Food and Chemical Toxicology 137 (2020) 111117

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Dose addition in chemical mixtures inducing craniofacial malformations in zebrafish (*Danio rerio*) embryos

Maria Zoupa[a], Edwin P. Zwart[b], Eric R. Gremmer[b], Ananditya Nugraha[b], Sharon Compeer[b], Wout Slob[c], Leo T.M. van der Ven[b,*]

[a] Laboratory of Toxicological Control of Pesticides, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, Attika, 44561, Greece
[b] Department of Innovative Testing Strategies, Center for Health Protection, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[c] Department of Food Safety, Center for Food, Prevention and Care, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

ARTICLE INFO

Keywords:
Dose addition
Zebrafish model
Craniofacial malformations
Mixture toxicity

ABSTRACT

A challenge in cumulative risk assessment is to model hazard of mixtures. EFSA proposed to only combine chemicals linked to a defined endpoint, in so-called cumulative assessment groups, and use the dose-addition model as a default to predict combined effects. We investigated the effect of binary mixtures of compounds known to cause craniofacial malformations, by assessing the effect in the head skeleton (M-PQ angle) in 120hpf zebrafish embryos. We combined chemicals with similar mode of action (MOA), i.e. the triazoles cyproconazole, triadimefon and flusilazole; next, reference compounds cyproconazole or triadimefon were combined with dissimilar acting compounds, TCDD, thiram, VPA, prochloraz, fenpropimorph, PFOS, or endosulfan. These mixtures were designed as (near) equipotent combinations of the contributing compounds, in a range of cumulative concentrations. Dose-addition was assessed by evaluation of the overlap of responses of each of the 14 tested binary mixtures with those of the single compounds. All 10 test compounds induced an increase of the M-PQ angle, with varying potency and specificity. Mixture responses as predicted by dose-addition did not deviate from the observed responses, supporting dose-addition as a valid assumption for mixture risk assessment. Importantly, dose-addition was found irrespective of MOA of contributing chemicals.

1. Introduction

Combined exposure of humans to multiple chemicals calls for methods for risk assessment of chemical mixtures. This is reflected in public concern regarding exposure to mixtures and the interest from a regulatory point of view, which has increased over the years (Kienzler et al., 2016). To date, while no harmonized strategy exists to predict cumulative risk, a basis to evaluate cumulative effects has been created through testing of multiple compounds in a mixture in experimental models, targeting various organs and toxicological endpoints (Boobis et al., 2008; Kienzler et al., 2016; McCarty and Borgert, 2006).

The discussion on harmonization of chemical mixture assessment relates to scientific factors complicating the issue, particularly regarding methods to determine the combined effect of chemicals in a mixture (Cassee et al., 1998; Monosson, 2005). In the case of simple similar action, chemicals do not interact and react similarly with the biological system, i.e. display a similar mode of action (MOA). The toxicity of these mixtures depends on the potency and dose of each individual compound, which has its own contribution to the toxicity. Simple dissimilar action applies to compounds in a mixture which do not interact but have a different MOA. Finally, complex situations, where compounds interact and influence each other, may result in synergism or antagonism. Overall, dose addition is considered as a suitable approach to human cumulative risk assessment (EFSA-PPR-Panel, 2013), because it logically follows in the case of simple similar MOA, it is most likely in simple dissimilar MOA, it is conservative in case of antagonism, while synergism has only been reported occasionally, and predominantly in models relevant to environmental risk assessment (Cedergreen, 2014). Ideally, for precise prediction of mixture effects, it is important to include information on MOAs of contributing compounds, which, however, will often not be available. To further elucidate this issue, experimental research in this area was implemented in the EU-H2020-project EuroMix.

The zebrafish (*Danio rerio*) embryo is a well described model in

* Corresponding author.
E-mail address: leo.van.der.ven@rivm.nl (L.T.M. van der Ven).

https://doi.org/10.1016/j.fct.2020.111117
Received 26 September 2019; Received in revised form 17 December 2019; Accepted 3 January 2020
Available online 09 January 2020

human and environmental toxicology, considered as an alternative and efficient model for screening of chemicals (Scholz et al., 2008; Zoupa and Machera, 2017). It combines the benefits of a whole organism and of *in vitro* models, including high throughput (large numbers of offspring), rapid embryonic development, and transparency. In Europe, it is exempted from registration up to 5 days post-fertilization (dpf) (European Commission, 1986).

Previously, we developed a method to evaluate mixture effects on the head skeleton in zebrafish embryos through the measurement of the angle formed by the Meckel's and palatoquadrate (M-PQ) cartilages (Staal et al., 2018). We here employ this M-PQ analysis to further expand the database of binary mixture testing, specifically aiming at investigation of dose-addition analysis for combination effects of compounds of (suspected) dissimilar MOA.

To do this, compounds of various classes were selected based on relevance to human exposure and on known effect(s) on the skeletal/craniofacial development (Nielsen et al., 2012; Kyriakopoulou et al., 2016). The latter criterium follows EFSA's strategy to group compounds in cumulative assessment groups (CAGs), which are defined by toxicological phenotype (target organ, specific effect) and mode/mechanism of action, in order to prioritize compounds for combination testing while limiting combinations with less relevance. Two widely used agricultural triazole fungicides, cyproconazole and triadimefon, were selected as reference for eight compounds with mostly dissimilar MOA compared to those triazoles (Table 1). Exposure to triazole compounds may result in craniofacial malformations, including cleft palate in humans (Giavini and Menegola, 2010), particularly due to inhibition of cytochrome P450 Cyp26 enzymes that are part of retinoic acid (RA) catabolism, and a subsequent imbalance of RA (Menegola et al., 2005; Okano et al., 2014). Flusilazole is the third included triazole, which, with its the same MOA and effects on craniofacial development (Menegola et al., 2005), served as a further positive control for dose addition under the condition of similar MOA.

A first compound with dissimilar MOA is TCDD. TCDD is a teratogen in mice, affecting palatal formation and development due to disturbance of TGF-α, EGF, TGF-β1 and TGF-β2 developmental pathways (Abbott and Birnbaum, 1990). Next, the dithiocarbamate thiram is known to induce palatal and skeletal malformation in mice, hamsters and rabbits (Robens, 1969). Dithiocarbamates also caused craniofacial malformations in developing zebrafish embryos, associated with perturbed expression of lysyl oxidase-like 3b (van Boxtel et al., 2011) and TGF-β1 signaling (van Boxtel et al., 2010). Dithiocarbamates are also heavy metal chelators, and altogether, their MOA is dissimilar when compared to triazoles. Similarly, other test compounds which were included because of known adverse effects on craniofacial development have other key MOAs, i.e. PPAR activation for PFOS (Das et al., 2015; Rosen et al., 2017), histone deacetylase (HDAC) inhibition for valproic acid (Kouraklis and Theocharis, 2002; Manivasagam et al., 2017), and disruption of reproductive hormone signaling for endosulfan and prochloraz (Altamirano et al., 2017; Hunter et al., 1999) (Andersen et al., 2002; Vinggaard et al., 2006). Fenpropimorph, a morpholine, may mainly be involved in sterol metabolism (Crowley et al., 1994; Georgopapadakou and Walsh, 1996). However, many compounds have multiple actions, including indirect off-target MOAs, which may eventually contribute to the effect. This may for instance be relevant for disruption of thyroid hormone signaling, which is a shared action among triazoles, PFOS, and thiram (Chen et al., 2019; Liu et al., 2011; Shi et al., 2009; Zhang et al., 2019), or nuclear receptor heterodimerization involving retinoic acid receptors in the case of valproic acid (van Breda et al., 2018). Still, the potency of a compound for each of its MOAs may differ, and the most potent action identifies thea key MOA. In this perspective, the assumption of dissimilar MOA for the combinations of included triazole with non-triazoles may be justified, and this enables the study of binary mixtures representing dissimilar MOA in the zebrafish embryo model.

Table 1
Test compound and single-compound exposure dose-ranges.

