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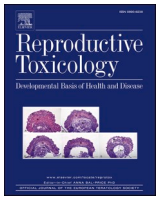

Determination of the zebrafish embryo developmental toxicity assessment (ZEDTA) as an alternative non-mammalian approach for the safety assessment of agrochemicals

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ABSTRACT

With the US Environment Protection Agency reducing requests for (and funding of) mammalian studies alongside the proposed elimination of requests by 2035, there is an urgent need for fully validated New Approach Methods (NAMs) to fill the resultant gap for safety assessment of agrochemicals. One promising NAM for assessing the potential for human prenatal developmental toxicity potential is the Zebrafish Embryo Developmental Toxicity Assessment, a bioassay that has been used by the pharmaceutical industry for more than a decade in early-stage drug safety assessment. Despite its promise, little data has been generated to assess the validity of ZEDTA for assessing Developmental and Reproductive Toxicity of new agrochemical products. Addressing this knowledge gap, we tested 67 compounds (insecticides, herbicides and fungicides) spanning multiple different chemical groupings and mechanisms of action. ZEDTA assay results were compared with the European Chemicals Agency (ECHA) Classification and Labelling (C&L) for mammalian hazard classification and with publicly available data to determine the ZEDTA's translation power. Overall, the ZEDTA assay had an effective detection capability of 65 % for sensitivity and 64 % for specificity as compared with the ECHA-C&L classification and publicly available data. Comparing the ZEDTA data there were both strengths and weaknesses in alignments for across the different chemical classes and chemical mechanisms of action. Overall, the data generated, show the performance of the ZEDTA assay was comparable with other bioassays highlighted as alternatives for mammalian assessment and holds good promise as a NAM for screening agrochemical prenatal developmental toxicity during new product human safety assessment.

1. Introduction

Regulatory approval of agrochemical compounds relies upon internationally accepted test methods for identification and characterisation of hazard potential for 'prenatal Developmental toxicity And Reproduction Toxicity (DART)', which have remained largely unchanged for decades. These tests employ a minimum of two mammalian species, one a rodent (typically the rat) and the other a non-rodent (typically the rabbit) [1] and comprise studies addressing fertility, embryofoetal development, pre- and post-natal development and multigenerational impacts. Assessing a chemical's potential for prenatal developmental toxicity alone requires the use of more than 80 adult mammals for each of the two test species (OECD 414) [2]. Moreover, this number does not include the hundreds of foetuses which form part of the assessment, nor does it include the preliminary studies in both non pregnant and pregnant animals necessary for dose range-finding purposes. In addition, for each compound, approximately 2600 animals are used in the two-generation reproductive toxicity study (OECD 416) which is the minimum regulatory requirement for progressing to product registration.

Due to the high numbers of mammals used, and the severity of the protocols involved, there has been considerable 3Rs (replacement,

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reduction and refinement) -focussed research centred on the development of alternative non-mammalian DART assessment methods, including the use of human cell cultures, whole organoid culture and computer modelling [2]. These approaches are collectively termed New Approach Methods (NAMs) and when used in isolation or as a combination aim to provide reliable and robust scientific data for human health risk assessment without the need for mammalian *in vivo* testing.

Various NAMs are already widely used across different industries for non-regulatory decision making in research and development. However, there has been a reluctance to formally adopt any of these methods as replacements to the established (mammalian) animal studies, and, as yet, there are no published OECD guidelines available for alternative DART assessment methods. Adding greater urgency to the NAM landscape, in Sept 2019, the United States Environment Protection Agency (USEPA), who are responsible for the registration of all new agrochemical products in the USA, announced it will reduce requests and funding for animal studies by 30 % by 2025, and eliminate all mammal study requests and funding by 2035. This further emphasises the growing need to develop viable and predictive animal alternatives to replace the use of mammals in agrochemical prenatal developmental toxicity (and other toxicity) assessment programmes.

Of the various NAMs proposed for agrochemical prenatal developmental toxicity testing which include the animal Whole Embryo Culture, Embryonic Stem Cells and Micromass test for a range of species have been developed and assayed for validity [3–6], the zebrafish embryo-larvae shows great promise. Indeed, zebrafish embryo-larvae have been used in developmental toxicity assessment by the pharmaceutical industry for over a decade [7–10] and offers several advantages over simpler model organisms or cell-based assays. These include rapid development of tissues and organs, high optical transparency allowing rapid non-invasive assessment on organ and tissues, and active metabolism in the later stages of embryo-larval development. The rapid development of the zebrafish also offers time and resource advantages over mammalian-based assays: zebrafish develop *ex vivo* from a fertilised embryo to hatch in 3 days and to independent feeding after a further 2 days, compared with, for example, (*in vivo*) uterine examination in the rat or rabbit at day-21 or day-29 of gestation, respectively [11].

Despite the relatively large volume of work assessing the validity of zebrafish embryo-larvae as a model for mammalian prenatal developmental toxicity screening following drug exposure, a comparatively small amount of research has focussed on its translational utility with respect to agrochemicals. Studies published previously have tended to focus on a small number of specific agrochemicals (e.g., profenofos or difenoconazole) [12,13] or on specific groups of related chemical derivatives (e.g., glycol ether alkoxy metabolites and triazole derivatives) [14]. Comparison of data between studies is further complicated by the use of different methodologies, such as differences in developmental exposure windows or the presence or absence of a chorion around the embryo [15]. To date, a comprehensive assessment of the translational power of zebrafish embryo-larvae across multiple representatives of different agrochemical classes and/or mechanisms of action using a standardised approach has not been undertaken.

To address this knowledge gap, here we employed a validated zebrafish embryo-larval test method, the Zebrafish Embryo Developmental Toxicity Assessment assay (or ZEDTA assay – Gustafson *et al.*, 2012; Ball *et al.*, 2016 [7,8]) to evaluate the effects of 67 agrochemicals and relevant comparators (tested blinded) on the development of zebrafish embryos. The compounds were selected to represent a wide range of chemical structures across a variety of indications and mechanisms of action, thus providing the most comprehensive analysis of agrochemicals in a zebrafish embryo-based test method to date (supplemental Table 1). The ability of the ZEDTA assay to predict the prenatal development hazard potential of a chemical was done by comparing the outcome of the assay with the developmental hazard classification for the chemical in Europe (European Chemicals Agency (ECHA) Classification & Labelling (C&L) inventory) and publicly accessible data,. In turn we assessed how the translational power of the model varies between agrochemical classes, chemical structures, molecular targets or primary/secondary modes of action; and highlight areas of uncertainty in its use, requiring further research activity.

2. Materials and methods

2.1. Test compounds

The test compounds employed in the current study, their intended or historic use (fungicide/herbicide/insecticide/pharmaceutical comparator), mechanism of action and CAS number are summarised in supplemental Table 1. Where possible, several representatives of a given class were tested to allow for a more thorough assessment of translation capability for that class of compounds between the mammalian and zebrafish assays. All test compounds were blinded (they were anonymised to the analyst) throughout the assessment and pre-classified as positive or negative based on compound specific hazard classifications for developmental toxicity obtained from the ECHA C&L Inventory or publicly accessible data, where the European CLP hazard classification criteria were used to categorize hazard potential (see results section). Compounds were supplied by Syngenta, with the exception of clethodim, emamectin, fenoxaprop-P-ethyl, sethoxydim, spiroxamine, tepraloxydim, bromoxynil and clethodim, that were obtained from Sigma-Aldrich (UK) and flumiclorac-pentyl, quizalofop-p-tefuryl that were obtained from LGC limited (UK).

2.2. Chemical reagents and consumables

All reagents for the bioassays were supplied by Sigma–Aldrich (St. Louis, MO), unless otherwise stated and all other consumables (microwell plates etc.) were supplied by VWR Ltd, (UK) unless otherwise indicated. Further details of the materials and experimental procedures are captured in the supplemental material and methods (S 1.1 – S 1.6).

2.3. Exposure concentration determination

Published adult fish LD_{50} values (where available) were used to establish the initial exposure concentration ranges (that were conducted in a 10-fold dilution series) (supplemental table 2). Concentration range settings are detailed in the supplemental material and methods (S 1.1).

2.4. Zebrafish brood stock and egg production

Breeding stocks of adult Wild Indian Karyotype (WIK) strain zebrafish were held at the University of Exeter (sourced from a historical line held by Brixham Environmental Laboratory, AstraZeneca and periodically outcrossed with WIK from the Zebrafish International Resource Centre, Oregon, USA) were used for the production of fertilized eggs. Details of husbandry conditions, adult feeding, system water, embryo collection and staging of embryos are detailed in supplemental materials and methods (S 1.2).

