicals defined as **active**, for which individual concentration-response relationships are shown in Figure 1 and Table 3, and 16 **potential modulators**, which had no or minimal activity in the ESCREEN.

Three separate mixtures were tested in this section, Table 4 shows the composition of the mixture of actives and Table 5 shows the composition of two mixtures containing potential modulators. Both modulator mixtures were tested alone and in combination with the mixture of actives.

1. A mixture comprising 13 active estrogenic agents

Figure 2 shows the concentration addition prediction and the concentration-response relationship obtained from experimental testing of a 13 component mixture of active chemicals.

For this mixtures the 'real-world' concentration was associated with an observed effect size of 50%, whilst an effect of 39% was predicted by CA for the same concentration. In general, the observed mixture response was well predicted by CA, however there was a small trend towards under-prediction at higher levels.

2. A mixture composed of 29 components, including actives and modulators

2a. Modulator mixture with phthalates at exposure levels

Modulators alone: mixture of 16 modulator components

Figure 3 shows the results of testing a mixture of 16 modulator chemicals, the design of which is shown in Table 5. When tested alone, the modulator mixture was itself active and was associated with an effect size of around 25% at the real-world' level.

Combined mixture: 29 component mixture comprised of 13 active and 16 modulator components

Figure 3 shows that the presence of an increasing concentration of the modulator mixture did not substantially affect the effect of the 1X, 'real-world' concentration of the actives mixture. At higher concentrations of the modulator mixture, a small positive shift may have been expected due to addition of the activity of the modulator mixture alone, the reason for this not being observed could be due to the increase being smaller than the inherent noise of the assay and due to the shape of the individual concentration-response relationships of the components responsible. Alternatively a small negative modulation of the actives mixture, by the mixture of modulators, may be present.

In this 29 component mixture, at the 'real-world' level, the activity of the actives mixture was not substantially altered from that predicted by CA and which was confirmed when the actives mixture was tested alone (Figure 1). This finding indicates that CA is a useful model for this more complicated exposure scenario.

2b. Modulator mixture with phthalates at reduced levels

Due to the estrogenic activity seen in the modulator mixture alone, a study was carried out to investigate whether the phthalates concentration in the modulator mixture explained the activity.

Figure 4 shows that reducing the level of three phthalates (see Table 5) abolished the activity of the modulator mixture, when tested alone. When the reduced phthalates concentration mixture was tested in combination with a fixed concentration of the actives mixtures a decline in response was observed, however this is most likely due to an experimental artefact on the singe plate used.

The value of this small study was in showing that the modulator mixture lacked activity when the phthalates concentration was reduced. Further interpretation, for example of the effect of the reduced phthalate concentration mixture on the actives mixture, would require further testing. The comparable dataset in the ERLUX assay, see Figure 8, is more complete and can be fully interpreted.

Figure 1: Concentration response relationships from single chemical testing in ESCREEN

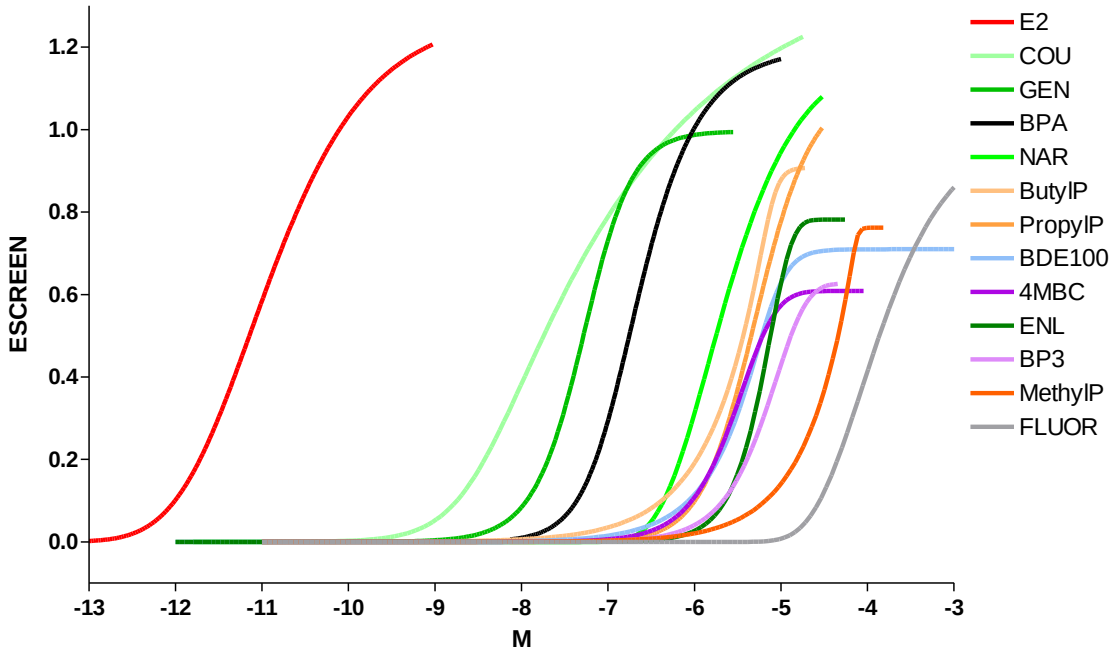


Figure shows the non-linear regression models that were fitted to the experimental data. Regression model parameters are given in Table 3.

Table 3: Regression modelling parameters for all active chemicals included in the mixtures tested in the ESCREEN assay.

Chemical name	*Concentration-response function*					*EC_{10} (M)*	*EC_{50} (M)*
	NLR model	*Tmax*	*T1*	*T2*	*T3*		
Estradiol	GL1	1.278	16.71	1.356	5.117	1.00E-12	7.23E-12
Coumestrol	GL2	1.416	26.32	3.081	0.1711	1.79E-09	1.81E-08
Genistein	GL1	0.9958	26.88	3.726	0.8301	1.15E-08	5.21E-08
Bisphenol A	GL2	1.198	26.26	3.799	0.5217	4.35E-08	1.76E-07
Naringenin	GL1	1.197	14.74	1.745	96.66	4.41E-07	1.77E-06
Butylparaben	GL1	0.9074	70.27	13.82	0.1233	4.18E-07	3.68E-06
Propylparaben	GL1	1.106	15.74	2.93	1.219	1.01E-06	4.54E-06
BDE-100	GL1	0.7097	34.74	6.808	0.2979	8.53E-07	6.01E-06
4MBC	GL2	0.6087	14.48	2.954	5.075	9.39E-07	6.19E-06
Enterolactone	GL2	0.7814	13.31	3.85	620.1	2.27E-06	7.56E-06
Benzophenone-3	GL2	0.6267	11.75	2.882	17.15	2.15E-06	1.31E-05
Methylparaben	GL1	0.762	168.8	40.98	0.04677	6.63E-06	4.58E-05
Fluoranthene	GL1	1	10.69	1.777	32.22	2.81E-05	0.000138

Substances are listed in order of their EC_{50}. **NLR**: non-linear regression; T_{max}, T1, T2, T3: derived parameters of the selected regression model; **GL1**: general logit I regression model, y= T_{max} * 1/(1+exp(-T1-T2*log10(x)))^T3, where T_{max}, T1, T2 and T3 are the parameters to be fitted; **GL2**: general logit II regression model, y= T_{max} * (1-1/(1+exp(T1+T2*log10(x)))^T3) where T_{max}, T1, T2 and t3 are the parameters to be fitted; **EC_{10}**: concentration evoking a 10% effect; **EC_{50}**: concentration evoking a 50% effect

Table 4. Composition of ESCREEN 'actives' mixture

Chemical	*Exposure level (M)*	*Mixture ratio*
Bisphenol A	2.00E-08	0.013812
Genistein	5.00E-09	0.003453
Estradiol	1.00E-12	0.000001
Propyl paraben	1.00E-08	0.006906
Fluoranthene	1.00E-09	0.000691
Naringenin	2.00E-07	0.138120
4MBC	8.00E-08	0.055248
Benzophenone-3	1.00E-06	0.690602
Methyl paraben	1.00E-07	0.069060
Butyl paraben	1.00E-08	0.006906
Enterolactone	2.00E-08	0.013812
Coumestrol	2.00E-09	0.001381
Polybrominated diphenyl ether-100	1.00E-11	0.000007

Figure 2: Concentration-response relationship in ESCREEN for 13 component mixture of active chemicals, fixed ratio design using real-world concentrations

Graph shows the concentration-response relationship for a 13 component mixture of active chemicals designed with a fixed mixture ratio based on exposure levels, see Table 4 (n=5). Each black circle represents mean data from one experimental plate (two replicate wells), thick black line indicates a fitted sigmoid model (4 parameter hill, GraphPad Prism) with 95% confidence intervals (dotted line). The human exposure concentration is indicated by a vertical line, labelled "RW" on the x-axis.

The predicted response, using the concentration addition (CA) model, is shown as a thick red line. The prediction is split above 55% to indicate extrapolation of the model using two different approaches for mixture components that showed sub-maximal effects: 1) component is assumed to make no effect (thin red line); 2) component is assumed to have a maximum effect (thick red line).

Inset shows a TU plot, see legend to Figure 9 for explanation.

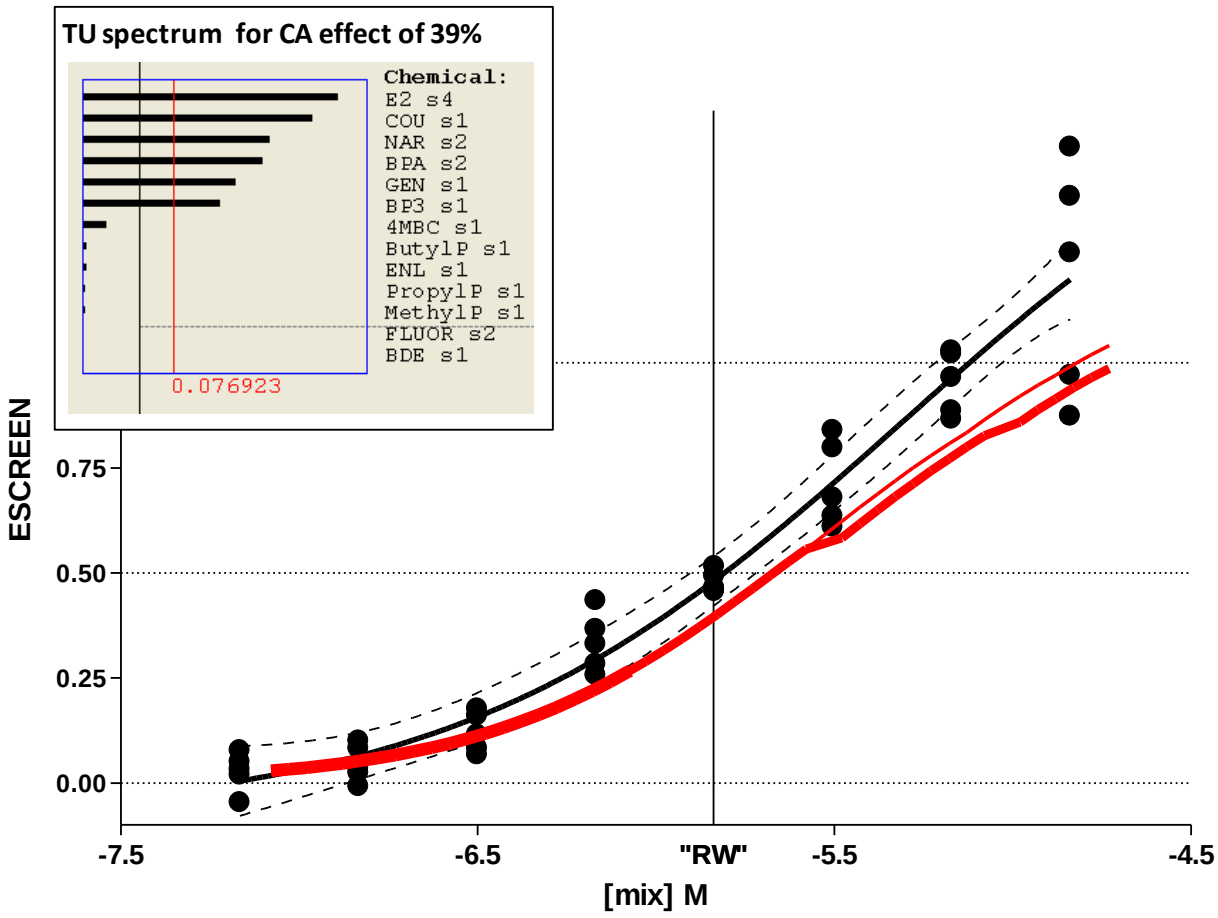


Table 5. Composition of ESCREEN modulator mixtures

Chemical group	*Chemical name*	*MOD1**		*MOD2**		*Difference in concentration between MOD1 and MOD2***
		Exposure level (M)	*Mixture ratio*	*Exposure level (M)*	*Mixture ratio*	
PCBs	PCB008	9.00E-09	0.003467	9.00E-09	0.009850	
	PCB126	2.50E-12	0.000001	2.50E-12	0.000003	
	PCB153	2.40E-09	0.000925	2.40E-09	0.002627	
	PCB180	1.20E-09	0.000462	1.20E-09	0.001313	
Heavy metals	Mercury	2.50E-09	0.000963	2.50E-09	0.002736	
	Cadmium	7.60E-09	0.002928	7.60E-09	0.008318	
	Lead	2.10E-07	0.080899	2.10E-07	0.229829	
Poly aromatic hydrocarbons (PAH)	BaP	9.00E-10	0.000347	9.00E-10	0.000985	
Heterocyclic amines (HCA)	MeIQx	2.00E-11	0.000008	2.00E-11	0.000022	
	PhIP	2.00E-10	0.000077	2.00E-10	0.000219	
Phthalates	DEHP	1.00E-06	0.385234	1.00E-08	0.010944	reduced 100-fold
	BBP	4.90E-07	0.188765	4.90E-09	0.005363	reduced 100-fold
	DEP	3.60E-08	0.013868	3.60E-08	0.039399	
	DBP	2.30E-07	0.088604	2.30E-08	0.025172	reduced 10-fold
Antioxidants	BHA	5.60E-08	0.021573	5.60E-08	0.061288	
	BHT	5.50E-07	0.211879	5.50E-07	0.601933	

*MOD1 contains phthalates at the human exposure level, see Figure 3; MOD2 contains phthalates at a reduced concentration such that they are no longer estrogenic, see Figure 4.