Compound	full name	CAS	Purity	Chemical class	Mode of action relative to reference compound[a]	Dose -ranges (μM)[b]
Cyproconazole	2-(4-Chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)-2-butanol	94361-06-5	97.2%	triazole	similar	0, 10, 20, 30, 60, 100, 300
Triadimefon	1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)butan-2-one	43121-43-3	not listed	triazole	similar	0, 0.3, 1, 3, 10, 30, 60, 100, 300
Flusilazole	1-((bis(4-fluorophenyl)methylsilyl)methyl)-1H-1,2,4-triazole	85509-19-9	analytical standard	triazole	similar	0, 1, 3, 5.73, 6, 10, 20, 30, 60, 100
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin	1746-01-6	not listed	dioxin	dissimilar	0, 0.1, 0.3, 1, 3, 10 nM
Thiram	dimethylcarbamothioylsulfanyl- N,N-dimethyl dithiocarbamaat	137-26-8	not listed	dithiocarbamate	dissimilar	0, 0.00811, 0.003, 0.01, 0.03, 0.1, 0.3, 1
VPA	2-Propylpentanoic acid	99-66-1	98%	organic acid	dissimilar	0, 10, 30, 100, 200, 300, 500
Prochloraz	N -propyl- N -[2-(2,4,6-trichlorophenoxy)ethyl]-1 H - imidazole-1-carboxamide	67747-09-5	98.6%	imidazole	dissimilar	0, 0.1, 0.3, 1, 3, 10, 30
Fenpropimorph	cis-4-[(RS)-3-(p-tert-butylfenyl)-2-methylpropyl]-2,6-dimethylmorfoline	67564-91-4	not listed	morpholine	dissimilar	0, 0.1, 0.3, 1, 3, 10, 30, 60, 100
PFOS	1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-Heptadecafluoro-1-octanesulfonic acid	1763-23-1	98%	perfluorinated compound	dissimilar	0, 0.3, 1, 3, 10, 30
Endosulfan	1,2,3,4,7,7-hexachloor-8,9,10-trinorborn-2-een-5,6-yleendimethylsulfiet	115-29-7	99.4%	organochlorine compound	dissimilar	0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10

[a] Reference compounds are cyproconazole and triadimefon.
[b] In μM unless otherwise stated.

2. Materials and methods

2.1. Compounds

The compounds tested, purities, and their dose-ranges are listed in Table 1. All tested chemicals were purchased from Sigma Aldrich, Zwijndrecht, Netherlands, except for TCDD (Toronto Research Chemicals, Toronto, Canada), fenpropimorph (Dr. Ehrenstrofer, GmbH, Germany), and PFOS (AVCR, Karlsruhe, Germany). All substances were dissolved in DMSO solvent (CAS number 67-68-5, purity: 99,9%) that was also used as a solvent control at 0.1%.

2.2. Zebrafish husbandry, embryo collection and scoring

The zebrafish (*Danio rerio)* were maintained and bred in an automatic Zebtec flow-through system (Tecniplast S.p.A, Buguggiate, Italy) in the National Institute for Public Health and the Environment (RIVM) facility. pH was maintained between 7.5 ± 0.5, Tm at 27.5 ± 0.5 °C and conductivity at 500 ± 100 μS. The photoperiod was set at a day-night cycle of 14–10 h. Zebrafish were fed 3 times a day, twice with SDS (Special Diet Services, Tecnilab-BMI BV, the Netherlands) 100 CAT. 824856, 200 CAT 824862, 300 CAT 824867 or small granules (CAT 824876) and once with frozen artemia (Superfish). For breeding purposes, ten females were isolated in a 3.5-L tank and fed frozen artemia three times a day for a period of 4 days, to improve egg production. The evening prior to mating, two females and two males were joined in a 3.5-L tank. Spawning was triggered when the lights turned on and eggs were collected within 30 min. Egg collection was performed in Petri dishes and successfully fertilized eggs of good quality, as evaluated under a stereomicroscope (Leica M8), kept for further use. Eggs at cleavage stage were exposed in 24-well plates containing one egg in 2 mL of test medium (Dutch Standard Water (Staal et al., 2018) with 0.1% DMSO (Merck, Darmstadt, Germany).

Zebrafish embryo development and teratological assessment were performed on 3- and 5-dpf under the stereoscope and as previously described (Hermsen et al., 2011). Concisely, embryo development was scored on the following endpoints: tail detachment, somite formation, eye development, movement of the embryo, heartbeat, blood circulation, embryo pigmentation, pectoral fin, protruding mouth and hatching. Next, teratological effects were recorded based on presence of pericardial edema, yolk sac edema, eye edema, head malformation, absence/malformation of sacculi/otoliths, tail malformations, heart malformations, modified chorda structure, scoliosis, rachischisis, and yolk deformation. These developmental and teratological scores were used to determine concentration ranges for the exposures aiming at analysis of head malformation, which was further investigated as the key endpoint.

2.3. Zebrafish alcian blue staining and cartilage morphometric analysis

Alcian blue staining method was used to visualize the cartilage skeletal structures of the zebrafish embryos at 5 dpf. Methodology was based on previous protocols (Cohen et al., 2014; Kimmel et al., 1998) with minor modifications. Briefly, after exposure embryos were euthanized by rapid cooling and fixed in 4% PFA for 2 h at room temperature (RT). Embryos were washed with phosphate buffered saline (PBS) and bleached with 3% H_2O_2 (Sigma, cat no. 216763) and 0.5% KOH (Sigma, cat no. P1767) for 35 ± 5 min. Additional PBS washes were followed by overnight staining at 4 °C with 0.01% alcian blue (Sigma, CAT no 05500) and 60 mM $MgCl_2$ (Sigma, CAT no M2670) in 70% ethanol solution. After staining, embryo washes were performed in 80% ethanol/10 mM $MgCl_2$, 50% ethanol/10 mM $MgCl_2$ and 25% ethanol solutions. Excess staining was reduced by additional bleaching with 3% H_2O_2 and 0.5% KOH. Embryos were washed with 25% glycerol/0.1% KOH and stored in 50% glycerol in 0.1% KOH until further use. For the cartilage measurement, stained embryos were transported in slits of a silica gel block or a 3D-printed device (Wittbrodt et al., 2014) and the head photographed in a ventral view under the stereoscope (Leica microscope camera system, Olympus C5050 ZOOM digital camera). Morphometric measurement of M-PQ angles, illustrated in Fig. 1, was performed on alcian blue stained craniofacial cartilages drawn and measured in Adobe Photoshop (vCC 2018). Cartilage measurements of embryos exposed to the compounds of interest were compared to 0.1% DMSO control groups.

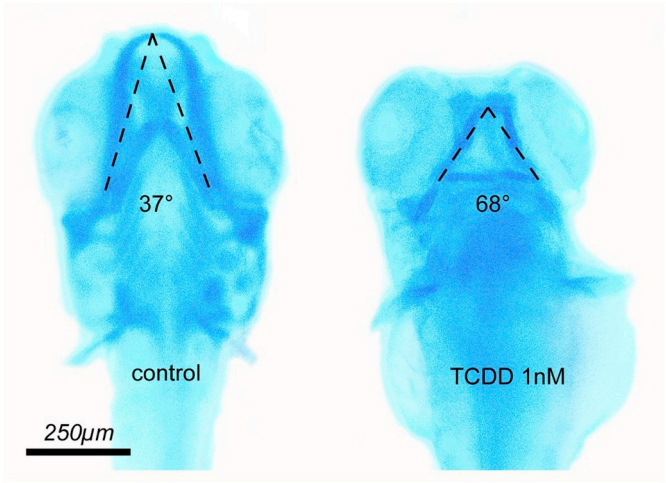

Fig. 1. M-PQ angle measurements in 5dpf zebrafish embryos in 0.1% DMSO (solvent control, **A)** and a representative toxicant (1 nM TCDD, **B)**. Measurements were performed on alcian blue stained craniofacial cartilages.

2.4. Exposures

Zebrafish embryos were exposed to fourteen binary mixtures, combining cyproconazole or triadimefon as reference compound with one of the second compounds triadimefon, flusilazole, TCDD, thiram, VPA, prochloraz, fenpropimorph, PFOS, or endosulfan, alongside to 0.1% DMSO solvent control groups. Details of the applied method for mixture design has been described previously (Staal et al., 2018). In short, mixtures were designed as a range of combinations of (near-) equipotent concentrations (see below), where the concentration of the second compound was calculated using it's relative potency factor (RPF) to the reference compound. RPFs were modeled using a dedicated function in the PROAST software (see below) by comparing the benchmark concentrations (BMC) of the two compounds. PROAST finds optimal parallel fits for the compounds, and the BMCs are then calculated at the distance between both fitted curves (on log-dose scale), or, equivalently, by the ratio of the BMDs of both compounds (note that this ratio does not depend on the value of the BMR, or CES).

The RPF values produced from the combined single compound analyses were further used to calculate a range of combination concentrations expected (based on dose addition) to result in responses covering the intermediate part of the single dose-response curve of the reference compound. Each targeted mixture concentration was achieved through combining half the concentration of the reference compound with half the concentration of the second compound (after adjustment by the RPF), i.e. 0.5*conc of R + 0.5*conc of B*RPF). Some near-equipotency combinations, e.g. 1:3 and 3:1, were included to account for estimation errors in the RPF from the preceding experiments.