2.5. Test compound preparation and embryo-larval exposure

Test Compounds were initially dissolved in 100 % DMSO to either 20 mM or 200 mM and stored in separate aliquots at 4°C until used in the bioassays (within 2 months). Each aliquot was used only once avoiding repeated freeze-thaw cycles in DMSO. On the day of use, exposure solutions were prepared in Danieau's medium with a final DMSO concentration of 0.5 %, according to the methods of Gustafson *et al.*, (2012) and Ball *et al.*, (2014) [7,8], described in further detail in the supplemental materials and methods (S 1.3 and S 1.4). Visual inspection of DMSO stock solution and exposure solutions were conducted at the time of solution preparation and at 5dpf.

On the day of exposure, embryos were staged according Kimmel *et al.*

1995[16], and exposures commenced at 4–6 hours post -fertilisation (hpf) and were carried out in 24 well plates with one embryo per well in 1 ml. Embryo-larvae were then cultured at 28°C (± 1) with 14:10 light dark cycle (same conditions as the adults animals) until 5dpf, when they were assessed. Two independent replicates were conducted for each compound as a minimum. If there was a difference in the Teratogenic or Non-teratogenic classification, then a third replicate was performed to determine the overall classification. If there was an alteration in the dosing concentrations due to the initial replicate results (see supplemental materials and methods) then the altered dosing concentration was performed in a minimum two independent replicates to obtain an consistent and overall classification.

2.6. Assessment of chemical effects on development and teratogenic index (TI) ratio calculation

Embryo viability and morphological assessments were conducted using the endpoints detailed in Gustafson *et al.*, 2012 [7] and Ball *et al.*, 2014 [8] and also using a numerical system described previously by Panzica-Kelly *et al.*, 2010 [17] to determine the No Observed Adverse Effect level (NOAEL) and Lowest Observed Adverse Effect Level (LOAEL) as detailed in supplemental materials and methods (S 1.5). Example images of jaw malformations and associated scoring values assigned are shown in supplemental Figure 1. Assessments of morphogenesis were carried out under weak anaesthesia (1 mM buffered MS222) using a Leica M205C stereomicroscope equipped with a Leica DMC4500 digital camera (Leica, Germany). In addition, the teratogenic index (TI) was calculated for each compound by dividing the LC_{25} by the NOAEL. A TI value of less than 10 was considered non-teratogenic, while a TI ratio of 10 or greater was considered teratogenic as determined in Brannen et al. [18]. Compounds were subsequently classified as Teratogenic (T) or Non-Teratogenic (NT) based on the determined TI value. Any compound that did establishment a LOAEL (either by morphological impact or lethality) at the dose range investigated and if there was no determination of any compound present within the developing embryo then the compound was classified as undetermined.

After morphological scoring, the fork length of each animal was measured using a captured image on a Leica LAS X core and LAS X measurements® and then humanely killed using an anaesthetic overdose (overdose via benzocaine [6 mM] for 20 minutes and then destruction of the brain). Full details of the assessment process are provided in the supplemental materials and methods section (S 1.5).

2.7. Statistical analysis

Differences in total body length between treatments were assessed using Minitab 16 Statistical software (Minitab, Inc www.minitab.com 2010). Initially, normality was assessed through the application of Levene's and Bartlett's tests. If the data were normally distributed, treatments were compared by 1-way ANOVA and Tukey's test. Where data were not normally distributed, the Kruskal-Wallis test was applied followed by Mann-Whitney tests to compare each treatment group. Treatment groups were regarded as significantly different (at $p < 0.05$) against all control groups (Danieau's medium, solvent and negative control).

2.8. Comparison of zebrafish embryo-larval and mammalian DART classification

The zebrafish TI was compared against the EU hazard classification for developmental toxicity in mammals, accessible at https://echa.europa.eu/information-on-chemicals/registered-substances (last accessed April 2024). Compounds with either H361 (suspected of damaging fertility or the unborn child) or H360 (may damage fertility or the unborn child) hazard classifications were categorized as positive. Where compounds were only classified as impacting fertility (e.g. H360F or H361f), these were classified as negative for developmental toxicity. Where C&L entries did not exist, compound developmental toxicity hazard potential was determined from evaluating the publicly available data. Based on this comparison, compounds were marked as True Positives (TP), True Negatives (TN), False Positives (FP) or False Negatives (FN). Overall ZEDTA assay detection capability/accuracy was assessed via calculations on the percentage of compounds correctly identified as TPs and the percentage of compounds correctly classified as TNs. In addition, the following predictive capabilities of the ZEDTA assay were determined:

The sensitivity, determined via the formula:

$$TP \div (TP + FN)$$

The specificity, determined via the formula:

$$TN \div (TN + FP)$$

positive predictive value (PPV), determined via the formula:

$$TP \div (TP + FP)$$

negative predictive value (NPV), determined via the formula:

$$TN \div (TN + FN)$$

and balanced accuracy value (BAC), to allow account for any uneven distribution of both positive and negative classified compounds determined via the formula:

$$(PPV + NPV) \div 2$$

2.9. Bioanalysis of chemical uptake in zebrafish embryo-larvae

Uptake of the test chemical was determined in the whole bodies of embryos following a 24-hour exposure (6–30hpf), the period encompassing early organogenesis. For this, embryos were exposed to the three highest non-lethal test concentrations of the test chemical and to a corresponding solvent control (0.5 % DMSO). Embryos were then washed thoroughly, extracted and whole-body compound concentration measured using liquid chromatography with tandem mass spectrometry (LC–MSMS). Full details on the bioanalytical methods are provided in the supplemental materials and methods (S 1.6).

3. Results

3.1. Bioanalysis of whole-body compound concentration

The results of the bioanalysis of compound uptake into larvae and chemical stability in aqueous solution are detailed in supplemental table 3 in the Supplementary Information and supplemental materials and method (S 1.6). Table 1 gives the levels of chemical uptake into the embryo-larvae at the LOAEL and the NOAEL (μM). Eighty five percent of the compounds were detectable in zebrafish embryo-larvae using our standard LC-MSMS method. The remaining 10 compounds were not detected due to either a lack of method sensitivity for that compound or absence of compound uptake. Of those compounds where uptake was measurable, the uptake ranged between 3 % and 14,000 % of the external concentration after 24 hours of exposure. Compound stability, as indicated by measurement of exposure solutions concentrations at 0 and 5 days ranged between 1 % and 124 % of the nominal medium concentrations (supplemental table 3).

3.2. ZEDTA assay results

The results of the ZEDTA are summarised in Table 1 and are described for each mechanism of action class in the following sections below. Groups are listed in order of the number of representative test compounds assessed in the ZEDTA, starting from the largest group

Table 1

Summary of the assessed agrochemicals assessed, grouped by mechanism of action, chemical class, CAS number, ECHA C&L inventory classification, ZEDTA classification, LC_{25} value, LOAEL and NOAEL exposure concentrations (μM), chemical uptake as percentage and the estimated uptake as μM at the NOAEL and LOAEL exposure concentrations were available. Red filled boxes denote a teratogenic (positive) classification while green filled boxes denote a non-teratogenic classification (negative). Colour classification applied to both the ECHA C&L classification and the ZEDTA classification. Pale peach boxes denote where there was no classification by ZEDTA. White boxes denote where there was no classification by ECHA C&L inventory. TP – true positive, TN – true negative, FN – false negative, FP – false positive. Pale yellow coloured boxes denotes fungicide, pale blue coloured boxes denotes insecticide, pale red coloured boxes denotes herbicide indication, pale orange coloured boxes denotes pharmaceutical inidication.

