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Adverse effects in the fish embryo acute toxicity (FET) test: a catalogue of unspecific morphological changes versus more specific effects in zebrafish (*Danio rerio*) embryos

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Abstract

Background: The Fish Embryo Acute Toxicity (FET) test with the zebrafish (*Danio rerio*) embryo, the OECD test guideline (TG) 236, has been designed as an alternative for acute fish toxicity testing such as the OECD Acute Fish Toxicity Test (TG 203). To provide equivalent sensitivity to the acute fish test, the original FET test was designed to use only four morphological core endpoints: coagulation of the embryo, lack of somite formation, lack of heart beat, and non-detachment of the tail. These endpoints were selected due to (1) their association with mortality, directly or indirectly, (2) improve the practicality for screening by well-trained technical staff, and (3) the endpoints being relatively simple morphological alterations.

Results: With the growing need to understand the developmental toxicity of compounds found in the environment, the FET protocol has repeatedly been extended to a multitude of additional morphological endpoints that also allow the monitoring of teratogenicity. As the extensive use of the FET test has generated a multitude of observations in the scientific literature, a harmonisation of the terminology used for the description of the morphological effects seen after chemical exposure has become necessary.

Conclusion: For this end, the present communication provides an overview of both common and selected more specific morphological effects seen in zebrafish embryos after exposure to a wide variety of chemical substances together with suggestions for a harmonised nomenclature.

Keywords: Zebrafish, OECD TG 236, Embryo toxicity, FET test, Teratogenicity, Spinal defects, Lordosis, Kyphosis, Scoliosis, Craniofacial deformation, Yolk malabsorption

Background

Increasing amounts of anthropogenic compounds entering the environment have led to the need for reliable and accurate acute toxicity tests [1]. Most regulatory-accepted models for environmental hazard identification and risk assessment of chemicals, pharmaceuticals,

biocides, additives, and effluents are based on testing with vertebrate models such as rodents and fish [2]. According to the European Chemicals Agency [3], the Acute Fish Toxicity (AFT) test (OECD Test Guideline 203; [4]) is used for the prospective assessment of individual chemicals for environmental classification according to the Globally Harmonised System of Classification, Labeling and Packaging of Chemicals (GHS), a Predicted No-Effect-Concentration (PNEC), and one potential element of the toxicity criterion for the assessment of Persistence, Bioaccumulation and Toxicity (PBT) assessment [5]. In

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an attempt to improve the assessment of the status of European waters under the Water Framework Directive [6], fish are also utilised for the monitoring of the quality of effluents and surface waters [7]. As a consequence, the AFT test must be conducted in accordance with, for example, OECD Test Guideline 203 [4] or similar guidelines [8, 9] and is, by far, the most frequently used vertebrate test for aquatic toxicity assessment.

The frequent use of the AFT test has given rise to both economic and ethical concerns since the early 1980s [10], as the existing data are frequently of poor quality [11], and newer alternative assays are deemed more applicable to long-term hazard identification and risk assessment in an environmental context [12, 13]. With the implementation of the European chemical policy REACH (Registration, Evaluation, Authorization and Restriction of Chemicals [14]) and the European Cosmetics Directive [15], there is a clear mandate to strongly promote the development of alternative methods according to the 3Rs principle for “Replacement, Reduction, and Refinement” [16] and to preferentially use data generated by alternative methods whenever validated methods are available [14]. All these considerations are calling for the replacement of acute toxicity and teratogenicity testing traditionally founded on animal models by adverse outcome pathway (AOP)-based biomarker studies [17, 18] and in vitro models [19] along with more 3R-compatible in vivo models [13, 20]. Most importantly, alternative in vitro assays and in vivo protocols using, e.g., fish embryos are also amenable to high-throughput testing [21–24].

Whereas various European countries as well as Canada and the USA continue requiring conventional AFT testing for whole effluent testing [9], Germany replaced AFT testing in 2003 by a standardised 48 h test with zebrafish (*Danio rerio*) embryos [25], and UK also discontinued AFT testing for animal welfare reasons [26]. In an attempt to comply with upcoming EU regulations, the German Environment Agency submitted a draft zebrafish embryo toxicity (FET) test to the OECD Test Guidelines Programme [27]. In July 2013, the FET test was adopted by OECD as Test Guideline 236 [28]. Although ECHA so far refuses to accept the FET test as a stand-alone alternative to OECD TG 203 for regulatory purposes [29], the FET test has found wide acceptance in science and even proved to have a higher sensitivity and better correspondence to humans than other whole organism models [30–32].

Thus, since its implementation, a considerable database on morphological changes in zebrafish embryos following exposure to chemical contaminants has been built [2, 33]. However, there is a striking variability with regard to the endpoints assessed, and many authors list an ill-defined set of observations, but neglect other changes,

which could be expected. Even worse, there is a significant inconsistency in the nomenclature used, making it difficult to compare the results presented in the literature. Lordosis, for example, is a term frequently used for all types of back curvatures and deformations, whilst these should be divided into lordosis (hyperextension of the back), kyphosis (hunchback) and scoliosis (sideways curvature). Whereas some publications correctly identify lordosis as a hyperextension of the back [34–37], other communications use this term to describe what was actually scoliosis [38]. One paper correctly determined lordosis, but scoliosis was annotated as “spinal column flexure (tail defect)”, and alterations noted as kyphosis rather appear as a break in the spinal column than a curvature [39].

To contribute to harmonisation of observations and terminology, the present study provides a catalogue of both more frequently found and less common morphological alterations in zebrafish embryos after exposure to chemicals. To cover a multitude of modes-of-action and different effects, a broad array of 48 chemicals was tested. The resulting catalogue aims to improve the future analyses of observations in FET experiments as well as providing reference for the correct identification of normal versus altered development of the zebrafish embryo.

Methods

Chemicals and materials

All compounds tested were purchased at a minimum purity grade of 98%. Paraquat, mercury (II) chloride, carbaryl, colchicine, rifampicin, clofibrate, sulfisoxazole, and taxol were obtained by Carbosynth (Carbosynth, Compton, UK), rotenone, tebuconazole, and ibuprofen were obtained from TCI (Eschborn, Germany), 4-enevalproic acid was obtained from Santa Cruz Biotechnology (Dallas, USA), acrylamide, carbamazepine, copper (II) sulphate, dibutyl maleate, 3,4-dichloroaniline, 2,2-dimethylvaleric acid, dinitro-*o*-cresol, 2,4-dinitrophenol, ethanol, 2-ethylbutyric acid, 2-ethylhexanoic acid, hexachlorophene, hexanoic acid, luviquat, malathion, merquat, 2-methylhexanoic acid, 6-methyl-5-heptene-2-one, 2-methylpentanoic acid, 1-methyl-4-phenyl-pyridinium iodide (MPP+), 1-octanol, paracetamol, PCB 180, 4-pentenoic acid, prochloraz, 2-propylheptanoic acid, sodium chloride, sodium tetradecyl sulphate (STS), tolbutamide, triclosan, triethylene glycol, 2,3,6-trimethyl phenol, triphenylphosphate, valproic acid, and zinc pyrithione were purchased from Sigma-Aldrich (Deisenhofen, Germany), and dimethyl sulfoxide (DMSO) was ordered from Honeywell International (Offenbach, Germany). Detailed information on test compounds can be found in Additional file 1: Tables S1 and S2.