Exposure to mixtures was performed in the same way as for single substances, i.e. under static conditions, starting within the first 2 h post fertilization, and terminating at 5 dpf for evaluation of the head skeleton. Single compound exposures were always replicated along with the mixture as a reference for the mixture dose response.

2.5. Statistical analysis

Statistical analysis was performed by dose-response modelling using PROAST software package v65.5–67.0 (https://www.rivm.nl/en/proast), available as plug-in module in the R statistical software package, as web application (https://proastweb.rivm.nl/), and as integrated part in the EuroMix toolbox (https://mcra-test.rivm.nl/EuroMix/WebApp/#/). For head cartilage morphology analysis, M-PQ-angle data were fitted using the $y = a\{(c - (c - 1) \exp(-bx^d)$ model, and the $BMC_{50}\}$ calculated. BMC_{50} is the concentration where the M-PQ angle of the exposed embryo increased by 50% compared to background M-PQ angle. The parameters that are estimated in the exponential and Hill functions are: *a,* the background value, i.e. the expected value of the M-PQ angle at zero exposure; *b,* the potency of the tested compound; c, the maximum effect, i.e. upper plateau value relative to the background; and *d,* the steepness of the curve (Slob, 2002). In the modelling, concentrations of the second compound in the mixture are expressed as equivalents of the reference compound.

Dose addition from mixtures studies can be evaluated in two ways. The first way is by estimating the RPF based on the single compounds only and plot the single-compound responses (and fitted curve) against dose in terms of the reference compound (using the estimated RPF). Mixture responses added in this same plot should be close to the single-compound responses, in case dose addition holds. If dose addition does not hold, mixture responses will be (systematically) located on either side of the single-compound responses.

This visual assessment can be complemented with a quantitative (and more direct) evaluation, i.e. through assessment of the "ratio of overlap", which comprises.

Estimation of the RPF using both the single-compound and the mixture data. When dose addition applies (i.e. mixture and single-compound responses are close when plotted against dose in terms of the reference), fitting the curve to all single-compound and mixture responses will result in a similar estimate of the RPF as found using the single doses only. However, when dose addition does not apply, and mixture and single-compound responses are not close, fitting the curve to all responses will result in a compromise between the single-compound and the mixture responses, as the latter cannot be made close to the former for any RPF value. As a result, the RPF estimate will be biased compared to the single-compound estimate. Hence, in case of dose addition, the confidence interval of the second RPF estimate (all responses) is expected to overlap with the confidence interval of the single-compounds RPF estimate, whereas in case of deviation from dose addition, it can be expected that the overlap between both confidence intervals will be smaller or that there is no overlap at all. The overlap of the confidence intervals of both RPF estimates may be quantified by the ratio of overlap (BMDL of the higher interval divided by the BMDU of the lower interval). A relatively large value (> 1) of the ratio of overlap indicates a relatively large deviation from dose addition, while a ratio smaller than one means that there is no evidence of deviation from dose addition.

3. Results

3.1. Skeletal analysis of M-PQ angle in exposure experiments

Skeletal analysis of the M-PQ angle at 5dpf was performed in all embryos of the single compound and mixture exposure experiments. An example of a single compound effect compared to the solvent control is presented for the case of TCDD in Fig. 1. Zebrafish embryos exposed to 1 nM TCDD show an increase of M-PQ angle of 31° when compared to the control. Effects in M-PQ angle generally occurred at a wide range of non-lethal concentrations, with exception of some cases, notably with PFOS, where the effective concentrations for the effect of interest were close to lethality, limiting the specific observations. In the case of thiram, general skeletal malformations interfered with M-PQ angle increases, particularly at the higher effective concentrations. Delayed hatching, such as observed at high concentrations of VPA, also hampered correct visualization of the head skeleton, but this could be solved by manual dechorionation. Other off-target effects, such cardiac effects with TCDD, were also observed, however without interfering with M-PQ analysis. More detailed descriptions of compound-induced phenotypes were provided previously (Staal et al., 2018).

3.2. Single compound dose-response analysis

Replicate dose-response experiments were performed for each of the 10 selected compounds, with the aim to investigate the reproducibility of the estimated potency (BMD). For each compound the dose-response data and BMD analysis for separate and combined the replicate studies is shown in Appendix 1. BMD CIs were derived for each replicate study, by fitting the dose-response model on each combined data-set with "study" as a covariate (Slob, 2002; Slob and Setzer, 2014). The study-specific BMD CIs for cyproconazole and triadimefon are presented as examples in Fig. 2, with 16 and 12 replicate experiments, respectively. The results show that not all BMD CIs overlap, which implies that the

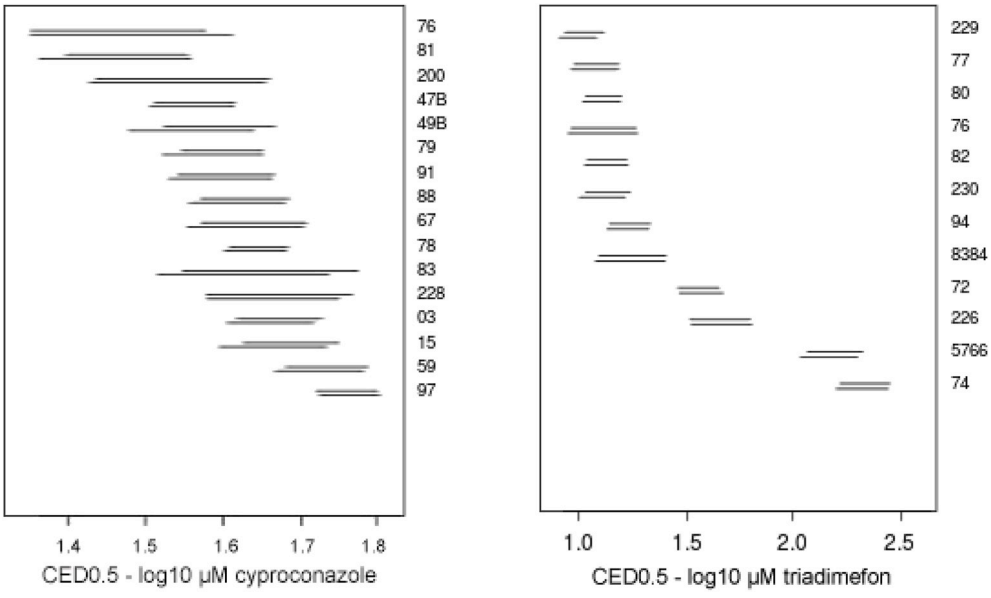

Fig. 2. Graphical representation of BMC (=CED) confidence intervals (CI) derived from cyproconazole (left) and triadimefon (right) covariate analysis of 16 and 12 independent experiments, respectively (experiment numbers in the right-hand legend). Each pair of lines represents the confidence interval (CI) related to the exponential (upper) and Hill (lower) model. CIs were calculated at the 50% effect level, and depicted along the exposure concentration (X-axis, log10 of concentration in µM). See Appendix 1 for dose response analyses of the experiments.

Table 2
BMCs confidence intervals (CI) at CES = 50%.

Compound	BMC CI (μM)
Cyproconazole	41.6–47.9
Triadimefon	16.4–20.9
Flusilazole	11.5–17.1
TCDD	0.76–1.1 nM
Thiram	33–167 nM
VPA	220–265
Prochloraz	12.6–25.2[a]
Fenpropimorph	27.9–36.8
PFOS	43.5–103[a]
Endosulfan	1.6–4.1

BMC CIs derived from combined analysis of all included experiments (see Appendix 1), showing the lowest BMDL-highest BMDU in the exponential and Hill models. All values are in μM, except in nM for TCDD and thiram.

[a] Extrapolated values, which occur in cases with limited achieved effect sizes within the exposure dose range.

studies were not always true replicates (i.e. there were differences in unknown experimental factors with an impact on the response), even though we aimed the studies to have similar experimental conditions. For the other compounds between two and five replicate studies were performed, resulting in non-overlapping BMD CIs in various cases as well (see Appendix 1). Consequently, an estimated RPF from a previous study cannot be used in the subsequent mixture study that aims to evaluate dose addition: the RPF needs to be estimated in the mixture study itself, to avoid false conclusions.