MOA	Indication	Compound	Chemical Class	CAS	ECHA C&L Inventory Hazard Statement Code	ECHA C&L Inventory Hazard Category	ECHA C&L Inventory Harmonised or Self Classification	ECHA C&L Developmental Toxicity potential	ZEDTA Developmental Toxicity potential	Classification	LC_{25} (μM)	LOAEL (μM)	NOAEL (μM)	Uptake as % of external medium conc	Uptake as LOAEL (μM)	Uptake as NOAEL (μM)
C14-demethylase in sterol biosynthesis	Fungicide	Cyproconazole	Triazole	94361-06-5	H360D	Repr. 1B	Harmonised	Positive	Positive	TP	>200	50	10	165		19.5
	Fungicide	Diniconazole-M	Triazole	83657-18-5	H361(D)	Repr. 2	Self	Positive	Positive	TP	8.125	2.5	0.25	>1500	105.9	3.4
	Fungicide	Epoxiconazole	Triazole	133855-98-8	H360DF	Repr. 1B	Harmonised	Positive	Positive	TP	39.988	12.5	2.5	1330		31.4
	Fungicide	Metconazole	Triazole	125116-23-6	H361d	Repr. 2	Harmonised	Positive	Positive	TP	>20	5	1	1400		13
	Fungicide	Myclobutanil	Triazole	88671-89-0	H361d	Repr. 2	Harmonised	Positive	Positive	TP	125	50	10	360		37.3
	Fungicide	Penconazole	Triazole	66246-88-6	H361d	Repr. 2	Harmonised	Positive	Positive	TP	32.5	10	1	1600	281.3	15.9
	Fungicide	Propiconazole	Triazole	60207-90-1	H360D	Repr. 1B	Harmonised	Positive	Positive	TP	>25	2.5	0.25	>590	22.7	1.5
	Fungicide	Triadimenol	Triazole	55219-65-3	H360	Repr. 1B	Harmonised	Positive	Positive	TP	>100	10	1	270	29.4	2.4
	Fungicide	Flutriafol	Triazole	76674-21-0	H361d	Repr. 2	Self	Positive	Negative	FN	63.957	100	10	150	209.4	14
	Fungicide	Ipconazole	Triazole	125225-28-7	H360D	Repr. 1B	Harmonised	Positive	Negative	FN	20	5	2.5	3500		18.8 (0.5μM)
	Fungicide	Hexaconazole	Triazole	79983-71-4	None	None	Harmonised	Negative	Positive	FP	31.25	12.5	2.5	>400		20.3
	Fungicide	Prothioconazole	Triazolinthione	178928-70-6	None	None	Harmonised	Negative	Positive	FP	3.25	1	0.1	>400	4	
	Fungicide	Simeconazole	Triazole	149508-90-7	NA	NA	Not Applicable (no entries in the C&L inventory).	Negative	Positive	FP	125	50	10	106		11.2
Inhibition of ACCase	Herbicide	Quizalofop-P-tefuryl	Aryloxyphenoxy-propionates (FOPs)	119738-06-6	H361fd	Repr. 2	Harmonised	Positive	Positive	TP	5	1.25	0.25	17	0.8	
	Herbicide	Quizalofop-P-ethyl	Aryloxyphenoxy-propionates (FOPs)	100646-51-3	NA	NA	Not Applicable (no entries in the C&L inventory).	Negative	Negative	TN	1.5	1	0.5	N/A		
	Herbicide	Tralkoxydim	Cyclohexanediones (DIMs)	87820-88-0	None	None	Harmonised	Negative	Negative	TN	100	50	25	30		10.8
	Herbicide	Sethoxydim	Cyclohexanediones (DIMs)	74051-80-2	NA	NA	Not Applicable (there are no entries in the C&L inventory). Call is based on USDA	Negative	Negative	TN	>100	>100	100	27		27.2
	Insecticide	Spirodiclofen	Tetronic and tetramic acid derivatives (TAs)	148477-71-8	H361f	Repr. 2	Harmonised	Negative	Negative	TN	3.25	10	1	1700		342.8
	Insecticide	Spiromesifen	Tetronic and tetramic acid derivatives (TAs)	283594-90-1	None	None	Self	Negative	Negative	TN	3.25	10	1	>3500	350.5	40.8
	Herbicide	Clethodim	Cyclohexanediones (DIMs)	99129-21-2	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	7		8
	Herbicide	Fluazifop-P-butyl	Aryloxyphenoxy-propionates (FOPs)	79241-46-6	H361d	Repr. 2	Harmonised	Positive	Negative	FN	2.5	2.5	0.5	81	4.1 (5μM)	
	Herbicide	Tepraloxydim	Cyclohexanediones (DIMs)	149979-41-9	H361fd	Repr. 2	Harmonised	Positive	Negative	FN	>100	>100	100	20		18.8
	Herbicide	Fenoxaprop-P-ethyl	Aryloxyphenoxy-propionates (FOPs)	71283-80-2	None	None	Harmonised	Negative	Positive	FP	2.5	2.5	0.25	165	4.1	
nAChR competitive modulators	Insecticide	Thiacloprid	Neonicotinoids	111988-49-9	H360FD	Repr. 1B	Harmonised	Positive	Positive	TP	1000	100	50	25	22	
	Insecticide	Clothianidin	Neonicotinoids	210880-92-5	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	7		7.5
	Insecticide	Imidacloprid	Neonicotinoids	105827-78-9	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	13		13
	Insecticide	Sulfoxaflor	Sulfoximines	946578-00-3	None	None	Harmonised	Negative	Undetermined	N/A	>100	>100	100	No LCMS MS method		
nAChR allosteric modulators - Site I	Insecticide	Spinosad	Spinosyns	168316-95-8	None	None	Harmonised	Negative	Negative	TN	16.25	50	5	>200	203.6	11.7
Inhibition of Photosynthesis at PSII - Histidine 215 Binders/Uncouplers	Herbicide	Bromoxynil	Nitriles	1689-84-5	H361d	Repr. 2	Harmonised	Positive	Positive	TP	32.5	100	10	41	4.1	
	Herbicide	Bromofenoxim	Nitriles	13181-17-4	None	None	Harmonised	Negative	Negative	TN	0.163	5	0.5	>14000		9.4
Inhibition of Photosynthesis at PSII - Serine 264 Binders	Herbicide	Linuron	Ureas	330-55-2	H360Df	Repr. 1B	Harmonised	Positive	Positive	TP	>50	12.5	2.5	1200		28.6
	Herbicide	Monolinuron	Ureas	1746-81-2	None	None	Harmonised	Negative	Negative	TN	32.5	100	10	126	99	17.4
Δ 14-reductase and Δ 8-> Δ 7-isomerase in sterol biosynthesis	Fungicide	Fenpropimorph	Morpholines	67564-91-4	H361d	Repr. 2	Harmonised	Positive	Positive	TP	31.25	12.5	2.5	>500		47.8
	Fungicide	Spiroxamine	Spiroketal-amines	118134-30-8	H361d	Repr. 2	Harmonised	Positive	Positive	TP	32.5	10	1	>800	152.7	8.1
	Fungicide	Fenpropidin	Piperidines	67306-00-7	None	None	Self	Negative	Positive	FP	>20	5	1	>820	136.8 (10μM)	8.2
	Fungicide	Aldimorph	Morpholines	91315-15-0	H360	Repr. 1B	Self	Positive	Undetermined	N/A	>10	>10	10	No LCMS MS method		
DNA / RNA synthesis	Fungicide	Quinolin-8-ol	Isoxazoles	148-24-3	H360D	Repr. 1B	Harmonised	Positive	Positive	TP	8.125	2.5	0.25	No data		
	Fungicide	Hymexazole	Isoxazoles	10004-44-1	H361d	Repr. 2	Harmonised	Positive	Negative	FN	>100	>100	100	29		36.2
	Fungicide	Octhilinone (2-octyl-2H-isothiazol-3-one)	Isothiazolones	26530-20-1	None	None	Harmonised	Negative	Negative	TN	1.625	5	0.5	N/A		
Inhibition of HPPD	Herbicide	Bicyclopyrone	Triketones	352010-68-5	H360D	Repr. 1B	Harmonised	Positive	Negative	FN	>100	>100	100	110		146.8
	Herbicide	Mesotrione	Triketones	104206-82-8	H361d	Repr. 2	Harmonised	Positive	Negative	FN	>100	>100	100	N/A		
	Pharmaceutical	Nitisinone	Triketones	104206-65-7	None	None	Self	Negative	Positive	FP	>100	10	1	31	31.4 (100)	
Complex II: succinate-dehydrogenase	Fungicide	Isopyrazam	pyrazole-4 - carboxamides	881685-58-1	H360D	Repr. 1B	Harmonised	Positive	Positive	TP	>0.5	0.5	0.05	>2700	28.4	1.7
	Fungicide	Penflufen	pyrazole-4 - carboxamides	494793-67-8	None	None	Harmonised	Negative	Negative	TN	1.625	5	0.5	890	47.8	4.1
Mitochondrial complex II electron transport inhibitor	Insecticide	Cyenopyrafen	beta-Ketonitrile derivatives	560121-52-0	None	None	Self	Negative	Negative	TN	1.625	5	0.5	2111	105.6	
Sodium channel modulators	Insecticide	Cypermethrin	Pyrethroids, Pyrethrins	67375-30-8 (alpha)	None	None	Harmonised	Negative	Positive	FP	48.593	1	0.1	NA		
	Insecticide	Deltamethrin	Pyrethroids, Pyrethrins	52918-63-5	None	None	Harmonised	Negative	Positive	FP	>5	0.5	0.05	2100	10.5	
Complex III cytochrome bc1 (ubiquinol oxidase) at Qo site	Fungicide	Dimoxystrobin	Oximinoacetamides	149961-52-4	H361d	Repr. 2	Harmonised	Positive	Negative	FN	0.185	0.5	0.05	530		0.2
	Fungicide	Metominostrobin	Oximinoacetamides	133408-50-1	None	None	Self	Negative	Negative	TN	26.39	100	10	110	152.5	8.2
Inhibition of Cellulose Synthesis	Herbicide	Isoxaben	Benzamides	82558-50-7	None	None	Harmonised	Negative	Negative	TN	>10	>10	10	>110		99.2
	Herbicide	Chlorthiamid	Nitriles	1918-13-4	None	None	Harmonised	Negative	Positive	FP	>200	50	10	No LCMS MS method		
Glutamate-gated chloride channel (GluCl) allosteric modulators	Insecticide	Abamectin	Avermectins, Milbemycins	73989-17-0 (classification from 71751-41-2)	H361d	Repr. 2	Harmonised	Positive	Positive	TP	3.25	1	0.1	>200	2	
	Insecticide	Emamectin	Avermectins, Milbemycins	155569-91-8 (CAS benzoate)	None	None	Harmonised	Negative	Positive	FP	3.25	1	0.1	220	1.9	
Inhibition of DHOD	Pharmaceutical	Brequinar	Pharmaceutical (novel MOA)	96187-53-0	NA	NA	Self	Positive	Positive	TP	32.5	1	0.1	76	0.8	
	Pharmaceutical	Leflunomide	Pharmaceutical (novel MOA)	75706-12-6	H360D	Repr. 1B	Self	Positive	Positive	TP	7.757	1	0.1	2000	22.8	2.2
Inhibition of Very Long-Chain Fatty Acid Synthesis	Herbicide	Dimethachlor	α-Chloroacetamides	50563-36-5	None	None	Harmonised	Negative	Negative	TN	32.5	100	10	45	64.1	2.7
	Herbicide	Pyroxasulfone	Isoxazolines	447399-55-5	None	None	Self	Negative	Negative	TN	>100	>100	100	140		154.3
Inhibitors of chitin biosynthesis affecting CHS1	Insecticide	Flufenoxuron	Benzoylureas	101463-69-8	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	>2800		283.2 (10M)
	Insecticide	Lufenuron	Benzoylureas	103055-07-8	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	>6000		9.5 (0.1μM)
Inhibition of PPO	Herbicide	Flumioxazin	N-Phenylimides	103361-09-7	H361d	Repr. 2	Harmonised	Positive	Positive	TP	48.593	10	1	50	5	
	Herbicide	Flumiclorac-pentyl	N-Phenylimides	87546-18-7	NA	NA	NA	n/a	Positive	n/a	16.25	5	0.5	58	29.2 (50μM)	
Inhibition of Microtubule Organization	Herbicide	Carbetamide	Carbamates	16118-49-3	H360D	Repr. 1B	Harmonised	Positive	Negative	FN	>100	>100	100	18		20.8
	Herbicide	Chlorpropham	Carbamates	101-21-3	None	None	Harmonised	Negative	Positive	FP	50	50	5	No LCMS MS method		
Chordotonal organ nicotinamidase inhibitors	Insecticide	Flonicamid	Flonicamid	158062-67-0	None	None	Harmonised	Negative	Negative	TN	>100	>100	100	5% at 100uM and 50% at 10uM		5
Chordotonal organ TRPV channel modulators	Insecticide	Pymetrozine	Pyridine azomethine derivatives	123312-89-0	H361fd	Repr. 2	Harmonised	Positive	Negative	FN	>100	>100	100	11		11.7
Photosynthesis inhibitor	Fungicide	Cyanamide	NA	420-04-2	H361fd	Repr. 2	Harmonized	Positive	Negative*	FN	>100	>100	100	No LCMS MS method		
GABA-gated chloride channel blockers	Insecticide	Fipronil	Phenylpyrazoles (Fiproles)	120068-37-3	None	None	Harmonised	Negative	Positive	FP	>100	1	0.1	1050	9.8	

