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# The zebrafish embryo as a model for chemically-induced steatosis: A case study with three pesticides

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ABSTRACT

There is an increasing incidence and prevalence of fatty liver disease in the western world, with steatosis as the most prevalent variant. Known causes of steatosis include exposure to food-borne chemicals, and over-consumption of alcohol, carbohydrates and fat, and it is a well-known side effect of certain pharmaceuticals such as tetracycline, amiodarone and tamoxifen (drug-induced hepatic steatosis). Mechanistic knowledge on chemical-induced steatosis has greatly evolved and has been organized into adverse outcome pathways (AOPs) describing the chain of events from first molecular interaction of a substance with a biological system to the adverse outcome, intrahepatic lipid accumulation. In this study, three known steatosis-inducing pesticides (imazalil, clothianidin, and thiacloprid) were tested for their ability to induce hepatic triglyceride accumulation in the zebrafish (*Danio rerio*) embryo (ZFE) at 5 days post fertilization, both as single compounds and equipotent binary mixtures. The results indicate that the ZFE is very well applicable as a higher tier testing model to confirm effects in downstream key events in AOPs, that is, chemically-induced triglyceride accumulation in the whole organism and production of visible steatosis. Moreover, dose addition could be concluded for binary mixtures of substances with similar and with dissimilar modes of action.

## 1. Introduction

Steatosis is the most prevalent form of fatty liver disease occurring in the human population, with an increasing incidence and prevalence in the western world (Kaiser, Lipscomb, and Wesselkamper, 2012; Palekar et al., 2006). Besides alcohol consumption, known causes are related to food intake (overconsumption of carbohydrates and fat), food-borne chemicals, and it is a well-known side-effect of several pharmaceuticals (drug-induced hepatic steatosis) such as tetracycline, amiodarone and tamoxifen (Idilman, Ozdeniz, and Karcaaltincaba, 2016). Hepatic steatosis is characterized by accumulation of triglycerides in hepatocytes, microscopically visible as small or large lipid-containing intracellular vesicles and is considered an early stage of a pathophysiological process that via the occurrence of steatohepatitis may culminate in cirrhosis and liver cancer (Reddy and Rao, 2006). Mechanistic knowledge on chemical-induced steatosis has greatly evolved and has been organized into adverse outcome pathways (AOPs) describing the chain of events from first molecular interaction of a substance with a biological system (molecular initiating event, or MIE) ultimately to the adverse outcome (AO), steatosis (Gijbels and Vinken, 2017; Verhoeven et al., 2024; Vinken, 2015). These MIEs include for instance pregnane X receptor (PXR) activation, peroxisome proliferation-activated receptor alpha (PPARα) inactivation, and liver X receptor (LXR) activation (Gijbels and Vinken, 2017; Vinken, 2015). In the AOPs related to these MIEs, activation of the nuclear receptor PXR leads to induction of CD36 (fatty acid translocase) and increase of fatty acid influx from peripheral tissues, which in turn leads to both increased fatty acid influx and *de novo* fatty acid synthesis, and ultimately to hepatic triglyceride accumulation (Lichtenstein et al., 2020a, 2020b; Verhoeven et al., 2024). PPARα antagonism leads to peroxisomal acyl-CoA oxidase (ACOX1) downregulation, then inhibition of microsomal fatty acid beta-oxidation, and then to hepatic triglyceride accumulation (Lichtenstein et al., 2020a, 2020b; Verhoeven et al., 2024). Comparable to the effect of PXR activation, activation of LXR leads to upregulation of the fatty acid translocase CD36 (Rada et al., 2020), and the same follow-up events. In all cases the downstream result of triglyceride

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accumulation is histopathologically observable steatosis.

Each step in the AOP requires relevant and specific testing models. As such, chemical activation of relevant pathways and subsequent induction of triglyceride accumulation has been demonstrated earlier *in vitro* using human hepatocarcinoma cells, HepaRG, exposed to a set of pesticides (Table 1) commonly found in the food chain, with both single substances as well as mixtures (Lichtenstein et al., 2020). The used pesticides were imazalil (IMZ, also known as Enilconazole; CAS nr. 35554-44-0), an imidazole fungicide widely used in agriculture, thiacloprid (THI; CAS nr. 111988-49-9), and clothianidin (CTD; CAS nr. 210880-92-5), which are both broad range neonicotinoid insecticides introduced as replacement for among others organophosphate and carbamate insecticides, in view of their lower acute toxicity in mammals. Mechanistically, IMZ and THI are activators of PXR whereas CTD acts as an antagonist for PPARα.