Table 2 shows the BMC CIs obtained by a dose-response analysis of all replicate studies combined per compound. Apparently, TCDD and VPA are the most and least potent compounds, respectively, in inducing craniofacial developmental effects, with the reference compounds cyproconazole and triadimefon taking intermediate positions. In the case of prochloraz and PFOS, the BMC CIs are extrapolated values, due to the fact that the maximally achieved effect size was lower than the applied CES = 50%, which may relate to the specificity of the effect of these compounds (see Discussion).

3.3. RPF estimation based on two-compounds analyses and subsequent mixture design

Next, we combined the data from each second compound with the reference compounds cyproconazole or triadimefon, to obtain an estimate of the RPF, with confidence interval. The resulting RPF estimates, given in Table 3, were used to design 14 mixture experiments through calculation of a range of combination concentrations at equipotency, covering the intermediate part of the single dose-response curve of the reference compound.

Table 3
RPFs/CIs of compound combinations used in the binary mixture experiments.

Reference compound	Second compound	RPF	RPF-CI
Cyproconazole	Triadimefon	1.2	0.88–1.5
Cyproconazole	Flusilazole	4.5	3.4–5.4
Cyproconazole	TCDD	45053	37569–54540
Cyproconazole	Thiram	529.4	392.1–767.9
Cyproconazole	VPA	0.18	0.14–0.21
Cyproconazole	Fenpropimorph	1.4	1.2–1.7
Cyproconazole	PFOS	0.53	0.35–1.5
Triadimefon	TCDD	27439	22269–35738
Triadimefon	Thiram	996.8	599.8–1686.3
Triadimefon	VPA	0.09	0.08–0.1
Triadimefon	Prochloraz	2.4	1.6–3.4
Triadimefon	Fenpropimorph	1.0	0.7–1.4
Triadimefon	PFOS	2.1	1.2–3.5
Triadimefon	Endosulfan	1.8	0–5.7

3.4. Mixture experiments

The results from the mixture studies were analyzed in combination with simultaneously executed single compound data, as presented in Figs. 3 and 4, which have cyproconazole and triadimefon as the reference compound, respectively. In these plots, the second compound and the mixture are expressed as equipotent concentrations of the reference compound after application of the RPF. Since the dose-response model is fitted to these equipotent concentrations, the responses to both single compounds are expected to follow that same curve. When dose addition applies, the responses to the mixtures should follow that curve as well. Thus, dose addition is (approximately) confirmed if the mixture responses (approximately) follow the fitted curve.

The main conclusion from Figs. 3 and 4 is that the mixture responses follow the fitted curve in all cases. This is true for similar and dissimilar MOA (triazole-triazole and triazole-non-triazole combinations), which indicates that dose-addition applies irrespective of similarity of MOA.

As a further observation, similar to single compound analyses, the maximum response varies among cases, probably due to specificity of the effect (see Discussion). In the case of PFOS, the maximum response was higher in the combination with triadimefon as compared to the combination with cyproconazole, indicating some experimental variability. Finally, similar non-specific effects as observed in the single compound exposures also occurred in the mixture experiments, and may be associated with more scatter in the responses.

Further analysis of the single compound RPFs without and with mixture shows that these always overlap (ratio < 1; Table 4). This supports the visual observation from Figs. 3 and 4 that combinations of compounds behave as predicted by dose addition, both for combinations of similar and dissimilar MOA.

4. Discussion

In this study, dose-addition was investigated in binary mixtures of compounds known to induce head skeleton malformations, including cleft palate, through measurement of the M-PQ angle in the head of 120hpf zebrafish embryos. Based on visual assessment of the dose-response plots it can be concluded that the mixture responses can indeed be predicted by dose addition for all compounds considered, irrespective of the MOA. The ratio of overlap of RPF confidence intervals was used an approach for further quantitative assessement of dose-addition, through comparison of the RPF confidence interval resulting from all dose-response data (including the mixtures) with that from the single compound data only, by taking the ratio of the upper bound of the lower interval to the lower bound of the higher interval. In case of deviation from dose-addition, the ratio of overlap will be relatively large. However, we found that the ratio of overlap was smaller than one in all 14 studied binary combinations of compounds, both with similar and with dissimilar mode of action. This confirms the conclusion from the visual assessment of the dose-response fits that dose addition applies irrespective of MOA for the studied category of compounds.

4.1. Evaluation of single compound effects

The compounds included in the study were pesticides, environmental pollutants, and pharmaceuticals, and were selected based on known effects on craniofacial development in rodents (Nielsen et al., 2012; Kyriakopoulou et al., 2016). Although the formation of the head skeleton differs between mammals and zebrafish, the underlying molecular pathways are conserved (Duncan et al., 2017), and phenotypic expressions of head skeleton malformations in zebrafish embryos, with the M-PQ angle as a read-out, may therefore be considered as a valid model for craniofacial malformations in mammals. Otherwise,

irrespective of translatability of the effect among animal classes, the M-PQ angle appears to be a practical parameter to quantify toxicological effects on head skeleton development (Staal et al., 2018). All 10 compounds considered affected the M-PQ angle, albeit with different potencies, and with differences in the maximally achievable effect.

The BMD variation within the independent experiments was generally very limited, and reflects the robustness of the study design. In contrast, the inter-experimental variation in estimated BMDs could mount up to more than a 10-fold difference in the cases of thiram and fenpropimorph (Appendix 1), and thus may indicate that circumstantial experimental factors, including actual toxicant concentrations in the exposure medium, ambient conditions, post-exposure handling of embryos and inter-observer variation, affect the final outcome. Although such unintended experimental factors were aimed at to be well-

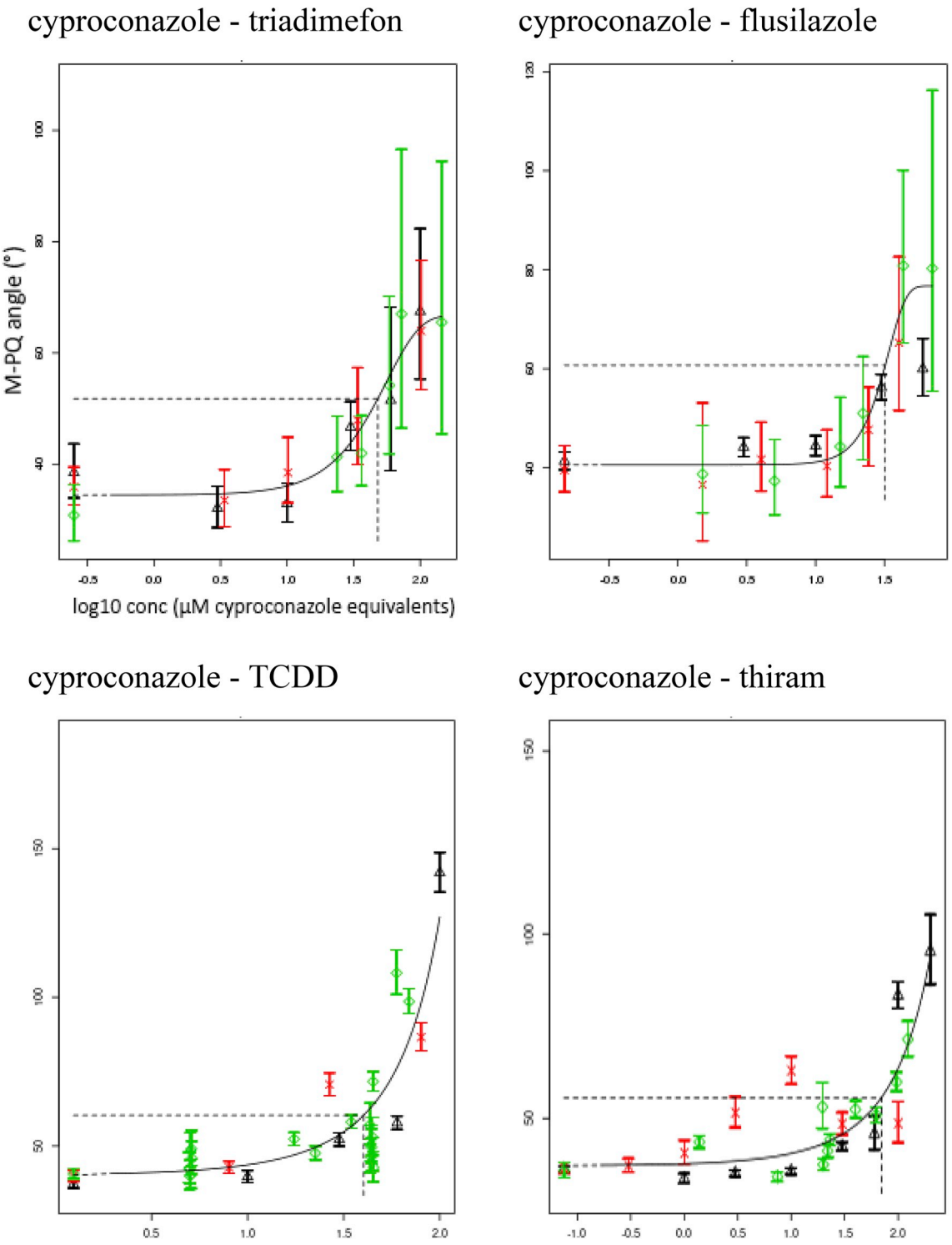

Fig. 3. Dose-response mixture effects on M-PQ angle in zebrafish embryos, with cyproconazole as the reference compound (black triangles/lines) combined with either triadimefon, flusilazole, TCDD, thiram, VPA, fenpropimorph, or PFOS, as second compound (red crosses/lines). The new RPF reads for each combination are shown in Table 4 for quantitative evaluation of dose addition. Dose addition is visually supported when there is no systematic deviation of the mixture (green diamonds/lines) from the overall dose response fit. Axis titles in the first plot can be read throughout.