*Indicates where the dose range increased to 1000μM with no impact on lethality or developmental morphological formation.

tested. Full details of the morphological malformations seen are shown in supplemental Table 4. Assessment of the combined controls (Supplemental Table 6) showed that there was a background abnormality rate ranging from 1.3 % to 2.2 % with majority of the malformations presenting within the arches/jaw structure. There was no observed correlation between the LogP physiochemical property value and the observed classification of the compound. This lack of correlation was also true of the percentage uptake value and the percentage stability value (Supplemental Figure 2A-C).

3.2.1. C14-demethylases inhibitors

The largest number of compounds tested (13) belonged to the C14-demethylase inhibitors, which predominantly contains the triazole chemical class. Of these, 8/10 (80 %) of the C&L developmentally toxic classification 'positives' were identified as positives in the ZEDTA assay. All induced a jaw malformation as the only morphological phenotype, or alongside other structural alterations, for example, significant neural tube malformations (e.g. penconazole at 10μM), reduced body and fin length (e.g. propiconazole at 25μM) or heart defects, such as pericardial oedema (e.g. cyproconazole at 50μM). The most potent triazole compound with the lowest LOAEC was diniconazole, with jaw and neural malformation occurring at an external exposure concentration of 2.5μM.

All of the TP triazoles demonstrated relatively high compound embryo-larval penetration, with body concentrations ranging from 165 % to 1600 % of the nominal external medium concentration. Furthermore, all c14-demethylases demonstrated a high level of aqueous stability (≥89 %), with the exception of cyproconazole which had a stability of 54 % after 5 days in the exposure medium. Two triazoles, namely ipconazole and flutriafol, were classified as non-teratogenic in the ZEDTA assay contrasting with a positive *in vivo* prenatal developmental toxicity hazard classification, despite showing good stability in the exposure media and relatively high uptake into the embryo-larvae (average 3500 % and 150 %, respectively).

The three c14-demethylases fungicides considered to be non-developmentally toxic by ECHA C&L classification (hexaconazole, prothioconazole and simeconazole) all caused malformation of the jaw, neural development (in the case of hexaconazole and simeconazole), and impacted on multiple other tissues/structures. Uptake of the compounds into the embryo ranged from 100 % to 400 % of the nominal exposure concentration and the compounds were relatively stable over 5 days of exposure. Of note, hexaconazole had a NOAEL of 2.5μM (based on measured concentrations in the exposure medium) and prothioconazole a NOAEL of 0.1 μM, equal and lower, respectively, to the lowest NOAEL value of all the c14-demethylase fungicide TPs tested.

3.2.2. Inhibitors of acetyl CoA carboxylase (ACCase)

The next most represented chemical class tested the acetyl CoA carboxylase (ACCase) inhibitors (10), included both herbicides (8) and insecticides (2), from four chemical classes. Of these, three have a hazard classification for prenatal developmental toxicity and seven are not classified as developmentally toxic in mammals. Of the C&L positive compounds, quizalofop-P-tefuryl was the only compound classed as a TP in the ZEDTA assay, causing impacts on the jaw and notochord development at 1.25μM, This response was seen despite this compound being relatively unstable in the culture medium (measured concentrations in the exposure medium were less than 1 % of nominals at 5 days) and with relatively low-level embryo-larval uptake (17 %). The of the two FNs, fluazifop-p-butyl showed poor stability (the measured concentrations in the exposure medium was 1 % of nominal after 5 days).

Of the compounds considered to be non-developmentally toxic in mammals, 6/7 were classified in the ZEDTA assay accordingly. Importantly, these compounds also generally showed moderate to high uptake, with the exception of clethodim where the internal concentration in the embryo-larval was only 6 % of that in the external medium. It should be noted that although uptake was not measurable for quizalofop-p-ethyl, lethality was observed confirming tissue exposure, thus ruling out poor tissue penetration as the reason for the lack of any morphological effect seen in ZEDTA.

One ACCase inhibitor only was classified as a FP, namely fenoxaprop-p-ethyl. Exposure resulted in malformation of multiple structures including the jaw, neural tube cranial-facial features, fins and heart, reduced hatching and lethality with a LOAEL of 2.5μM. Fenoxaprop-p-ethyl had low stability (3 %) in aqueous solution but had relatively high uptake (at 165 % of the external medium concentration, 4μM internal concentration, at 24 hours).