The study with HepaRG cells was initiated to test the dose-addition hypothesis for exposure to combinations of chemicals in the cumulative assessment group (CAG) for liver steatosis (Alarcan et al., 2022; Kennedy et al., 2020; Nielsen et al., 2012). This hypothesis could be confirmed in this model, but, although the results with HepaRG cells proved informative for the mechanistic analysis (Lichtenstein et al., 2020a, 2020b), this model is deficient of *in vivo* kinetics and tissue interactions, and cannot inform on histopathological effects in the whole liver. Therefore, and imperative to further downstream testing in the AOP, inclusion of a higher-tier whole-organism model in a toxicological assessment strategy for a better prediction of human *in vivo* chemical-induced steatosis is required. The zebrafish (*Danio rerio*) embryo (ZFE) is a well-recognized whole-organism model in human- and environmental toxicology, and is considered an efficient alternative model for rodent studies for screening of chemical-induced toxicity (Scholz et al., 2008; Zoupa et al., 2020). Also in the study of human liver disease, zebrafish embryos are considered a valuable tool as the development and maturation of the digestive system is fast (≥72 hours post fertilization (hpf)), and the liver of zebrafish shows a great similarity to humans in terms of cellular composition, function, presence of signalling pathways, metabolism, and response to liver toxicants (Driessen et al., 2013; Goessling and Sadler, 2015). Specifically, Driessen et al. (2013) confirmed steatotic lipid accumulation in the liver of zebrafish embryos as histopathologically observable lipid vacuoles (in H&E staining) after exposure to reference substances (Driessen et al., 2013); in cryosections of adult zebrafish liver, steatotic lipid accumulation was also shown using Oil-red-O staining. The molecular signalling for both pathways to steatosis which are relevant to this study (interaction with the receptors PPAR and PXR) was found to be functionally expressed in ZFE, and has been extensively characterized across various life stages of the zebrafish (Bainy et al., 2013; Ibabe et al., 2002; Leboffe et al., 2020). Their transparent nature renders the embryos particularly useful for testing strategies involving (fluorescent) imaging techniques, and zebrafish embryos up to 5 days post-fertilization (dpf) are exempt from legal laboratory animal registration in Europe (Directive 2010/63/EU). This further adds to the ease of use of zebrafish embryos in toxicological (hazard) assessment strategies, and supports its application as a higher tier model in specific AOPs.

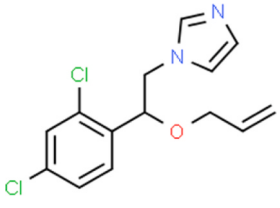
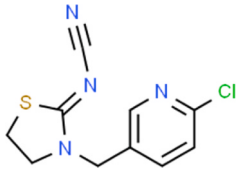
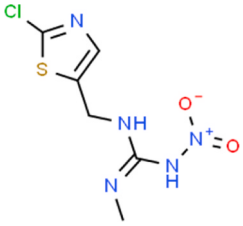
Therefore, in the present study, ZFE was used to confirm induction of steatosis with the same set of substances used in the HepaRG study (IMZ, THI, CTD) (Alarcan et al., 2022; Alarcan et al., 2020; Lichtenstein et al., 2020a, 2020b), and, additionally, test the same dose-addition hypothesis as previously confirmed with HepaRG cells. For these purposes, we adapted an existing protocol for testing of chemical-induced steatosis in zebrafish embryos, involving labelling of intrahepatic lipids with the fluorescent dye LipidGreen2 (Chun et al., 2013; den Broeder et al., 2017). Following protocol optimization, the target set of chemical substances was tested, and compared with fructose as a reference positive control (Sapp et al., 2014). The selected pesticides were tested individually for their potency to induce hepatic lipid accumulation, as well as in equipotent binary mixtures combining similar and dissimilar mechanisms of action. The dosing scheme for the assessment of intrahepatic lipid accumulation was chosen starting from the lowest dose showing non-specific toxicity in the zebrafish embryotoxicity test (ZFET; (Hermsen et al., 2011)) in 4–6 half-log steps down. As the approach is aimed at contributing to hazard assessment through dose-response analysis, no comparison was made to environmental concentrations. Chemical analysis of toxicant concentrations in the exposure solution and in the embryos was performed to obtain information on kinetic processes. Collectively, the current study contributes to the development of new approach methodologies (NAMs) for testing of chemical-induced steatosis in the related AOP, as well as the further substantiation of CAG-based assessment of mixture effects.

## 2. Materials & methods

### 2.1. Chemicals

In this study, three compounds were used: imazalil (IMZ; CAS nr. 35554-44-0), thiacloprid (THI; CAS nr. 111988-49-9), and clothianidin (CTD; CAS nr. 210880-92-5); all were obtained as Pestanal analytical standard grade from Sigma-Aldrich (Zwijndrecht, the Netherlands). All further chemicals were obtained from Sigma-Aldrich, unless otherwise noted. Stock solutions of all compounds were made in DMSO. Just prior to exposure, stock solutions were diluted in Dutch Standard Water (DSW; containing $NaHCO_3$ (100 mg/l), $KHCO_3$ (20 mg/l), $CaCl_2$ * 2 $H_2O$ (200 mg/l) and $MgSO_4$ * 7 $H_2O$ (180 mg/l) in demineralized water), with a final concentration of 0.1 % DMSO in all conditions.

**Table 1**
Test substances.

| Substance name | Imazalil | Thiacloprid | Clothianidin |
|---|---|---|---|
| Abbreviation | IMZ | THI | CTD |
| Molecular structure |  |  |  |
| CAS nr | 35554-44-0 | 111988-49-9 | 210880-92-5 |
| Log Kow* | 4.10 | 2.33 | 0.64 |

* Predicted data, generated using the US Environmental protection agency's EPISuite software. Source: chemspider.com.

