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Deliverable D3.5

Scientific article ready for submission describing the results of the in vitro testing of mixtures

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Deliverable D3.5

PART 1

Scientific article ready for submission describing the results of the in vitro testing of mixtures regarding liver toxicity

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1. Introduction

This deliverable provides an instruction on the EuroMix testing strategy for testing compounds grouped based on their Mode of action (MoA) and their mixtures regarding the liver specific endpoint steatosis as well as an overview of the results obtained by following an AOP-wise testing strategy.

To address one major question of mixture testing in EuroMix, namely how compounds with similar and dissimilar MoA act in combination, different acting compounds were selected for detailed mixture testing. These compounds were solely chosen based on their MoA (NR activation for steatosis) to have compounds with preferably clear similar or dissimilar MoA. Following derivation of relative potency factors (RPF) from the dose-response curves of lipid accumulation testing of the single compounds, mixture testing was based on equipotent combinations of chemicals A, B and C (AB, AC, BC and ABC). Here, A and B are proposed to have a similar MoA while C has a dissimilar MoA compared to A and B. By using the Benchmark Dose modelling software PROAST, mixture experiments were analyzed for dose addition or deviation from dose addition (e.g. synergism etc.).

2. Testing procedure

2.1 Choice of compounds to be tested in vitro

For compounds which are already grouped in cumulative assessment groups (CAGs) like CAG1 (target organ liver) and grouped in CAG2B (fatty acid changes) by EFSA, the selection is based on data for dietary exposure and hazard information. Thus, the choice of compounds to be considered for testing is based on the exposure driven approach and the likelihood of co-exposure as described in detail in Deliverable 3.2 “How to select the chemicals for mixture testing based on the EuroMix concept” and by Crépet et al. (2018).

Additionally, in silico approaches like a CAG2-specific EuroMix QSAR model and a molecular docking simulation developed as described in D 2.2 “Report on the use of in silico methods for the prioritization of substances and mixtures thereof”, could be used to select compounds for further analysis. If a compound is positive in the EuroMix QSAR model and in simulation of the steatosis-specific nuclear receptor docking, AOP-wise testing should be considered.

For the purpose of EuroMix, 126 substances of the EuroMix inventory list have been identified for steatosis (EuroMix Chemical Inventory) (see Deliverable 2.1 “Report describing cumulative assessment groups for a broad range of chemicals, based on information extracted from literature and databases”). As described in Deliverable 5.1, the compounds forming the main mixtures were selected due to data indicating combined exposure and to their potential to cause steatosis (e.g.: Imazalil) and grouped regarding their mode of action as listed in Table 1.

Table 1: Overview of compounds considered for mixture testing according to their MoA

similar acting compounds; main MoA: PXR activation	
Imazalil	activation of PXR, AhR, CAR, RAR
Thiacloprid	activation of PXR and PPARγ, PPARa-antagonism
Fenpyroximate	activation of PXR
T0901317	activation of PXR, LXR
similar acting compounds; main MoA: PPAR activation	
2-propylheptanoic acid	activation of PPARa/g, RXR, GR, RAR
2-propylhexanoic acid	activation of PPARa/g, RXR, GR, RAR
dissimilar acting compounds; nuclear receptor independent	
Cyclosporin A	no nuclear receptor activation
Clothianidin	PPARa-antagonism

In this deliverable the results of the in vitro testing of different compounds and their binary and ternary mixtures are described. The mixture design follows the EuroMix approach, where compound A and compound B are proposed to have a similar MoA while compound C has a dissimilar MoA compared to A and B. Representative for all possible mixtures of compounds listed in Table 1, this deliverable will focus on the results of the mixture testing of Thiacloprid and Imazalil (as A and B compounds) and Clothianidin (C compound).

2.2 Tentative Adverse Outcome Pathway (AOP)-wise testing for steatosis

EuroMix is a Horizon2020 EU-project which aims to develop an AOP-wise and quantitative test strategy (bioassay toolbox) regarding combined mixture effects of food relevant residues and contaminants. An AOP describes, in a linear fashion, linkages (key events relationships) between chemically-induced adverse effects (key events, KEs) at various levels of biological organization, progressing from a molecular initiating event (MIE) to an adverse outcome (AO) that is relevant to risk assessment and regulatory decision-making (see Figure 1). A toolbox which addresses all relevant molecular initiating events and key events of the AOP steatosis was developed and evaluated using cyproconazole, a fungicide inducing steatosis in rodents (Luckert et al. 2018). This toolbox addresses the nuclear receptor activation (molecular initiating event) as well as different molecular and cellular key events (gene and protein expression, lipid accumulation) downstream the AOP.

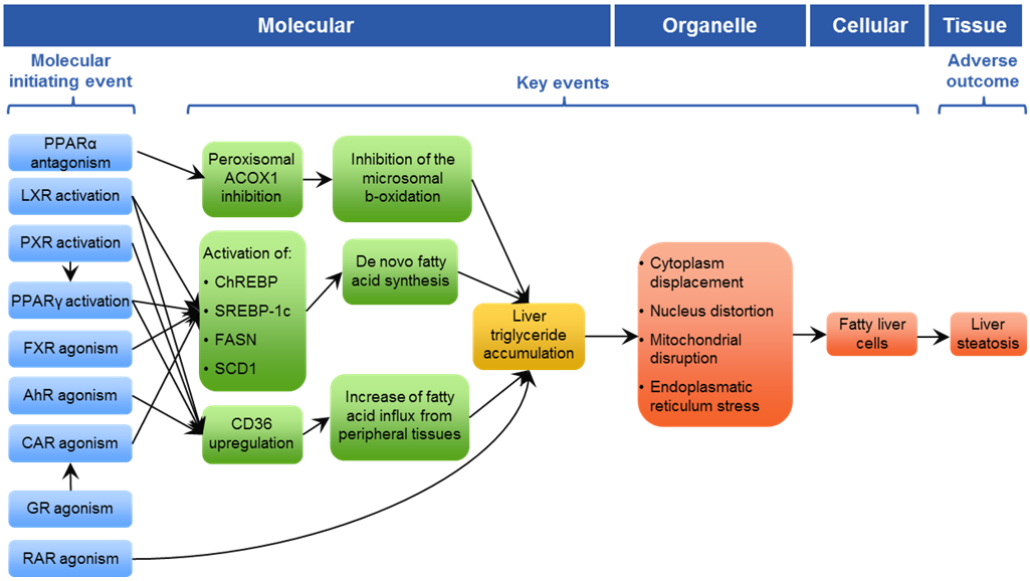


Figure 1 : AOP for liver steatosis

2.2.1 In vitro test system

In the EuroMix project an *in-vitro* assay toolbox was developed to evaluate the role of mode of action (MoA) and key events of chemicals (Deliverable 3.2). The bioassay toolbox contains optimal *in-vitro* assays to detect MoA for liver toxicity and maps to molecular initiating events (MIE) and key events (KE) of the AOP for steatosis. Assay evaluation was performed in human HepaRG hepatocarcinoma cells exposed to the model compound cyproconazole, a fungicide inducing steatosis in rodents as described in Luckert et al. (2018).