All test solutions were freshly prepared prior to use in standardised dilution water (OECD TG 203, [28]); in cases of limited water solubility, the solutions were prepared in 0.1% DMSO. Carbaryl had to be dissolved in 0.5% DMSO, and triclosan was prepared in 0.1% ethanol (Additional file 1: Table S3). MPP⁺ iodide, PCB 180, rifampicin, sulfisoxazole, and taxol were dissolved in 1% DMSO concentrations. For compounds requiring solvents, a solvent control was also added to the exposure regime at the equivalent concentration required by the compound. The stock solutions of carbamazepine and sodium tetradecyl sulphate were heated up to 90 °C and 60 °C, respectively, to dissolve. The stock solutions of 2,4-dinitrophenol, dinitro-*o*-cresol, copper (II) sulphate pentahydrate, paracetamol, paraquat, and sodium tetradecyl sulphate were stored in the dark (Additional file 1: Table S3). The stock solution for colchicine had to be prepared freshly every day, as the compound is highly reactive. Mercury (II) chloride, carbamazepine, copper (II) sulphate, dibutyl maleate, 3,4-dichloroaniline, dinitro-*o*-cresol, 2,4-dinitrophenol, ethanol, luviquat, malathion, merquat, 6-methyl-5-heptene-2-one, 1-octanol, prochloraz, sodium chloride, STS, triclosan, triethylene glycol, 2,3,6-trimethyl phenol, and DMSO were tested as part of the OECD TG 236 validation phases, with chemical analytics of water samples having been conducted [40–42].

Fish

Adult wild-type zebrafish of the ‘Westaquarium’ strain were obtained from breeding facilities at the Aquatic Ecology and Toxicology Group at the Centre for Organismal Studies (University of Heidelberg; licenced under no. 35-9185.64/BH). Fish maintenance, breeding conditions, and egg production were described in detail elsewhere [1] and are in accordance with internationally accepted standards.

Fish embryo acute toxicity (FET) tests (OECD TG 236)

The acute toxicity of the test substances was determined according to OECD TG 236 [43]. In brief, freshly laid eggs [<1 h post-fertilisation (hpf)] were transferred to 50 ml crystallising dishes filled with the respective test solutions. After control of fertilisation success, eggs were individually transferred to 24-well plates (TPP, Trasadingen, Switzerland) with 2 ml of test solution per well. All test vessels had been pre-incubated (saturated) with the test solutions for at least 24 h. Subsequently, well plates were sealed with self-adhesive foil (SealPlate™ by EXCEL Scientific, Dunn, Asbach, Germany). Well plates were placed in an incubator at 26.0 ± 1.0 °C under a 10/14-h dark/light regime. The test medium was renewed each day (semi-static exposure), and all developmental alterations of the embryos were documented at 24, 48, 72, and

96 hpf and in some cases at 120 and 144 hpf (according to OECD TG 236 [43] and Nagel [44], respectively). FET tests with a minimum mortality rate of 30% in the positive control (4 mg/L 3,4-dichloroaniline) and a maximum effect rate of 10% in the negative control (dilution water) at 96 hpf were classified as valid.

In addition to the endpoints specified by OECD TG 236, (1) coagulation of fertilised eggs, (2) lack of somite formation, (3) non-detachment of tail bud, and (4) lack of heart beat (OECD, 2013), any other observation was recorded as further lethal or sublethal endpoints. Common examples were reduced heart beat or reduced blood flow, inhibited or missing pigmentation, delayed or altered development, modified movement(s), distortion of the spine, and formation of various types of oedemata. In case of evidence for delayed toxicity, the standard exposure duration of 96 h given in OECD TG 236 (OECD, 2013) was extended to 120 or even 144 hpf for more obvious expression of observations. However, the developmental stage at the end of the experiments never exceeded the limits for unprotected developmental stages set by the current EU animal welfare legislation [45, 46]. FET tests were run in four replicates, for compounds with previously conducted small range-finding studies, two full FET tests were accepted, if these did not vary from the previous findings. The embryos were analysed under an Olympus CKX41 inverted microscope (Olympus, Hamburg, Germany), and images were captured using an Olympus C5040 AUD camera.

Data analysis

To better highlight the area(s) of interest, micrographs were optimised (e.g., adjustment of light intensity) using Photoshop CS5 (Adobe, Munich, Germany). Lethal concentrations (LC) and effect concentrations (EC) were calculated at effect levels of 10 and 50% based on probit analysis using linear maximum-likelihood regression with ToxRat™ (ver. 2.10.03; ToxRat™ Solutions, Alsdorf, Germany), with both lethal and sublethal effects included into the calculation of EC and LC values [13].

Results and discussion

Formal toxicity data derived from fish embryo acute toxicity (FET) tests

The toxicity of the test compounds was determined after 96 h of exposure as both EC and LC at the 10 and 50% levels (Table 1). The five test compounds dissolved in 1% DMSO (MPP⁺ iodide, PCB 180, rifampicin, sulfisoxazole, and taxol) due to poor solubility did not show any effects. Overall, it became evident that where 96 and 120/144 hpf values were provided, the toxicity of the compounds showed only minor variation. Whereas DMSO, for example, was markedly less toxic at 144 than at 96 hpf, other

Table 1 Acute and sublethal toxicity data from the fish embryo tests with zebrafish (*Danio rerio*) as computed by ToxRat™

	n	Toxicity 96 h (mg/L)				Toxicity 120 h (mg/L)/144 h (mg/L) ^a			
		EC ₁₀	EC ₅₀	LC ₁₀	LC ₅₀	EC ₁₀	EC ₅₀	LC ₁₀	LC ₅₀
Acrylamide	4	75.400	94.000	166.600	199.300				
Carbamazepine	3	42.700	81.330	138.760	164.920	<i>53.400</i>	<i>62.600</i>	<i>124.500</i>	<i>147.400</i>
Carbaryl	2	2.200	2.400	6.600	12.200				
Clofibrate	2	200.000	300.000	600.000	1100.000				
Colchicine	4	23.100	32.400	32.500	41.400				
Copper (II) sulphate	3	0.400	0.700	0.600	0.800	<i>0.200</i>	<i>0.600</i>	<i>0.600</i>	<i>0.800</i>
Dibutyl maleate	3	0.400	0.600	0.500	0.700				
3,4-Dichloroaniline	3	1.200	1.900	1.600	2.400				
Dimethyl sulfoxide	3	14,460.000	20,100.000	27,200.000	36,560.000	<i>20,250.000</i>	<i>24,610.000</i>	<i>25,390.000</i>	<i>33,880.000</i>
2,2-Dimethylvaleric acid	2	56.600	64.300	71.700	77.200	54.400	62.500	71.700	77.200
Dinitro-o-cresol	3	0.300	0.400	0.400	0.500	<i>0.300</i>	<i>0.400</i>	<i>0.400</i>	<i>0.500</i>
2,4-Dinitrophenol	3	2.000	2.700	3.400	4.100	1.600	2.400	2.000	2.700
Ethanol	3	5800.00	7840.000	8650.000	11,3000.000				
2-Ethylbutyric acid	3	20.100	47.100	68.500	95.700	32.300	58.300	61.200	83.700
2-Ethylhexanoic acid	3	5.600	20.800	24.200	40.000	7.200	16.400	24.200	38.200
Hexachlorophene	2	0.004	0.005	0.007	0.008				
Hexanoic acid	2	62.500	69.400	75.300	76.100	61.900	67.000	74.600	77.200
Ibuprofen	2	4.700	10.800	31.700	37.300				
Luviquat	3	0.300	0.500	0.400	0.900	<i>0.400</i>	<i>0.600</i>	<i>0.300</i>	<i>0.800</i>
Malathion	3	0.800	1.200	2.800	3.500	<i>0.800</i>	<i>1.100</i>	<i>2.000</i>	<i>2.600</i>
Merquat	3	0.200	0.300	0.300	0.500	<i>0.200</i>	<i>0.300</i>	<i>0.200</i>	<i>0.500</i>
2-Methylhexanoic acid	2	46.500	53.900	75.200	84.100	28.700	42.600	75.300	84.100
6-Methyl-5-heptene-2-one	3	54.500	81.700	76.100	139.700				
Methylmercury (II) chloride	3	0.020	0.030	0.030	0.040	<i>0.0100</i>	<i>0.020</i>	<i>0.030</i>	<i>0.030</i>
2-Methylpentanoic acid	3	35.900	53.500	59.800	77.000	43.200	54.400	52.300	67.800
1-Methyl-4-phenyl-pyridinium iodide	3	No effects observable up to solubility limits (up to 1% final DMSO concentration) ^b							
1-Octanol	3	8.700	12.400	13.700	18.900	<i>10.100</i>	<i>15.100</i>	<i>13.200</i>	<i>18.400</i>
Paracetamol	2	200.000	300.000	1000.000	1200.000				
Paraquat	2	328.700	353.200	933.800	1161.400	384.700	545.900	721.100	855.000
4-Pentenoic acid	2	52.100	60.200	90.800	107.100	52.200	60.100	63.500	103.500
PCB 180	3	No effects observable up to solubility limits (up to 1% final DMSO concentration) ^b							
Prochloraz	3	1.700	3.800	2.300	4.500	<i>1.500</i>	<i>3.200</i>	<i>2.200</i>	<i>4.300</i>
2-Propylheptanoic acid	3	3.400	4.100	10.000	13.500	2.400	3.100	8.900	11.500
Rifampicin	3	No effects observable up to solubility limits (up to 1% final DMSO concentration) ^b							
Rotenone	2	0.004	0.007	0.006	0.010				
Sodium chloride	3	1090.00	3370.000	2860.000	5910.000				
Sodium tetradecyl sulphate	3	0.300	0.300	0.300	0.300	<i>0.200</i>	<i>0.300</i>	<i>0.300</i>	<i>0.300</i>
Sulfisoxazole	3	No effects observable up to solubility limits (up to 1% final DMSO concentration) ^b							
Taxol	3	No effects observable up to solubility limits (up to 1% final DMSO concentration) ^b							
Tebuconazole	2	2.300	5.300	15.000	17.300				
Tolbutamide	2	54.300	116.900	223.200	278.600				
Triclosan	3	0.100	0.200	0.200	0.300				
Triethylene glycol	3	31,220.000	35,700.000	45,220.000	54,220.000	<i>26,400.000</i>	<i>32,300.000</i>	<i>45,000.000</i>	<i>52,200.000</i>
2,3,6-Trimethyl phenol	3	9.800	12.300	10.500	14.100				
Triphenylphosphate	2	0.300	0.500	1.400	1.600				
4-ene-Valproic acid	2	24.700	37.400	41.900	56.100	28.400	33.100	49.800	68.900
Valproic acid	3	3.100	9.100	26.200	56.900	6.200	11.800	33.300	62.700
Zinc pyrithione	3	0.002	0.005	LC > solubility limits					