### 2.2. Zebrafish husbandry, embryo collection and scoring

Wild-type (AB-strain) 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. The pH was maintained at 7.5 ± 0.5, temperature at 27.5 ± 0.5 °C (adults) and 30.0 ± 0.5 °C (embryos), and conductivity at 500 ± 100 μS. The photoperiod was set at a light/dark 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) (depending on age), and once with frozen artemia (Superfish). For breeding purposes, females were separated in a 3.5 L tank and fed frozen artemia three times a day for a period of 4 days, to stimulate egg production. The evening prior to mating, four females and four males were joined in a 1.7 L sloped breeding tank. Spawning was triggered by the onset of light, and eggs were collected within 30 min. after spawning. Eggs were transferred to Petri dishes and successfully fertilized eggs of good quality (symmetrical development; ≤10 % coagulated eggs in total), 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 (DSW) with 0.1 % DMSO (Merck, Darmstadt, Germany) (Staal et al., 2018). Development and teratology of exposed embryos were assessed at both 3- and 5-dpf (day post-fertilization) under the stereomicroscope in a zebrafish embryo toxicity test (ZFET), as previously described (Hermsen et al., 2011). In brief, 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 non-embryotoxic concentration ranges for the subsequent experimentation.

### 2.3. Exposure

For the steatosis-induction experiments, embryos were exposed to four concentrations of the test compounds plus a blank control, starting at 72 hpf (48 h exposure) or 96 hpf (24 h exposure), in 6-wells plates with a target number of 10 embryos per well per condition; exposures were repeated in 3–5 independent experiments (Table 2A). To answer the question whether the cumulative response to mixtures of compounds is predicted by dose addition, binary, equipotent mixtures were designed and applied as described previously (van der Ven et al., 2022), see also Section 2.6, Table 2B. Exposure to 2 % fructose for 48 h was included (Table 2A) as positive control for the development of steatosis (protocol adapted from (Sapp et al., 2014). In line with Sapp and co-workers, embryos were exposed to 2 % fructose from 72 hpf onwards until staining at 120 hpf. To prevent fungal growth, medium for these experiments was supplemented with fungizone (250 ng/mL), ampicillin (25 μg/mL) and kanamycin (5 μg/mL), and compared to embryos exposed to medium containing only the antifungal treatment as control.

### 2.4. LipidGreen2 staining

Hepatic lipid accumulation as a result of chemical exposure was studied using the cell permeable dye LipidGreen2 (Calbiochem, Merck, Darmstadt, Germany) (Chun et al., 2013; den Broeder et al., 2015). Fluorescent staining was optimized for incubation time and LipidGreen2 concentration for embryos at 120 hpf. At 120 hpf all embryos were euthanized by instant cooling on ice. After removal of exposure media, embryos were fixed with 2 mL paraformaldehyde solution (PFA; 4 %) in PBS for at least 1 h at room temperature (RT). Subsequently, embryos were washed 3 times to remove residual PFA, and incubated in a LipidGreen2 solution (1 μL LipidGreen2/10 mL) in PBS for 30 min on a shaking platform in the dark. Following staining, embryos were washed twice (15 min/wash in PBS) and left o/n at 4 °C to preserve the fluorescent signal. The next day, the fluorescence was assessed using an imaging system for automated detection and positioning of the embryo (VAST BioImager; Union Biometrica, Holliston MA, USA), attached to the stage of a Leica DM4 B fluorescent microscope. In the resulting images, the intensity of fluorescence in the liver area was recorded as average Gray value (Gy) using ImageJ software (version 1.53c) by manual outlining of the region of interest. The average fluorescence of a section of dorsal muscle was selected to correct for background fluorescence ([Avg Gy Liver]-[Avg Gy muscle]) = [corrected Gy].

**Table 2A**
Exposure conditions.

| Compound | Nominal conc. | Number of experiments | Number of replicate embryos per dose group* | Exposure duration |
|---|---|---|---|---|
| Imazalil | 0.1, 0.3, 1, 3 μM | 5 | 7–10 | 24 h |
| Thiacloprid | 10, 30, 100, 300 μM | 4 | 7–10 | 24 h |
| Clothianidin | 100, 300, 600, 1000 μM | 3 per time point | 8–10 | 24 and 48 h |
| Fructose | 2 % | 3 | 6–10 | 48 h |
| Solvent control | 0.1 μl/mL DMSO | Each exp | 8–10 | 24 and 48 h |
| Anti biotic control medium | fungizone (250 ng/mL), ampicillin (25 μg/mL) and kanamycin (5 μg/mL) | 3 | 6–10 | 48 h |
| Mixtures | | | | |
| IMZ-THI | 1:1 ratio equipotent | 3 | 8–10 | 24 h |
| IMZ-CTD | 1:1 ratio equipotent | 3 | 8–10 | 24 h (IMZ), 48 h (CTD) # |

* The target number of embryos per experiment was 10, in some cases a lower number of embryos had to be used due to an insufficient total number of embryos produced, or incidental mortality of individual embryos.
# exposure to CTD: 72 – 120 hpf, combined exposure to IMZ and CTD from 96–120 hpf.

**Table 2B**
Mixture conditions.

| Compound | Mixture | Nominal concentration in equipotent mixture | Exposure duration | Calculated RPF* |
|---|---|---|---|---|
| Imazalil | IMZ – THI | 0.002, 0.006, 0.021, 0.064, 0.11 μM | 24 h | 2346 |
| Thiacloprid | IMZ – THI | 5, 15, 50, 150, 250 μM | 24 h | 1 |
| Imazalil | IMZ – CTD | 0.075, 0.25, 0.75, 1.25, 2.5 μM | 24 h | 200 |
| Clothianidin | IMZ – CTD | 15, 50, 150, 250, 500 μM | 48 h | 1 |

* See Section 2.6 for details.