2.2.2 In-vitro bioassay-toolbox to asses liver steatosis

Many different cell based assays were available to assess key events of the AOP with different effort in terms of cost and time. Within the EuroMix Project several assays were chosen and tested to reflect some of the molecular events for MoA analysis and the major adverse outcome on cellular level (lipid accumulation). Based on the proof of principle of the AOP-wise testing strategy tested with cyproconazol described by Luckert et al. 2018 and in Deliverable 3.2, the listed assays and procedures were summarized as a bioassay toolbox and used for the assessment of mixture effects.

<u>HepaRG cell culture and exposure to test compounds</u>

Undifferentiated HepaRG cells were purchased from Biopredic International (Saint Grégoire, France). Cells were seeded at a density of approximately 25,000 cells/cm^2 and cultivated in William's Medium E with 2 mM glutamine (PAN-Biotech, Aidenbach, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS; FBS Good Forte EU approved, PAN-Biotech, Aidenbach, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany) and 5 x 10^{-5} M hydrocortisone hemisuccinate (Sigma-Aldrich, St. Louis, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. For differentiation, cells were maintained in culture medium for 14 days after seeding, followed by another 14 days of cultivation in the above-mentioned culture medium containing additionally 1.7% DMSO. For treatment with pesticides, differentiated HepaRG cells were first adapted to treatment medium (culture medium containing only 2% FBS and 0.5% DMSO) for 48 h. After adaptation, cells were exposed to indicated pesticides in treatment medium for 24 h or 72 h with a final DMSO concentration of 0.5%.

Cultivation of HepG2 cells

The human hepatocellular carcinoma cell line HepG2 was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, PAN-Biotech, Aidenbach, Germany) supplemented with 10% (v/v) fetal calf serum (PAN-Biotech, Aidenbach, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged at a confluence of about 80-90% and seeded at a density of 60,000 cells/cm^2.

Cell viability testing: WST-1 assay

To determine cytotoxic concentrations for compounds of interest in HepaRG cells as well as in HepG2 cells, the commercially available WST-1 assay can be performed. The assay relies on the cellular enzymatic reduction of a substrate (tetrazolium salts) to a product (formazan) which can then be spectrophotometrically quantified to determine cell viability. Cell viability testing is not directly relevant for AOP-wise liver steatosis testing but a prerequisite for all cell based assays.

Reporter gene assays for nuclear receptor activation

To determine activation of human nuclear receptors PXR, PPARα, PPARγ, PPARδ, LXRα, FXR, RXRα, RARα and CAR, GAL4/UAS-based transactivation assays were performed in HepG2 cells. This assay uses a fusion protein of the ligand-binding domain (LBD) of the nuclear receptor and the DNA-binding domain (DBD) of the yeast-specific GAL4 transcription factor. In combination with a luciferase reporter construct driven by multiple copies of the GAL4-responsive upstream activating sequence (UAS), this enables analyses of ligand binding to the respective receptor and the resulting transactivation potential of the nuclear receptor. To investigate PXR- CAR- or VDR-mediated induction of CYP2B6 promoter activity as well as activation of nuclear receptors AhR and GR, classical reporter gene assays were performed also in HepG2 cells. These assays rely on specific DNA sequences responsive to the respective transcription factor of interest which are cloned in front of a luciferase reporter gene.

Analysis of mRNA expression levels

HepaRG cells were differentiated in 12-well plates and treated with different concentrations of pesticides or solvent control (0.5% DMSO) for 24 h. Cells were washed and lysed with RLT buffer (RNeasy Mini Kit, Qiagen, Hilden Germany). Total RNA was extracted according to the manufacturer protocol. The RNA was quantitated spectrophotometrically and RNA integrity was estimated. For first-strand cDNA synthesis, 2 µg of total RNA were reverse-transcribed into cDNA, using the SuperScript First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer instructions using oligo dT primers for the reaction. Primers for genes of interest were designed using NCBI's Primer-BLAST tool. SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, USA) was used to measure the expression of 69 genes linked to liver steatosis, nuclear receptor activation and hepatotoxicity (see Luckert et al. (2013)). Expression levels of the target genes were normalized to the reference gene B2M which was found to be stably expressed throughout treatments. RNA from three independent, biological replicates was used. Each cDNA was analyzed at least in duplicate by real-time PCR. Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). Statistical significance of differences in expression was assessed by the non-parametric Kruskal-Wallis test followed by Dunn's test. A p value < 0.05 was assumed statistically significant. The statistical calculation was based on $2^{-\Delta CT}$ values.

Protein extraction and data-independent acquisition (DIA) analysis

HepaRG cells were differentiated in 15 cm dishes and exposed to compound treatment or solvent control (0.5% DMSO). After 72 h cells were harvested, centrifuged, supernatants were removed and cell pellets were snap-frozen in liquid nitrogen and stored until further analysis. Cells were lysed, heated, sonicated and centrifuged. Protein concentration in the extracts was determined. 1 µg peptide samples were further separated by reversed-phase chromatography on a high pressure liquid chromatography (HPLC) column and connected to a nano-flow HPLC. Mass spectra were acquired in a data-independent acquisition (DIA-MS) manner using the OT Fusion (Thermo Scientific, San Jose). High resolution survey MS scans were acquired in the Orbitrap to monitor peptide ions across the mass range of 350–1600 m/z, followed by a sequential quadrupole isolation of variable windows for higher energy collision dissociation (HCD) MS/MS scans and detection in the Orbitrap. DIA data were analyzed using a spectral library by discovery-based LC-MS/MS analysis from the same and additional in-house samples and the background proteome from UniProtKB/Swiss-Prot specific to Homo sapiens. Single, binary and ternary mixtures treatments were compared against solvent control treated samples. Proteins were further selected corresponding to the steatosis-relevant gene transcripts examined at the mRNA expression level. Target proteins with an abundance change of 1.5 or higher and a p value of 0.05 or lower were considered as statistically relevant.

Quantification of Nile Red staining (AdipoRed)

HepaRG cells were differentiated in 96-well plates and treated with single, binary and ternary mixtures for 72 h. For the detection of lipid accumulation in HepaRG cells after exposure to compounds of interest, cells were stained with Nile red. Accumulation of Nile red is a measure for intracellular triglyceride accumulation and is spectrophotometrically quantified.

Quantification of Nil Red staining (High content screening)

HCA/HCS (High Content Analysis / Screening) technology combines automated fluorescence microscopy, microplate reader measurements and image processing. This approach quantitatively evaluate the phenotypical effect of chemicals on multiple molecular toxicity pathways simultaneously (e.g. cell death, oxidative stress, lipid storage etc.) at single cell level and via an HTS (High Throughput Screening) analysis. Therefore, HepaRG cells were differentiated in 96-well plates and treated with single, binary and ternary mixtures. After 72 h of exposure cells were fixed, washed and stained with Nile red (neutral lipids) and DAPI (nuclei). Both dyes were quantified using image analysis.