Data are given as mean ± SD from n independent replicates

^a Data for 144 h in italics

^b For data on solubility limits, see Additional file 1: Table S2

compounds showed more diverse changes: 2-methylhexanoic acid, e.g., did not show any change in acute toxicity between 96 and 120 hpf, and sublethal toxicity was lower at 120 than at 96 hpf. Paraquat, on the other hand, showed less symptoms of sublethal toxicity at 120 than at 96 hpf, whereas its acute toxicity was higher at 96 than at 120 hpf. Although the FET test has very frequently been conducted over the past years, effect and lethal concentrations (EC and LC, respectively) resulting from these tests are only rarely found in the open literature, but have frequently been incorporated into online databases such as EnviroTox (<http://www.EnviroToxdatabase.org>) or ECOTOX (<https://cfpub.epa.gov/ecotox>). In many cases, however, only exposure concentrations, single values, or concentrations of interest are given or represented graphically. Illustrating the point, out of 48 substances of diverse chemical classes and applications examined in the present study, the LC₅₀ value for only 6 of them had previously been published, and only for one compound, the EC₅₀ value was available. This points towards a large gap in the current scientific standing, as these values often build the foundation for many higher tier assays, such as behavioural tests and more specific and mechanistic toxicity assessment with adult animals.

Lethal and sublethal effects in the Fish Embryo Acute Toxicity (FET) tests

Table 2 provides a summary of all observations recorded during the analysis of morphological endpoints. It should be noted that the gastrulation arrest observed with hexachlorophene was added as a sub-type of the first core endpoint (“coagulation”) listed by OECD TG 236 (OECD, 2013). For reasons of a more detailed description, the fourth core endpoint (“lack of heart beat”) of OECD TG 236 was subdivided into partial or complete lack of both heart beat and blood flow as well as blood congestion (formation of blood islands within extended blood vessels).

Compounds such as 2,2-dimethylvaleric acid, hexanoic acid, 2-propylheptanoic acid, and 4-ene-valproic acid caused a large number of the developmental alterations (termed as “endpoints”) observed (18, 18, 17, and 19 endpoints, respectively). Another compound found to induce a large number of endpoints was valproic acid, inducing yolk deformation, reduced yolk resorption, reduction of pigmentation, lordosis, craniofacial deformations, and 11 other endpoints. In contrast, there were compounds causing only a limited set of alterations, part of which also varied significantly in severity. For example, compounds such as 2,4-dinitrophenol (6), luviquat (6), malathion (7), merquat (7), sodium chloride (7), and triclosan (6) caused overall fewer endpoints. Finally, for MPP+, PCB 180,

rifampicin, sulfisoxazole, and taxol, neither acute nor sublethal toxicity could be recorded at concentrations up to the solubility limits (Table 1).

Likewise, with respect to specific endpoints, some endpoints were seen after exposure to the majority of tested compounds. As is evident from Table 2, formation of oedemata could be observed with any of the 45 substances tested positive. Therefore, oedemata in zebrafish appear to be of very little mechanistic value and have to be categorised as an unspecific side effect of both acute and sublethal toxicity [47, 48], although oedemata were listed as changes typical of cardiotoxicity [49]. Oedemata have also been described after exposure to petroleum oils [50], nanomaterials [51], parabens [52], endocrine disruptors [53], flame retardants [54, 55], pesticides [56], and numerous other classes of compounds. Likewise, physicochemical parameters such as temperature are capable of inducing oedemata in zebrafish embryos [57].

Other endpoints, such as otolith deformation and eye malformations, were caused by only 4 and 6 of the 50 compounds tested, respectively. According to the literature, otolith deformation has only been reported after exposure to bisphenol A [58], carbamazepine, diclofenac, and metoprolol [59], as well as ethanol and acetaldehyde [60], whereas various kinds of eye malformation are commonly observed in zebrafish embryos.

When conducting FET tests, complete lists of deviations from the normal embryonic development must be compiled to fully exploit the power of this test and to add to a deeper insight into the mechanisms of action of the compound(s) in question. Only a complete list of observations can finally be utilised to determine the next steps to take in the compound’s risk assessment and for planning of further tests with adults. The present work has provided the proof of concept that certain endpoints occur with a higher frequency than others, leading to the question towards the underlying mechanisms of action and biological pathways. Further research should aim to determine these, providing a greater insight into the underlying toxicology of the compounds causing these more specific endpoints.

Normal development of zebrafish embryos

Figures 1, 2, 3, 4, and 5 illustrate the normal development of zebrafish embryos over 120 h. Briefly, after 24 h (Fig. 1), the principle organisation of the embryo is already discernible, and anatomical structures such as somites, notochord, otoliths, and eye anlage as well as the heart anlage can be localised. Overall body length can be measured, and the embryo shows first erratic movements (tail curling).