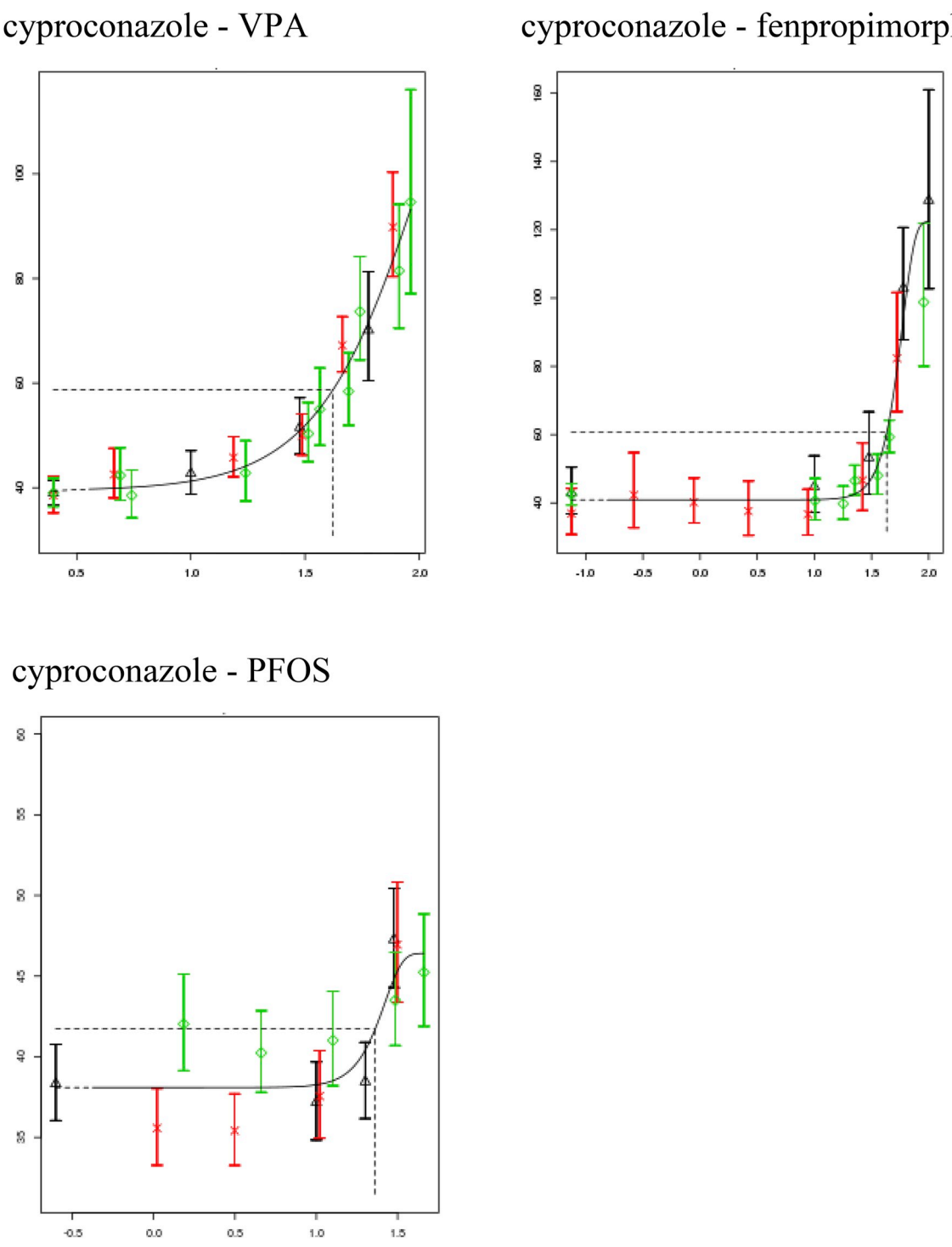

Fig. 3. (*continued*)

controlled, differences apparently still occurred. The relatively high throughput of the zebrafish embryo model allows to perceive such variation, which may go unnoticed in experimental models with lower throughput. A consequence is that comparing potencies of independently performed mixture and single compound exposures may lead to false conclusions, and thus that the RPF used for predicting the mixture responses should always be estimated in the mixture study itself, and not in an independent (e.g. preceding) study. When results from zebrafish studies are directly used for risk assessment, the consequence is that repeated experiments in zebrafish embryos would be needed for a more secure derivation of the BMDL.

Although the M-PQ angle represents a practical read-out, each included compound may not specifically target this endpoint, or have the formation of the head skeleton as a primary target at all. This is reflected in the variation of maximally achieved increase of the M-PQ angle, which was high for the triazoles, TCDD and VPA, but limited for prochloraz, fenpropimorph, and PFOS (Appendix 1). The size of the effect may be limited by additional toxicities masking the craniofacial phenotype, e.g. through lethality (observed with PFOS) or severe overall skeletal malformation as was the case with thiram. This is in line with observations with the developmental and teratological scores, which were used for concentration range-finding, and which revealed

compound-specific toxicological profiles (not shown), with for instance scoliosis as a specific and consistently observed effect after thiram exposure, whereas yolk sac edema was observed with VPA but not with thiram. Still, although development of the head skeleton may not be the specific key target of each included compound, the observed dose-addition in all binary mixtures indicates that even such compounds do contribute to mixture effects in the investigated domain. Another consideration is that the same compound with limited effects in craniofacial development may be more active in other systems in the organism, and thus can contribute to mixture effects in different CAGs.

Potency differences can be the result of differing toxicokinetics among compounds, determining at what concentration a compound, or its active metabolite, will arrive at the internal target. Physicochemical characteristics will vary among the used classes of compounds, and affect initial passage over the chorion, absorption in the embryo proper, or binding to proteins. However, generally, the internal concentrations in zebrafish embryos will be quickly equilibrated against external aquatic concentration for most compounds, irrespective of the presence