3.2.3. Nicotinic acetylcholine receptor (nAChR) modulators

Of the five modulators (including both competitive and allosteric modulators) of nAChR insecticides tested, only thiacloprid was predicted as developmentally toxic in the assay(NOAEL 100μM external), and this compound was classified in accordance with the ECHA C&L hazard classification as a TP. In addition, three nAChR modulators were also classified as TNs in the ZEDTA showing no observable impacts, including for the highest tested concentration of 100μM. Having said this, in the case of imidiclopride and clothianidin internal concentrations were comparatively low (13μM and 7μM, respectively). Sulfoxaflor, classified as negative under ECHA C&L also showed no effects in the ZEDTA assay but with LC-MSMS analysis not being possible due to the hydrophobic nature of the compound it was marked as unclassified in the ZEDTA.

3.2.4. Photosystem II inhibitors (PSII) inhibitors (nitriles/ureas)

All four of the PSII inhibitors, targeting either histidine 215 or serine 264, were correctly classified in the ZEDTA, when compared with the classification using ECHA C&L developmental toxicity hazard criteria. Of the two ZEDTA positives, exposure to linuron resulted in malformations in the jaw and neural structures at external concentrations as low as 12.5μM and exposure to bromoxynil induced malformation of the fins and jaw and a reduction in length at 10μM.

Similarly, the two predicted non-teratogenic PSII inhibitors, monolinuron and bromofenoxim, were also correctly classified as TNs although compound uptake was relatively low in the case of bromofenoxim, at 9.4 % of the external medium concentration.

3.2.5. Sterol biosynthesis inhibitors

Of the four sterol biosynthesis inhibitors assessed, two were classified as positive in the ZEDTA assay (TPs), one as a FP and one was undetermined. Of the two TPs assessed in the embryo-larvae, spiroxamine showed malformation of the somites at an external media concentration 10μM, whilst fenpropimorph exposure impacted the jaw and neural structures at an external media concentration of 2.5 μM.

Fenpropidin was classed as a FP, with a ZEDTA NOAEL based on a reduced heart rate at just an external media concentration of 5μM, and malformations of the jaw, somites and neural structures were seen at 20μM. Uptake of fenpropidin (as with the other sterol biosynthesis inhibitors) was high, with measured internal concentrations up to 820 % of the corresponding external medium concentration. The fourth compound, aldimorph, which is classified as developmentally toxic in mammals had no morphological impact in the ZEDTA at ≤ 10μM. This compound proved difficult to measure via LC-MSMS due to its highly hydrophobic nature.

3.2.6. DNA/RNA synthesis inhibitors

Two of the three DNA/RNA inhibitors tested were classified in the ZEDTA assay, matching C&L hazard classification in mammals. Of these, quinolin-8-ol (8-Hydroxyquinoline) was a TP and exposure resulted in malformation of the notochord, body shape and jaw structure at 2.5 mM (external concentration). Hymexazol showed an uptake into the embryo-larvae of 29 % of the external concentration, but was a false negative in the assay. Despite octhilinone (2-octyl-isothiazol-3-one) being incompatible with the generic analytical method, it induced lethality thus confirming its uptake.

3.2.7. HPPD inhibitors

One of the three HPPD inhibitors tested was correctly classified according to the ECHA C&L hazard criteria. Bicyclopyrone and mesotrione were both classified as FNs, albeit uptake could not be confirmed in the case of mesotrione (this compound was not compatible with generic LC-MSMS method), whilst bicyclopyrone displayed no malformations up to, and including, an external concentration of 100μM.

The pharmaceutical product nitisinone was found to be a TP, with malformation of the jaw observed at 10μM (external concentration). Interestingly, exposure to nitisinone at 100μM also resulted in an extension of the sinus venosus.

3.2.8. Mitochondrial complex II inhibitions

Both succinate-dehydrogenase fungicides (isopyrazam, penflufen) and the beta-ketonitrile derivative insecticide (cyenopyrafen) acting upon complex II resulted in a ZEDTA classification which matched their ECHA C&L classifications. Isopyrazam (TP) at 0.5μM (external medium concentration) caused malformations of the jaw, fin, heart, facial and neural structures. Cyenopyrafen and penflufen showed no developmental effects in the assay (TN), however penflufen was lethal at just 5μM also a very high uptake into the embryo-larvae (890 % of the external exposure concentration). Cyenopyrafen also showed high embryo-larval uptake.

3.2.9. Sodium channel modulators (pyrethroids)

The two pyrethroids, both sodium channel modulators (cypermethrin and deltamethrin) of which both were considered non-developmentally toxic in mammals were classified as FPs in the ZEDTA. The various malformations were observed included the jaw, neural, facial structures, body shape, with the lowest LOAEL of 1 μm (external medium) shown in the case of cypermethrin. Uptake for deltamethrin was determined at over 100 % (2100 %) of the external media concentration. The applied method for LC-MSMS analysis prevented the determination of the level of cypermethrin uptake into the embryo-larvae.

3.2.10. Inhibitors of mitochondrial complex III

Metominostrobin showed high embryo-larval uptake and was classified as negative (TN) in the ZEDTA, matching the in vivo mammalian hazard classification. Dimoxystrobin, however, was classified as FN, causing head and tail abnormalities as well as lethality at external concentrations as low as 0.5μM

3.2.11. Inhibitors of cellulose synthesis

For the two cell wall biosynthesis inhibitors tested, both previously classified according to ECHA as non-developmentally toxic, one isoxaben was classified as a TN (exposed to a nominal external medium concentration of 10μM the published fish LC_{50} value is >1 mg/ml) and the other chlorthiamid was found to be teratogenic (FP), inducing jaw malformation at an external media concentration of 100μM, with reduced hatching success also seen at 200μM.

3.2.12. Glutamate-gated chloride channel (GluCl) allosteric modulators

Of the two chloride channel modulators were tested in the ZEDTA, abamectin (TP) was found to reduce hatching at 1μM as well as cause malformations in the heart, body shape, jaw, neural, facial, heart and fin structures, and emamectin, was classified as a FP inducing malformations of the jaw, heart and neural structures at external concentrations as low as 1μM. Both these chloride channel modulators had good to high-level uptake in the zebrafish embryo. The chemical stability was low for emamectin and not determined for abamectin.

3.2.13. Dihydroorotate dehydrogenase [DHOD] (novel mode of action for fungicides and pharmaceuticals)

Here, both of the DHOD pharmaceuticals tested were classified as TPs consistent with the ECHA C&L classification, with brequinar exposure resulting in malformation of the jaw structure at just 1μM (external concentration), and exposure to leflunomide similarly resulting in jaw malformation, and abnormalities in other structures, at 1μM (external concentration).

3.2.14. Very long chain fatty acid (VLCFA) inhibitors

Both tested VLCFA inhibitors (pyroxasulfone and dimethachlor) were classified as TNs in accordance with the ECHA C&L classifications.

3.2.15. Inhibitors of chitin biosynthesis, type 0 (benzoylureas)

The two tested benzoylureas (lufenuron and flufenoxuron) were classified as non-teratogenic in accordance with their ECHA C&L classifications, showing no evidence of morphological abnormalities up to maximum external media concentration of 100μM, despite very high levels of uptake (>2800 % and >6000 % of the external media concentration, respectively).

3.2.16. Protoporphyrinogen oxidase inhibitors [PPO] (N-phenylphthalimides)

Of the two PPOs tested, flumioxazin was classified as a TP in accordance with the ECHA C&L classification, with malformation of the jaw, heart and neural structures and a LOAEL of 10μM (external medium concentration). Interestingly, these animals also exhibited a clear reduction in the number of circulating erythrocytes and an impact on heme production at an external concentration of only 1μM, (does not contribute to the TI). This specific effect has also been reported for another PPO inhibitor butafenacil [19]. Flumiclorac-pentyl caused disruption to the jaw, neural tube, cranial-facial structure, fins and heart at an exposure medium concentration of only 5μM. The absence of ECHA C&L Inventory Harmonised or Self Classification for Flumiclorac-pentyl meant that no final classification has been determined.

3.2.17. Microtubule inhibitors

For the two carbamate microtubule inhibitors tested, the findings in the ZEDTA did not concur with their ECHA C&L developmental toxicity classifications. Chlorpropham was found to be a FP with a LOAEL of 50μM (external medium concentration) inducing malformations in multiple tissues, whereas carbetamide, conversely, was a FN. The standard LC-MSMS analysis for chlorpropham did not detect this compound to allow for assessments on its uptake or stability. For carbetamide this was present in the embryo at 18 % (of the nominal external concentration) and was also stable in solution at 83 % of the external dosing concentration during the assessment.