### 2.5. Analytical chemistry

For translation of the nominal concentration of the pesticides in the medium to an intraembryonic effect concentration, the internal concentration in the embryo, as well as the nominal medium concentration around the time of the assay were assessed. Following exposure for 24 h

(IMZ, THI, CTD) or 48 h (CTD) embryos were euthanized by instant cooling on ice. Per timepoint and concentration, 10 embryos were pooled, rinsed with ice cold DSW for 15 s, collected in a pre-weighed Eppendorf tube, dried with a non-fibrous tissue, extracted in 500 µl 1:1 acetonitrile:methanol (ACN:MeOH), and stored at −20 °C until further preparation. For liquid chromatography separation, a Vanquish (Thermo Fisher Scientific) UHPLC system was used. Separation was established on an Accucore Phenyl Hexyl column (100 ×2.1 mm 2.6 µm). A flow of 0.5 mL/min was applied with the following gradient: 1 % solvent (10 mM ammonium formiate in ACN:MeOH 1:1 v/v +0.1 % formic acid) for 1 minute, with a gradient curve 5–99 % in 9 minutes, hold at 99 % for 1.5 minutes. Re-equilibration was followed by returning to 1 % solvent in 0.01 minute and stabilization at 1 % solvent for 4.49 minutes. Data acquisition was performed on an Exploris 120 Orbitrap (Thermo Fisher Scientific). Utilizing a full scan MS1 set at 60,000 FWHM and data dependent MS2 fragmentation 15,000 FWHM acquisition in both positive and negative mode. When required, samples were diluted 10 times in 10 mM ammonium formiate and injected using an injection volume of 10 µl. Quantitative measurements were determined based on a calibration curve of the corresponding analyte of interest ranging from 10, 25, 50, 75 and 100 ng/mL or a tenfold increment of this curve.

### 2.6. Data analysis and statistics

Because we were interested in the potency of the tested substances, in order to derive equipotent doses for the mixture analysis, this work was designed as a dose-response study. Dose-response analysis enables a more precise estimation of potency because it enables definition of a point of departure at intermediate, non-tested doses. Furthermore, it considers the responses in a full data-set as a whole, thus avoiding imprecision due to methodological errors when comparing single dose groups. For the purpose of concentration-response analysis, data in the ZFET (morphology and teratology scores) as well as lipid accumulation were analysed using the PROAST software tool (v70.0; RIVM, Bilthoven, the Netherlands, https://www.rivm.nl/en/proast/), as a package in R statistical software v3.6.0–4.0.0 (RIVM, Bilthoven, the Netherlands) (Slob, 2002). The PROAST tool analyses the input data for the optimal fit using an exponential function from a nested family of 1–4 parameter models and their variants, with the following parameters: a = background value (the corrected fluorescence in the liver of unexposed embryos), b = potency of the compound, c = maximum fold-change relative to the background response value, d = shape of the curve (slope)); this fit is tested for a statistically significant difference from the no effect model y=a, using the log-likelihood ratio test by means of the Akaike information criterion (AIC) (Hardy et al., 2017), and is shown in the heading of the output figure. A benchmark concentration (BMC or critical effect dose (CED)) can be derived from the dose-response fit at a defined critical effect size (CES). A $CED_{05}$ (CED at CES = 5 %) was derived for the ZFET to estimate the highest concentration without signs of embryotoxicity and malformations. The steatosis experiments were analysed using the pooled data of all replicate experiments with experiment number as covariate, to find reproducibility among replicate experiments. To compare the potency of the individual compounds, a $CED_{20}$ was used (CED at CES = 20 %). Given that the CEDs for the various compounds represent equipotent doses, the ratio of the CED of a given compound compared to another (reference) compound was used to express relative potencies and defined as the relative potency factor (RPF). This RPF was then used to design the mixture experiments for two separate binary equipotent mixtures as described earlier (van der Ven et al., 2022), Table 2B). The means of the individual datapoints in the fructose experiments were compared to the means of appropriate controls using a Student's t-test in GraphPad Prism (v9.5.1), a *p*-value of ≤0.05 is considered statistically significant.

## 3. Results

### 3.1. ZFET

To avoid that chemical-induced developmental delay, or other morphological or developmental effects would confound the detection or localization of intrahepatic lipid accumulation, all substances were tested in a zebrafish embryo toxicity test (ZFET). The results derived from the ZFET indicated no lethality, no developmental delay, and no teratological or morphological anomalies following exposure from 2 – 120 hpf to nominal medium concentrations up to 1000 µM CTD, 300 µM THI, or 3 µM IMZ (Supplementary data). These concentrations were used to guide the choice of upper exposure limits for assessment of lipid accumulation in subsequent experiments.

### 3.2. Chemical-induced steatosis

In subsequent experiments, ZFE were exposed to known steatosis-inducing chemicals (IMZ, THI, CTD) for 24 h, or 2 % fructose as positive control. Exposure to 2 % fructose resulted in a clear increase in intrahepatic lipid accumulation compared to the medium control (Fig. 1). Upon exposure to IMZ and THI for 24 h (96–120 hpf) a concentration-dependent increase in fluorescence in the liver region, indicating hepatic lipid accumulation, was observed (Fig. 2A,B). In contrast, exposure to up to 1 mM CTD for 24 h during the same time frame did not result in measurable hepatic lipid accumulation (data not shown). However, at the exposure duration of 48 h (starting at 72 hpf), a modest, but statistically significant, concentration-dependent increase in lipid accumulation was observed (Fig. 2C). Extending the exposure duration for IMZ and THI to 48 h resulted in overall toxicity (data not shown), therefore differed exposure durations were applied per substance. The critical effect dose (CED) at CES=20 % change in lipid accumulation derived from the PROAST curve fit, summarized in Table 3, indicated that IMZ (CED: 0.99 µM; Fig. 2A) was more potent than THI (CED: 143 µM; Fig. 2B), which was more potent than CTD (CED: 750 µM; Fig. 2C). Table 3 also shows that the upper and lower limits of CED, respectively CED-U and CED-L, are rather close (ratios <10) for IMZ and THI, supporting the quality of these data-sets (limited uncertainty); the CED-U/L ratios for CTD is much higher, indicating a higher uncertainty in this analysis.