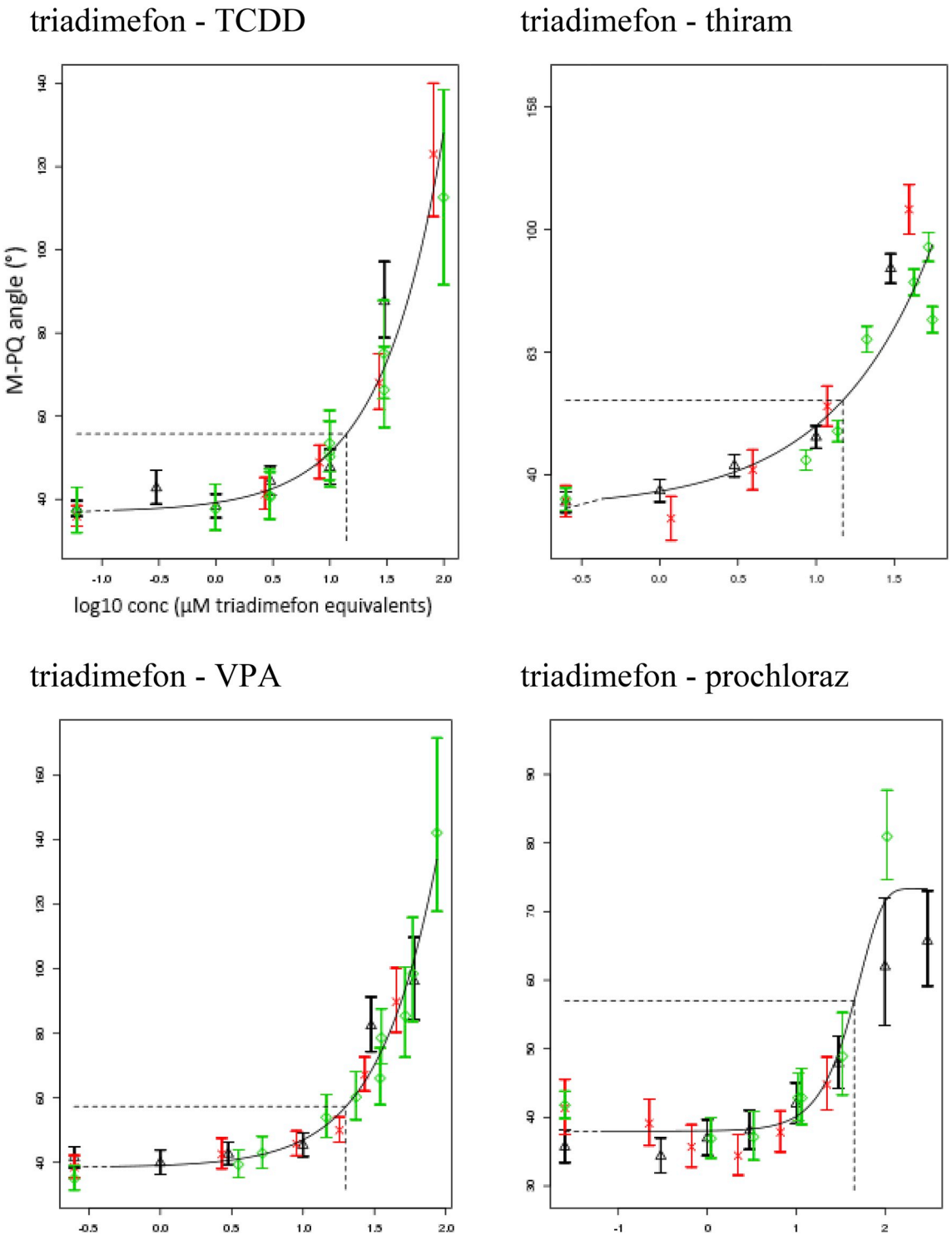

Fig. 4. Same as Fig. 3, but with triadimefon as reference compound (black triangles/lines) and TCDD, thiram, VPA, prochloraz, fenpropimorph, PFOS and endosulfan as second compound (red crosses/lines). Mixtures in green (diamonds and lines).

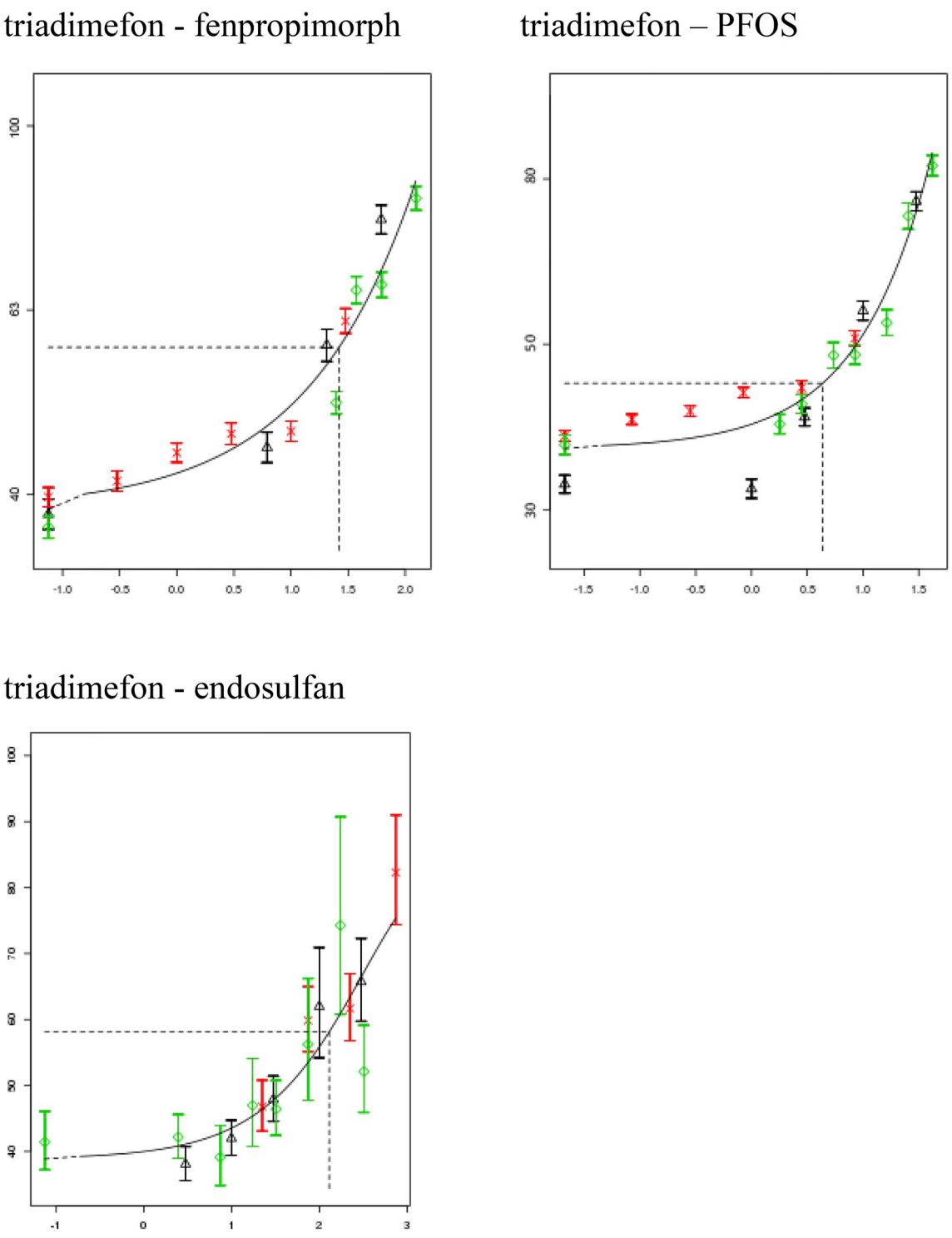

Fig. 4. (*continued*)

of the chorion; moreover, such an established equilibrium will often even lead to bioconcentration of the compound (Rorije et al., in preparation). Further, the kinetics of interaction with the initial biological target will differ per class of compounds, determining its primary MOA. The relative high potency of a compound like TCDD is thus explained by its high affinity to the zfAHR2 (Andreasen et al., 2002). Differing binding affinities also occur within a given class of compounds for a common molecular target (Cotterill et al., 2019), which may explain the potency differences of the triazole flusilazole as compared to its family members cyproconazole and triadimefon.

4.2. Evaluation of mixture experiments

The two triazole reference compounds cyproconazole and triadimefon are known to have the same MOA (Robinson et al., 2012). We found that these two compounds have similar potency, as well as a similar target specificity, i.e. a similar observed effect-phenotype and a maximal achieved size of the effect on the M-PQ angle. Based on this, combinations of either reference compound with the same second compound can be considered as mutual controls. Indeed, such pairs of experiments generally showed high similarity, and are thus mutually confirmative.

Table 4
Effect of mixtures on RPF of single compounds.

	RPF CI without mixture		RPF CI with mixture data		Ratio of Overlap[a]
	Lowest	Highest	Lowest	Highest	
Reference compound: Cyproconazole					
RPF - Triadimefon	2.35	5.72	2.29	5.09	0.46
RPF - Flusilazole	3.14	4.13	3.6	4.78	0.87
RPF - TCDD	23700	31900	23300	30300	0.78
RPF - Thiram	82.8	3740	1040	1830	0.28
RPF - VPA	0.14	0.20	0.13	0.18	0.81
RPF - Fenpropimorph	0.633	0.944	0.763	1	0.81
RPF - PFOS	0.9	1.1	0.79	2.13	0.42
Reference compound: Triadimefon					
RPF - TCDD	14300	23300	22600	32600	0.97
RPF - Thiram	315	476	277	522	0.60
RPF - VPA	0.059	0.08	0.08	0.1	0.93
RPF - Prochloraz	0.84	2.73	1.38	3.31	0.51
RPF - Fenpropimorph	0.32	0.59	0.33	0.68	0.55
RPF - PFOS	0.29	0.53	0.21	0.37	0.77
RPF - Endosulfan	61	143	47.2	121	0.50

[a] Ratio of overlap indicates overlap of RPF confidence intervals without and with mixture. Overlapping intervals (ratio < 1) indicate that including the mixture responses does not affect the estimate of the RPF, which supports the hypothesis the mixture responses can be predicted by dose addition.4.