**dilution factor was chosen such to reduce the phthalate concentration in the highest tested concentration ("10X") to at least 10-fold less than the lowest concentration at which estrogenicity was considered likely

Figure 3: Concentration-response relationships in ESCREEN for a mixture of modulators alone and in combination with a fixed concentration of the actives mixture

Graphs shows the concentration-response relationships for a 16 component mixture of modulator chemicals alone (black circles and lines, n=3) and in the presence of a fixed concentration of the 13 component mixture of active chemicals shown in Figure 2 (total of 29 components, grey circles and lines, n=2). Both mixtures have a fixed mixture ratio based on human exposure levels. Circles indicate mean data from one experimental plate (two replicate wells), solid lines indicate a fitted sigmoid model (4 parameter hill, GraphPad Prism) or a linear regression model with 95% confidence intervals (dotted line).

The human exposure concentration for the mixture of modulators is indicated by a vertical line, labelled "RW" on the x-axis. The expected effect of the fixed concentration of the active mixture is indicated by a horizontal dotted line, labelled "expected:" on the y-axis. The intersection of these two lines indicates the effect of the 29 component mixture at the human exposure level.

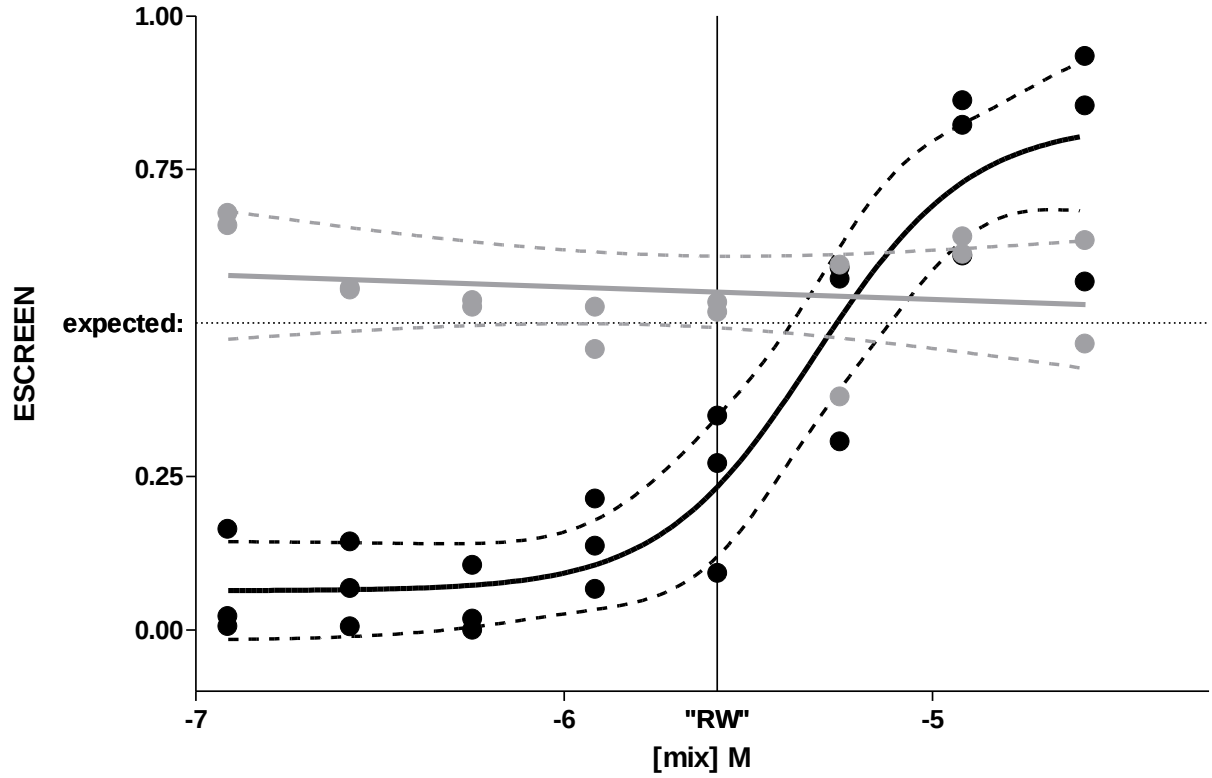
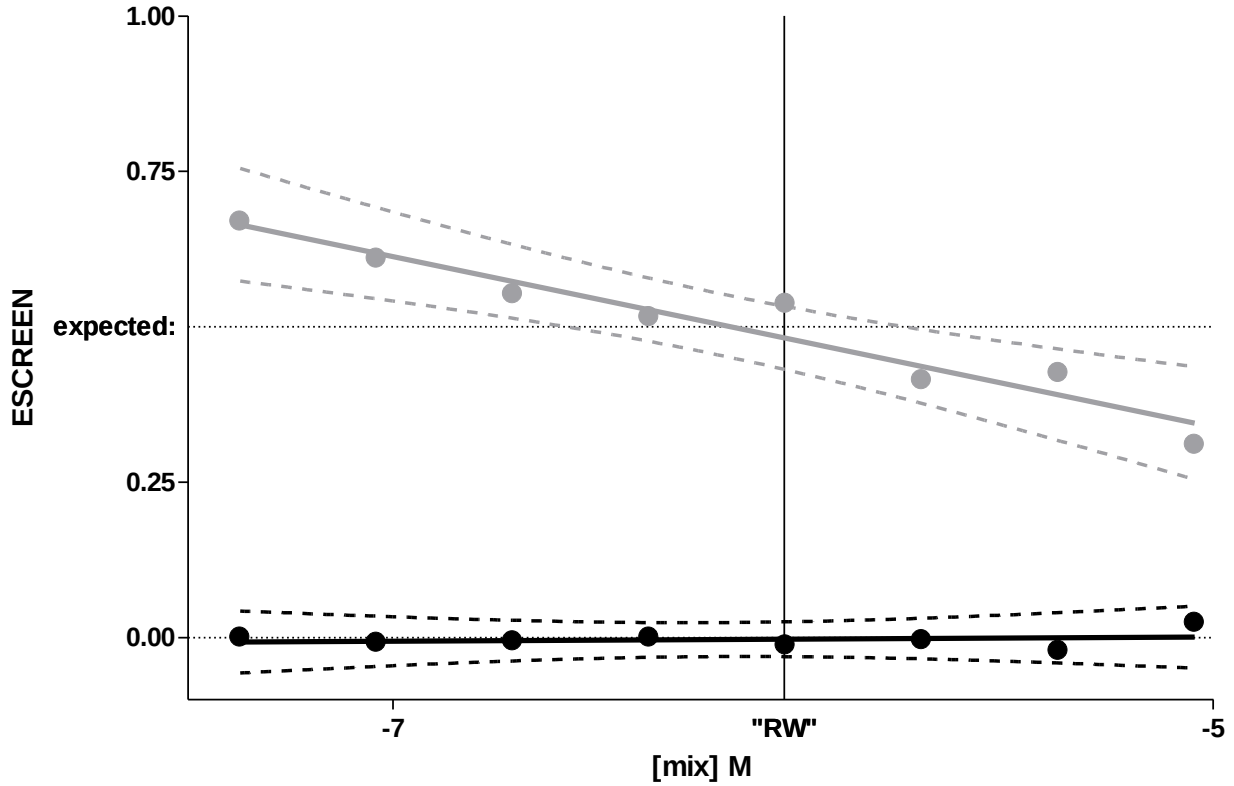


Figure 4: Concentration-response relationships in ESCREEN for a mixture of modulators (reduced phthalate concentration) alone and in combination with a fixed concentration of the actives mixture

Graphs shows the concentration-response relationships for a 16 component mixture of modulator chemicals with a reduced phthalate concentration alone (black circles and lines, n=1) and in the presence of a fixed concentration of the 13 component mixture of active chemicals shown in Figure 2 (total of 29 components, grey circles and lines, n=1). Both mixtures have a fixed mixture ratio based on exposure levels. Circles indicate mean data from one experimental plate (two replicate wells), solid lines indicate a fitted sigmoid model (4 parameter hill, GraphPad Prism) or a linear regression model with 95% confidence intervals (dotted line

The human exposure concentration for the mixture of modulators is indicated by a vertical line, labelled "RW" on the x-axis. The expected effect of the fixed concentration of the active mixture is indicated by a horizontal dotted line, labelled "expected:" on the y-axis. The intersection of these two lines indicates the effect of the 29 component mixture at the human exposure level.



B. ERLUX

The mixture experiments performed in ERLUX included testing of 14 chemicals defined as **active**, for which individual concentration-response relationships are shown in Figure 5 and Table 6, and 15 **potential modulators**, which had no or minimal activity in the ERLUX.

Three separate mixtures were tested in this section, Table 7 shows the composition of the mixture of actives and Table 8 shows the composition of two mixtures containing potential modulators. Both modulator mixtures were tested alone and in combination with the mixture of actives.

These mixtures were designed using the same principles as used for the equivalent mixtures tested in the ESCREEN, see above, but the groupings as 'active' or potential modulator' differed for one component, benzo[a]pyrene. Benzo[a]pyrene was inactive in the ESCREEN (and was thus tested there as a potential modulator) but was active in the ERLUX and was thus included in this mixture of active chemicals. The inclusion of an extra component has only small implications for the mixture composition, see tables 4 and 7.

1. A mixture of 14 estrogenic agents

Figure 6 shows the concentration-response relationship for a 14 component mixture of estrogenic chemicals tested in the ERLUX assay. In the ERLUX, the 'real-world' concentration of this actives mixture was associated with an observed effect size of 45%, compared to a predicted effect of 59% according to CA. Overall, the response was generally well predicted by CA, however there was a small over-prediction at most levels, although not towards the lower concentration ranges.

2. A 29- component mixtures, comprised of actives and modulators

2a. Modulator mixture with phthalates at exposure levels

Modulators alone: mixture of 15 modulator components

Figure 7 shows the results of testing a mixture of modulator compounds in ERLUX. The design of the modulator mixture is shown in Table 8.

As seen for ESCREEN, Figure 7 shows that the modulator mixture was estrogenic when tested alone. In ERLUX however the activity of the mixture was only apparent at concentrations greater than the 1X, real-world level.

Combined mixture: 29 component mixture comprised of 14 active and 15 modulator components

Figure 7 shows that, when the concentration of modulators was increased in the presence of a fixed 'real-world' concentration of the actives mixture, a small positive increase in effect occurred, consistent with a superimposition of the effect of the modulator mixture, as measured when tested alone.

At real-world concentrations of both actives and modulators, see Figure 7, a small increase in effect, of around 10% compared to the actives tested alone at this concentration, is present.

2b. Modulator mixture with phthalates at reduced levels

Modulators alone: mixture of 15 modulator components

Figure 8 shows that reduction of the phthalates concentration in the modulator mixture abolished the estrogenic activity of the mixture when tested alone.

Combined mixture: 29 component mixture comprised of 14 active and 15 modulator components

Figure 8 shows that the reduced phthalate modulator mixture lacks any modulatory effect on the mixture of actives, and shows that CA is a good predictor of outcome in this complicated scenario, since the effect of the actives was adequately predicted (see Figure 6) and this effect is not shifted by the presence of the modulators (when phthalate concentration is reduced).

3. Comparison of toxic unit plots between assays

Figure 9 shows a comparison of TU plots for both ESCREEN and ERLUX assays for the mixtures tested in this task, and for comparison also shows TU plots for two mixtures tested in previous tasks using a different mixture ratio approach, namely ratios of equi-effective concentrations rather than ratios of 'RW' concentrations.

The TU plots for both assays show that, as expected, the mixture composition is not balanced; this is due to the use of real-world levels to set the mixture ratio. The mixture effect is primarily driven by six components, estradiol, coumestrol, naringenin, bisphenol A, genistein and benzophenon-3; which explain greater than 95% of the cumulative TU. The order of importance of these components varies a little between the two assays (see Figure 9A, B), but the overall conclusion is the same.

Figure 5: Concentration response relationships from single chemical testing in ERLUX

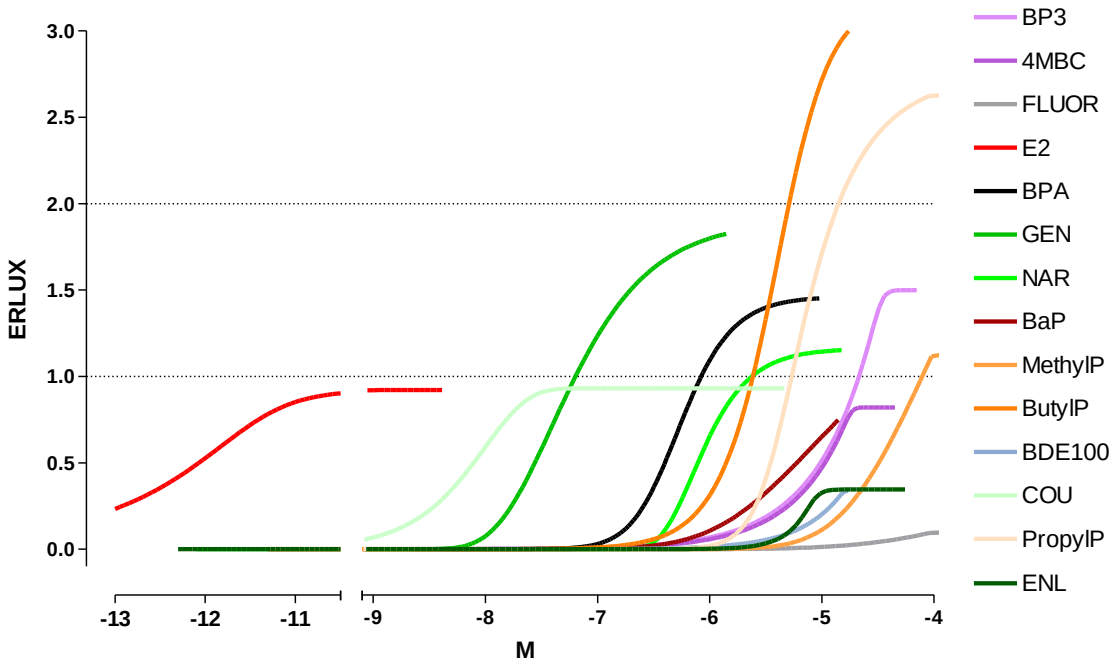


Figure shows the non-linear regression models that were fitted to the experimental data. Regression model parameters are given in Table 6.