Quantification of lipid accumulation via GC-FID

HepaRG cells were differentiated in 12-well plates and treated with single, binary and ternary mixtures for 72 h. Cells were washed, harvested and triglycerides were extracted according to a method described by Hutchins et al. (2008). GC-FID is used to measure changes in the levels of triglycerides in HepaRG cells. GC-FID analysis allows determining the nature of the triglycerides that are most affected upon exposure of HepaRG cells to the compounds of interest.

Quantification of mitochondrial respiration modulation

Mitochondrial respiration in HepaRG was characterized as an indicator of cellular metabolism and fitness in response to the exposure to single, binary and ternary mixtures using Agilent Seahorse XF24 Analyzer (Agilent Seahorse Bioscience, Santa Clara, CA USA). The XF24 equipment allowed measurement of oxygen consumption rate (OCR) in treated and control HepRG cells providing information on key parameters of mitochondrial respiration. For this purpose oligomycin (2 µM) was used to block ATP synthase, µM carbonyl-cyanide-4-(trifluoromethoxy) phenyhydrazone (FCCP, 1 µM) was used to make the inner mitochondrial membrane permeable for protons and allow maximum electron flux through the electron transport chain, and rotenone (0.5 µM) and antimycin A

(0.5µM) were used together to inhibit complexes I and III, respectively. Through use of mitochondrial inhibitors, four key mitochondrial respiration parameters were measured: basal, ATP production-linked, maximal, and proton leak-linked OCR. Based on this data also spare respiratory capacity and non-mitochondrial respiration are calculated, as described previously by Luckert et al. (2018).

Estimation of relative potency factors, dose addition and statistical approach

Dose-response modeling and relative potency factor (RPF) analysis were performed based on the BMD approach using the R-based software package PROAST. Response data were provided as relative data normalized to the solvent control and submitted to PROAST as continuous, summary data containing mean, standard deviation and sample size in tab-delimited text files. Data were statistically analyzed by fitting the exponential 4-parameter model $y = a\left[c - (c-1)\exp\left(-bx^{d}\right)\right]$ to the data. This model adequately describes a large variety of toxicological dose-response data (Slob and Setzer 2014). For RPF analysis dose-response data of the single compounds were obtained in dose-finding experiments. Based on these dose-response curves fitted with mentioned model, the RPFs were calculated for a benchmark response of 50% (BMR_{50}) and their confidence intervals. The corresponding two-sided 90% RPF confidence interval given by RPFL (lower bound of the RPF confidence interval) and RPFU (upper bound of the RPF confidence interval) were calculated. Afterwards, the mixture experiments were designed according to the estimated RPFs in 1:1 ratios of equipotency. For mixture analysis the dose-response data for the mixture and the single compounds were compared using the same BMD approach as for the RPF analysis. First the single compounds were analyzed again for recalculation of the RPF to recognize differences between experiments. Afterwards the analysis was repeated including the mixture data and the RPFs were compared as previously described by Kienhuis et al. (2015) and Staal et al. (2018).

In Table 2 the chosen *in-vitro* assays are listed that are deemed suitable for liver steatosis detection and are included in the bioassay toolbox for AOP-wise testing of compounds and their mixtures concerning steatosis.

Table 2 : Bioassay toolbox included *in-vitro* assays to detect liver steatosis

Assay	**Endpoint**
WST-1 cell viability assay	Prerequisite testing: cytotoxicity
Reporter gene assays for nuclear receptor activation	Key event analysis: activation of PXR, PPARα, PPARγ, PPARδ, LXRα, FXR, RXRα, RARα, CAR, AhR, GR and PXR- CAR- or VDR-mediated induction of CYP2B6 promoter activity
qPCR	Key event analysis: gene expression analysis of ACOX1, ChREBP, SREBP, FASN, SCD and CD36 as well as drug-metabolizing enzymes
Proteomic analysis	Quantification of chemical-induced changes in protein abundance
Mitochondrial respiration	Key event analysis: oxygen consumption rate, basal and uncoupled respiration, ATP levels, proton leakage
Nile red staining (AdipoRed assay)	Key event analysis: triglyceride accumulation
High content screening	Key event analysis: quantification of neutral lipid accumulation and phospholipidosis
GC-FID analysis	Key event analysis: triglyceride and fatty acid accumulation

2.3 Dose-range findings of the individual compounds for mixture studies

Based on the single compound testing of Clothianidin (CTD), Imazalil (IMZ) and Thiacloprid (THI), effect doses are known. The next step was then to calculate RPFs for the establishment of the mixtures design. In mixtures we are interested in potencies as a fixed characteristic not in effect doses which are not a fixed characteristic of a compound. Consequently, the dose selection for the dose-finding study was based only on how to best estimate the potency of the compound. Among the different endpoints for lipid accumulation, the measurement of triglyceride levels by GC-FID was considered as the most accurate indicator for *in vitro* steatosis condition. All three compounds induced a dose-dependent accumulation of triglycerides with following potency THI < CTD < IMZ (Figure 2). Based on these data, RPFs were determined as follows; CTD is 1.7 times (RPF 1.717; CI 1.03-3.2) more potent than THI (RPF 1), while IMZ is 11 times (RPF 10.873; CI 66-24.75) more potent than THI (RPF 1). In comparison to CTD (RPF 1), IMZ is 6.5 times more potent (RPF 11/ RPF1.7). Due to the relatively high confidence intervals (CI) for IMZ, these RPFs were used as preliminary RPFs to design the equipotent doses for the mixture experiments. The exact RPFs are then recalculated during the analysis of the mixtures for the individual key events.

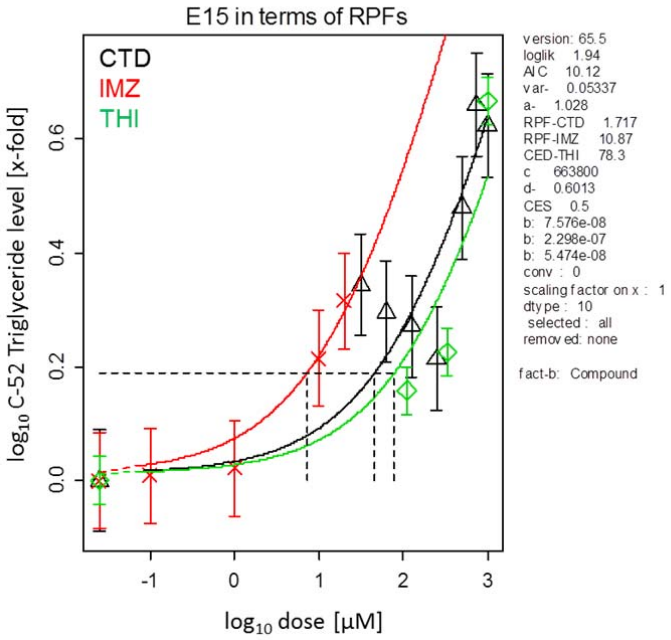


Figure 2 : Dose-response modelling for RPF determination. Mean values of C52 triglyceride level as a function of dose, related to clothianidin (CTD; black triangles), imazalil (IMZ; red crosses) and thiacloprid (THI; green diamonds).