3.2.18. Chordotonal organ disruptors (nicotinamidase inhibitors and TRPV channel modulators)

Flonicamid exposure resulted in no lethality or morphological impact in the ZEDTA, and was thus classified as a negative. However, on a cautionary note and, rather oddly, the compound was present at 5μM in the embryo-larvae across the entire external dose range investigated (1, 10 and 100μM), representing only 5 % uptake up to and including highest test concentration. Conversely, pymetrozine, where the embryo-larval body content was 11 % of the external medium concentration, was classified as a false negative conversely with its ECHA C&L developmental toxicity classification.

3.2.19. Photosynthesis inhibitors

Cyanamide, the only photosynthesis inhibitor tested, was found to be negative in the ZEDTA, however, bioanalysis was not possible (cyanamide was not compatible with our generic LC-MSMS method) and as such tissue exposure could not be confirmed. When the external exposure concentration was increased to 1000μM there was still no effect on viability or developmental morphology. Cyanamide was thus classified as a false negative against the ECHA C&L developmental toxicity classification.

3.2.20. GABA-gated chloride channel antagonists (phenylpyrazole)

Fipronil a phenylpyrazole class of GABA-gated chloride channel antagonists, was tested. This was classified as a FP.

3.3. Summary of ZEDTA assay performance

Overall, comparing the ZEDTA assay results with the ECHA C&L developmental toxicity classifications (or alterntive soruces of data when where not unavailable in the ECHA C&L developmental toxicity classifications), the ZEDTA identified 20 teratogen positives out of 31 compounds in accordance with their developmental toxicity hazard classifications in the ECHA C&L developmental toxicitu classifications, giving a sensitivity value of 65 % and a PPV of 63 %. The ZEDTA also identified 21 non-teratogens out of 33 as classified as non-teratogens classified by the ECHA C&L developmental toxicity classifcations giving a specificity value of 64 % and a NPV of 66 % (3 of the 67 compounds were excluded from classification). An overall BAC value of 64 % was generated from this study set of compounds.

Assessing the translational abilty between zebrafish and mammalian assay outcome based upon compound mechanisms of action (Fig. 1), the ZEDTA appeared more predictive for representatives of the C14 demethylases (triazoles and triazolinthione), ACCase inhibitors, nAChR modulators and PSII herbicides [78 % senstivity and 69 % specificity, BAC 67 %], and less predictive with respect to other categories such as the sodium channel modulators (pyrethroids) and HPPD inhibitor. Caution should be applied here, however as the number of compounds tested for a given mechanisms of action differed in turn likely affecting conclusions for the latter chemical categories, where the numbers of chemical in these groups was low.

4. Discussion

A major challenge when assessing the translational power of NAMs, especially those attempting to quantify complex and multifactorial effects, as in the case for prenatal developmental toxicity, is ensuring confidence in their predictive capability. Furthermore, making judgement on when the weight of evidence available is sufficient to classify a compound as having an 'adverse developmental effect' can be somewhat subjective because it needs to be determined in relation to the toxicity observed in the dam. Coupled with the complexities surrounding the issue of the nature of dose-response curves, interpretations on adverse effect concentrations between different bioassays, and different routes of chemical administration applied, this makes harmonising effects analyses between studies difficult. It is also the case that studies assessing multiple chemical classes and multiple representatives of each class are also few and far between. Daston *et al.*, (2014) proposed a list of 20 chemicals for use as reference compounds based upon their known developmental effects in mammals, including humans [20], however, these chemicals were largely drawn from the pharmaceutical or environmental chemical sectors, and covered a limited range of mechanisms of toxic action and chemical properties. Deriving such a list for the agrochemical sector is particularly challenging as there is limited information on the effects of these chemicals in appropriate models, and/or measurement of impact on human exposure. Here we used the ECHA C&L classification scheme (https://echa.europa.eu/home) and publicly accessible data to determine our test set of developmentally and non-developmentally toxic agrochemicals for assessment of the ability of the ZEDTA to detect for developmental hazard in mammals. We also sought to ensure compound coverage across as wide a range of agrochemical mechanisms of action as possible to assess the relative strengths and weaknesses of the ZEDTA assay based on biological and chemical features. Multiple representative compounds across several important groups of agrochemical mechanisms of action were included wherever possible to help ensure firm conclusions regarding the predictive value of the ZEDTA assay for those compounds could be drawn. The use of ECHA C&L classification is based on the hazard properties of a compound, used for classification and labelling within Europe. Utilizing the C&L inventory for mammalian prenatal developmental toxicity classifications where available is conservative, as suspected developmental toxicants (category 2 – H361) which contain only some evidence in experimental animal models are classed as positive. In addition, the ECHA C&L classification system does not always distinguish between compounds impacting fertility or development and a general H360/H361 may be applied.

4.1. Overall performance of the ZEDTA assay for detecting developmental effects reported for mammalian models

Assessing the zebrafish-based classification (ZEDTA) with the reported developmental NOAELs from standard (regulatory) mammalian species (Table 2 and supplemental table 5) showed ZEDTA had a greater sensitivity, but lower (differing) specificity compared to that identified for studies in rats and rabbits. This might suggest that the ZEDTA has a greater capacity for identifying and detecting positives for developmental effects in mammals, but also potentially for more false positives. An additional NAM with a higher specificity (but low sensitivity) could be applied as a follow on assessment to the ZEDTA to identify the false positives; for example the rat Whole Embryo Culture (WEC) [21]. The high value of the NPV (66 %) of the ZEDTA also indicates a high probability for predicting of negative teratogenic compounds for mammalian species. Overall, these data suggest that the ZEDTA, which offers considerably greater throughput and far lower compound requirement, can provide an effective initial vertebrate NAM bioassay for compound screening prior to a higher tier mammalian tests, in turn offering considerable value in reducing mammal use in the human risk assessment of new agrochemical products.

4.2. Performance of ZEDTA assay for each mechanism of action tested

4.2.1. C14-demethylases (triazoles and triazolinthione)

For the C14-demethylase inhibitors 80 % were identified as positives using the ZEDTA although the 3 reportedly non-teratogenic compounds in mammals were classified as FPs. These fungicides act by targeting the enzyme lanosterol C14-demethylase, but they can also bind to non-target cytochrome P450 enzymes critical in drug metabolism and reproduction [22]. As a critical step in the biosynthesis pathways of sterols, including cholesterol, interference in their activity impacts multiple bioactive molecules in mammals including steroid hormones [23] and it has been suggested that its mechanism for teratogenic effect centres on the inhibition of embryonic retinoic acid degradation via CYP26 [24]. A number of these compounds have been associated with embryotoxicity and/or some craniofacial abnormality, although these effects are often seen at maternally toxic doses and tend to occur at a relatively low incidence [25–36]. In mammals, for example, penconazole has been demonstrated to induce cervical ribs in rats and microphthalmia in the rabbit foetus, propiconazole caused skeletal variations and cleft palate in rat foetus while epoxiconazole caused the formation of cleft palates in the rat studies [26,28,32] below maternal toxic concentration. Here we observed multiple developmental malformations in zebrafish embryo-larvae including jaw and fin defects alongside body length effects all of which could be indicative of an impact on development processes. These morphological impacts on development of the embryo-larval zebrafish have also been demonstrated previously for exposure to triazole compounds [37,38]

Two of the three C14-demethylase compounds classified as FPs in the ZEDTA, prothioconazole and hexaconazole have both been classed as developmentally toxic in mammals but only at maternally toxic concentrations [39,40]. Here, internal body concentrations were 4 times higher than the external concentrations after just 24 hours of exposure, and as such the effects observed could indicate exposure that approaches levels that would be lethal in older animals. Indeed, the reported fish acute LD_{50} values for hexaconazole and prothiconazole are 19μM and

5μM, respectively, which are at or below the internal concentrations reported for teratogenic treatment levels here (e.g. internal LOAELs concentrations of 50 μM [extrapolated] and 4μM, respectively).

Two triazoles, namely ipconazole and flutriafol, were classified as FNs in the ZEDTA assay. Flutriafol is considered positive as a teratogen in mammals as it is reported to delay ossification in pups. Bone ossification in the larval zebrafish at 5dpf, however, is extremely limited and visible only through specific staining methods [41] and as such any changes in bone mineralisation are unlikely to be a detectable endpoint in 5dpf zebrafish.