Table 6: Regression modelling parameters for all active chemicals included in the mixtures tested in the ERLUX assay.

Chemical name	*Concentration-response function*					*EC_{10} (M)*	*EC_{50} (M)*
	NLR model	*Tmax*	*T1*	*T2*	*T3*		
Estradiol	GL1	1.148	111	10.05	0.2025	5.70E-13	3.54E-12
Coumestrol	GL2	0.9309	14.6	2.594	476.1	1.43E-09	7.84E-09
Genistein	GL1	1.904	19.11	2.034	56.84	1.11E-08	2.77E-08
Bisphenol A	GL1	1.462	25.03	3.896	1.655	1.64E-07	3.97E-07
Naringenin	GL1	1.162	25.79	3.595	39.33	3.88E-07	7.80E-07
Butylparaben	GL1	3.178	23.77	4.455	0.772	4.58E-07	1.41E-06
Propylparaben	GL2	2.743	36.37	6.705	0.3383	1.80E-06	3.51E-06
Benzo[a]pyrene	GL1	0.8922	16.99	3.196	0.8849	8.66E-07	5.11E-06
Benzophenone-3	GL1	1.499	144.9	32.42	0.0623	1.55E-06	9.70E-06
4MBC	GL1	0.8201	235.6	49.75	0.0422	1.83E-06	1.07E-05
BDE-100	GL1	2.527	10.19	2.536	1.001	5.32E-06	2.70E-05
Methylparaben	GL1	2.684	7.958	1.539	5.563	9.31E-06	3.21E-05
Fluoranthene	GL1	1	4.846	0.685	20.1	0.000101	0.006546
Enterolactone	GL1	0.4435	57.05	11.32	0.5092	5.09E-06	n.a.

Substances are listed in order of their EC_{50}. **NLR**: non-linear regression; T_{max}, T1, T2, T3: derived parameters of the selected regression model; **GL1**: general logit I regression model, y= T_{max} * 1/(1+exp(-T1-T2*log10(x)))^T3, where T_{max}, T1, T2 and T3 are the parameters to be fitted; **GL2**: general logit II regression model, y= T_{max} * (1-1/(1+exp(T1+T2*log10(x)))^T3) where T_{max}, T1, T2 and t3 are the parameters to be fitted; **EC_{10}**: concentration evoking a 10% effect; **EC_{50}**: concentration evoking a 50% effect; **n.a.** : EC50 not available since ENL model had a maximum limit below the 50%

Table 7. Composition of ERLUX 'actives' mixture

Chemical name	*Exposure level (M)*	*Mixture ratio*
Bisphenol A	2.00E-08	0.013803
Genistein	5.00E-09	0.003451
Estradiol	1.00E-12	0.000001
Propyl paraben	1.00E-08	0.006902
Fluoranthene	1.00E-09	0.000690
Naringenin	2.00E-07	0.138035
4MBC	8.00E-08	0.055214
Benzophenone-3	1.00E-06	0.690174
Methyl paraben	1.00E-07	0.069017
Butyl paraben	1.00E-08	0.006902
Enterolactone	2.00E-08	0.013803
Coumestrol	2.00E-09	0.001380
Poly brominated diphenyl ether-100	1.00E-11	0.000007
Benzo[a]pyrene	9.00E-10	0.000621

Figure 6: Concentration-response relationship in ERLUX for 14 component mixture of active chemicals, fixed ratio design using real-world concentrations

Graph shows the concentration-response relationship for a 14 component mixture of active chemicals designed with a fixed mixture ratio based on exposure levels, see Table 7 (n=4). Each black circle represents mean data from one experimental plate (six replicate wells), thick black line indicates a fitted sigmoid model (4 parameter hill, GraphPad Prism) with 95% confidence intervals (dotted line). The human exposure concentration is indicated by a vertical line, labelled "RW" on the x-axis.

The predicted response, using the concentration addition (CA) model, is shown as a thick red line. The prediction is split above 55% to indicate extrapolation of the model using two different approaches for mixture components that showed sub-maximal effects: 1) component is assumed to make no effect (thin red line); 2) component is assumed to have a maximum effect (thick red line).

Inset shows a TU plot, see legend to Figure 7 for explanation.

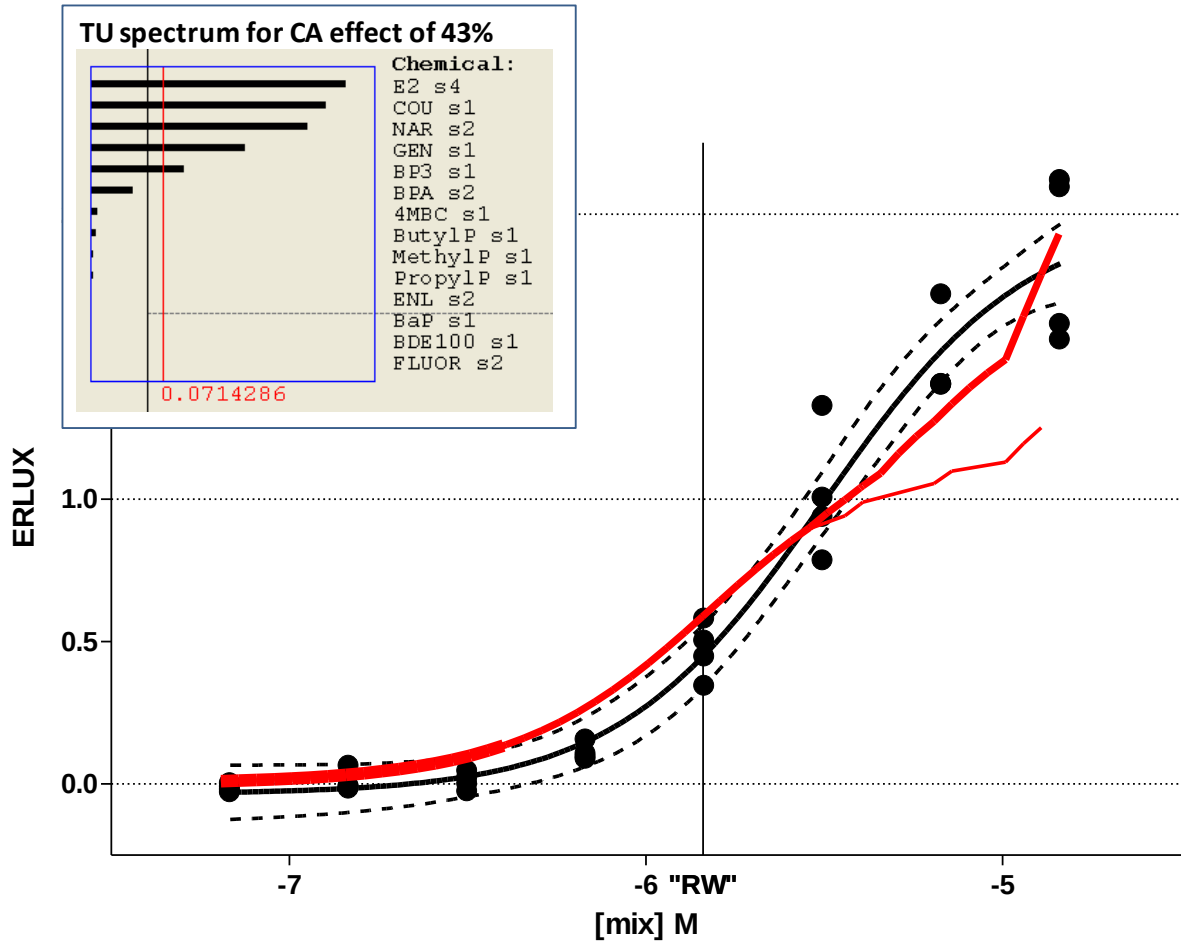


Table 8. Composition of ERLUX modulator mixtures

Chemical group	*Chemical name*	*MOD1**		*MOD2**		*Difference in concentration between MOD1 and MOD2***
		Exposure level (M)	*Mixture ratio*	*Exposure level (M)*	*Mixture ratio*	
PCBs	PCB008	9.00E-09	0.003468	9.00E-09	0.010222	
	PCB126	2.50E-12	0.000001	2.50E-12	0.000003	
	PCB153	2.40E-09	0.000925	2.40E-09	0.002726	
	PCB180	1.20E-09	0.000462	1.20E-09	0.001363	
Heavy metals	Mercury	2.50E-09	0.000963	2.50E-09	0.002840	
	Cadmium	7.60E-09	0.002929	7.60E-09	0.008632	
	Lead	2.10E-07	0.080927	2.10E-07	0.238522	
Heterocyclic amines	MeIQx	2.00E-11	0.000008	2.00E-11	0.000023	
	PhIP	2.00E-10	0.000077	2.00E-10	0.000227	
Phthalates	DEHP	1.00E-06	0.385368	1.00E-08	0.011358	reduced 100-fold
	BBP	4.90E-07	0.188830	4.90E-09	0.005566	reduced 100-fold
	DEP	3.60E-08	0.013873	3.60E-09	0.004089	reduced 10-fold
	DBP	2.30E-07	0.088635	2.30E-08	0.026124	reduced 10-fold
Antioxidants	BHA	5.60E-08	0.021581	5.60E-08	0.063606	
	BHT	5.50E-07	0.211952	5.50E-07	0.624700	

*MOD1 contains phthalates at the human exposure level, see Figure 7; MOD2 contains phthalates at a reduced concentration such that they are no longer estrogenic, see Figure 8.

**dilution factor was chosen such to reduce the phthalate concentration in the highest tested concentration ("10X") to at least 10-fold less than the lowest concentration at which estrogenicity was considered likely

Figure 7: Concentration-response relationships in ERLUX for a mixture of modulators alone and in combination with a fixed concentration of the actives mixture

Graph shows the concentration-response relationships for a 15 component mixture of modulator chemicals alone (black circles and lines, n=2) and in the presence of a fixed concentration of the 14 component mixture of active chemicals shown in Figure 4 (total of 29 components, grey circles and lines, n=3). Both mixtures have a fixed mixture ratio based on exposure levels. Circles indicate mean data from one experimental plate (six replicate wells), solid lines indicate a fitted sigmoid model (4 parameter hill, GraphPad Prism) or a linear regression model with 95% confidence intervals (dotted line).

The human exposure concentration for the mixture of modulators is indicated by a vertical line, labelled "RW" on the x-axis. The expected effect of the fixed concentration of the active mixture is indicated by a horizontal dotted line, labelled "expected:" on the y-axis. The intersection of these two lines indicates the effect of the 29 component mixture at the human exposure level.

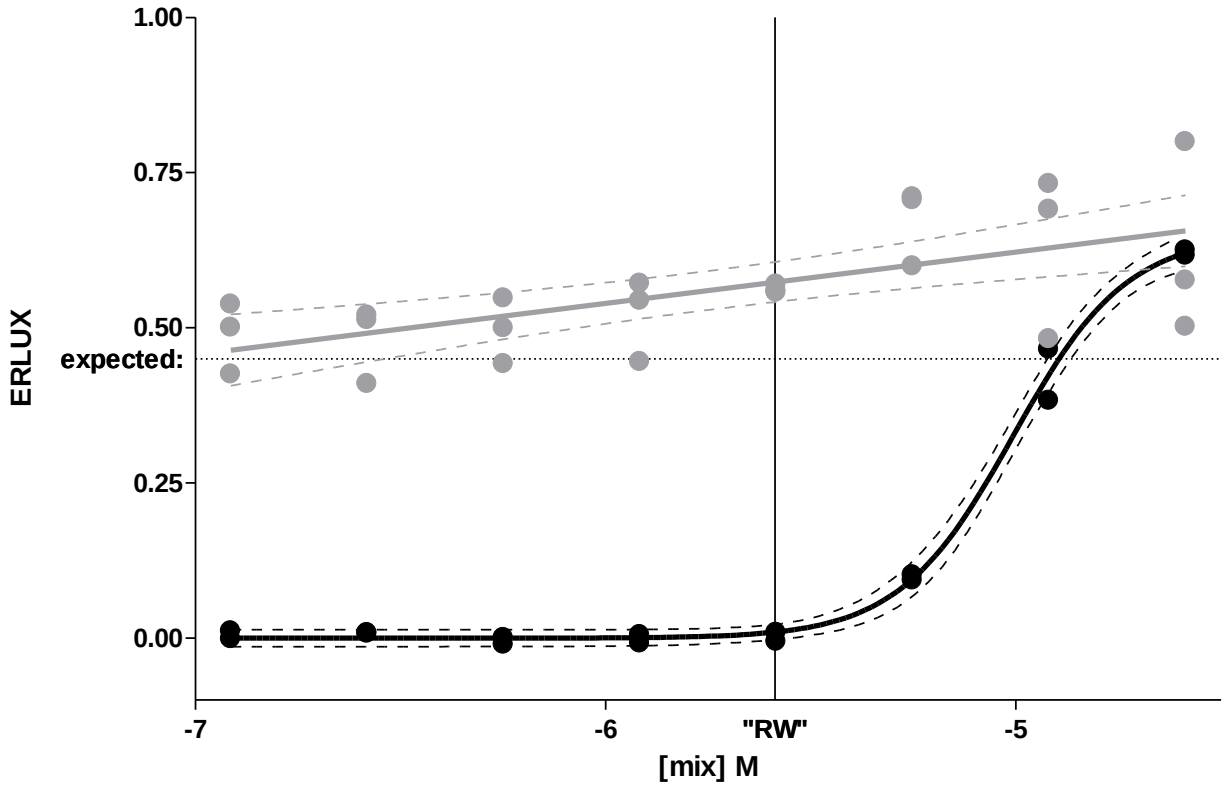


Figure 8: Concentration-response relationships in ERLUX for a mixture of modulators (reduced phthalates concentration) alone and in combination with a fixed concentration of the actives mixture

Graphs show the concentration-response relationships for a 15 component mixture of modulator chemicals with a reduced phthalate concentration alone (black circles and lines, n=2) and in the presence of a fixed concentration of the 14 component mixture of active chemicals shown in Figure 4 (total of 29 components, grey circles and lines, n=3). Both mixtures have a fixed mixture ratio based on exposure levels. Circles indicate mean data from one experimental plate (six replicate wells), solid lines indicate a fitted sigmoid model (4 parameter hill, GraphPad Prism) or a linear regression model with 95% confidence intervals (dotted line).