Table 2 (continued)

Compound	Coagulation	Gastrulation arrest	Lack of tail detachment	Lack of somite formation	Impaired/missing heart-beat	Impaired/missing blood flow	Blood congestion	Formation of oedemata	Delayed development	Delayed hatching	Impaired/missing pigmentation	Reduced yolk resorption	Yolk deformation	Impaired fin development	Altered spontaneous movement	Tremor (> 96 hpf)	Craniofacial malformation	Otolith malformation	Eye malformation	Spinal cord malformation	Lordosis	Kyphosis	Scoliosis
Rotenone	■	□	■	□	■	■	□	■	■	■	■	□	□	□	■	□	□	□	□	□	□	□	□
Sodium chloride	■	□	■	□	■	■	□	■	■	□	□	□	□	□	□	□	□	□	□	■ ^s	□	□	□
Sodium tetradecyl sulphate	■	□	■	■	■	■	□	■	■	□	□	□	□	□	□	□	■	□	□	■ ^s	□	□	□
Sulfisoxazole #	□	□	■	□	□	■	■	■	■	□	□	□	□	□	□	□	■	□	□	■	■	□	□
Taxol #	□	□	■	□	□	■	■	■	■	□	□	□	□	□	■	□	□	□	□	■	□	■	□
Tebuconazole	■	□	■	□	■	■	□	■	■	■	□	□	□	□	■	□	■	□	□	■	■	□	□
Tolbutamide	□	□	■	□	■	■	■	■	■	□	■	□	□	■	□	■	■	□	□	■	■	□	□
Triclosan	■	□	■	■	■	■	□	■	■	□	□	□	□	□	□	□	□	□	□	□ ^s	□	□	□
Triethylene glycol	■	□	■	□	■	■	□	■	■	■	□	□	□	□	□	□	■	□	■	■ ^s	□	□	□
2,3,6-Trimethyl phenol	■	□	■	□	■	■	□	■	□	□	□	□	□	□	□	□	■	□	□	■ ^s	□	□	□
Triphenylphosphate	■	□	■	□	■	■	□	■	■	□	□	□	□	□	■	□	■	□	□	■	■	□	□
4ene-Valproic acid *	■	□	■	□	■	■	■	■	□	■	■	■	■	■	□	■	■	■	■	■	■	■	□
Valproic acid *	■	□	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■
Zinc pyrithione #	□	□	■	□	■	■	■	■	■	■	□	□	□	■	□	□	□	□	■	■	□	□	□

The four core endpoints listed by OECD TG 236 (OECD, 2013) are indicated in bold. Frequency of occurrence: ■—frequent and/or at concentrations < LC₁₀; ■ — ≤ 20% of exposed organisms and/or only at concentrations > LC₁₀; □ —not observed for this compound

* Additional determination at 120 and/or 144 hpf (cf. Table 1); # no symptoms of acute lethality at highest solubility in a final DMSO concentration of 1 %; ^s no differentiation for specific spinal cord deformations; endpoints grouped under general “spinal cord malformation”

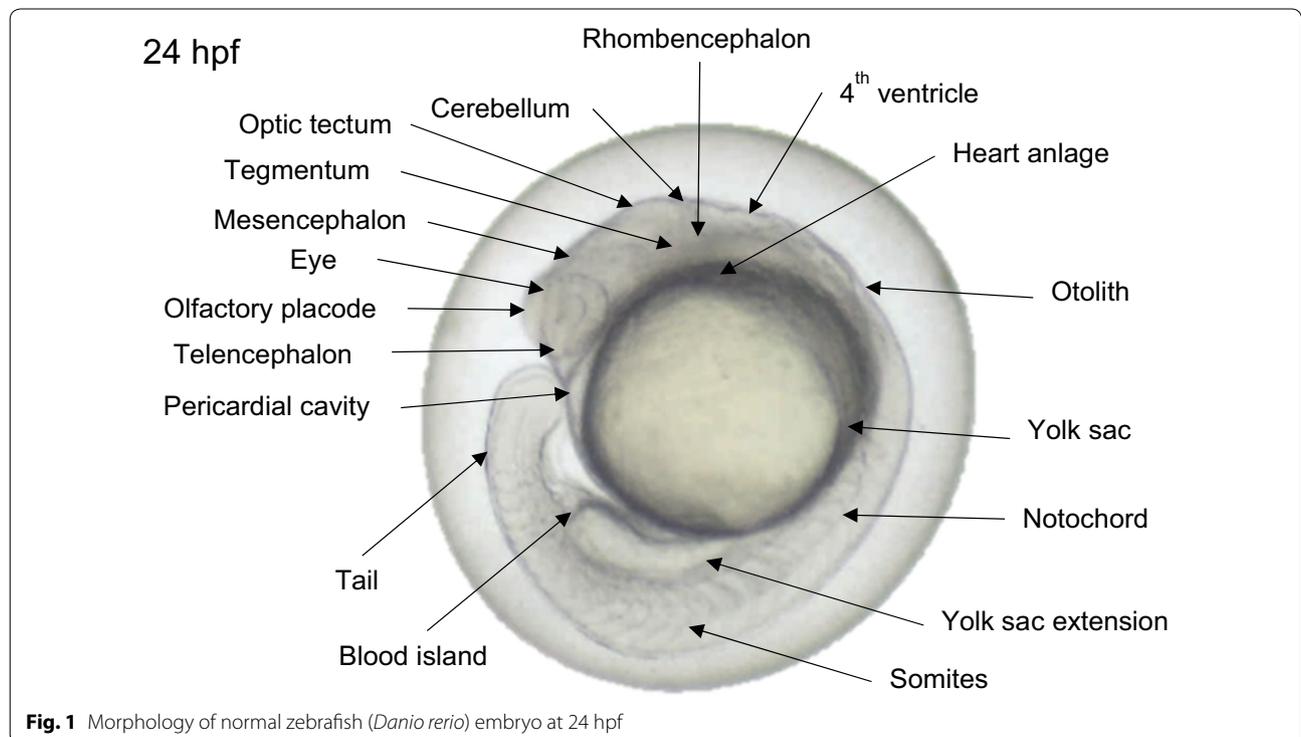
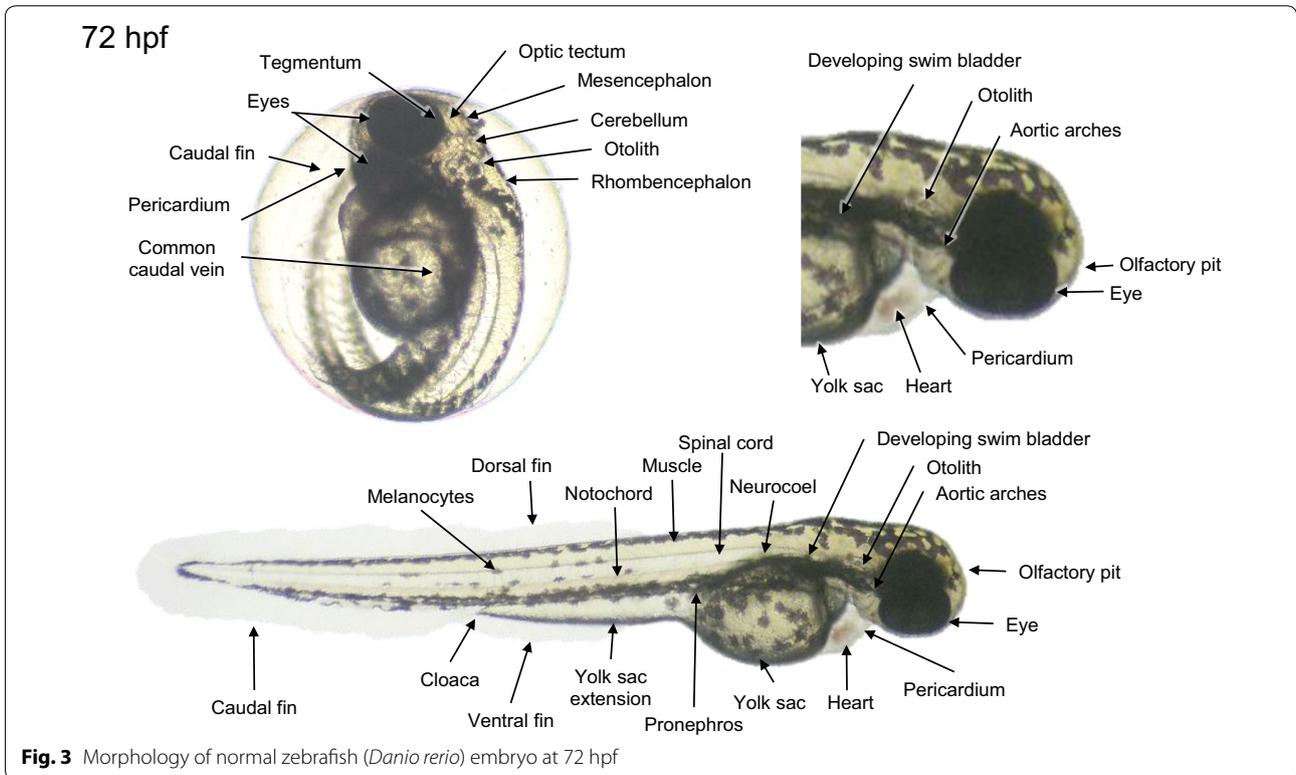
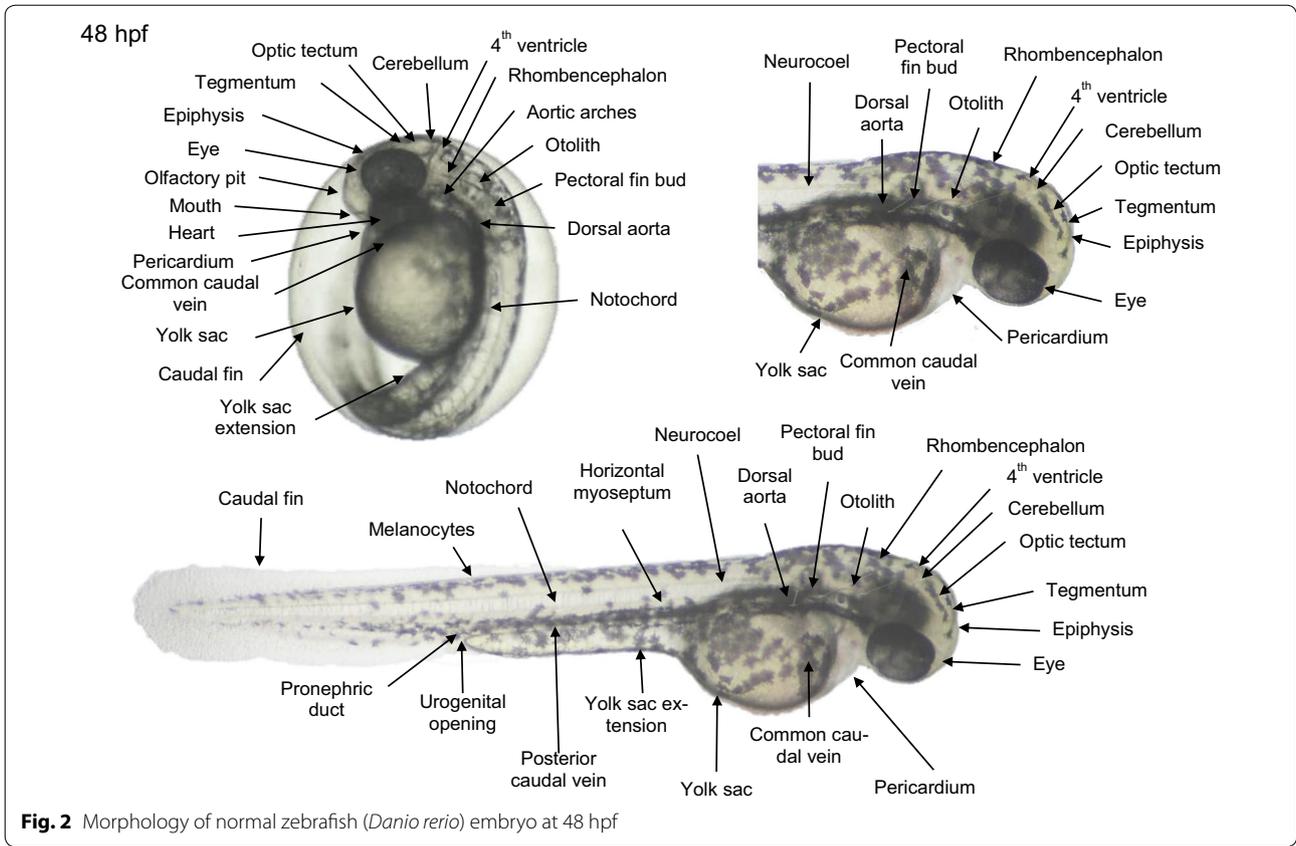