In mammalian test models also the compound has to undergo maternal transfer via a placental membrane to reach the developing embryo. The latter transport barrier might explain the differences seen in the translational ability for cypermethrin (FP) and propiconazole (TP) where the placental foeto/maternal diffusion ratio is 0.5 for cypermethrin but higher (0.7) for propiconazole (TP) i.e. this lower diffusion rate of cypermethrin in mammals might be sufficient to protect the foetus from a teratogenic effects in mammals [42].

4.2.2. *Inhibitors of acetyl CoA carboxylase*

In eukaryotes Acetyl-CoA carboxylases (ACCs) are large multi-domain enzymes, which are inhibited by classes of inhibitors including the FOPs (Aryloxyphenoxypropionates) and DIMs (Cyclohexanediones) herbicides, and tetronic and tetramic acid derivative (TA) insecticides [43]. Most of the ACCase inhibitors tested were considered to be non-developmentally toxic in mammals according to the ECHA C&L system and of these 6/7 were classified as such in the ZEDTA. Only one of these compounds showed low uptake (clethodim) but this was still sufficient to achieve tissue exposure effect level. Interestingly, the data obtained here for clethodim differ to those reported previously in zebrafish. Neurological developmental effects of clethodim have been reported at a LOAEL of 27μM which coincides with our concentration range [44]. However, in that study exposure was initiated at 2hpf rather than 6hpf (as in our study) which could offer an explanation for the differing results obtained. For spiromesifen an exposure at 10μM, we observed unusual uncontrolled spontaneous movements in embryo-larvae at 4dpf (before the exposure became lethal) which has also a behavioural phenotype reported (spontaneous and increased movements) in rats exposed to spiromesifen [45].

One non-teratogenic ACCase inhibitor, Fenoxaprop-P-ethyl, was classified as a positive (FP) in the ZEDTA as it caused multiple morphological abnormalities at an exposure of 2.5μM (4μM internal concentration). This compound is not classified for development toxicity by ECHA, however developmental effects were observed at maternally toxic dose levels [46]. Given the relatively high uptake observed compared to the nominal exposure concentration in zebrafish embryo-larvae, this may be a case where tissue exposure is elevated to a level that might equate with a maternally toxic level in mammals.

In contrast with the high predictivity rate for non-developmentally toxic ACCases, only one compound considered to be developmentally toxic in mammals, quizalofop-P-tefuryl, was identified as such in the ZEDTA, where it had effects on the jaw and notochord development at 1.25μM, and even though it was a relatively unstable compound in the medium. Quizalofop-p-terfuryl and quizalofop-p-ethyl are both rapidly broken down by hydrolysis to quizalofop acid and tetrahydrofurfuryl alcohol, but the metabolites are considered to be less toxic than the parent compound [47]. Here, exposure to quizalofop-P-tefuryl resulted in particularly notable defects in the jaw and notochord. In rat, this compound has been reported to induce curved tail, cleft pallet and abdominal wall defects at maternally toxic concentrations with indications of reproductive impacts (testicular) in a 2-generation study [48]. It is thought that this effect is mediated through an interaction with peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptor proteins that are relatively well conserved in zebrafish [49]. Tepraloxydim and fluazifop-P-butyl were both classified as FNs in the ZEDTA, differing to their classification in mammals, but both these compounds showed moderate level of uptake. Interestingly, both of these compounds did not show developmental effects when assessed in rabbits but did so in rats [50]. This may indicate differential species sensitivity, in turn highlighting the value of multispecies assessment and

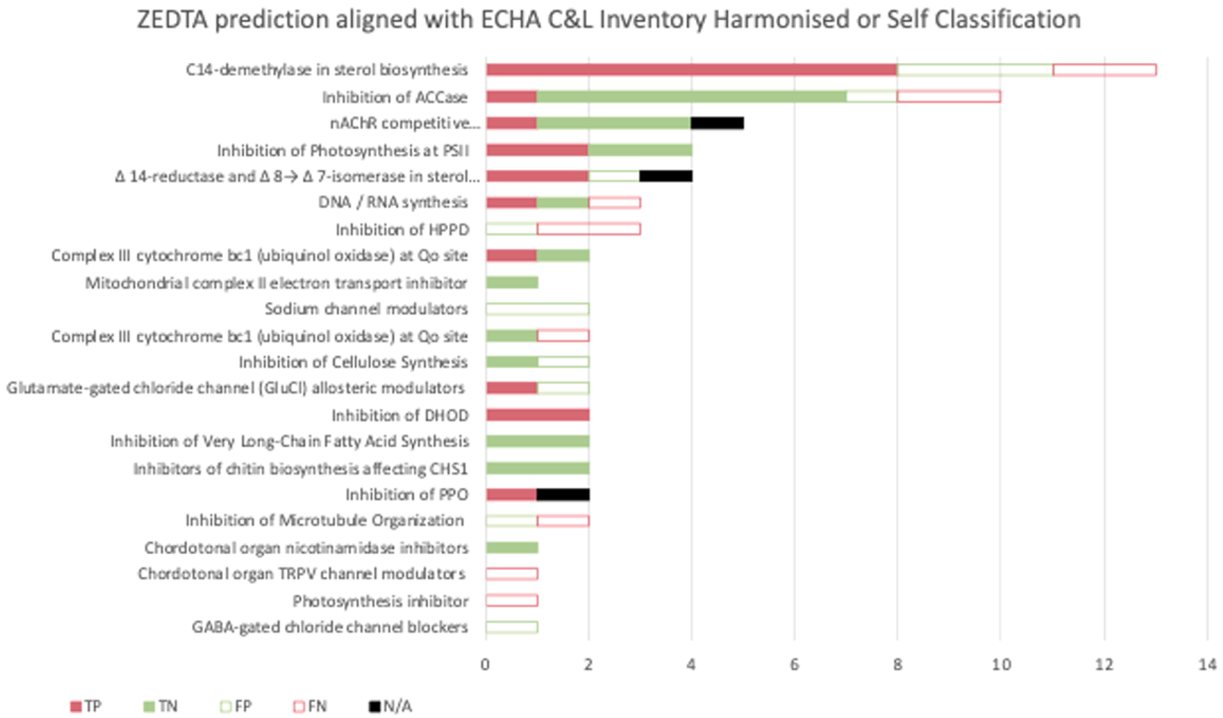

Fig. 1. Comparison of ZEDTA classifications against those for the ECHA C&L developmental toxicity classification grouped by compound mechanism of action. X-axis shows the total compound number for each class. Red solid: True positive (TP), Green solid: True negative (TN); Red open: False positive (FP), Green open: False negative (FN). Black: Not applicable between the ZEDTA classificational and the ECHA C&L developmental toxicity classifications.

a weight of evidence-based approach in DART studies for human risk assessment.

4.2.3. nAChR modulators

All of the nAChR allosteric and competitive modulators tested in the ZEDTA were classified in accordance with the ECHA DART classification system. The only TP the neonicotinoid thiacloprid impacts fertility in rats, and this effect is believed to occur through altering ovarian aromatase levels in pregnant females in addition to increases in pelvic dilation and skeletal variations in rat developmental toxicity studies, in the absence of maternal toxicity [51,52]. Zebrafish are sensitive to oestrogen from early development (1dpf) and exposure to exogenous oestrogen and/or alterations in the oestrogen biosynthesis can induce gene expression alteration that can impact multiple development targets including the retinoic acid receptor [53].

All other nAChR insecticides successfully tested were identified as TNs in the ZEDTA in full accordance with the ECHA DART data classification for mammals. It should be emphasised however, that clothianid and imdicloprid showed relatively low compound uptake at 7 % and 13 % of the external medium exposure concentrations, respectively.

4.2.4. PSII (nitriles/ureas)

All four PSII herbicides were classified in the ZEDTA assay (2 TPs and 2TNs), in accordance with that indentified in the ECHA C&L developmental toxicity classification. Of the two TPs, in mammalian studies, linuron induces multiple impacts on male reproductive tissues and has an anti-androgenic mechanism, while bromoxynil potentially has a mode of action of thyroid toxicity impacting on weight gain and delayed eye opening and has various skeletal impacts along with hydrocephalus, enophthalmia and, micropththalmia [54,55]. Multiple morphological abnormalities were observed after exposure to both of these compounds in the ZEDTA although no corresponding impacts on eye development were noted in the case of bromoxynil. The ZEDTA assay was able to differentiate between the parent compound bromofenoxim and the active metabolite bromoxynil.