The human exposure concentration for the mixture of modulators is indicated by a vertical line, labelled "RW" on the x-axis. The expected effect of the fixed concentration of the active mixture is indicated by a horizontal dotted line, labelled "expected:" on the y-axis. The intersection of these two lines indicates the effect of the 29 component mixture at the human exposure level.

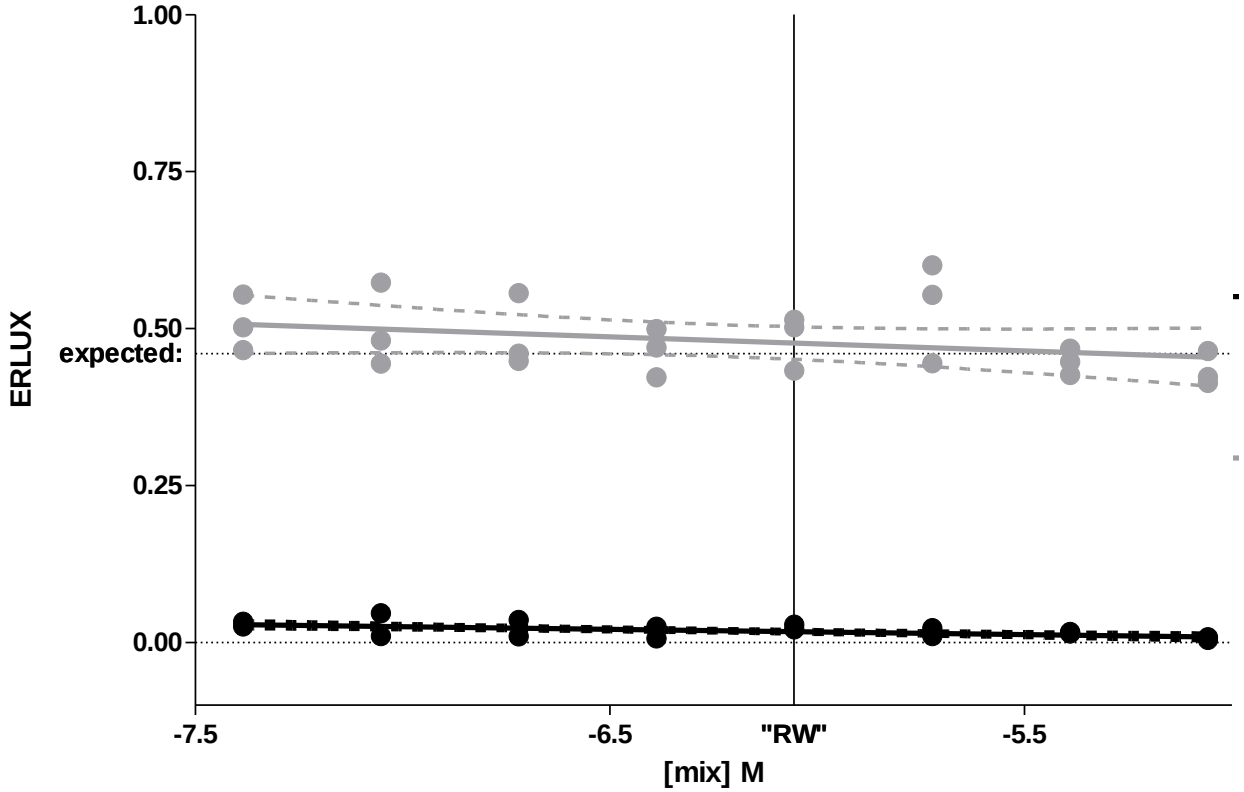


Figure 9: Comparison of TU distributions

See figure on following page

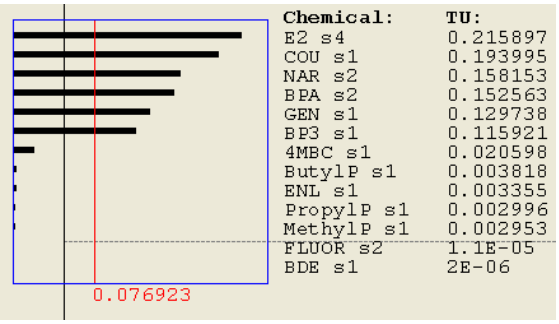
Figure shows toxic unit (TU) distributions for mixtures designed using fixed ratios of RW concentrations and tested in ESCREEN (A) and ERLUX (B).

For comparison, plots C to F show TU distributions for mixtures tested in previous tasks and designed using fixed, equieffective mixture ratios. Each plot shows the TU for each mixture component (horizontal black bar), at the stated effect level. A vertical red line indicates the TU expected for a completely balanced mixture. C, D: TU distributions for an ESCREEN mixture designed using ratios of 25% effect levels (EC25s) and shown at the effect level matching the mixture design (25%, C) and at a higher effect level (50%, D). E, F: TU distributions for an ERLUX mixture designed using ratios of 10% effect levels (EC10s) and shown at the effect level matching the mixture design (10%, E) and at a higher effect level (25%, F).

A. ESCREEN

13 components, "RW"

TU for 39% ("RW")

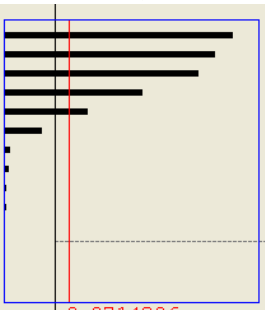


Chemical:	TU:
E2 s4	0.215897
COU s1	0.193995
NAR s2	0.158153
BPA s2	0.152563
GEN s1	0.129738
BP3 s1	0.115921
4MBC s1	0.020598
ButylP s1	0.003818
ENL s1	0.003355
PropylP s1	0.002996
MethylP s1	0.002953
FLUOR s2	1.1E-05
BDE s1	2E-06

B. ERLUX

14 components, "RW"

TU for 43% (just below "RW")

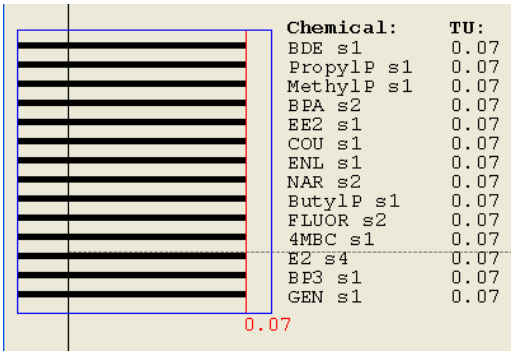


Chemical:	TU:
E2 s4	0.2511699
COU s1	0.2311686
NAR s2	0.2132067
GEN s1	0.1524653
BP3 s1	0.0914513
BPA s2	0.0419045
4MBC s1	0.0066031
ButylP s1	0.005931
MethylP s1	0.0027055
PropylP s1	0.002295
ENL s2	0.000932
BaP s1	0.0001665
BDE100 s1	3E-07
FLUOR s2	2E-07

C. ESCREEN

14 components, equieffect at 25%

TU for 25%

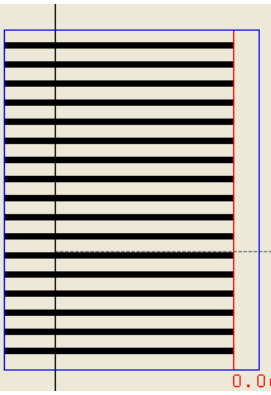


Chemical:	TU:
BDE s1	0.07
PropylP s1	0.07
MethylP s1	0.07
BPA s2	0.07
EE2 s1	0.07
COU s1	0.07
ENL s1	0.07
NAR s2	0.07
ButylP s1	0.07
FLUOR s2	0.07
4MBC s1	0.07
E2 s4	0.07
BP3 s1	0.07
GEN s1	0.07

E. ERLUX

17 components, equieffect at 10%

TU for 10%

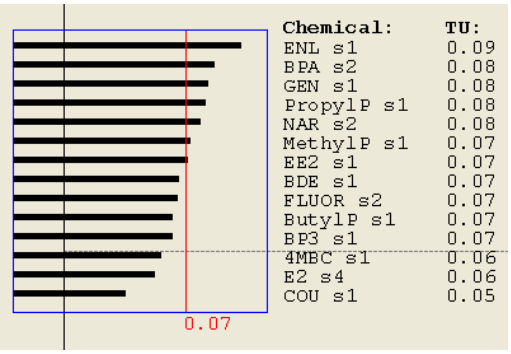


Chemical:	TU:
MethylP s1	0.06
EE2 s1	0.06
TON s1	0.06
4MBC s1	0.06
BaP s1	0.06
BPA s2	0.06
GAL s1	0.06
FLUOR s2	0.06
E2 s4	0.06
BP3 s1	0.06
ButylP s1	0.06
NAR s2	0.06
GEN s1	0.06
PropylP s1	0.06
COU s1	0.06
BDE100 s1	0.06
ENL s2	0.06

D. ESCREEN

14 components, equieffect at 25%

TU for 50%

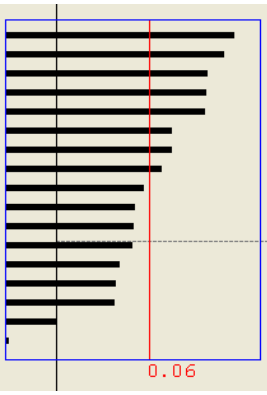


Chemical:	TU:
ENL s1	0.09
BPA s2	0.08
GEN s1	0.08
PropylP s1	0.08
NAR s2	0.08
MethylP s1	0.07
EE2 s1	0.07
BDE s1	0.07
FLUOR s2	0.07
ButylP s1	0.07
BP3 s1	0.07
4MBC s1	0.06
E2 s4	0.06
COU s1	0.05

F. ERLUX

17 components, equieffect at 10%

TU for 25%



Chemical:	TU:
NAR s2	0.09
PropylP s1	0.09
GEN s1	0.08
ENL s2	0.08
BPA s2	0.08
MethylP s1	0.07
ButylP s1	0.07
GAL s1	0.06
EE2 s1	0.06
BaP s1	0.05
BDE100 s1	0.05
COU s1	0.05
4MBC s1	0.05
E2 s4	0.05
BP3 s1	0.04
FLUOR s2	0.02
TON s1	0

C. MDA-kb2 assay (ARLUX)

1. Testing of anti-androgenic mixtures reflecting human tissue levels

The anti-androgenic mixtures, designed according to human tissue levels included compounds that exhibited anti-androgenic activity in the presence of 0.25 nM DHT. These also included chemicals which were androgenic when tested without DHT.

Substances included in these mixtures were bisphenol A (BPA), methyl paraben (MeP), ethyl paraben (EtP), *n*-butylparaben (n-BP), *n*-propylparaben (n-PP), benzophenone 2 (BP2), benzophenone 3 (BP3), 3-benzylidene camphor (3BC), 4-methylbenzylidene camphor (4MBC), butylated hydroxy anisole (BHA), butylated hydroxyl toluene (BHT), Tonalide (AHTN), Galaxolide (HHCB), perfluorooctane sulfonate (PFOS), polychlorinated biphenyls 118, 126, 138, 153 and 180 (PCBs 118, 126, 138, 153, 180), polybrominated diphenyl ether 100 (BDE100) and benzo(α)pyrene (BaP).

The non-linear regression fits for the individual compounds included in the mixtures are shown in Figure 10. The corresponding non-linear regression models used, with their regression parameters, are presented in Table 9.

To elucidate whether the presence of inactive (i.e. non-anti-androgenic) chemicals within the mixture can affect the mixture activity, additional mixtures were tested, that did not only consist of these active anti-androgens, but also inactive chemicals, including the chemicals that were tested as potential effect modulators (task 3, see chapter 4). These were mercury chloride ($HgCl_2$), cadmium chloride ($CdCl_2$), lead nitrate (Pb), bis(2-ethylhexyl)phthalate (DEHP), dibutylphthalate (DBP) and perfluorooctanoic acid (PFOA). Additional "inactives" that were tested were octyl-methoxycinnamate (OMC), 6:2 fluorotelomer alcohol (6:2 FTOH) and 8:2 fluorotelomer alcohol (8:2 FTOH). Furthermore, genistein was included in the mixture, which showed androgenic activity in the presence of 0.25 nM DHT.