There is some quality variation among the mixture experiments, in the sense of how informative the dose-response data are. For example, the combination experiment with cyproconazole and VPA resulted in multiple and gradually increasing responses, both for the single compounds and the mixtures, whereas the study of cyproconazole-PFOS showed only a few responses large enough to be distinguishable from the statistical noise. As already noted, this can be explained by off-target effects leading to general toxicity and lethality of the embryo at high concentrations, preventing larger effects on the M-PQ angle to occur.

Dose-addition appears to be adequate for predicting combination effects for craniofacial malformations in zebrafish embryos, as concluded from visual observation of the dose-response analyses, and the ratio of overlap being smaller than 1 in all cases. This was observed in a wide range of similar and dissimilar MOA combinations. Deviating combination effects, that might result from synergy or antagonism (Cassee et al., 1998), were not observed. This suggests that upstream events in the AOP network describing the processes leading to craniofacial malformations contribute equally to the adverse outcome and eventually merge along the pathway. A follow-up challenge is to exactly describe and quantify such interactions, which may help to model and predict combination effects without experimental work, and which may be achieved through ontology analysis of combined MOAs (Desprez et al., 2019; Wang et al., 2019).

4.3. Evaluation of methodology for risk assessment purposes

As discussed, M-PQ measurement in zebrafish embryos is a valid model for assessment of craniofacial malformations, including cleft palate, in mammals. However, a caveat of this model is in toxicokinetics, in view of the different routes of exposure, which is via the placenta in the mammalian embryo and directly from ambient water in the fish embryo, with absorption via skin, gills and orally, depending on the stage of development (Rubinstein, 2006). Also, metabolic transformation with (in)activation of compounds depends on the stage of development in fish embryos (Alderton et al., 2010; Jones et al., 2010; Otte et al., 2017), whereas (in)activation of compounds for the mammalian embryo will be mainly determined by maternal metabolic activity. When the mixture exposure results reported here, or toxicological results from the zebrafish embryo model per se, are to be extended beyond a proof-of-principle conclusion and implemented in human risk assessment, these factors require further consideration for extrapolation of toxicological effects from the zebrafish embryos to humans. Still, the model can be used to further study mixture exposure issues, e.g. to evaluate if dose addition can predict responses of complex mixtures. For that matter, throughput of the model may be improved by replacing the elaborate procedure of staining by implementing a transgenic fluorescent skeleton model (Hammond and Moro, 2012) and automate imaging (Pulak, 2016).

Overall, the application of dose-response modelling appears to be a robust method for comparative potency assessment of individual compounds and for evaluation of the dose addition hypothesis. The same method was successfully applied in other experimental models, similarly concluding that dose addition applied for skin sensitizers in the local lymph node assay (Kienhuis et al., 2015) and for AHR ligands in HepG2 cells (Knebel et al., 2018). The extensive set of mixture studies analyzed in this research indicates that the methodology can even be applied to compounds that perform suboptimal in the experimental model, such as PFOS.

The overall conclusion of this study supports the validity of EFSA's default of dose-addition in risk assessment of mixtures.

Author contributions section

Maria Zoupa analyzed data and wrote the article.
Edwin P. Zwart designed and performed experiments.
Eric R. Gremmer designed and performed experiments.
Ananditya Nugraha designed and performed experiments.
Sharon Compeer designed and performed experiments.
Wout Slob advised experimental design, analyzed and interpreted data and co-authored the article.
Leo T.M. van der Ven conceived the study, interpreted the data and wrote the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was undertaken within the H2020-project EuroMix - WP3: Bioassay toolbox and mixture testing (www.euromixproject.eu), which was funded by the European Commission, European Union (Grant Agreement 633172), and by the Dutch Ministry of Health, Welfare and Sports (VWS), Netherlands (project 5.1.2: Knowledge base and policy advise on CMRS substances).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111117.

References

Abbott, B.D., Birnbaum, L.S., 1990. TCDD-induced altered expression of growth factors may have a role in producing cleft palate and enhancing the incidence of clefts after coadministration of retinoic acid and TCDD. Toxicol. Appl. Pharmacol. 106, 418–432.
Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547–557.
Altamirano, G.A., Delconte, M.B., Gomez, A.L., Alarcon, R., Bosquiazzo, V.L., Luque, E.H., Munoz-de-Toro, M., Kass, L., 2017. Early postnatal exposure to endosulfan interferes with the normal development of the male rat mammary gland. Toxicol. lett. 281, 102–109.
Andersen, H.R., Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M., Bonefeld-