4.2.5. Sterol biosynthesis inhibitors

Of the sterol biosynthesis inhibitors were classified, fenpropimorph and spiroxamine were identified according to the ECHA C&L developmental toxicity classification as TPs, whereas fenpropidin was classified as a FP, differing to that for the ECHA DART classification. In mammals, treatment with spiroxamine and fenpropimorph are associated with craniofacial malformations, specifically cleft palate, at doses also inducing maternal toxicity [56,57]. Notably, exposure of zebrafish embryo-larvae to fenpropimorph resulted in a clear impact on jaw development (and neural tube) at $\geq$ 12.5 μM, a result which concurs with previous data in zebrafish where jaw defects have also been reported for exposure to 27–38μM fenpropimorph [58]. Fenpropidin, found to be a FP in the ZEDTA showed high uptake (uptake 8x higher than the external concentration) resulting in an internal concentration which broadly coincides with the recorded LOAEL of 90 mg/kg in in rats [59].

Table 2
Predictivity values and BAC score of each species against the ECHA classification based on the published NOAEL concentrations for developmental toxicity in both rat and rabbit (see supplemental table 5 for details and references).

		Rat	Rabbit	ZEDTA
Sensitivity	TP/(TP+FN)	43 %	18 %	65 %
Specificity	TN/(TN+FP)	89 %	93 %	64 %
Exclusion rate	% not included	15 %	18 %	4 %
% Correct (excluding non-returned)		**65 %**	**55 %**	**64 %**
PPV	TP/ TP+FP	81 %	71 %	63 %
NPV	TN/TN+FN	59 %	52 %	66 %
BAC	PPV+NPV/2	70 %	62 %	64 %

4.2.6. Complex II inhibitors

Full concordance was observed between the ZEDTA results and the ECHA C&L developmental toxicity for the mitochondrial complex II inhibitors.

4.2.7. Chemical classes where less than 3 representative compounds were tested

As stated above, it is more difficult to assess the predictive value of the ZEDTA against mammalian DART data for compounds where the numbers of compounds tested for a given group was low. Nonetheless, amongst these diverse chemical classes, a number of noteworthy observations were made.

Notably, the following groups showed full concordance between the ZEDTA outcome and the ECHA C&L developmental toxicity classification: all three DNA/RNA synthesis inhibitors namely Quinolin-8-ol (8-Quinolinol), as a TP and both hymexazol and Octhilinone (2-octyl-isothiazol-3-one) as TNs; both pharmaceutical DHOD compounds as TPs (leflunomide and brequinar [the same mode of action as leflunomide]); both VLCFA inhibitors (dimethachlor and pyroxasulfone) as TNs; and both benzoylureas chitin biosynthesis inhibitors, namely lufenuron and flufenoxuron, as TNs. Other chemical classes varied more. Members of the HPPD class, for example, all classified differently in the ZEDTA compared to the ECHA C&L developmental toxicity classification. However, for one of these compounds (nitisinone) there have been isolated reports of developmental defects in mammals at high exposure doses [60,61], casting doubt in the ECHA C&L developmental classification. Uncertainty around the definitive classification of dimoxystrobin as a developmental toxicant has also been raised [62]. These cases further highlight the difficulty faced when selecting test compounds for use as part of an inter- assay validation exercise.

Interspecies (in)sensitivity is also an important factor when comparing translation between two animal models. Beaudegnies *et al.,* (2009), for example reported that certain HPPD inhibitors exhibit large differences in binding strength to HPPD in different species [63] and this could be the case for seeing differing responses between the zebrafish and mammals. An additional complication here is that the zebrafish possess two paralogous gene orthologues for *hppd*. Expression of these orthologues (*hpda* and *hpdb*) are detected from 10hpf (*hpdb*) and 24hpf (*hpda*) up to at least 72hfp with *hpda* is expressed predominantly in the liver (at 5dpf) while *hpdb* is expressed predominantly in the fin tip epidermis. The primary site of *Hppd* activity is in the liver of mammals. One hypothesis that might explain the results seen might be that bicyclopyrone and mesotrione may interact with *hpda* in the liver but as the build-up of tyrosine metabolites takes time to cause impact on development and the full metabolic activity of the liver in the larval zebrafish is not realised by 5dpf. Conversely nitisinone may have a higher affinity/preference for the *hpdb* ortholog in the zebrafish leading to the altered development of the jaw and fin epidermis (by 5dpf) as seen in the ZEDTA responses. The differing preference for the orthologue may relate to the differences in binding affinity of the compound to the HPPD enzyme or for the specific orthologue [64–68]. A higher sensitivity of fish to certain MOAs has also been highlighted. Published data on the effects of pyrethroids in the zebrafish has indicated they may be particularly sensitive to the development effects of these compounds. Fipronil, for example, has been reported to cause malformations in the notochord and muscle fibre disorganisation at concentrations as low as $\geq$ 0.23μM in zebrafish [69]. It has been suggested that this sensitivity is due to pyrethroids acting as GlyR antagonists in fish, rather than their primary mechanism of action as GABA antagonists [69] and this secondary pharmacology is perhaps more likely when tissue exposures are especially high, as was the case here.

As discussed previously, at 5dpf, there is relatively little bone ossification in zebrafish. This being the case, where the driving force behind a teratogenic classification is associated with the impairment of skeletal development, the ZEDTA may not be the most suitable model for such assessment. Examples include for the microtubule inhibitor carbetamide

(FN here), which has been reported to affect ossification in rabbits and rats [70,71] as is also the case for two aforementioned triazoles, ipconazole and flutriafol. Another major factor likely has an influence of the translational power is in the dynamics and bioavailability of test compounds to the embryo-larvae in the ZEDTA that are exposed via the water versus that dosed via the diet in mammals. Although internal uptake measurements were undertaken for the ZEDTA studies – these measures are for the whole organism taken at very early during development and do not provide any tissue specific data. The tissue exposure factor clearly complicates ZEDTA versus the ECHA C&L developmental toxicity classification cross species effect concentration/dose comparison and is very likely a reason for some of the differences in classification of compounds seen here. The use of automated microinjection systems or carrier molecules to provide precise *in ovo* /embryo-larval chemical delivery might help to align dosing levels in the ZEDTA with those against mammals [72].

5. Conclusions

The ZEDTA is used currently as a screen for pharmaceuticals and other bulk chemicals prior to their assessment with regulated animal models [18,73,74], a range of different NAMs have been proposed and reviewed for determination of risk assessment of agrochemical compounds [75,76]. Here through an assessment of 67 agrochemicals with a wide range of chemical structures, a variety of modes of action, and product uses, overall, we found good concurrence with the ECHA C&L developmental toxicity classification (or alternative) hazard classifications for prenatal developmental toxicity. This shows the ZEDTA can provide a strong supporting guidance when making decisions for to taking an agrochemical forward for development. Ultimately, under current guidelines, regulatory testing will be required for safety assessment, but the ZEDTA can be used to screen out agrochemicals more efficiently at an early stage that have clear developmental toxicity.

The ZEDTA's predictive power varied according to the primary mechanism of action of the agrochemical tested. Six chemical groupings in particular showed full (100 %) predictivity, although caution is applied here as in many of these cases, a relatively small number of compounds were tested. Where the numbers of compounds tested for a given group was greater than 4, there was a BAC score was 74 %). In contrast, for some groups with other mechanism of action groups there was poor translation. For several groups (HPPD inhibitors, microtubule inhibitors and photosynthesis inhibitors) none of the compounds classified according to the ECHA C&L hazard classifications for prenatal developmental toxicity in the ZEDTA. These differences between the bioassay results likely include differences in the amount of the compounds reaching the given target organ, given the different exposure routes. Adding further to this, better understanding on the differences in transport efficiently and compound form (i.e. parent verses metabolites) between maternal and foetal systems will both better support which compounds the ZEDTA can most effectively be applied and for which ones alternative NAMs may be required.

CRediT authorship contribution statement

Tyler Charles R: Writing – review & editing. **Wolton Kathryn:** Conceptualization, Project administration, Writing – review & editing. **Ball Jonathan:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Tochwin Anna:** Investigation. **Winter Matthew J:** Writing – review & editing. **Trznadel Maciej:** Investigation. **Currie Richard:** Writing – review & editing, Funding acquisition. **French Julian M:** Writing – review & editing. **Hetheridge Malcolm J:** Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jonathan Ball reports financial support, equipment, drugs, or supplies, and writing assistance were provided by Syngenta Jealott's Hill International Research Centre. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.reprotox.2025.108837.

Data availability

Data will be made available on request.

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