1.1 Prediction modelling

Based upon the concentration-response relationships for the individual mixture components (Table 9), the expected mixture effects were calculated according to either the model of concentration addition (CA) or of independent action (IA). The predictions were based on mixture ratios that reflect the concentrations found in human tissue. The human tissue levels used for calculation of the mixture ratios were extracted from the data gathered in task1, see chapter 2 and appendix 1. Tissue levels and mixture ratios for the compounds in the four mixtures are shown in Table 10.

1.2 Mixture testing

Mixture of 21 anti-androgenic chemicals at ratios reflecting average human tissue levels

The results from prediction modelling and testing of the 21 component mixture of anti-androgenic chemicals at a fixed mixture ratio, designed according to average human tissue levels (scenario I), is shown in Figure 11. Testing of the mixture showed that it acted according to CA.

The the sum of the actual tissue concentrations of all agents included in the mixture was 50.5 nM as indicated in Figure 11. At this concentration, the ARLUX assay did not show any observable effects.

The curves showing the individual effects of the compounds within the mixture in Figure 11 further reveals which chemicals had the major effect on the anti-androgenic response of the mixture (BPA>PFOS>BaP etc.).

Mixture of 21 anti-androgenic and 10 non-anti-androgenic chemicals at ratios reflecting average human tissue levels

The results from prediction modelling and testing of the 21+10 component mixture at a fixed mixture ratio, designed according to average human tissue levels (scenario II), is shown in Figure 12A. For the CA and IA predictions only the anti-androgenic effects of the 21 active anti-androgens were used for the calculations. Testing of the mixture showed a deviation from the predicted effect, with a shift of the concentration response to lower concentrations.

Examination of the data of all individual compounds, including the “inactive” chemicals, revealed that two compounds, DEHP and DBP, were present at cytotoxic concentrations within this mixture. Within this assay system, cytotoxicity has the same effect on the assay readout as anti-androgenicity, but can be distinguished from anti-androgenicity from testing the compound in the absence of DHT. Due to this technical similarity of anti-androgenic and cytotoxic responses it was possible to take both effects into consideration for prediction modelling. Figure 12B) shows that, after including the effects of DEHP and DPB into the model, the tested mixture data corresponded well with the CA prediction.

The curves showing the individual effects of the compounds within the mixture in Figure 12 suggest that the toxic effects of DEHP and DPB for the present mixture ratios are even more pronounced than the effects of the dominant anti-androgens (BPA, PFOS).

The total concentration of all components in this mixture was 706 nM as indicated in Figure 12.

Mixture of 21 anti-androgenic and 10 non-anti-androgenic chemicals at ratios reflecting high human tissue levels

Next, a new mixture of the 21 anti-androgenic chemicals was designed. For this mixture, not average, but maximum exposure levels were used for the design of the mixture ratios, reflecting “high level”

exposure (scenario III). The results from prediction modelling and testing of the "high level" 21 component mixture are shown in Figure 13. The results from mixture testing showed that this mixture acted according to CA, with some changes in the major contributors for the mixture effects (BP3>BPA>PB2 etc.).

The concentration of all components together amounted to 1.14 mM, as indicated in Figure 13.

Mixture of 21 anti-androgenic and 10 non-anti-androgenic chemicals at ratios reflecting high human tissue levels

The "high exposure scenario" was expanded for the next mixture, including 21 anti-androgenic compounds as well as ten "inactive" chemicals, all at fixed mixture ratio reflecting high level exposure (scenario IV). The results from prediction modelling and testing of the "high level" 21+10 component mixture are shown in Figure 14.

Testing of the mixture showed a smaller impact of cytotoxicity of DEHP and DBP in this exposure scenario and the mixture results were in agreement with the CA prediction.

The concentration of all chemicals together in this mixture was 3.74 mM, as indicated in Figure 14.

1.3 Point of departure index (PODI) for mixtures of 21 anti-androgenic compounds reflecting average and high human tissue levels

The risk units (RUs) for each compound within the anti-androgenic mixtures at average and high human exposure levels were determined. The RU summation curves for both scenarios are shown in Figure 15 (A) for average and B) for high exposure). Both graphs show, that not all compounds contributed equally to the mixture effect. Only 5-6 chemicals produced the major part of the mixture effect, whereas the curves level out for the rest of the compounds present in the mixture, indicating only minor contribution to the effect.

For the average exposure scenario, the major contributors were BPA, BaP, PFOS and BP2, followed by PCBs 138 and 126. In the high exposure scenarios, BP2 and BP3 cause the largest proportion of the effect, followed by BPA, PFOS, HHCB and BaP.

Furthermore, the PODI (sum of RUs) for the average exposure mixture was 0.0207 and the high exposure RUs added up to 0.3827. Both PODI values are below 1, thus no effect was to be expected for both mixtures at the actual tissue level concentrations (see also above and Figures 11 to 14).

Table 11 shows the RUs together with the respective RU summation data for both scenarios.

Figure 10: Concentration-response relationships for antiandrogenic compounds included in the 21 compound mixture.

Non-linear regression fits for the indicated compounds. More detail on regression models and model parameters is given in Table 9.

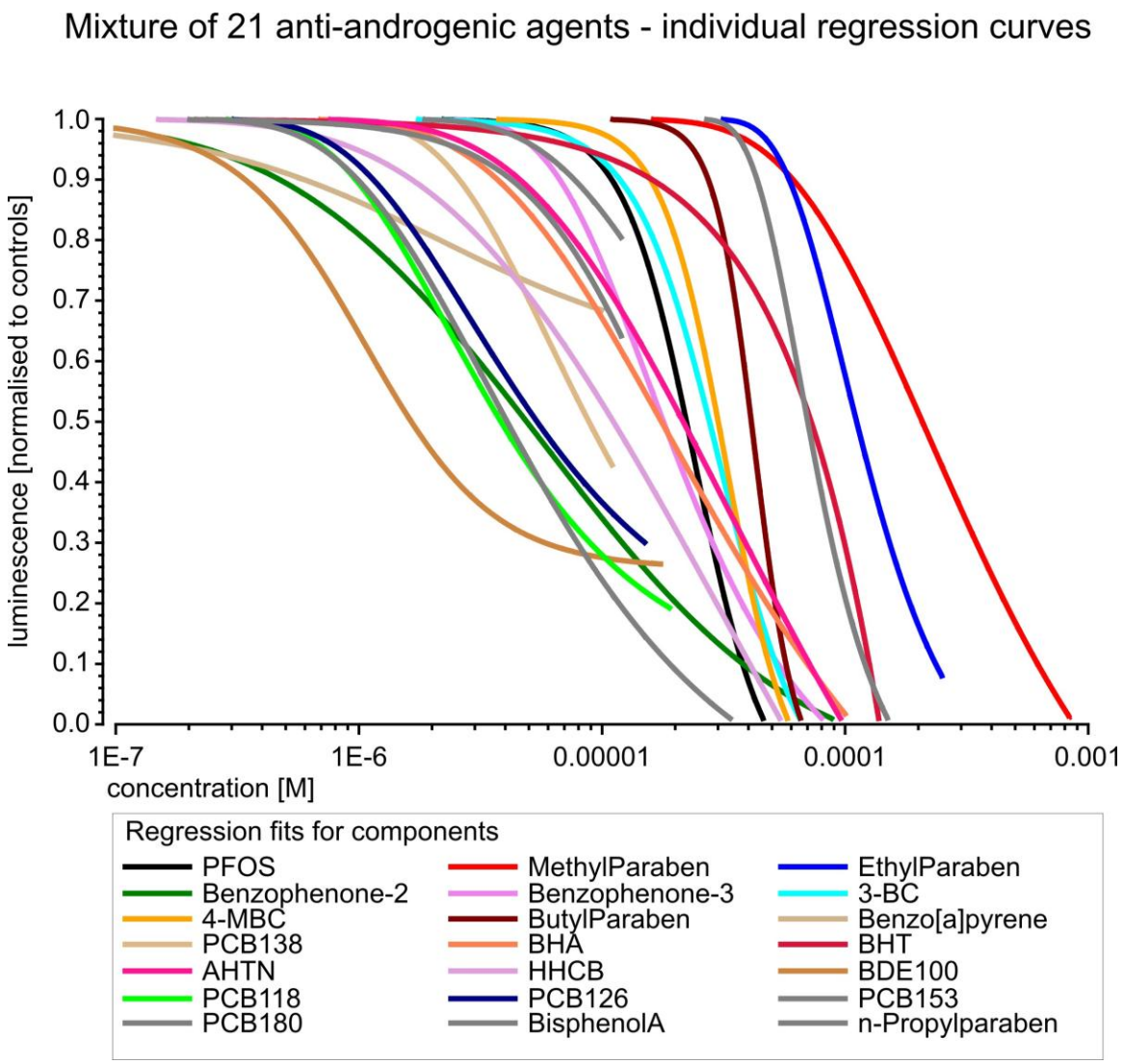


Table 9: Regression modelling data from all compounds included in the tested mixtures. The table contains the respective regression model which fit the data best and the corresponding model parameters. Furthermore, the EC_{10} and EC_{50} values for each compound, as derived from the regression analysis are given.

Compounds (in order of EC_{50})		*Concentration-Response Function*					*EC_{10}*		*EC_{50}*	
	RM	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_{min}$	$\hat{\theta}_{max}$	*M [CI]*		*M [CI]*	
BDE 100	logit	-22.58	-3.78	-	0.26	1	3.40E-7	[2.32E-7 - 4.99E-7]	1.64E-6	1.20[E-6 - 2.25E-6]
Benzophenone-2	probit	-6.83	-1.22	-	-0.07	1	4.71E-7	[2.34E-7 - 9.49E-7]	4.90E-6	[2.99E-6 - 8.01E-6]
PCB 118	Weibull	-12.08	-2.14	-	0.07	1	9.69E-7	[1.68E-7 - 1.28E-6]	3.84E-6	[3.09E-6 - 5.04E-6]
Benzo[a]pyrene	logit	-11.11	-1.96	-	0.60	1	5.94E-7	[2.97E-7 - 1.19E-6]	n.d.	n.d.
Bisphenol A	Weibull	-9.38	-1.71	-	-0.19	1	9.88E-7	[5.54E-7 - 1.51E-6]	4.07E-6	[3.48E-6 - 5.91E-6]
PCB 126	Weibull	-10.98	-1.98	-	0.11	1	1.14E-6	[8.81E-7 - 1.45E-6]	5.40E-6	[4.09E-6 - 5.94E-6]
HHCB	Weibull	-3.89	-0.87	-	-1.29	1	1.65E-6	[9.77E-7 - 3.05E-6]	1.11E-5	[8.88E-6 - 1.32E-5]
PCB 138	Weibull	-11.73	-2.25	-	0.01	1	2.58E-6	[1.71E-6 - 3.25E-6]	8.83E-6	[7.56E-6 - 9.95E-5]
BHA	Weibull	-6.54	-1.38	-	-0.42	1	3.71E-6	[2.61E-6 - 5.10E-6]	1.75E-5	[1.56E-5 - 2.11E-5]
PCB 180	logit	-16.96	-3.56	-	0*	1	4.23E-6	[2.60E-6 - 1.08E-5]	n.d.	n.d.
AHTN	Weibull	-5.56	-1.24	-	-0.73	1	4.59E-6	[2.48E-6 - 6.18E-6]	2.16E-5	[1.60E-5 - 2.66E-5]
Benzophenone-3	Weibull	-11.96	-2.49	-	-0.17	1	6.69E-6	[4.98E-6 - 8.99E-6]	1.79E-5	[1.51E-5 - 2.11E-5]
PCB 153	Weibull	-8.32	-1.79	-	0*	1	7.69E-6	[3.06E-6 - 1.13E-5]	n.d.	n.d.
PFOS	logit	-31.45	-6.81	-	-0.14	1	1.09E-5	[8.46E-6 - 1.42E-5]	2.22E-5	[2.01E-5 - 2.46E-5]
3-BC	logit	-25.60	-5.67	-	-0.15	1	1.18E-5	[8.59E-6 - 1.78E-5]	2.77E-5	[2.52E-5 - 3.09E-5]
4-MBC	logit	-33.79	-7.53	-	-0.14	1	1.58E-5	[1.10E-5 - 2.27E-5]	3.00E-5	[2.51E-5 - 3.60E-5]
BHT	G. logit II	-827	-217	0.011	-0.12	1	1.60E-5	[7.13E-6 - 2.09E-5]	7.24E-5	[6.31E-5 - 8.94E-5]
n-Butyl paraben	logit	-52.40	-11.99	-	-0.09	1	2.73E-5	[2.06E-5 - 3.62E-5]	4.11E-5	[3.59E-5 - 4.71E-5]
n- Propylparaben	Weibull	-21.52	-5.14	-	-0.16	1	4.34E-5	[3.50E-5 - 5.38E-5]	7.01E-5	[6.02E-5 - 8.18E-5]
Ethyl paraben	Weibull	-15.72	-3.93	-	-0.13	1	5.88E-5	[4.59E-5 - 6.71E-5]	1.11E-4	[1.03E-4 - 1.19E-4]
Methyl paraben	Weibull	-6.43	-1.76	-	-0.41	1	6.10E-5	[4.60E-5 - 7.50E-5]	2.08E-4	[1.77E-4 - 2.40E-4]

EC50, EC10: concentration provoking 50% and 10% effect (antiandrogenicity), respectively. Values in brackets denote the upper and lower limits of the approximate 95% confidence interval; the column “RM” indicates the mathematical regression function as defined at Scholze

et al. (2001): $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}, \hat{\theta}_{max}$ estimated model parameters , given for concentrations expressed in M (rounded values), $\hat{\theta}_{max}$ were set to the fixed value 1 relating to the mean value of the DHT controls; n.d. = not determined.

Figure 11: Predicted and observed concentration-response relationship for an antiandrogenic mixture with mixture ratios according to average human exposure levels (scenario I).