3. Results

3.1 Nuclear receptor activation / MoA

Activation of different nuclear receptors is considered to constitute the molecular initiating event of liver steatosis (Mellor et al. 2016; Vinken 2015), as delineated in the AOP for liver steatosis (Figure 1). Activation of a large set of nuclear receptors, namely AhR, CAR, FXR, GR, LXRα, PPARα, PPARγ, PPARδ, PXR, RARα and RXRα was monitored using luciferase-based reporter assays in human HepG2

cells. As listed in Table 3, receptor activation pattern differs between the three investigated substances. Dose-dependent statistically significant induction of reporter activities was observed.

Table 3: Nuclear receptor activation pattern of Thiacloprid, Imazalil and Clothianidin

Assay	Activation		
	Thiacloprid	Imazalil	Clothianidin
PXR	+	+	-
PXR-CYP2B6	+	+	-
CAR	-	+	-
RXRα	-	-	-
RARα	-	+	-
LXR	-	-	-
FXR	-	-	-
PPARα	-	-	-
PPARα antagonism mode	-	+	+
PPARγ	+	-	-
PPARδ	-	-	-
AhR	-	-	-

Reporter gene assays were used to analyze the activation of the ligand binding domain (LBD) of different nuclear receptors (CAR, FXR, LXRα, PPARα, PPARγ, PPARδ, PXR, RARα, RXRα), the receptor-mediated induction of a specific DNA response element (AhR), or the human CYP2B6 promoter (PXR-CYP2B6). For this purpose, HepG2 were transfected with the appropriate plasmids and exposed to indicated pesticide concentrations, solvent control (SC; 0.5% DMSO) or to the respective positive control for 24 h. (+ indicates a nuclear receptor activation, - indicates no activation).

In summary, THI and IMZ were defined as compounds acting in a more similar mode of action in contrast to CTD which could be defined as compound mainly acting in a dissimilar action compared to THI and IMZ. Based on the activation pattern and on initially calculated RPF, equipotent mixtures of Thiacloprid, Imazalil and Clothianidin were tested in all reporter gene assays activated by at least one compound, namely PXR, PXR-CYP2B6, CAR, RAR, PPARα and PPARγ.

If only one compound activates the investigated nuclear receptor, dose response curves correlate between single and mixture dose response curves. In all cases were two compounds activate the same the nuclear receptor dose addition assumption could be assumed.

3.2 PCR-based gene expression analysis

A screening approach with relevant genes involved in liver steatosis, nuclear receptor activation, and hepatotoxicity was first undertaken to select relevant target genes for mixtures testing. THI had mostly effects on *CYP* genes, up-regulating for instance *CYP3A* family or *CYP2B6*. CTD up-regulated *CYP1A2*, *CYP3A4* and *CYP3A5* but down-regulated *CYP2E1*, *NR0B2* and *PCCA*. IMZ affected the expression of many genes. With few exceptions, a global down-regulation of *CYP* genes was observed while a very potent up-regulation of *IL6* was reported. A subset of relevant genes involved in liver steatosis, proposed in the AOP, were barely regulated, except for IMZ which showed transcriptional deregulation on a few target genes such as *ACOX1* or *SCD*.

Considering the results from the screening, a small selection of genes (mostly *CYP*) was chosen for mixtures testing (Figure 3). Selection based on genes which were deregulated by both compounds in case of binary mixtures and by at least two compounds in case of ternary mixture.

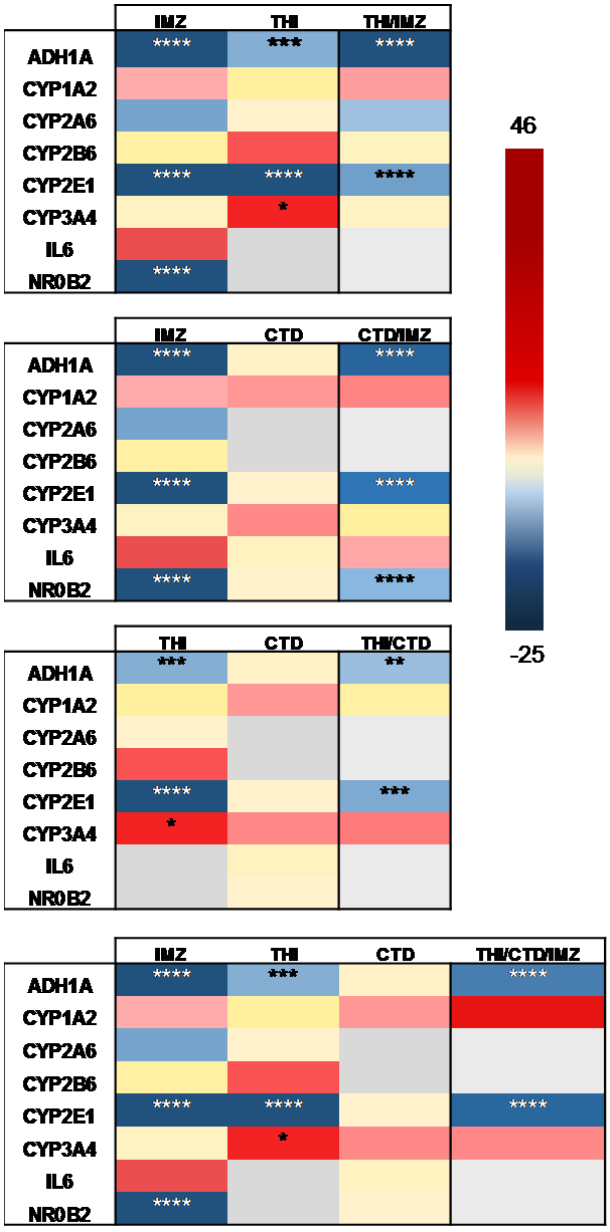


Figure 3: Representative result of the gene expression analysis linked to liver steatosis, hepatotoxicity and nuclear receptor activity of the single compounds and the mixtures in their highest concentration. Based on the PCR array screening, genes deregulated with a fold change almost equally or higher than 2 were selected for PCR analysis in cells treated with of Imazalil, Thiacloprid and Clothianidin and their binary and ternary mixture. The heat map represents the mean fold changes of three independent, biological replicates. Fold changes ≥ 2 and ≤ -2 are highlighted in red and blue, respectively. Values which are not determined are marked in grey. Statistical significance of differences in expression based on $2^{-\Delta\Delta CT}$ values was assessed by the nonparametric Kruskal-Wallis test followed by Dunn's test (* p< 0.05; ** p< 0.01; *** p< 0.001; **** p< 0.0001 in comparison to control).