Fig. 1 Morphology of normal zebrafish (*Danio rerio*) embryo at 24 hpf



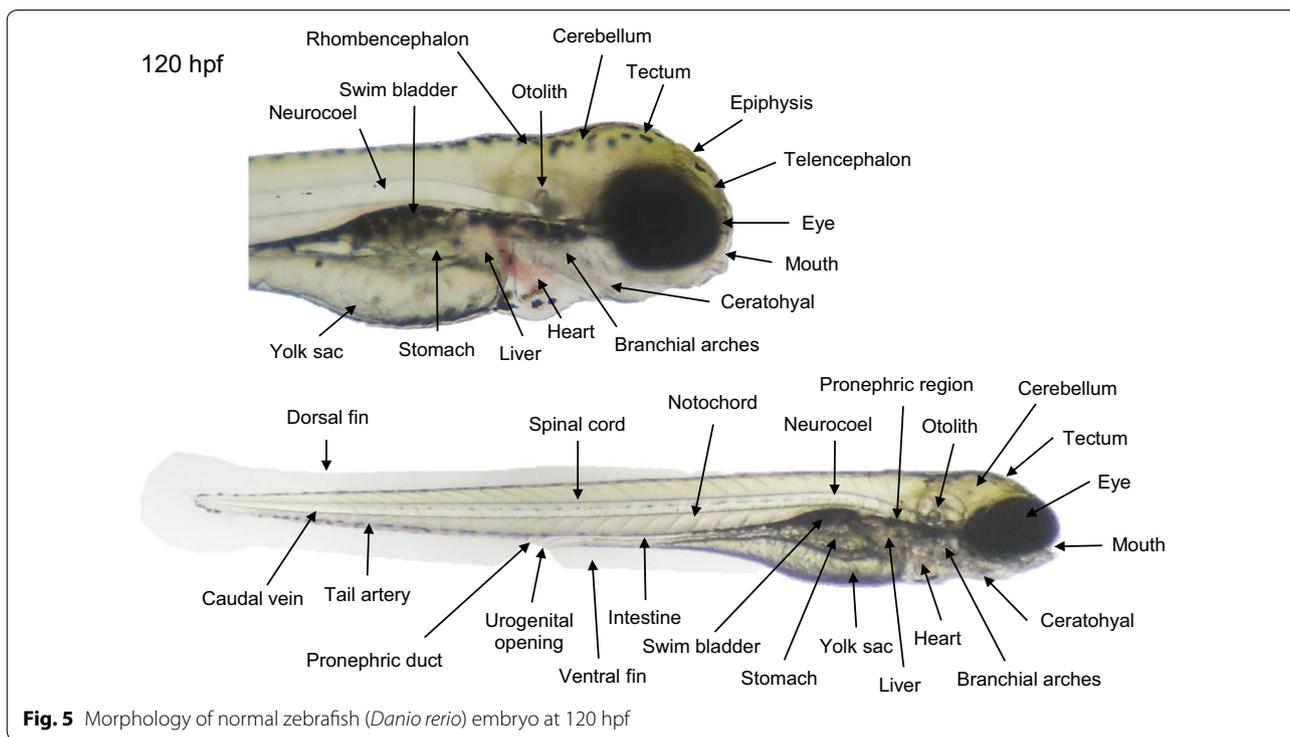
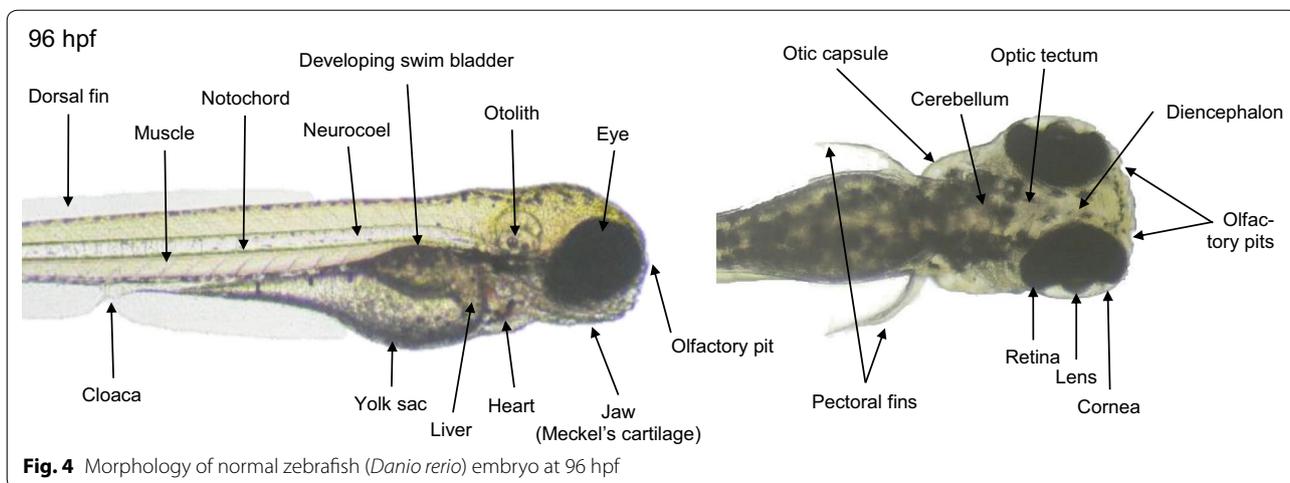