Jorgensen, E.C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol. Appl. Pharmacol. 179, 1–12.
Andreasen, E.A., Hahn, M.E., Heideman, W., Peterson, R.E., Tanguay, R.L., 2002. The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor. Mol. Pharmacol. 62, 234–249.
Boobis, A.R., Ossendorp, B.C., Banasiak, U., Hamey, P.Y., Sebestyen, I., Moretto, A., 2008. Cumulative risk assessment of pesticide residues in food. Toxicol. lett. 180, 137–150.
Cassee, F.R., Groten, J.P., van Bladeren, P.J., Feron, V.J., 1998. Toxicological evaluation and risk assessment of chemical mixtures. Crit. Rev. Toxicol. 28, 73–101.
Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9, e96580.
Chen, X., Fang, M., Chernick, M., Wang, F., Yang, J., Yu, Y., Zheng, N., Teraoka, H., Nanba, S., Hiraga, T., Hinton, D.E., Dong, W., 2019. The case for thyroid disruption in early life stage exposures to thiram in zebrafish (Danio rerio). Gen. Comp. Endocrinol. 271, 73–81.
Cohen, S.P., LaChappelle, A.R., Walker, B.S., Lassiter, C.S., 2014. Modulation of estrogen causes disruption of craniofacial chondrogenesis in Danio rerio. Aquat. Toxicol. (Amst.) 152, 113–120.
Cotterill, J.V., Palazzolo, L., Ridgway, C., Price, N., Rorije, E., Moretto, A., Peijnenburg, A., Eberini, I., 2019. Predicting estrogen receptor binding of chemicals using a suite of in silico methods - complementary approaches of (Q)SAR, molecular docking and molecular dynamics. Toxicol. Appl. Pharmacol. 378, 114630.
Crowley, J.H., Lorenz, R.T., Parks, L.W., 1994. Fenpropimorph affects uptake of uracil and cytosine in Saccharomyces cerevisiae. Antimicrob. Agents Chemother. 38, 1004–1007.
Das, K.P., Grey, B.E., Rosen, M.B., Wood, C.R., Tatum-Gibbs, K.R., Zehr, R.D., Strynar, M.J., Lindstrom, A.B., Lau, C., 2015. Developmental toxicity of perfluorononanoic acid in mice. Reprod. Toxicol. 51, 133–144.
Desprez, B., Birk, B., Blaauboer, B., Boobis, A., Carmichael, P., Cronin, M.T.D., Curie, R., Daston, G., Hubesch, B., Jennings, P., Klaric, M., Kroese, D., Mahony, C., Ouedraogo, G., Piersma, A., Richarz, A.N., Schwarz, M., van Benthem, J., van de Water, B., Vinken, M., 2019. A mode-of-action ontology model for safety evaluation of chemicals: outcome of a series of workshops on repeated dose toxicity. Toxicol. In Vitro 59, 44–50.
Duncan, K.M., Mukherjee, K., Cornell, R.A., Liao, E.C., 2017. Zebrafish models of orofacial clefts. Dev. Dynam. 246, 897–914.
EFSA-PPR-Panel, 2013. Scientific Opinion on relevance of dissimilar mode of action and its appropriate application for cumulative risk assessment of pesticides residues in food. EFSA J. 11, 3472–3512.
European Commission, 1986. Council Directive 86/609/EEC of November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Off. J. Eur. Union L358 1–28 18.12.1986.
Georgopapadakou, N.H., Walsh, T.J., 1996. Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob. Agents Chemother. 40, 279–291.
Giavini, E., Menegola, E., 2010. Are azole fungicides a teratogenic risk for human conceptus? Toxicol. lett. 198, 106–111.
Hammond, C.L., Moro, E., 2012. Using transgenic reporters to visualize bone and cartilage signaling during development in vivo. Front. Endocrinol. 3, 91.
Hermsen, S.A., van den Brandhof, E.J., van der Ven, L.T., Piersma, A.H., 2011. Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies. Toxicol. In Vitro 25, 745–753.
Hunter, D.S., Hodges, L.C., Vonier, P.M., Fuchs-Young, R., Gottardis, M.M., Walker, C.L., 1999. Estrogen receptor activation via activation function 2 predicts agonism of xenoestrogens in normal and neoplastic cells of the uterine myometrium. Cancer Res. 59, 3090–3099.
Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae in vivo. Zebrafish 7, 23–30.
Kienhuis, A.S., Slob, W., Gremmer, E.R., Vermeulen, J.P., Ezendam, J., 2015. A dose-response modeling approach shows that effects from mixture exposure to the skin sensitizers isoeugenol and cinnamal are in line with dose addition and not with synergism. Toxicol. Sci. 147, 68–74.
Kienzler, A., Bopp, S.K., van der Linden, S., Berggren, E., Worth, A., 2016. Regulatory assessment of chemical mixtures: requirements, current approaches and future perspectives. Regul. Toxicol. Pharmacol. 80, 321–334.
Kimmel, C.B., Miller, C.T., Kruze, G., Ullmann, B., BreMiller, R.A., Larison, K.D., Snyder, H.C., 1998. The shaping of pharyngeal cartilages during early development of the zebrafish. Dev. Biol. 203, 245–263.
Knebel, C., Kebben, J., Eberini, I., Palazzolo, L., Hammer, H.S., Sussmuth, R.D., Heise, T., Hessel-Pras, S., Lampen, A., Braeuning, A., Marx-Stoelting, P., 2018. Propiconazole is an activator of AHR and causes concentration additive effects with an established AHR ligand. Arch. Toxicol. 92, 3471–3486.
Kouraklis, G., Theocharis, S., 2002. Histone deacetylase inhibitors and anticancer therapy. Curr. Med. Chem. Anti Cancer Agents 2, 477–484.
Kyriakopoulou D., K.N., Machera, K., Peijnenburg, A., Louca-Christodoulou, D., Hadjiloizou, P., Beronius, A., Håkansson, H., Hanberg, A., Eberini, I., Rorije, E., 2016. Report Describing Cumulative Assessment Groups for a Broad Range of Chemicals, Based on Information Extracted from (Literature) Databases. . https://doi.org/10.5281/zenodo.1065327.
Liu, S., Chang, J., Zhao, Y., Zhu, G., 2011. Changes of thyroid hormone levels and related gene expression in zebrafish on early life stage exposure to triadimefon. Environ. Toxicol. Pharmacol. 32, 472–477.
Manivasagam, S., Velusamy, T., Sowndharajan, B., Chandrasekar, N., Dhanusu, S., Vellaichamy, E., 2017. Valporic acid enhances the Atrial Natriuretic Peptide (ANP) mediated anti-hypertrophic activity by modulating the Npr1 gene transcription in H9c2 cells in vitro. Eur. J. Pharmacol. 813, 94–104.
McCarty, L.S., Borgert, C.J., 2006. Review of the toxicity of chemical mixtures: theory, policy, and regulatory practice. Regulatory toxicology and pharmacology : RTP (Regul. Toxicol. Pharmacol.) 45, 119–143.
Menegola, E., Broccia, M.L., Di Renzo, F., Massa, V., Giavini, E., 2005. Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives. Toxicol. In Vitro 19, 737–748.
Monosson, E., 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. Environ. Health Perspect. 113, 383–390.
Nielsen, E., Nørhede, P., Boberg, J., Krag Isling, L., Kroghsbo, S., Hadrup, N., Bredsdorff, L., Mortensen, A., Christian Larsen, J., 2012. Identification of Cumulative Assessment Groups of Pesticides 9 EFSA Supporting Publications.
Okano, J., Udagawa, J., Shiota, K., 2014. Roles of retinoic acid signaling in normal and abnormal development of the palate and tongue. Congenital. Anom. 54, 69–76.
Otte, J.C., Schultz, B., Fruth, D., Fabian, E., van Ravenzwaay, B., Hidding, B., Salinas, E.R., 2017. Intrinsic xenobiotic metabolizing enzyme activities in early life stages of zebrafish (Danio rerio). Toxicol. Sci. 159, 86–93.
Pulak, R., 2016. Tools for automating the imaging of zebrafish larvae. Methods 96, 118–126.
Robens, J.F., 1969. Teratologic studies of carbaryl, diazinon, norea, disulfiram, and thiram in small laboratory animals. Toxicol. Appl. Pharmacol. 15, 152–163.
Robinson, J.F., Tonk, E.C., Verhoef, A., Piersma, A.H., 2012. Triazole induced concentration-related gene signatures in rat whole embryo culture. Reprod. Toxicol. 34, 275–283.
Rorije, E., Groot, R., Den Ouden, F., Simeon, S., Bois, F., van der Ven, L.T.M., de Knecht, J., in preparation. Toxicity and Toxicokinetics of Some Alkoxy Alcohols and Acids in Zebrafish Embryos. (in preparation).
Rosen, M.B., Das, K.P., Rooney, J., Abbott, B., Lau, C., Corton, J.C., 2017. PPARalpha-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling. Toxicology 387, 95–107.
Rubinstein, A.L., 2006. Zebrafish assays for drug toxicity screening. Expert Opin. Drug Metabol. Toxicol. 2, 231–240.
Scholz, S., Fischer, S., Gundel, U., Kuster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment–applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. Int. 15, 394–404.
Shi, X., Liu, C., Wu, G., Zhou, B., 2009. Waterborne exposure to PFOS causes disruption of the hypothalamus-pituitary-thyroid axis in zebrafish larvae. Chemosphere 77, 1010–1018.
Slob, W., 2002. Dose-response modeling of continuous endpoints. Toxicol. Sci. 66, 298–312.
Slob, W., Setzer, R.W., 2014. Shape and steepness of toxicological dose-response relationships of continuous endpoints. Crit. Rev. Toxicol. 44, 270–297.
Staal, Y.C.M., Meijer, J., van der Kris, R.J.C., de Bruijn, A.C., Boersma, A.Y., Gremmer, E.R., Zwart, E.P., Beekhof, P.K., Slob, W., van der Ven, L.T.M., 2018. Head skeleton malformations in zebrafish (Danio rerio) to assess adverse effects of mixtures of compounds. Arch. Toxicol. 92, 3549–3564.
van Boxtel, A.L., Gansner, J.M., Hakvoort, H.W., Snell, H., Legler, J., Gitlin, J.D., 2011. Lysyl oxidase-like 3b is critical for cartilage maturation during zebrafish craniofacial development. Matrix Biol. 30, 178–187.
van Boxtel, A.L., Pieterse, B., Cenijn, P., Kamstra, J.H., Brouwer, A., van Wieringen, W., de Boer, J., Legler, J., 2010. Dithiocarbamates induce craniofacial abnormalities and downregulate sox9a during zebrafish development. Toxicol. Sci. 117, 209–217.
van Breda, S.G.J., Claessen, S.M.H., van Herwijnen, M., Theunissen, D.H.J., Jennen, D.G.J., de Kok, T., Kleinjans, J.C.S., 2018. Integrative omics data analyses of repeated dose toxicity of valproic acid in vitro reveal new mechanisms of steatosis induction. Toxicology 393, 160–170.
Vinggaard, A.M., Hass, U., Dalgaard, M., Andersen, H.R., Bonefeld-Jorgensen, E., Christiansen, S., Laier, P., Poulsen, M.E., 2006. Prochloraz: an imidazole fungicide with multiple mechanisms of action. Int. J. Androl. 29, 186–192.
Wang, R.L., Edwards, S., Ives, C., 2019. Ontology-based semantic mapping of chemical toxicities. Toxicology 412, 89–100.
Wittbrodt, J.N., Liebel, U., Gehrig, J., 2014. Generation of orientation tools for automated zebrafish screening assays using desktop 3D printing. BMC Biotechnol. 14, 36.
Zhang, W., Chen, L., Xu, Y., Deng, Y., Zhang, L., Qin, Y., Wang, Z., Liu, R., Zhou, Z., Diao, J., 2019. Amphibian (Rana nigromaculata)exposed to cyproconazole: changes in growth index, behavioral endpoints, antioxidant biomarkers, thyroid and gonad development. Aquat. Toxicol. (Amst.) 208, 62–70.
Zoupa, M., Machera, K., 2017. Zebrafish as an alternative vertebrate model for investigating developmental toxicity-the triadimefon example. Int. J. Mol. Sci. 18.