For the mixtures of THI and IMZ a strong down-regulation of *ADH1A* and *CYP2E1* was observed which corresponds to the gene expression pattern of the single compounds. Similarly, mixtures of THI and CTD led to a down-regulation of *ADH1A* and *CYP2E1*, too. Mixtures of CTD and IMZ showed a strong down-regulation for *ADH1A, CYP2E1* and *NR0B2*, which also corresponds to the single compound expression pattern. A comparable behavior was observed in the ternary mixtures.

In summary, the gene expression pattern caused by the single compounds was reflected by the pattern of the mixtures. Dose-response modelling of gene expression data revealed additivity independent of the mixture composition.

3.3 Protein abundance changes in HepaRG cells upon mixture exposure

Using a mass spectrometric-based assay, quantification of selected proteins corresponding to gene transcripts examined previously by gene array analysis was determined. A total of 37 proteins were monitored. CTD had no substantial effect on any of the investigated proteins. THI and IMZ show a dose dependent increase for e.g. CYP3A4 level protein while decreases for CYP2A6 which is in line with the gene expression analysis. In summary, the binary and ternary combinations show similar tendencies in the direction of regulation for the corresponding genes. Again expression pattern caused by the single compounds was reflected by the pattern of the mixtures.

3.4 Liver triglyceride accumulation

AdipoRed assay

After 72 h treatment with compounds alone or in mixtures, intracellular lipids were measured using Nile red staining (AdipoRed assay). All three pesticides induced a dose-dependent increase of intracellular lipids. All mixtures also showed a dose-dependent increase of intracellular lipids. Benchmark dose modelling confirms the dose addition assumption for all mixtures (as shown in Figure 4).

GC-FID measurement of triglycerides

After 72 h treatment with compounds alone or in mixtures, triglycerides level were measured. All three pesticides induced a dose-dependent increase of triglycerides with stronger effects on 56 C-atoms in the fatty acyl chains. Benchmark dose modelling confirms the dose addition assumption for all mixtures.

Assessment of triglyceride accumulation at the single-cell level

After 72 h treatment with compounds alone or in mixtures, triglyceride accumulation was quantified at the single-cell level using high-content imaging. All treatments also showed a dose-dependent increase of triglycerides and again, benchmark dose modelling confirms the dose addition assumption for all mixtures.

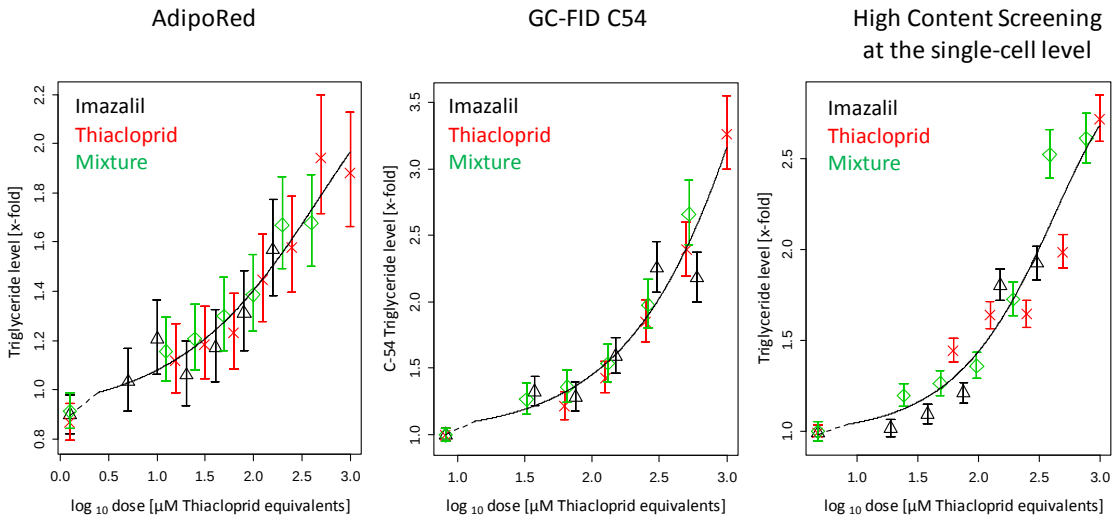
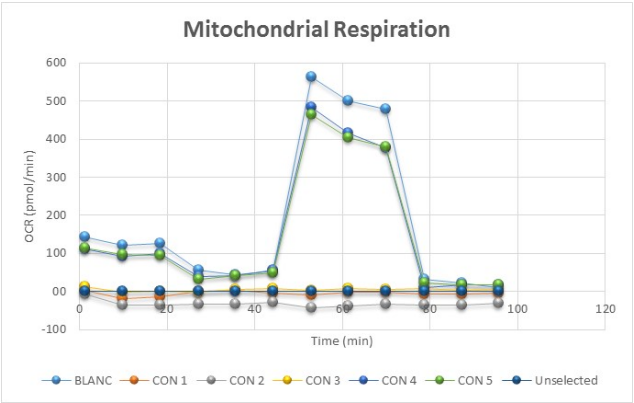


Figure 4: Representative concentration-response modeling of triglyceride accumulation, as determined by AdipoRed assay, GC-FID and high content screening for the binary mixture of IMZ and THI. Data were statistically analyzed by fitting the exponential 4-parameter model using PROAST v65.5.

3.5 Mitochondrial respiration

Observed findings are illustrated by the data of tertiary mixtures. Similar to the above findings dose additive effect could be noted at the level of mitochondrial respiration of treated cells (Figure 5Figure 5). Highest concentrations were toxic during the performed exposure that OCR was equal to zero.



Concentrations for ternary mixture of THI, CTD and IMZ			
RPF	**1**	**1.7**	**11**
Compound	**THI (µM)**	**CTD (µM)**	**IMZ (µM)**
Concentration 1	333.33	196.08	30.30
Concentration 2	166.67	98.04	15.15
Concentration 3	83.33	49.02	7.58
Concentration 4	41.67	24.51	3.79
Concentration 5	20.83	12.25	1.89
Concentration 6	10.4	6.13	0.95

Figure 5: Oxygen Consumption Rate (OCR) profile of HepaRG mitochondria upon treatment with tertiary mixtures of THI, CTD and IMZ.

Lower concentrations in the mixtures showed dose additive effect, but primarily after adding the FCCP as an ECT modulator which abolished the linkage between the respiratory chain and the phosphorylation system. By the collapse the inner membrane gradient, allowing the ETC to function at its maximal rate, derived maximal respiratory capacity showed measurable differences between control cells and lowest concentrations in the mixtures.