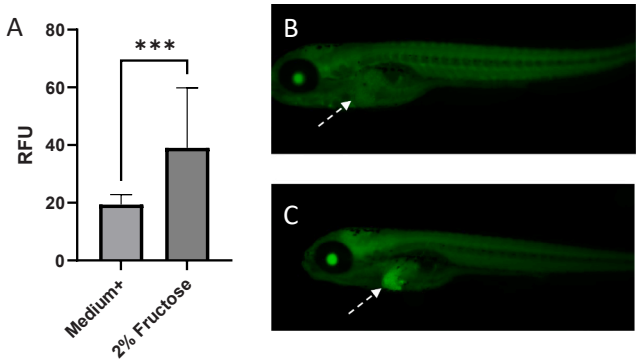



**Fig. 1.** Exposure of ZFE to 2 % fructose for 48 h results in a significant increase in intrahepatic lipid accumulation at 120hpf. A. Bar graph showing the increase in fluorescence upon exposure to fructose compared to the fluorescence in ZFE exposed to control medium containing antifungal treatment only. Data is presented as average fluorescence (Gray scale, background corrected) ± SD for the region of interest (liver), significance calculated using Student's t-test ***p≤0.001. N = 3, n = 17–19. B. Representative image of an embryo grown in control medium. C. Representative image of an embryo exposed to fructose for 48 h. The difference in fluorescence in the liver area (white arrow) of the fish is visually detectable.