Figure 2 provides an overview of the structures formed by 48 hpf; at this age, dechoriation can easily be performed [61] for better access to embryonic structures. At an age of 48 h, craniofacial features have further developed and can be utilised for assessing the disruption of cartilaginous structures [62]. Blood flow and an un-looped heart region can be discerned for the assessment of cardiac and circulatory malformations. Likewise, major sensory organs such as eye and ear are fully developed, and various components of the brain and the spine

above the notochord can be distinguished (Fig. 2). At 48 hpf, embryos show an increasing number of epidermal pigment cells. Furthermore, the caudal fin as well as the buds of the pectoral fins have formed.

After 72 hpf, the overall anatomy is mainly developed and the embryos are ready for hatch (Fig. 3). The S-shaped heart loop allows for effective circulation, and the curvature of the back enables the correct determination of modifications such as lordosis, scoliosis, and kyphosis. The fins have developed further, and after

hatching, the first behavioural alterations based on active swimming can be recorded.

In 96-h-old zebrafish embryos (Fig. 4), resorption has significantly reduced the volume of the yolk sac, the swim bladder has formed, and the intestinal tract is fully developed. After 96 h, all embryos should have hatched. After 120 h, embryos have almost completely resorbed the yolk, and facial features have flattened further to allow for active capturing of prey (Fig. 5), which effectively starts between 128 and 144 hpf [63].

Core endpoints of acute lethality in the development of zebrafish embryos

Developmental alterations observed during the FET tests of the compounds listed above are summarised in Figs. 6, 7, 8, and 9. Figure 6 illustrates coagulation, gastrulation arrest, and the lack of tail detachment and somite formation as 3 out of 4 core endpoints

of the OECD TG 236 for acute lethality. Coagulation frequently already occurs at 24 hpf (Fig. 6a) and is indicative of early death; only rarely, coagulation can be seen in later developmental stages: Zebrafish embryos exposed to colchicine clearly displayed active heart beat, but blood flow was severely reduced, the general development was delayed, and the body typically started coagulating from the tail and the yolk sac (Fig. 6b). With the majority of articles pertaining to FET results focusing on EC and LC values, coagulation is one of the more commonly mentioned endpoints. Gastrulation arrest (Fig. 6c) has often been considered as a precursor of coagulation, but may also be observed in embryos with severely delayed development, which is usually recorded as a sublethal endpoint. Lack of tail detachment also occurs in early development (Figs. 6d–f, with increasing severity) and can either be an indicator of general delayed

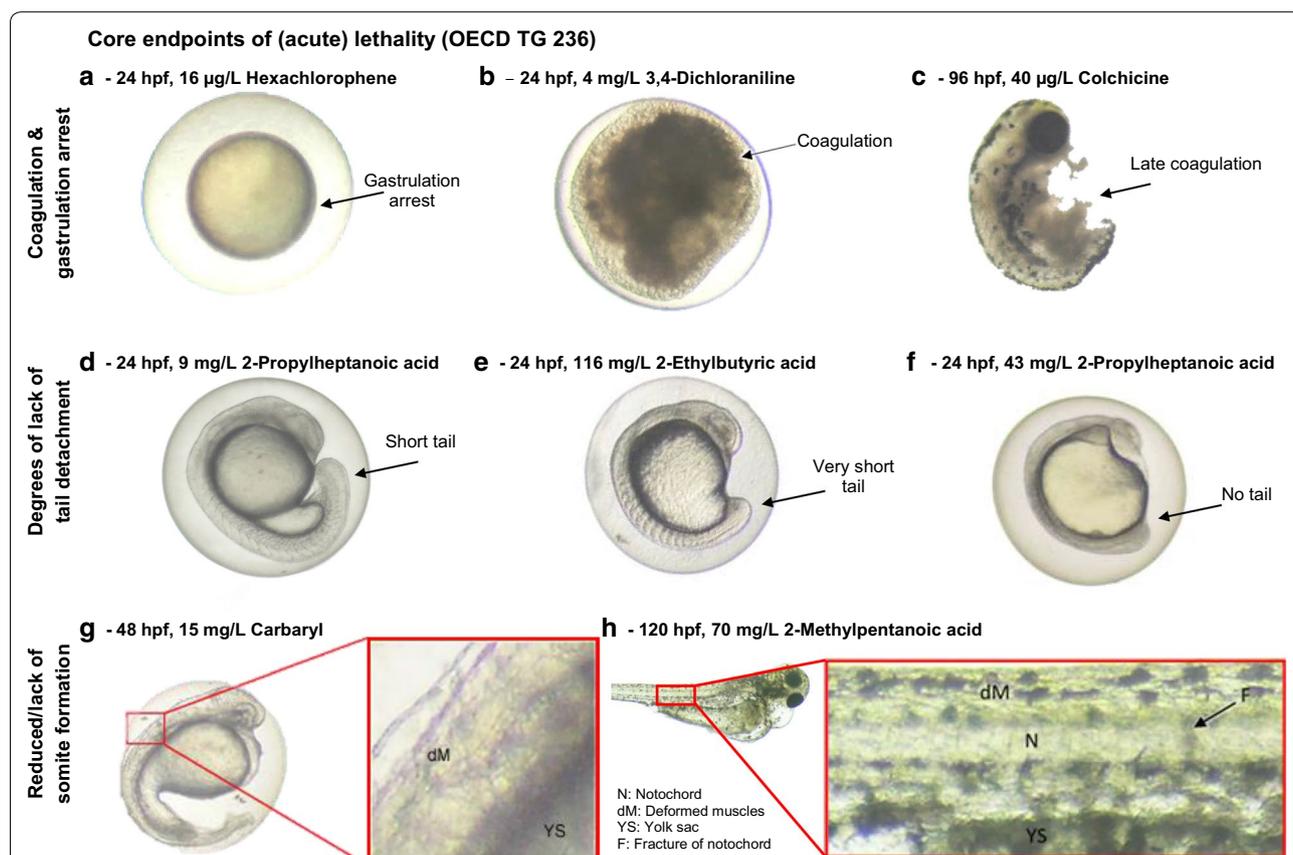
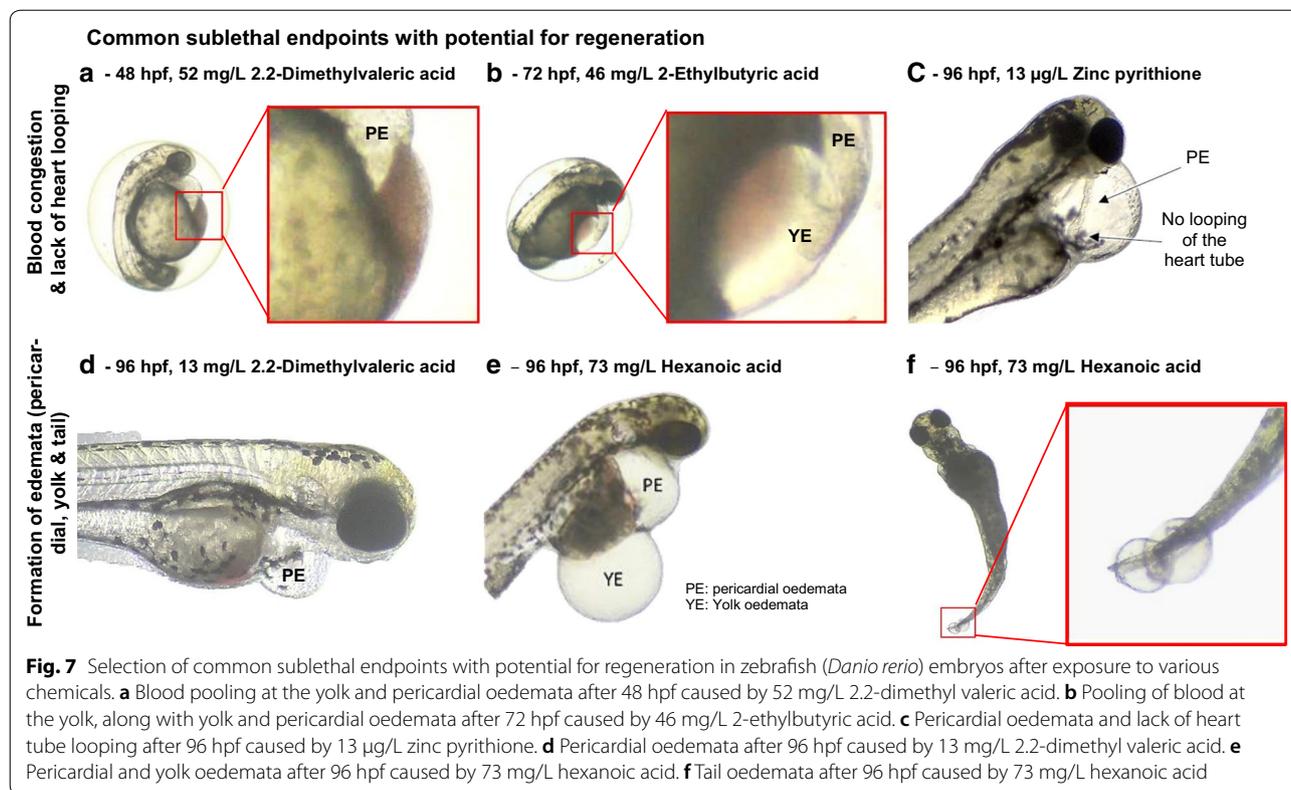