4. Summary and Conclusion

Main objective of the investigation of mixtures in the EuroMix project is to answer the question of how compounds with similar and dissimilar MoA act in combination and to establish an AOP-wise testing strategy to assess mixture effects in vitro.

Within the framework of Adverse Outcome Pathway (AOP), we investigated the *in vitro* combined effects of Imazalil (IMZ), Thiacloprid (THI), and Clothianidin (CTD) which were classified as similar and dissimilar acting compounds. Evaluation of mixtures of chemical compounds that are assumed to have a similar mode of action can be made by using relative potency factors (RPFs) (Committee et al. 2017). Therefore, relative potency factors (RPFs) were determined based on compounds-induced increase of triglycerides levels in human HepaRG cells. Equipotent mixtures of CTD/IMZ, THI/CTD, THI/IMZ and THI/CTD/IMZ were then established and the designed mixtures were further tested for nuclear receptors activation, gene regulation and protein expression, and triglyceride accumulation, according to the steatosis AOP.

Results show that IMZ activated PXR, CAR, RAR and AhR while antagonizing PPARα, THI activated PXR and PPARγ whereas CTD antagonized PPARα. We confirm previous studies that showed activation of human PXR by IMZ (Kojima et al. 2011; Lemaire et al. 2006; Yoshimaru et al. 2018).

Gene and protein expression analysis showed only minor changes on few cytochromes P450 (CYP), without affecting key genes/proteins proposed in the AOP. Due to the fact that THI activates both PXR and PPARγ we expect theoretically an up-regulation of *CD36* according to the AOP. However, no changes in *CD36* gene expression were observed. Our results suggest that the current AOP is not validated at every level of biological processes when tested with IMZ, THI and CTD. This outcome was previously observed by Luckert et al. 2018 who also reported some discrepancies in the gene regulations when the AOP was being tested for cyproconazole.

Excluding the genes proposed by the AOP, our findings on gene- and protein-regulation were consistent with the nuclear receptor activation that was associated. CYP3A4 is known to be under PXR regulation (Lehmann et al. 1998; Tolson and Wang 2010), while CYP1A2 has been described to be regulated AhR and CAR (Marx-Stoelting et al. 2017). In our study, THI activated PXR and induced both CYP3A4 gene and protein expression. IMZ activated PXR and AhR and induced CYP3A4 gene and protein expression as well as CYP1A2 gene expression. These effects were also observed in mixtures.

The steatosis AOP predicts that the activation of nuclear receptors will ultimately lead to the formation of fatty liver cells. In summary, all three pesticides induced triglyceride accumulation after interacting with one of the nuclear receptors included in the AOP. Additionally, all three tested compounds were classified in the cumulative assessment group for liver steatosis and by using this AOP-wise testing strategy their potential to induce fatty acid accumulation in human cells in vitro was confirmed.

In the EuroMix approach, the default assumption is that the model of dose addition applies to all substances that cause the same adverse outcome. This assumption could be confirmed during the analysis of mixture effects which revealed additivity for all the different combinations and endpoints that were tested (Table 4).

Table 4: Overall overview of the benchmark dose modeling regarding the dose addition assumption

Mixture	Nuclear receptor activation	Lipid accumulation			Gene expression
	e.g. PXR	GC-FID	AdipoRed	High Content Screening	e.g. ADH1A
THI/IMZ	DA	DA	DA	DA	DA
CTD/IMZ	DA	DA	DA	DA	DA
THI/CTD	DA	DA	DA	DA	DA
THI/CTD/IMZ	DA	DA	DA	DA	DA

DA= dose addition

Additionally, questions regarding the reproducibility among the different key events and laboratories could be answered. As shown in Table 5 for Imazalil as an example, the RPFs are not exactly the same and therefore depending on the assays. For assays which based on the same methodical background like the AdipoRed and the High content screening (both based on Nile red staining) the obtained RPFs show a high similarity.

Table 5 : Observed RPFs among the different sssays used in the AOP-wise testing for Steatosis

Assay	RPF of Imazalil (Thiacloprid is the reference (RPF=1))
Nuclear receptor activation e.g. PXR	23
PCR; e.g. ADH1A	82
PCR; e.g. CYP2E1	43
Fatty acid accumulation; AdipoRed	6.8
Fatty acid accumulation; High Content Screening	6.0
Fatty acid accumulation; GC-FID ($\bar{x}$C44-56)	28

However, the ratios of the RPFs of the three compounds (THI<CTD<IMZ) remained similar over the different levels of endpoints studied. Thus, the relationships of the RPFs of the investigated compounds are independent from the assay, the laboratory and independent from the key event.

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PART 2

Results of the in vitro testing of mixtures regarding craniofacial malformation

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Table of Contents

1. Introduction

This second part of the deliverable provides an instruction on the EuroMix testing strategy for testing compounds grouped based on their Mode of action (MoA) and their mixtures regarding the craniofacial malformation specific endpoint following the same approach of part 1

2. Testing procedure

2.1 Choice of compounds to be tested in vitro

Several compounds that have been tested *in vitro* but particular attention was given to A, B, C compounds, namely cyproconazole, triadimefon and valproic acid (VPA), as described in Deliverable 3.3 (paragraph 2.7).

In this deliverable the results of the *in vitro* testing of mixtures are described. The mixture design follows the EuroMix approach, where compound A and compound B are proposed to have a similar MoA while compound C has a dissimilar MoA compared to A and B.

2.2 Tentative Adverse Outcome Pathway (AOP)-wise testing for craniofacial malformations

As described in more detail in Deliverable 3.3 (paragraph 2.2) a tentative AOP for craniofacial malformations has been provided (see also figure 1)