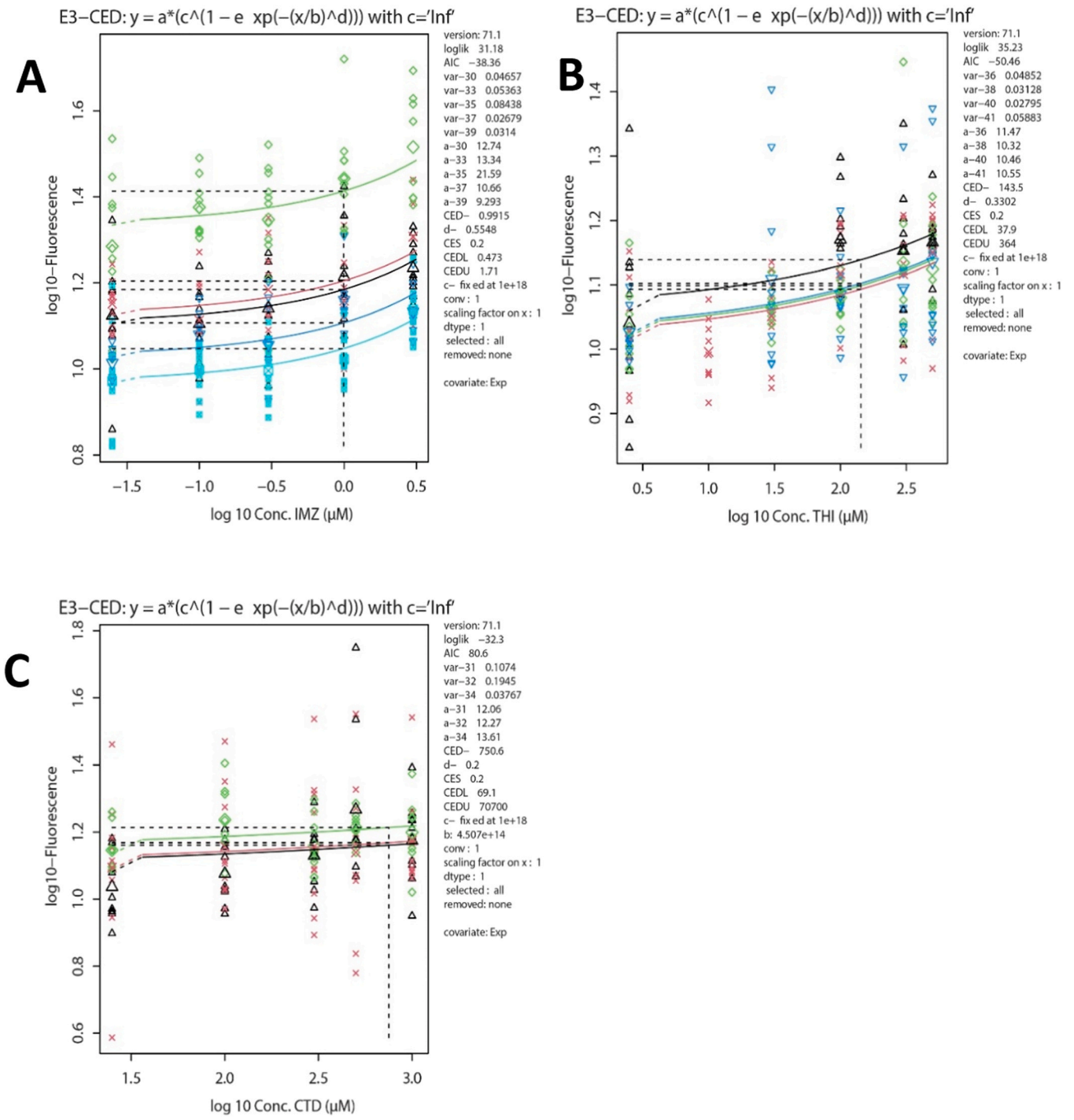



**Fig. 2.** Dose-response analysis of three test compounds IMZ (A), THI (B), CTD (C), with substance concentration on the X-axis and lipid green fluorescence in the liver on the Y-axis. Large symbols represent the geometric mean of each of 3–5 independent experiments (N, each independent replicate experiment shown separately with a different color, and provided with a different number 30–41 in the legends) with 7–10 embryos each (n; small symbols). CED (intersection dashed line) is presented at the 20 % effect level (CES). The parameters of the exponential function are explained in Methods (Section 2.6).

**Table 3**
Summary of dose-response results of the three test substances and mixtures.

| | CED20 (µM) | CED-L (µM) | CED-U (µM) |
|---|---|---|---|
| IMZ | 0.99 | 0.47 | 1.7 |
| THI | 143.5 | 37.8 | 364 |
| CTD | 750.6 | 69.1 | 70,700 |

CED20: Critical effect dose at a 20 % critical effect size; CED-L: Lower limit of critical effect dose; CED-U: upper limit of critical effect dose.

### 3.3. Mixture assessment

To evaluate whether mixtures of the current pesticides result in combined effects following the principle of dose addition, a range of concentrations of the individual compounds was applied to the embryos. In case dose addition applies, the fitted concentration-response of the sum of RPF-adjusted (equipotent) doses of the single compounds is indistinguishable from the (fitted) individual concentration-response data. Based on the potencies of the individual pesticide two binary

RPF-adjusted mixtures were composed (Table 2B). The mixture of IMZ and THI represented a mixture of substances with similar mode of action (MOA) (PXR activation), whereas the mixture of IMZ and CTD represented the mixture of substances with dissimilar MOA (PXR activation and PPARα antagonism, respectively). In both cases, the curve fit demonstrated that the principle of dose-addition applied as there was no statistically significantly difference between the fit of the mixture and of the individual components, which therefore showed as a single combined curve (Fig. 3).

### 3.4. Determination of internal exposure concentrations

To aid in the translation of the nominal medium concentrations used in these experiments to exposure levels used in other (animal) studies, the internal concentration of the pesticides was determined at the highest nominal exposure level following the 24 h (IMZ, THI) or 24 and 48 h (CTD) exposure (Table 4). The results demonstrate that in all three cases external exposure resulted in a measurable internal exposure in ZFE at 120 hpf. In case of exposure to IMZ, the nominal concentration of 3 μM added to the medium resulted in only 0.49 μM in the (pooled) medium fraction, whereas the internal concentration in the ZFE amounted to 20 μM (±3 μM). For THI the nominal concentration was more or less retrieved from the medium, and similar internal exposure levels were found in the ZFE. In case of CTD, the nominal concentration and the retrieved concentration in the (pooled) medium were similar, whereas the internal concentration in the ZFE amounted to half the medium concentration.

## 4. Discussion and conclusion

This research focused on the confirmation of chemically-induced hepatic triglyceride (TG) accumulation, histopathologically observable as steatosis, in zebrafish embryos (ZFEs). For this purpose, the same set of substances was used as in a preceding study in human hepatocarcinoma HepaRG cells, i.e., IMZ, THI, CTD (Lichtenstein et al., 2020a, 2020b). Next, dose addition was tested as binary combinations of substances with similar MOA (PXR activation, IMZ+THI), and with dissimilar mode of action (PXR activation and PPARα inhibition, IMZ+CTD). The method to assess hepatic TG accumulation was based on *in vivo* staining with the fluorescent dye LipidGreen2, which can then be visualized and quantified Chun and co-workers (Chun et al., 2013), demonstrated that LipidGreen2 staining upon exposure to steatosis-inducing substances overlaps with the correct anatomical location of the liver. For this purpose, they applied LipidGreen2 staining in a transgenic ZF reporter line expressing DsRed coupled to the liver-specific *l-fabp* promoter. For all three tested substances it holds that the MOAs are of relevance for humans and molecularly present in ZFE. The results demonstrated that it is possible to use LipidGreen2 staining to measure intrahepatic lipid accumulation as a result of exposure in ZFE at 120 hpf. This applies to single substances as well as binary mixtures of substances with dissimilar modes of action and varying ranges in maximal inducible increases in intrahepatic lipid accumulation. The latter point is illustrated by the findings with CTD which, compared to THI and IMZ, did induce only a minor yet significant increase in lipid accumulation. Albeit minimal, the dose-response data sufficed to perform BMD analysis and successfully demonstrated dose-addition in a mixture with IMZ, illustrating the sensitivity of the combined approach.