The graph shows the prediction and testing of the 21 component mixture at a fixed mixture ratio according to the average exposure levels found in human tissue. Antiandrogenic effects are predicted according to CA (green line) and IA (blue line). The light green area is an extrapolation of the CA model beyond the predictability due to the lack of full concentration response data for PCBs 118, 126, 138, 153, 180, BaP and BDE100. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The black circles show the data points of 3 independent experiments and the solid red line represents the non-linear regression fit for the data. The light grey lines represent the effects of the individual components within the mixture. The red arrow indicates the total concentration of the mixture as present in human tissue.

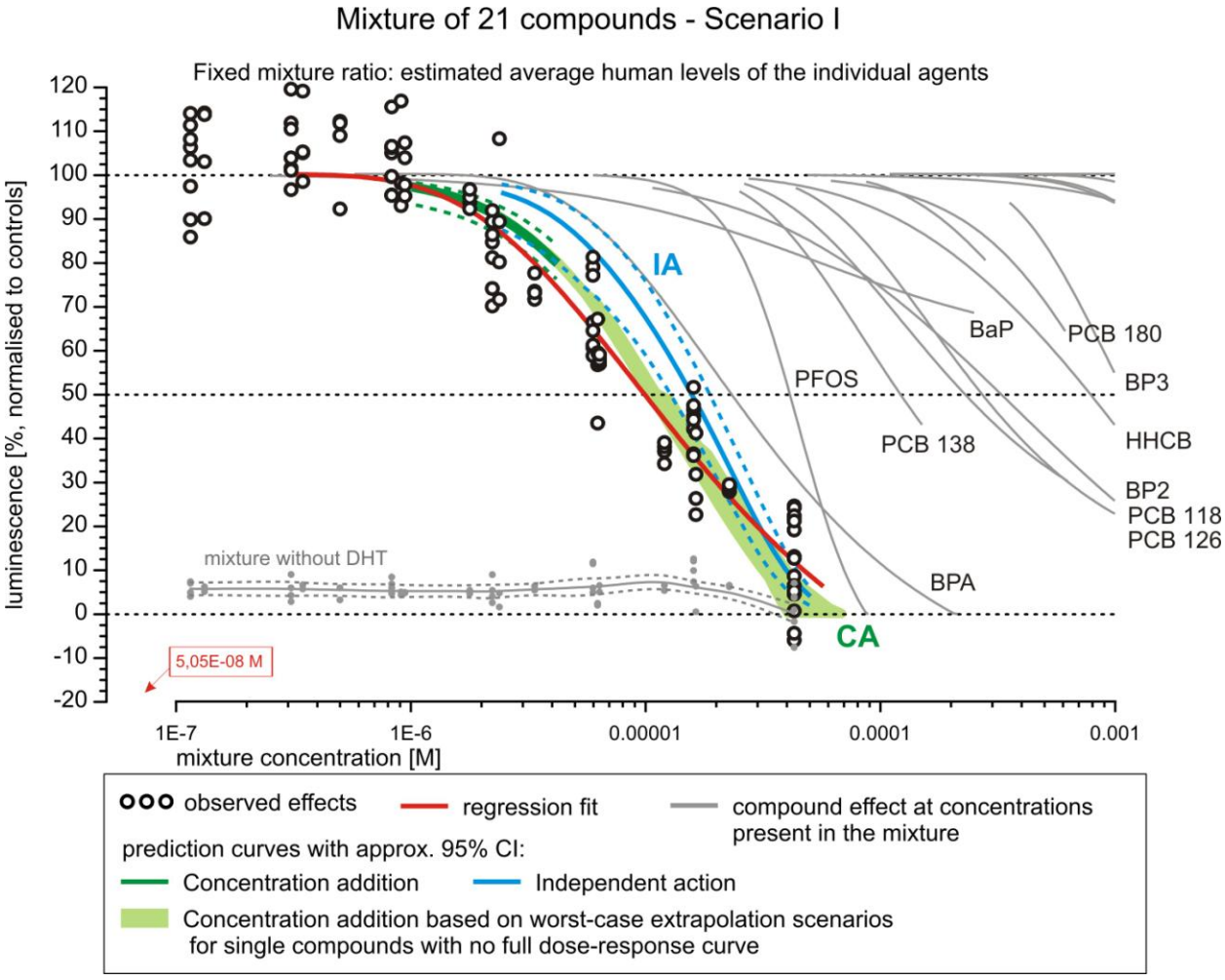
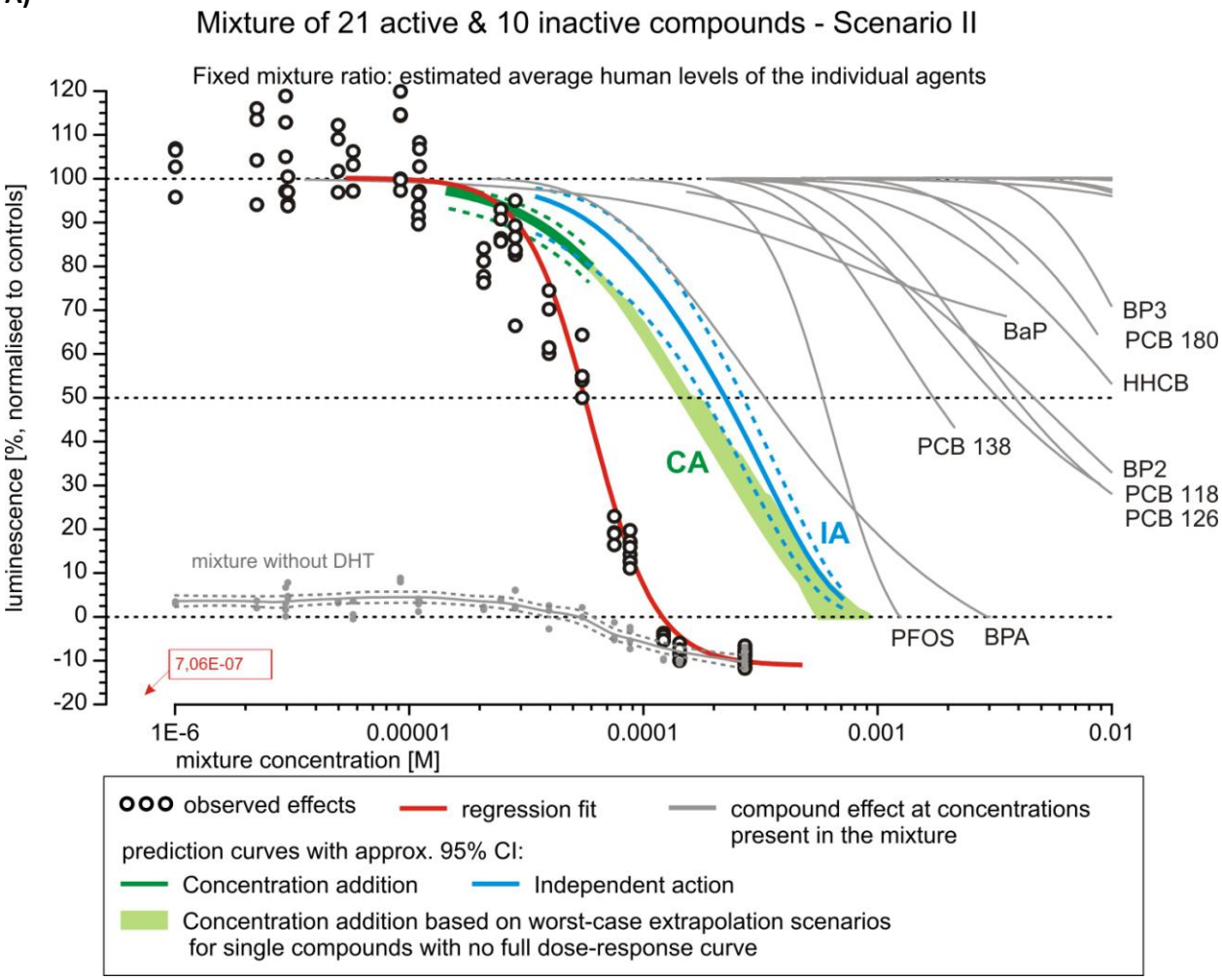


Figure 12: Predicted and observed concentration-response relationship for a mixture containing 21 anti-androgenic and 10 inactive compounds with mixture ratios according to average human exposure levels (scenario II).

The graphs show the prediction and testing of the mixture composed of 21 anti-androgenic and 10 non-active compounds. The mixture was designed at a fixed mixture ratio according to the average exposure levels found in human tissue. Both graphs show the predicted antiandrogenic effects according to CA (green line) and IA (blue line). The light green area is an extrapolation of the CA model beyond the predictability due to the lack of full concentration response data for PCBs 118, 126, 138, 153, 180, BaP and BDE100. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The black circles show the data points of 3 independent experiments and the solid red line represents the non-linear regression fit for the data. The light grey lines represent the effects of the individual components within the mixture. The red arrow indicates the total concentration of the mixture as present in human tissue. For prediction modelling in graph A) only the data for the 21 active anti-androgens have been taken into account. Graph B) shows the same mixture, but with the cytotoxic effects of the "inactive" compounds included in the prediction models. Individual cytotoxic effects for the compounds are shown in purple.

A)



B)

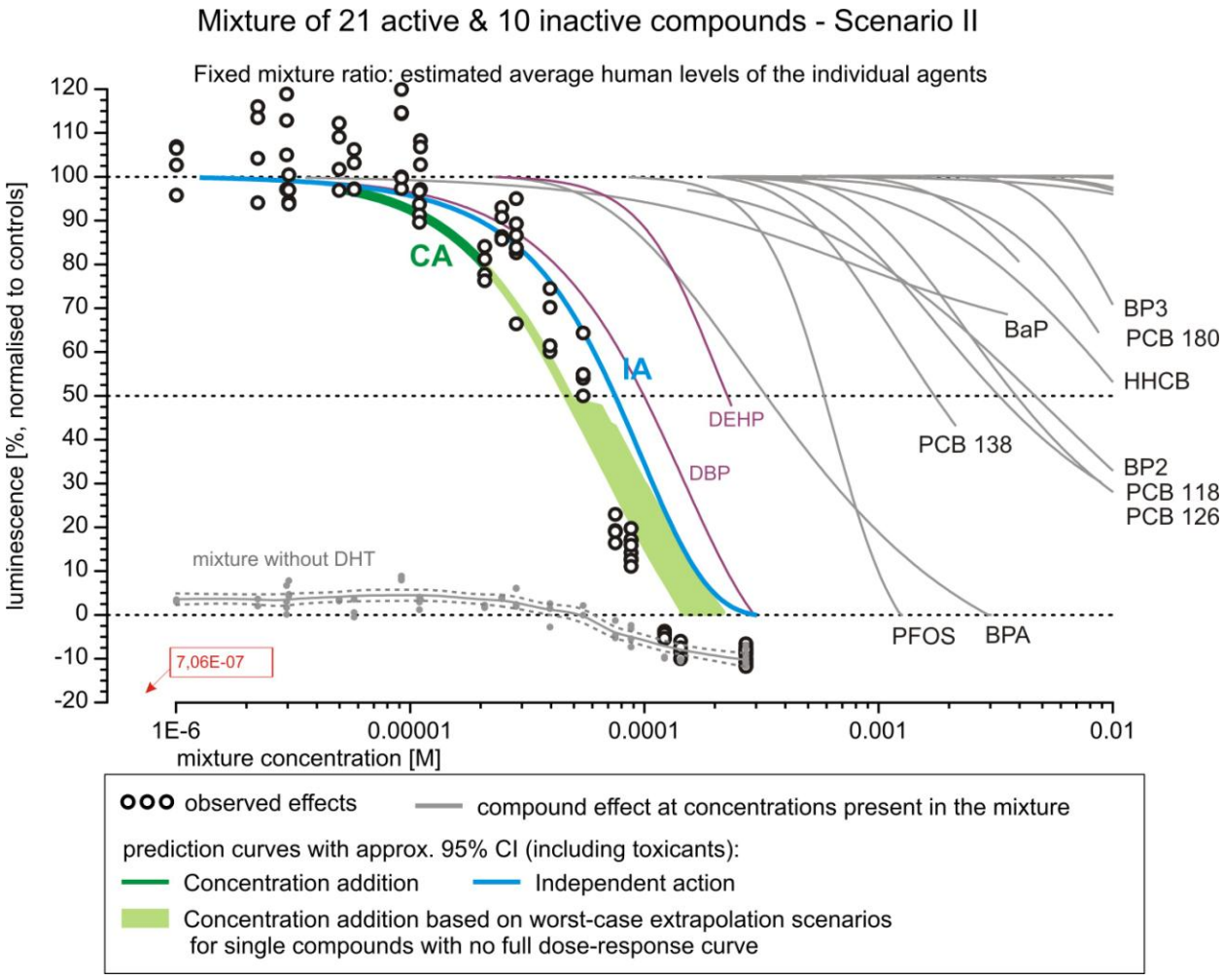

Mixture of 21 active & 10 inactive compounds - Scenario II
Fixed mixture ratio: estimated average human levels of the individual agents
luminescence [%, normalised to controls]
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
CA
IA
DEHP
DBP
BaP
BP3
PCB 180
HHCB
PCB 138
BP2
PCB 118
PCB 126
mixture without DHT
PFOS
BPA
7,06E-07
1E-6
0.00001
0.0001
0.001
0.01
mixture concentration [M]
observed effects
compound effect at concentrations present in the mixture
prediction curves with approx. 95% CI (including toxicants):
Concentration addition
Independent action
Concentration addition based on worst-case extrapolation scenarios
for single compounds with no full dose-response curve

Figure 13: Predicted and observed concentration-response relationship for an antiandrogenic mixture with mixture ratios according to high human exposure levels (scenario III).