Fig. 6 Overview of three out of the four core endpoints of acute lethality prescribed by OECD TG 236 (OECD 2013) in zebrafish (*Danio rerio*) embryos after exposure to various chemicals. **a** Gastrulation arrest after 24 hpf caused by 16 mg/L hexachlorophene. **b** Coagulation of a fertilised embryo after 24 hpf caused by 4 mg/L 3,4-dichloroaniline. **c** Late coagulation at 9 hpf caused by 40 µg/L colchicine. **d** Slightly reduced detachment of the tail after 24 hpf caused by 9 mg/L 2-propylheptanoic acid. **e** Barely detached tail after 24 hpf caused by 116 mg/L 2-ethylbutyric acid. **f** Complete lack of tail detachment after 24 hpf caused by 43 mg/L 2-propylheptanoic acid. **g** Reduced somite formation (dM) caused by 15 mg/L carbaryl exposure. **h** Fracturing of the notochord (F) and reduced somite formation (dM) caused by 7 mg/L 2-methylpentanoic acid. The fourth core endpoint, lack of heart beat, cannot be documented in static micrographs



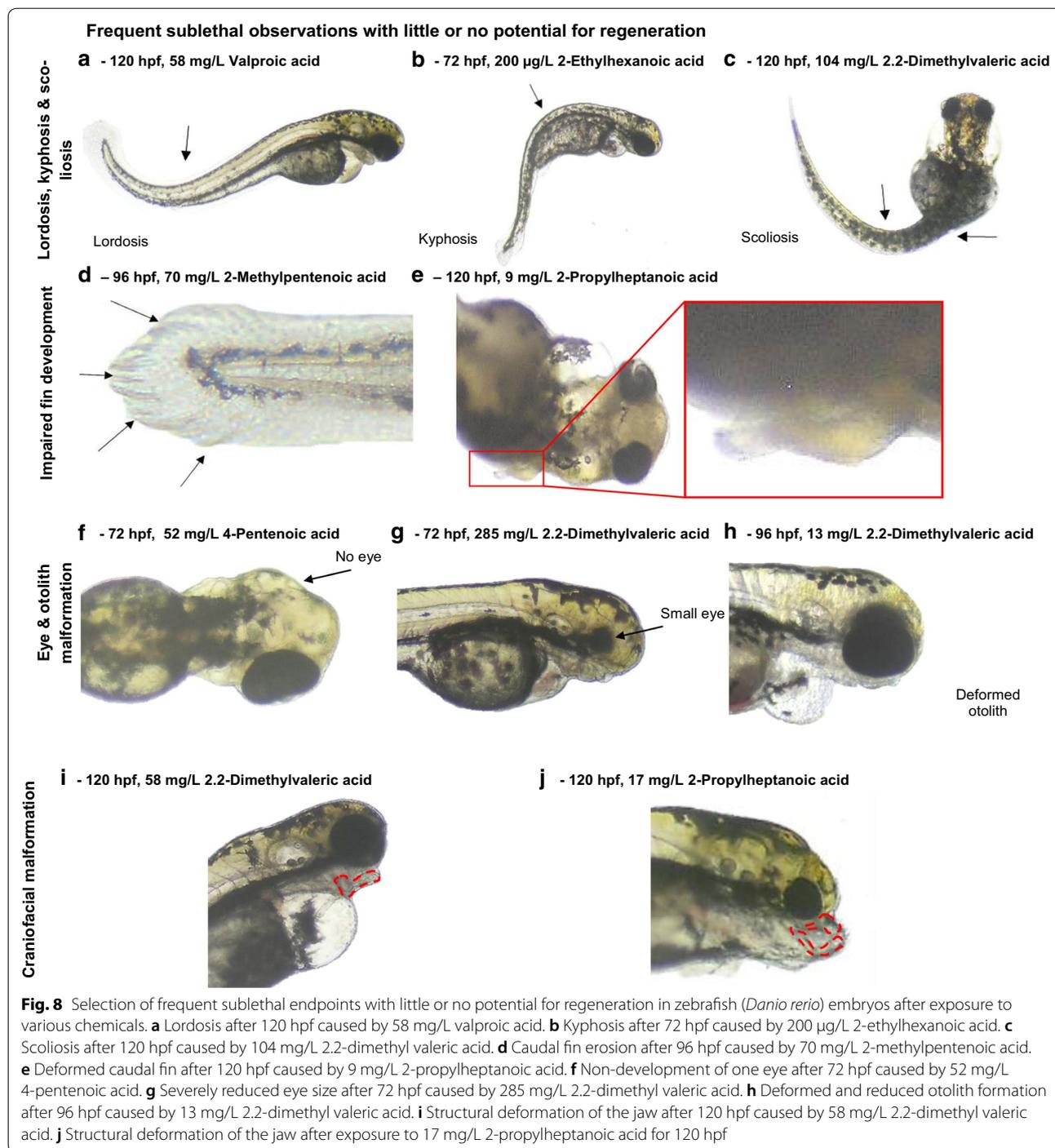
development or, in severe cases, develop into lethality. The underlying mechanisms for non-detachment of the tail are not yet understood, and more attention should be paid not only to its occurrence and severity, but also to its potential for recovery.

The core endpoints of acute toxicity as specified by OECD TG 236 further include lack of somite formation, an endpoint which becomes evident from 24 hpf. The insert in Fig. 6g illustrates the gradual disappearance of pylon-shaped somites (dM) above the yolk sac (YS) into a diffuse mass of tissues. Manipulation of the plane of focus also allows the detection of fractures of the spinal cord (Fig. 6h insert). Somites have been found to be involved in the development of the vertebrae, ribs, skeletal muscles, and skin, for example [64], indicating that correct formation of somites is vital for the later development of the embryo. Although the OECD protocol for the FET explicitly specifies somite formation as a key endpoint, reference to alterations of somite formation is scarce in the literature [65–68], which may be due to technical difficulties in revealing this endpoint and only Brannen et al. [69] recorded the number of somites.