Alternative methods to stain for intrahepatic lipid accumulation in ZFE are available, such as non-fluorescent staining using Oil-red, or fluorescent staining using Nile-red. A comparison between oil-red and LipidGreen2 has been made by Chun et al., (Chun et al., 2013) demonstrating that LipidGreen2 resulted in a less diffuse stain and therefore more accurate results. Nile-red has been applied in the recent publication by Le Mentec and co-workers (Le Mentec et al., 2023). Although the methodology appears similar, a side-by-side comparison of the performance of Nile-red vs LipidGreen2 is not yet available.

The results are in line with findings in human HepaRG cells (Alarcan et al., 2022; Lichtenstein et al., 2020a, 2020b) in terms of potency ranking of the three test compounds (IMZ>THI>CTD). The optimal exposure duration for IMZ and THI to induce the lipid accumulation was 24 h (Fig. 2A/B), whereas for CTD, an exposure duration of 48 h was required to achieve measurable TG accumulation (Fig. 2C). This difference may be explained because the mechanism of IMZ and THI, activation of the PXR pathway, is achieved instantaneously, whereas the mechanism of CTD, inhibition of PPARα, may require the turnover of existing intracellular ACOX1 (Kersten, 2014), which may take time. The chemical analysis (Table 4) indicated that the internal concentrations of CTD following 24 and 48 h exposure are similar, indicating that delayed uptake kinetics related to the physicochemical properties of CTD do not explain the required prolonged incubation period.

Given the differences in physicochemical properties, the internal concentration of the test substances was measured following the 24 h or 48 h exposure. These results, summarized in Table 4, indicate an important difference between nominal medium concentrations, actual medium concentrations and internal exposure levels for IMZ, largely related to physicochemical properties of the substance. From the results it is clear that internal exposure levels of this substance are about one order of magnitude higher than the free concentration in the medium, indicating bioaccumulation. This can be explained by the fat-soluble properties of IMZ (log Kow: 4.10). Moreover, the nominal medium concentration is somewhere in between, likely indicating loss of substance, compatible with bioconcentration in the embryos, but maybe also due to plastic-binding (Proenca et al., 2021). For THI (log Kow: 2.33) the nominal concentration, measured medium concentration, and internal exposure level were comparable. For CTD (log Kow: 0.64), the internal concentration was about half of the measured medium concentration, whereas the medium and nominal concentration were comparable. This can be explained by the rather hydrophilic nature of CTD. This signifies the importance to measure the internal concentration of a substance following the actual exposure for data interpretation and effect comparison between experimental models, as opposed to the nominal exposure levels. Having actual internal exposure levels instead of nominal concentrations is of particular importance for a reliable interspecies comparison with, e.g., QIVIVE and PBPK approaches (Punt et al., 2020).

As real-life exposure scenarios in humans do generally comprise mixtures of chemical substances, we assessed the effect of binary mixtures of the test substances. In mixture assessments, dose addition is generally considered a default scenario for substances with a similar MOA, but often debated for substances with dissimilar MOAs (More et al., 2019). In the past, dose addition has been shown to apply as a default to mixtures of substances with dissimilar MOAs (see e.g.: (van der Ven et al., 2022)). The current mixture data demonstrates that both the mixture of compounds with a similar MOA (IMZ-THI) and the mixture with dissimilar MOAs (IMZ-CTD) are subject to dose addition. Consequently, this further underlines that for cumulative risk assessment (CRA) grouping on the level of the adverse outcome may be most relevant.

The current results demonstrate that the zebrafish embryo provides an interesting model for the assessment of chemical-induced steatosis. The whole-organism aspect of the zebrafish embryo provides a clear advantage over the cellular models given that the complete physiology of the animal is present. Despite differences in metabolism between zebrafish and humans, CYP activity in ZFE has been demonstrated to be comparable or higher than humans (among others, CYP 3A4/5, 2C9 (Nawaji et al., 2020)). However, as only up to 70 % human genes coding for xenobiotic metabolism have a zebrafish orthologue (van Wijk et al., 2016), knowledge on metabolism of test substances and potential active metabolites is necessary for correct translation of the results to the human situation.

Altogether, when the AOP strategy for toxicological assessment of chemical substances is applied to predict steatotic effects in humans, the