The graph shows the prediction and testing of the 21 component mixture at a fixed mixture ratio according to high exposure levels found in human tissue. Antiandrogenic effects are predicted according to CA (green line) and IA (blue line). The light green area is an extrapolation of the CA model beyond the predictability due to the lack of full concentration response data for PCBs 118, 126, 138, 153, 180, BaP and BDE100. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The black circles show the data points of 3 independent experiments and the solid red line represents the non-linear regression fit for the data. The light grey lines represent the effects of the individual components within the mixture. The red arrow indicates the total concentration of the mixture as present in human tissue.

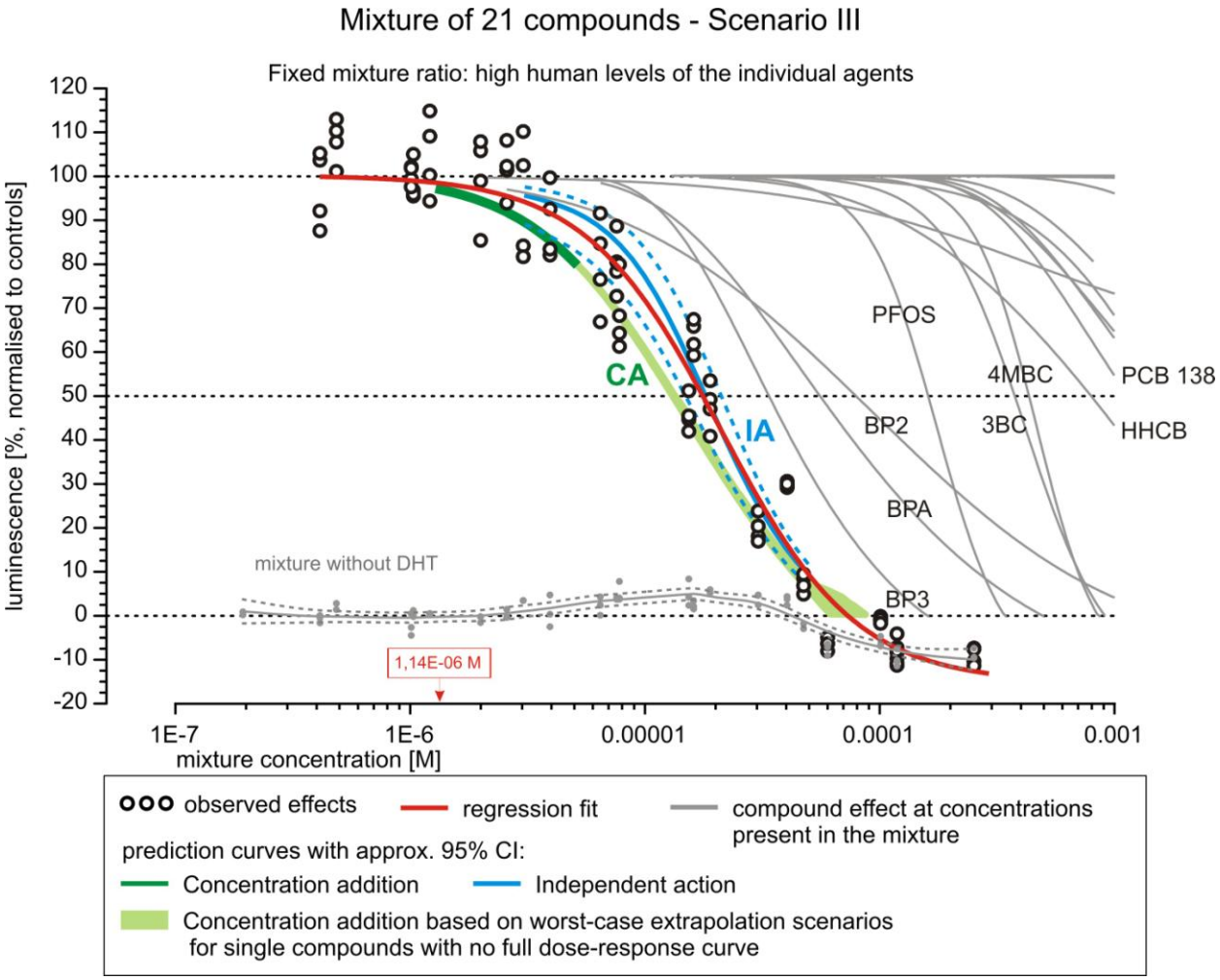


Figure 14: Predicted and observed concentration-response relationship for a mixture containing 21 anti-androgenic and 10 inactive compounds with mixture ratios according to high human exposure levels (scenario IV).

The graph shows the prediction and testing of the mixture composed of 21 anti-androgenic and 10 non-active compounds. The mixture was designed at a fixed mixture ratio according to high exposure levels found in human tissue. Antiandrogenic effects are predicted according to CA (green line) and IA (blue line). The light green area is an extrapolation of the CA model beyond the predictability due to the lack of full concentration response data for PCBs 118, 126, 138, 153, 180, BaP and BDE100. Dashed lines in the respective colours indicate the 95% confidence interval for the data fit and the predictions. The black circles show the data points of 3 independent experiments and the solid red line represents the non-linear regression fit for the data. The light grey lines represent the effects of the individual anti-androgenic components within the mixture. Purple lines show the individual cytotoxic effects of compounds within the mixture. The red arrow indicates the total concentration of the mixture as present in human tissue.

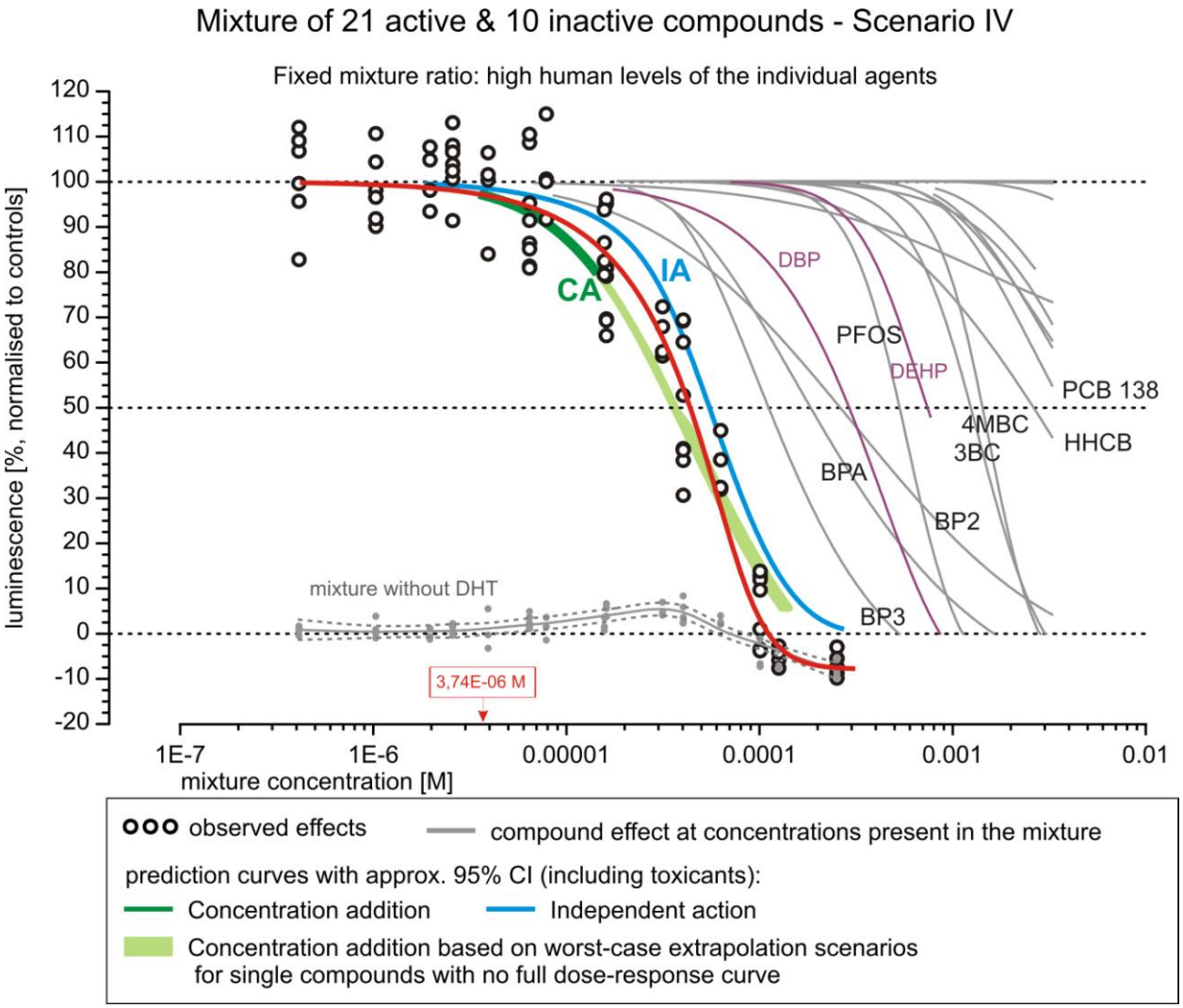


Table 10: Tissue levels and relative proportions of each compound (mixture ratio) for the examined mixtures.

Compounds (in order of EC_{50})	*average tissue levels [M]*	*Relative proportions (percentages, rounded values)*		*high tissue levels [M]*	*Relative proportions (percentages, rounded values)*	
		21 compounds average exposure	21 compounds +10 inactives average exposure		21 compounds high exposure	21 compounds +10 inactives high exposure
active compounds						
BDE 100	8.12E-13	0.0016%	0.0001%	1.59E-11	0.0014%	0.0004%
BP2	7.50E-10	1.49%	0.11%	7.00E-8*+	6.13%	1.87%
PCB 118	7.08E-10	1.40%	0.10%	2.83E-9	0.25%	0.076%
Benzo[a]pyrene	1.98E-9	3.92%	0.28%	5.55E-9	0.49%	0.15%
Bisphenol A	8.76E-9	17.35%	1.24%	8.28E-8	7.26%	2.22%
PCB 126	1.18E-9	2.34%	0.17%	3.54E-9	0.31%	0.095%
HHCB	7.10E-10	1.41%	0.10%	1.59E-8	1.39%	0.42%
PCB 138	3.63E-9	7.19%	0.51%	8.75E-9	0.77%	0.23%
BHA	5.09E-11	0.10%	0.0072%	1.02E-10	0.0089%	0.0027%
PCB 180	9.74E-10	1.93%	0.18%	1.21E-8	1.06%	0.32%
AHTN	1.77E-10	0.35%	0.25%	3.10E-9	0.27%	0.083%
BP3	8.09E-10	1.60%	0.11%	6.00E-7*	52.58%	16.06%
PCB 153	2.13E-9	4.23%	0.30%	1.67E-8	1.46%	0.45%
PFOS	2.70E-8	53.41%	3.82%	1.56E-7	13.71%	4.19%
3-BC	4.81E-10	0.95%	0.068%	8.32E-8	7.29%	2.23%
4-MBC	4.54E-10	0.90%	0.064%	7.86E-8+	6.89%	2.11%
BHT	4.99E-10	0.99%	0.071%	9.99E-10	0.088%	0.027%
n-Butyl paraben	2.46E-11	0.049%	0.0035%	1.09E-10	0.0095%	0.0029%
n- Propylparaben	2.34E-11	0.046%	0.0033%	2.34E-11	0.0021%	0.0006%
Ethyl paraben	2.21E-11	0.044%	0.0031%	8.17E-11	0.0072%	0.0022%
Methyl paraben	1.54E-10	0.31%	0.022%	3.53E-10	0.031%	0.0095%

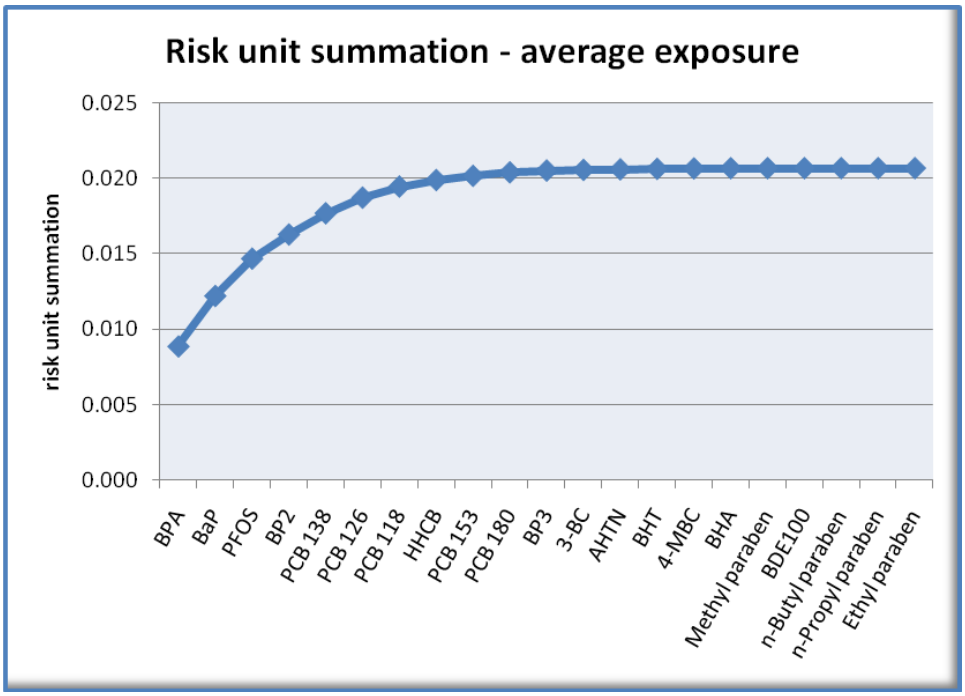
inactive compounds						
$HgCl_2$	1.84E-9	-	0.26%	7.00E-9	-	0.19%
$CdCl_2$	2.19E-9	-	0.31%	5.65E-9	-	0.15%
Pb NO_3	9.06E-8	-	12.82%	2.35E-7	-	6.29%
DEHP	3.00E-7	-	42.46%	4.80E-7	-	12.85%
DBP	2.00E-7	-	28.31%	3.59E-7	-	9.61%
PFOA	1.21E-8	-	1.71%	9.66E-8	-	2.59%
6:2 FTOH	1.37E-8	-	1.94%	1.10E-7	-	2.94%
8:2 FTOH	1.08E-8	-	1.52%	8.62E-8	-	2.31%
OMC	1.72E-8	-	2.44%	3.44E-8	-	0.92%
Genistein	7.59E-9	-	1.07%	1.18E-6	-	31.60%

*Adjusted values to avoid domination of the mixture effect. [+]No direct tissue levels available, thus the values were estimated from intake levels, compared to related compounds (BP2 with BP3 and 4-MBC with 3-BC).