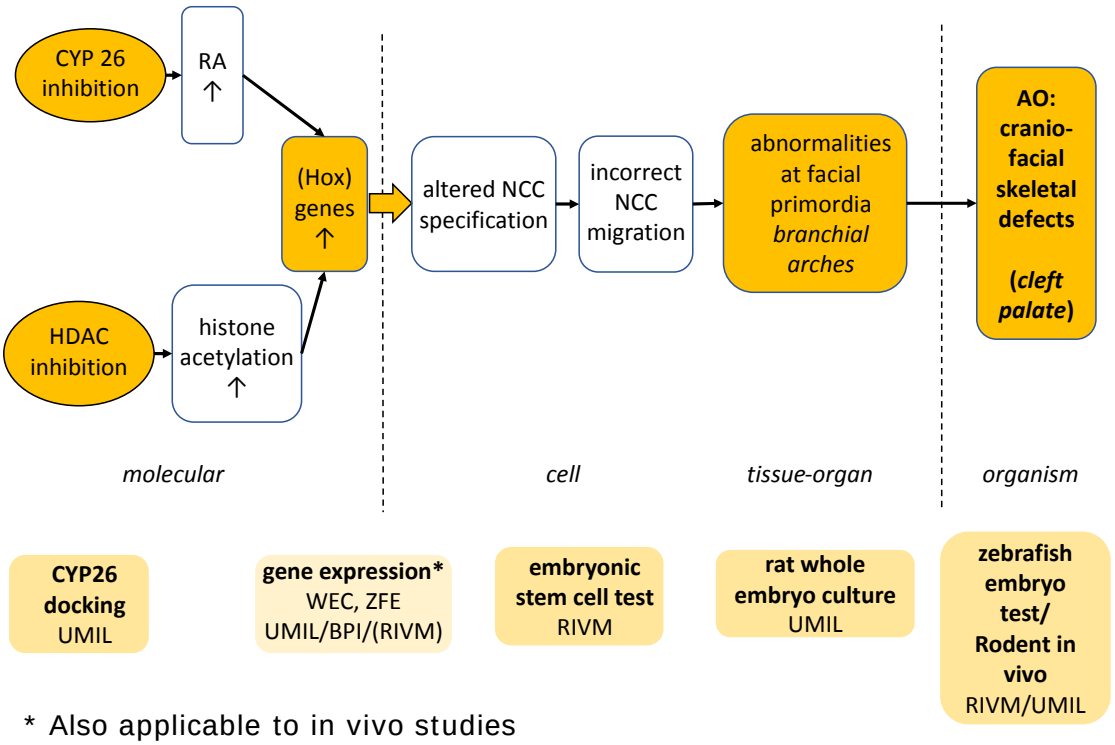

Figure 2.1 : tentative AOP for craniofacial malformation

2.2.1 In vitro test system

Available test systems have been described in Deliverable 3.3 (paragraph 2.4.1). In this report, the focus will be on the data from the ZebraFish Embryo (ZFE) assay and the rat Whole Embryo Culture (WEC) assay. Both assays are well established assays for which a detailed protocol is available.

2.3 Dose-response of the individual compounds for mixture studies and RPF definition

The single compounds have been tested both in the ZFE and in the WEC assay. Based on the dose-response curves RPFs have been estimated. Figures 2.2 and 2.3 report an example for ZFE and WEC, respectively.

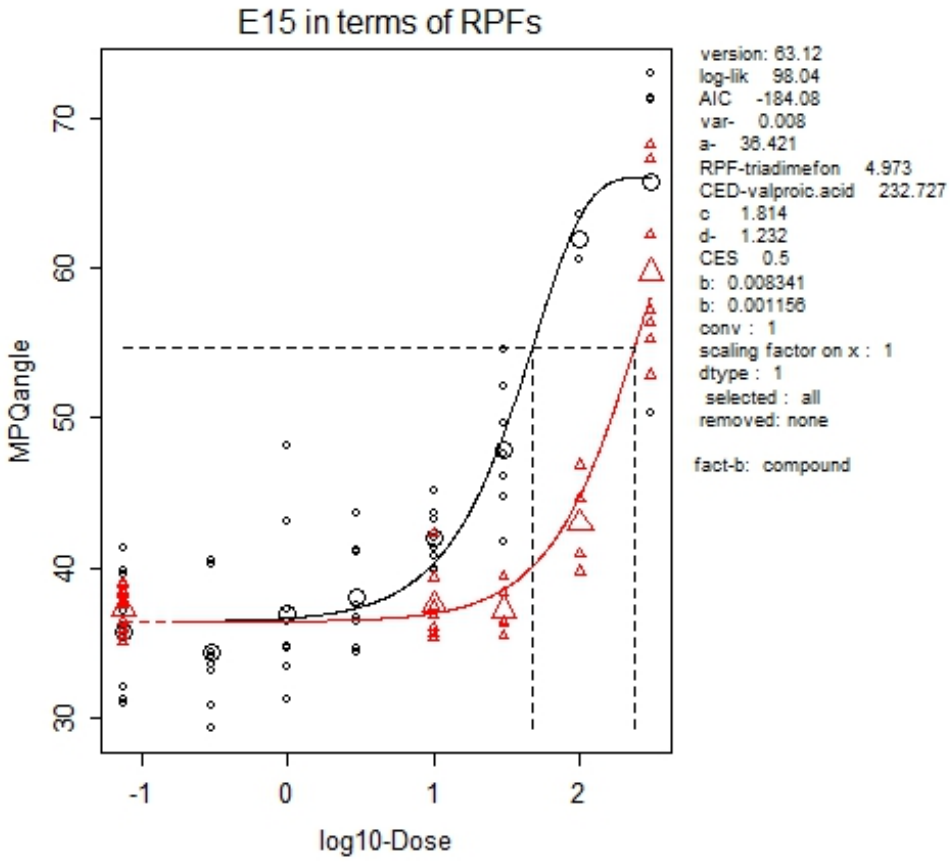

Figure 6.2 : Dose-response modelling for RPF determination for triadimefon (black circles) and VPA (red triangles) in ZFE.
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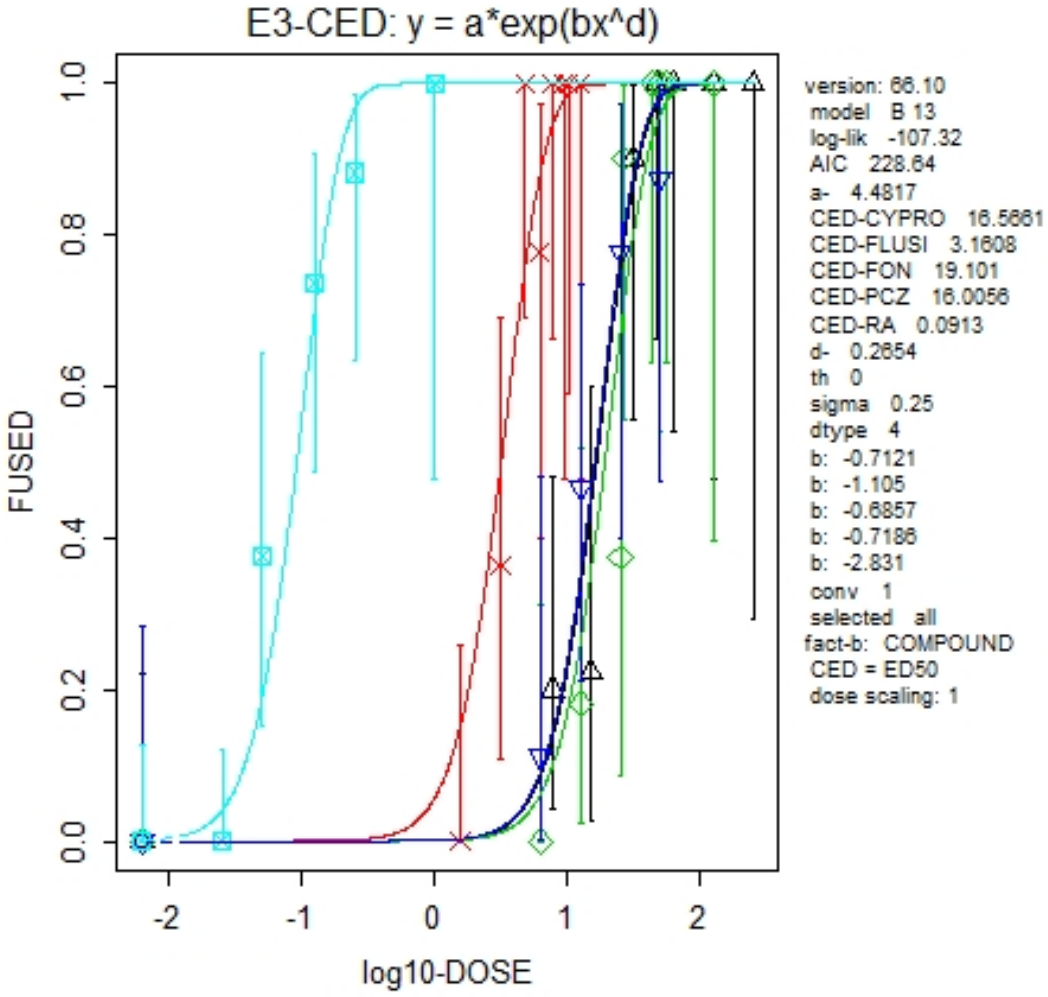