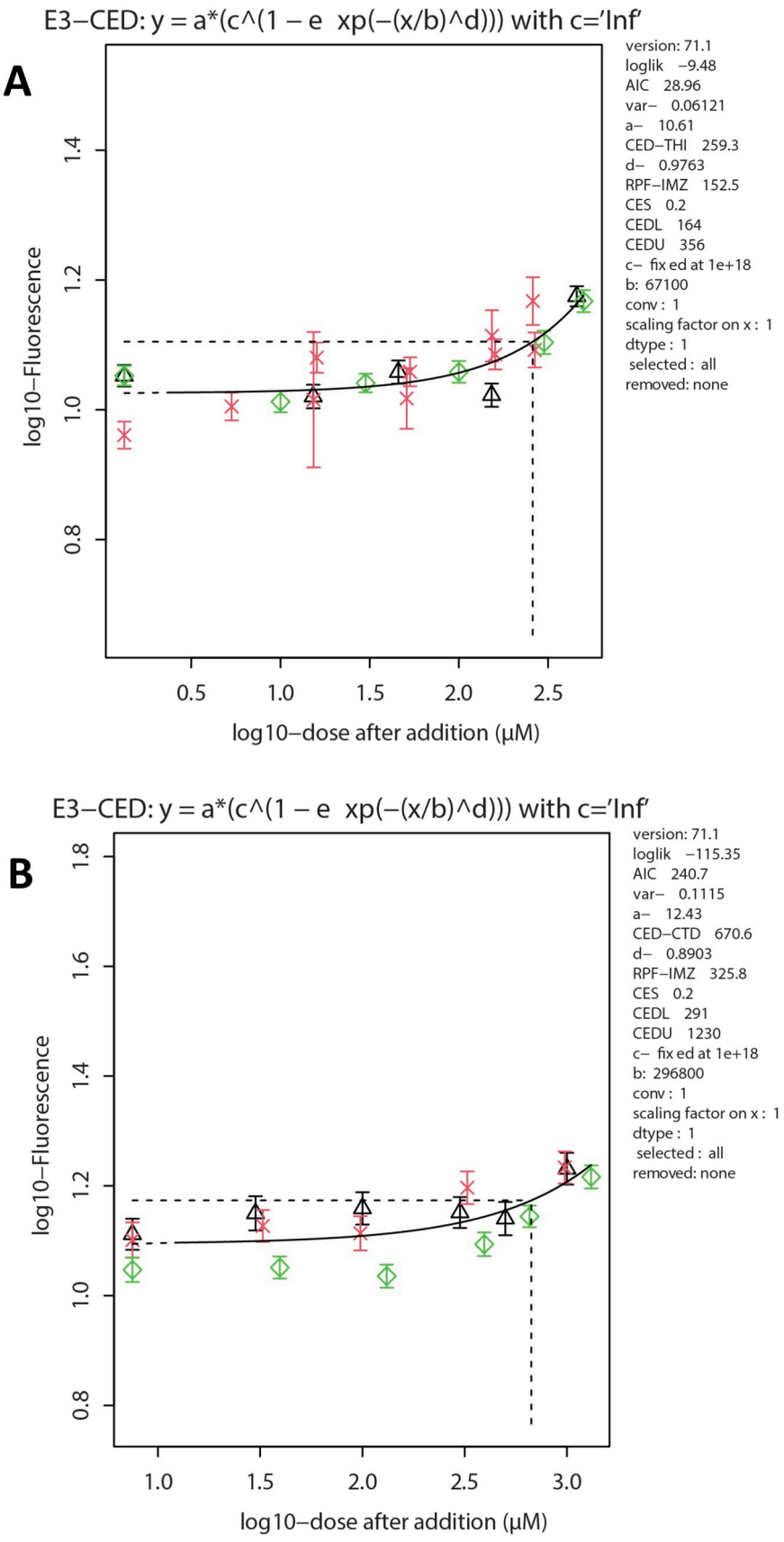



**Fig. 3.** Combined analysis of the effect of single compounds and binary equipotent pesticide mixtures on hepatic lipid accumulation. The graphs show combined concentration-response fits consisting of the individual components as well as their equipotent binary mixtures (3 **A**: IMZ (red), THI (black), mixture (green); RPF IMZ 152.5 3**B**: IMZ (red), CTD (black), mixture (green); RPF IMZ 325.8). The mixture responses (green diamonds) follow the same concentration-response curve as the individual components, indicating that in both cases the principle of dose addition applies.

**Table 4**
Internal concentration of pesticide vs nominal target medium concentration and actual medium concentration after 24/48 h exposure.

| | Nominal concentration (μM; medium) | Effective medium concentration (μM)[a] | Intraembryonic concentration (μM)[b] |
|---|---|---|---|
| IMZ | 3 | 0.49 | 20±3 |
| THI | 600 | 451 | 530±2 |
| CTD | 1000 | 1177 / 1237 | 489 ± 16 / 493 ± 43 |
| 24 h/ CTD 48 h | | | |

[a] Concentration of substance present in the medium at the end of exposure (IMZ, THI: 24 h, CTD: 24/48 h) measured by UPLC

[b] Intraembryonic concentration calculated from measured concentration to μg/g tissue (assuming 1 g tissue equals 1 mL) following exposure for 24 h (IMZ, THI) or 24 and 48 h (CTD).

zebrafish embryo is very well applicable as a higher tier testing model to confirm effects in downstream steps in the AOP, that is, chemically-induced TG accumulation in the whole organism and production of visible steatosis. Of note, hepatic fatty changes seen in long-term regulatory studies with the three pesticides could not be observed in rats in a study protocol with repeated-dose oral exposure for 28 days (Alarcan et al., 2021), highlighting the potential of zebrafish embryos as an alternative whole-organism model allowing for much shorter experimental setups, while yielding results in line with induction of steatosis in mammals. The zebrafish embryo may thus serve as an important upstream testing model in the AOP for steatosis. Furthermore, the steatotic effects are quantifiable in this model, and allow the assessment of mixture toxicology. Thus, a potency ranking of the tested substances could be achieved and appeared similar to the result in HepaRG cells, and dose-addition could be concluded for binary mixtures of substances with similar and with dissimilar modes of action. The current results indicate that the approach allows for testing of compounds with varying physical chemical properties. Further testing with a broader range of compounds would be required to assess the full (toxicological) applicability domain of the zebrafish embryo as model for chemically-induced steatosis.

## CRediT authorship contribution statement

**Angela de Haan:** Methodology, Investigation. **Albert Braeuning:** Writing – review & editing, Funding acquisition, Conceptualization. **Harm J. Heusinkveld:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Edwin P. Zwart:** Visualization, Methodology, Investigation. **Jimmy Alarcan:** Writing – review & editing, Funding acquisition, Conceptualization. **Leo T.M. van der Ven:** Writing – review & editing, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2024.153927.

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