Figure 15: Risk unit summations for antiandrogens

The graph shows the RU summation for the compounds included in the mixtures designed according to human tissue levels. RUs were determined for the average exposure (A) and high exposure scenario (B). For RU and RU summation values see also Table 11.

A)



B)

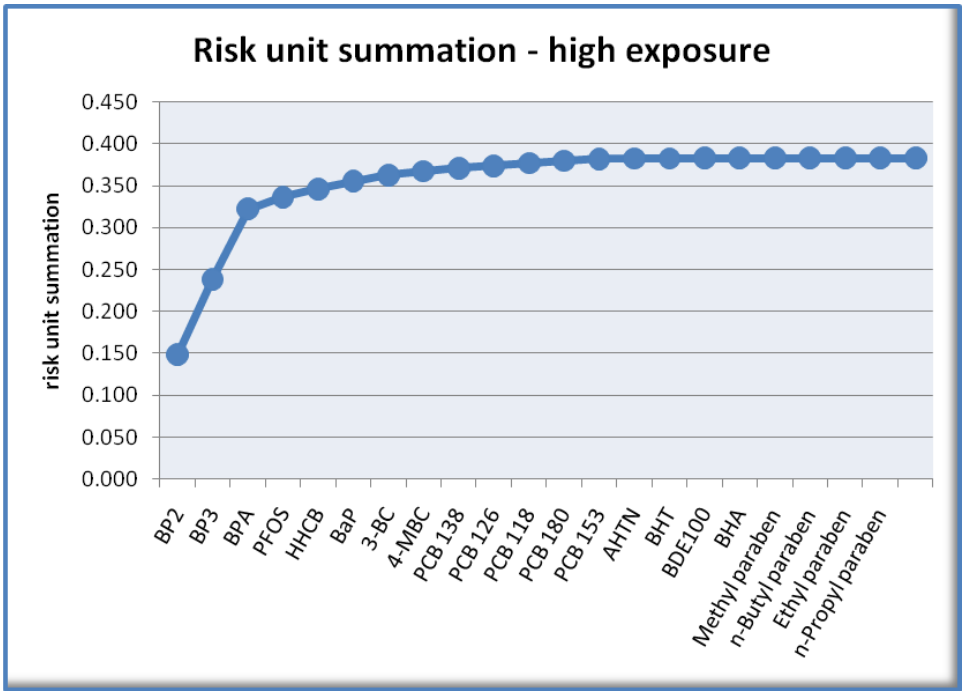


Table 11: Risk units and RU summation for the compounds included in mixtures designed according to average and high human tissue levels.

Risk unit summation - average exposure scenario			*Risk unit summation - high exposure scenario*		
Compounds (ranked by RU)	RU	RU summation*	Compounds (ranked by RU)	RU	RU summation*
BPA	8.87E-03	8.87E-03	BP2	1.49E-01	1.49E-01
BaP	3.34E-03	1.22E-02	BP3	8.97E-02	2.38E-01
PFOS	2.47E-03	1.47E-02	BPA	8.38E-02	3.22E-01
BP2	1.59E-03	1.63E-02	PFOS	1.44E-02	3.36E-01
PCB 138	1.41E-03	1.77E-02	HHCB	9.62E-03	3.46E-01
PCB 126	1.04E-03	1.87E-02	BaP	9.34E-03	3.55E-01
PCB 118	7.31E-04	1.94E-02	3-BC	7.05E-03	3.62E-01
HHCB	4.30E-04	1.99E-02	4-MBC	4.98E-03	3.67E-01
PCB 153	2.78E-04	2.02E-02	PCB 138	3.39E-03	3.71E-01
PCB 180	2.30E-04	2.04E-02	PCB 126	3.11E-03	3.74E-01
BP3	1.21E-04	2.05E-02	PCB 118	2.92E-03	3.77E-01
3-BC	4.07E-05	2.05E-02	PCB 180	2.86E-03	3.80E-01
AHTN	3.87E-05	2.06E-02	PCB 153	2.17E-03	3.82E-01
BHT	3.12E-05	2.06E-02	AHTN	6.75E-04	3.83E-01
4-MBC	2.88E-05	2.06E-02	BHT	6.24E-05	3.83E-01
BHA	1.37E-05	2.07E-02	BDE100	4.69E-05	3.83E-01
Methyl paraben	2.53E-06	2.07E-02	BHA	2.74E-05	3.83E-01
BDE100	2.39E-06	2.07E-02	Methyl paraben	5.79E-06	3.83E-01
n-Butyl paraben	8.99E-07	2.07E-02	n-Butyl paraben	3.98E-06	3.83E-01
n-Propyl paraben	5.39E-07	2.07E-02	Ethyl paraben	1.39E-06	3.83E-01
Ethyl paraben	3.75E-07	2.07E-02	n-Propyl paraben	5.39E-07	3.83E-01

* For RU summation, the RUs for all compounds were ranked. The highest value was chosen as initial RU. For subsequent values, the RU of the following compounds was added to the sum of the previous RUs, respectively.

Conclusions

ERLUX and ESCREEN

Mixtures of 29 components, including active and modulator components, have been tested in two estrogenicity assays, ESCREEN and ERLUX. The effects of the mixtures were generally well predicted by using the concentration addition (CA) model and data for the individual components tested alone.

Inclusion of modulator chemicals at concentrations relevant to the human exposure scenario showed minimal alteration of the effect of co-exposed active chemicals. This indicates that the negative modulation that was observed in the previous task (Task 3, see chapter 4) using different mixture ratios, is not observed when mixtures are based on human exposure concentrations.

Analysis of toxic unit distributions indicated that mixtures designed using human exposure levels are not balanced, i.e. certain components make more of a contribution to the overall effect than do others. The development of this approach in artificial, *in vitro* systems provides a solid basis from which the approach can be extended to more complex scenarios.

MDA-kb2 assay (ARLUX)

Four different scenarios for anti-androgenic mixtures at mixture ratios according to human tissue levels were tested in the MDA-kb2 assay. These included two 21 compound mixtures comprising anti-androgenic chemicals either at average or high exposure levels and two 31 compound mixtures which were composed of the 21 anti-androgens plus 10 non-anti-androgenic chemicals.

All 21- or 31-component mixtures tested acted according to concentration addition. For mixtures including chemicals without anti-androgenic activity, we found that other responses, such as cytotoxicity, have also to be taken into account for accurate mixture prediction.

None of the actual concentrations of the mixtures reflecting average or high exposure scenarios were within the active range of anti-androgenicity. However, whereas for the average tissue levels (scenarios I and II) the value was almost two orders of magnitude below the effective concentrations, it was less than an order of magnitude lower for the high tissue level scenarios (III and IV). These findings were also reflected in the PODI determined for both mixture scenarios, which was below one in both cases.

Determination of RUs allowed determination of the major contributors to the mixture effect for all mixture scenarios. RU analysis showed that usually 5-6 compounds had the major impact on the mixture effect, whereas the residual components participated only with minor effects.

General conclusions

In summary, the general aims of the studies of mixtures of food contaminants and additives that reflect human tissue levels proposed as task 4 of the project could be realised.

Chapter 6:
Discussion and Implications for Risk Assessment

Discussion

The need for considering the effects of mixtures

Due to the nature of the *in vitro* assays employed in this project, implications for the risk assessment of endocrine disrupters as a specific group of chemicals have to be discussed with care. Nevertheless, key findings of our project have significance in the context of generic issues and questions that often arise in chemicals risk assessment applied to mixtures.

Although humans are typically exposed to multi-component chemical mixtures, present in the surrounding environmental media (water, air, soil), in food or in consumer products, chemical risk assessment usually considers the effects of single substances in isolation. This approach could be justified if the exposure to mixtures did not bear the risk of an increased joint effect. This would be the case, for example, if only one chemical of the mixture is effective while the others are biologically inert, or if empirical evidence showed that the joint action of chemicals is typically not larger than the effect of the most toxic compound.

This project has produced good evidence that chemicals with common specific modes of action (receptor agonism or antagonism) work together to produce combination effects that are larger than the effects of each mixture component applied singly. It appears that the assumption underpinning chemical-by-chemical risk assessment is not met at the receptor level.

Predictability of mixture effects

One of the key aspirations of mixture toxicology has been to anticipate quantitatively the effects of mixtures of chemicals from knowledge about the toxicity of their individual components. This can be achieved by making the assumption that the chemicals in the mixture act in concert by exerting their effects without diminishing or enhancing each others' toxicity, the so-called non-interaction or additivity assumption. *Concentration addition* and *independent action* are the two concepts available for formulating the null hypothesis of additivity. Synergisms or antagonisms can then be defined in relation to this additivity assumption as upwards or downwards deviations, respectively.

The studies conducted in this project have yielded good evidence that *concentration addition* can give reasonable approximations for the prediction of combination effects when the effects of individual mixture components are known. Application of the competing concept of *independent action* led to underestimations of the experimentally observed effects.

Deviations from predicted additivity, indicative of synergisms or antagonisms, were only rarely observed, and were relatively small. There is no need for the experimental testing of each and every conceivable mixture, which would indeed make risk assessment unmanageable.

Application of assessment concepts in risk assessment practice

A question of importance to risk assessment and regulation is which of the two concepts, *concentration addition* or *independent action*, should be chosen for the interpretation of empirical data, or for anticipating mixture effects of untested combinations. Although both *concentration addition* and

independent action often provide good approximations of mixture effects, the issue of distinguishing between these concepts becomes important when the two concepts predict quantitatively different mixture toxicities, as was the case with mixtures of androgen receptor antagonists and estrogen receptor agonists.

Concentration addition is thought to be applicable to mixtures composed of chemicals with a similar mode of action. Conversely, *independent action* is applied to chemicals with diverse modes of action. It can be argued that the nature of the assays employed in this project (responses due to receptor activation or antagonism) precluded the applicability of *independent action* because truly independent events could not occur (with the possible exception of the Escreen assay). Even in systems representative of higher biological complexity, the practical relevance of *independent action* for the assessment of mixture effects can be called into question. The principle of strictly independent events may only rarely be relevant, due to converging signalling pathways and inter-linked subsystems. For these reasons, *concentration addition* can be viewed as being more broadly applicable.

In this project, we have in some cases evaluated the two concepts comparatively, side-by-side in the same experimental system. In the majority of cases, we found that *concentration addition* has provided more conservative mixture toxicity estimates, although the predictions derived from both concepts produced concentration estimates that differed by no more than a factor of 5.

For example, according to *concentration addition*, the EC_{50} of a mixture can be predicted based on the EC_{50} values of the individual components. Because such values are statistically highly reliable measures, documented in published toxicological studies and/or compiled in publically available databases, the calculation of an EC_{50} for a mixture derived from *concentration addition* usually does not pose particular problems. This principle can also be applied by utilizing points of departure (benchmark doses, NOAELs) in the Hazard Index or the Point of Departure Index approaches, as we have utilized for the work relating to Task 4 (see chapter 5).

In contrast, the use of *independent action* requires knowledge about the precise effects that each component would provoke if present individually at the concentration found in the mixture. This information is not readily available.

Thus, the concept of *concentration addition* is less demanding in terms of data requirements, which increases its applicability. Considering that the prediction differences between *concentration addition* and *independent action* are not very pronounced, and that *concentration addition* often produces the more conservative prediction, its use as the preliminary default concept for the assessment and prediction of mixture effects is well supported.

The contribution of single chemicals to an overall mixture effect in mixtures composed according to "real world" scenarios

Our studies with mixtures composed according to mixture ratios reflecting measured human tissue concentrations of estrogens and anti-androgens have shown that only a relatively small number of components explained most of an overall combined effect. If applicable to endpoints of toxicological

relevance for risk assessment, the implications of this observation could be very significant: The apparent complexity of real exposure scenarios could be reduced to a manageable group of relevant chemicals, possible health risks assessed more easily and significant risk reductions be achieved through targeted exposure reduction measures. However, for endocrine disrupters the issue cannot be resolved without information about the range of these chemicals that make up the internal exposure of humans. This information is currently only fragmentary. It remains to be seen whether the phenomenon also appears when a much larger number of chemicals is included for assessment, i.e. whether it is independent of the number of chemicals and thus can be generalized.

The knowledge gained as part of task 4 studies could be applied directly to efforts aimed at deriving measures of total internalized estrogenic or anti-androgenic load. Complex samples (extracts) from biological tissues or food items, all of unknown chemical composition, are beginning to be subjected to biotesting with *in vitro* assays (bioassay-directed fractionations). However, the measurement values that reflect internal load are unspecific in terms of the chemicals that produced the effects. Conversely, lists of chemicals and their levels do not reveal anything about their combined potency, making it impossible to explain internal loads in terms of specific combinations of individual chemicals. To bridge the gap between the above two types of data, it would be necessary to apply knowledge about the way in which chemicals act together in mixtures. The outcomes of the studies from task 4 show how *concentration addition* can play a vital role when used in concert with advanced chemical-analytical techniques in order to pinpoint the most important pollutants, which can then guide further investigations and/or risk management steps.

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