Figure 7.3 : Dose-response modelling for RPF determination for retinoic acid (reference) (light blu squares, flusilazole (red crosses), triadimefon (green diamonds), cyproconazole (blue triangles) and prochloraz (black triangles) in WEC.

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3. Results

Once that the dose responses curve have been established for individual compounds, mixture experiments have been carried out. Extensive summary data are reported in Deliverable 3.3 (para 2.4.3). Table 2.1 reports the data relevant for A, B, C compounds. Figures 2.4 and 2.5 report the graphs of some of the dose response curves analysed with the PROAST software for the BMD approach that led to the conclusions reported in table 2.1.

Table 2.1: summary results of mixture experiment with cyproconazole, triadimefon and VPA

compounds		**MOA**	**assays**	
1	**2**		**WEC**	**ZFE**
cyproconazole	triadimefon	similar	dose addition	dose addition
cyproconazole	valproic acid	dissimilar	dose addition	dose addition
triadimefon	valproic acid	dissimilar	dose addition	dose addition

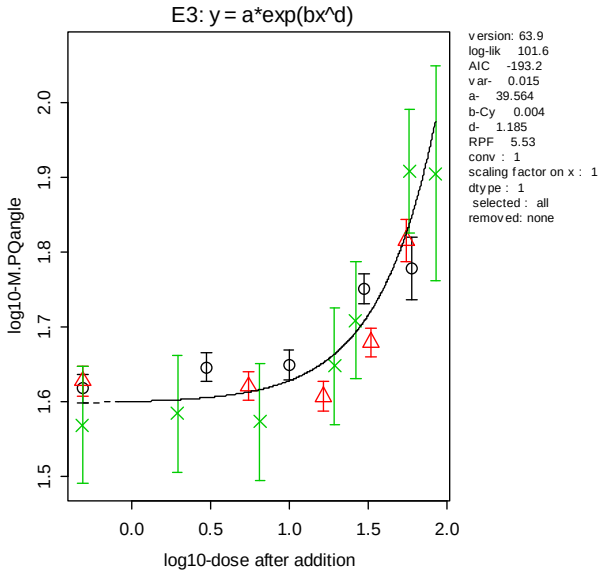

Figure 8.4 : Dose-response modelling triadimefon (red triangles), cyproconazole (black circles) and mixture (green crosses) in ZFE.

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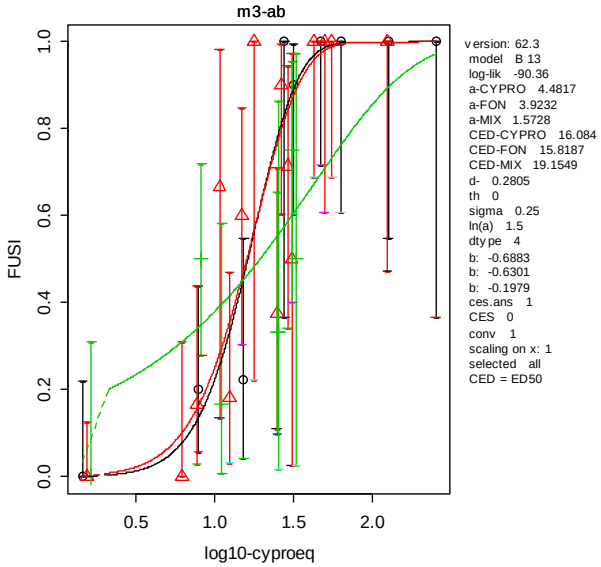

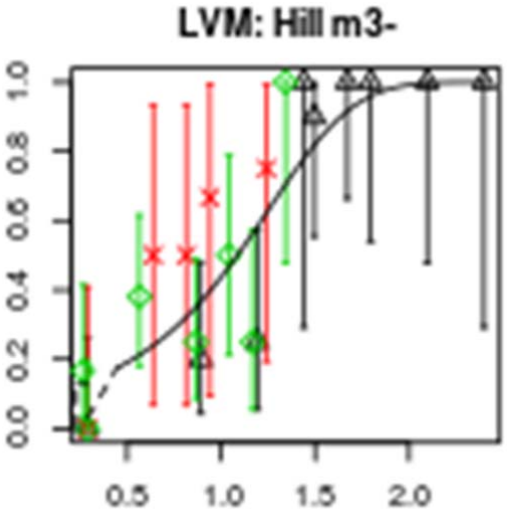

Figure 9. : Dose-response modelling in WEC. Top: triadimefon (red triangles), cyproconazole (black circles) and mixture (green crosses). Bottom: cyproconazole (red crosses), VPA (black triangles) and mixture (green diamonds)

4. Summary and Conclusion

Main objective of the investigation of mixtures in the EuroMix project is to answer the question of how compounds with similar and dissimilar MoA act in combination and to establish an AOP-wise testing strategy to assess mixture effects in vitro.

Within the framework of Adverse Outcome Pathway (AOP), we investigated the *in vitro* combined effects of cyproconazole, triadimefon and VPA which were classified as similar and dissimilar acting compounds. The relative potency factors (RPFs) were determined based on compounds-induced changes in the angle M-PQ (ZFE) or increase of craniofacial malformations (WEC)s. Equipotent mixtures were then established and the designed mixtures were further tested for the same end-points. It is to be noted that the data obtained with WEC are directly relevant for in vivo studies (see Deliverable 4.3, paragraphs 2.6).

In the EuroMix approach, the default assumption is that the model of dose addition applies to all substances that cause the same adverse outcome. This assumption could be confirmed during the analysis of mixture effects which revealed additivity for all the different combinations and endpoints that were tested (2.1).