Sublethal morphological alterations in zebrafish embryos with potential for regeneration

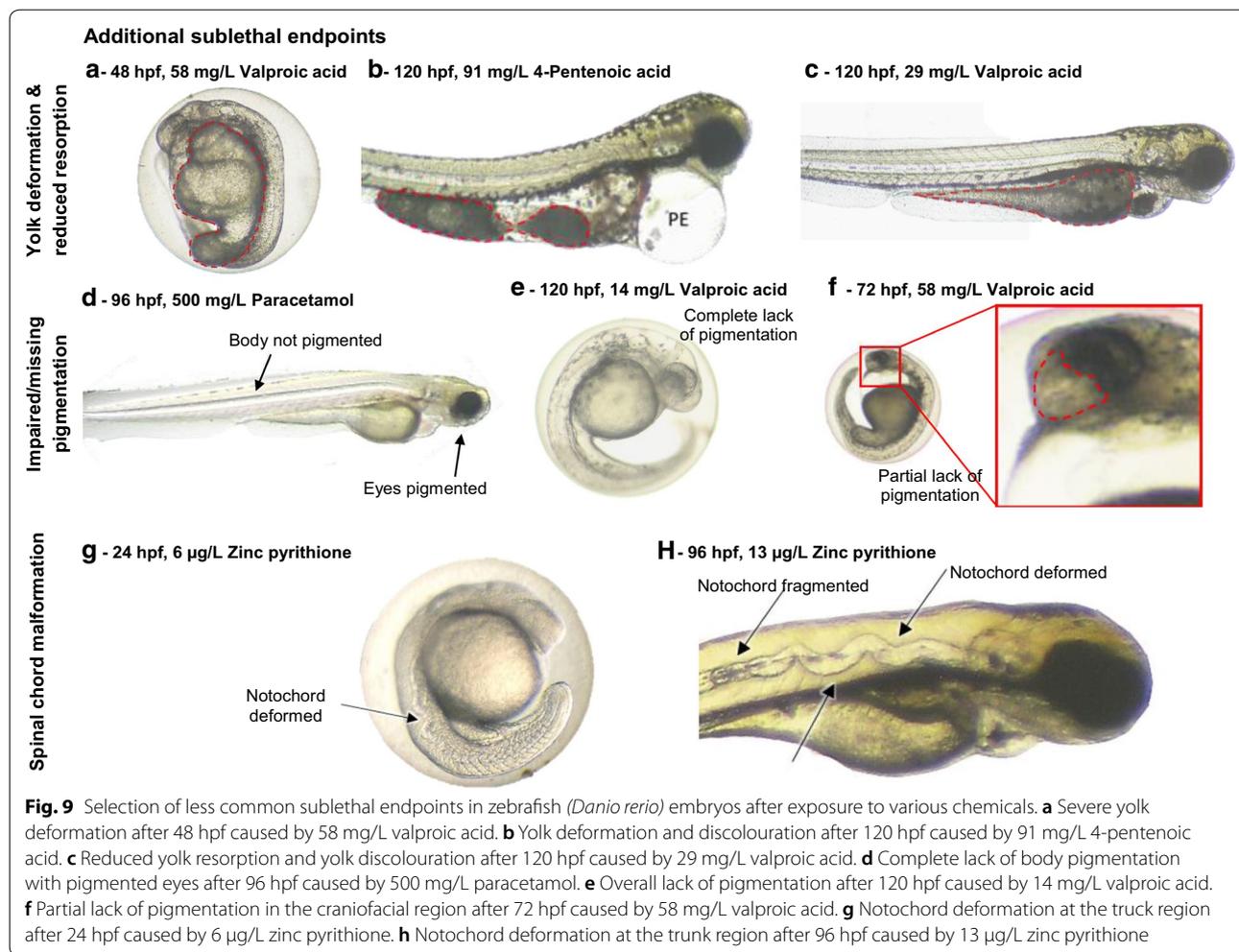
Figure 7 presents commonly observed sublethal endpoints with high recovery potential. To allow for a more detailed assessment, the OECD TG 236 core endpoint lack of heart beat was subdivided into partial and complete lack of heart beat, blood flow, as well as blood congestion. Lack of blood circulation can, by definition, not be captured in an image. In contrast, blood congestion characterised by the accumulation of blood cells, especially within pericardial (Fig. 7a) and yolk oedemata (Fig. 7b), can frequently be observed in conjunction with a severe reduction of both blood flow and heart-beat rate. Some authors (e.g., Brannen et al. [69]) did not differentiate cardiovascular effects, but summarised such effects under “cardiovascular function” and “heart rate”, thus hampering a direct comparison of results.

The literature on the development of the zebrafish cardiovascular system has described heart looping (Fig. 7c) in great detail [70–72], linking developmental alterations to oxidative stress [73], lipid metabolism [70], neural crest cell migration [71, 72], early cardiomyocyte differentiation [74], Ah receptor-mediated biotransformation [75], or retinoic acid metabolism and *hox* signalling [76–78]. However,



such observations have only rarely been mentioned in the (eco)toxicological literature [79]. In mild cases, pericardial oedemata (Fig. 7d) resemble enlarged pericardium, whereas severe cases can be identified as distended thin-walled cavities surrounding the heart (Fig. 7e: PE). Yolk oedemata (Fig. 7e: YE) may develop into cavities within the yolk itself or concentrate in the periphery underneath

the yolk sac. Oedemata are not restricted to pericardial and yolk regions, but may appear along the entire body (Fig. 7f), found among the most frequent observations in the present study (Table 2) and have, therefore, to be classified as unspecific. Likewise, oedemata have commonly been mentioned in the literature (for recent examples, see [80–82], and some authors differentiated between yolk,



pericardial, and facial oedemata [83], whereas others only listed “heart morphology” as a summary endpoint [69]. If not too severe, any kind of oedemata seen in zebrafish embryo development seems to be reversible.

Sublethal morphological alterations in zebrafish embryos with little potential for regeneration

Frequently occurring sublethal endpoints which rarely recover are given in Fig. 8. For instance, three types of spinal curvature can be differentiated: (1) lordosis, an inward curving of the spine (Fig. 8a), (2) kyphosis, an outward curving (Fig. 8b), and (3) scoliosis, a sideways curving (Fig. 8c). As discussed above, these are three distinctly different pathologies which should be treated as such (for other types of spinal cord deformations, see Fig. 9). The most frequently malformation mentioned in the literature is lordosis [34–37]. However, the term “lordosis” has also frequently been used as a generic term for any type of curvature of the spine [38]. Another study, however, correctly identified lordosis, but annotated “scoliosis”

as “spinal column flexure (tail defect)” and also listed “kyphosis” rather appears to be a break in the notochord, which we would suggest to call notochord fragmentation [39]. In some cases, “modified structure of chorda” [69], “body shape” [69], “notochord anomalies”, “tail anomalies”, and “kinks (wavy, curled, bend)” were employed to describe spinal cord malformations without further distinction [83]. No further publications could be found, directly discussing the different curvatures observed in zebrafish embryos, although—according to the present study—spinal curvature is a group of endpoints quite frequently observed (Table 2); with the literature providing the further examples [55, 84–90].

Further effects with limited regeneration potential include impaired fin development such as the tailfin fraying (Fig. 8d) and underdeveloped pectoral fins (Fig. 8e). Common summarising surrogate terms are “fin morphology”, “tail morphology” [69], and “tail malformation” [91], which, however, do not allow a separation between changes of the spinal cord and the fin(s) itself.

Figure 8 also illustrates the examples of reduced eye development (Figs. 8f, g) and lack of otolith formation (Fig. 8h). In literature, these endpoints are commonly referred to as “eye malformation” or “deficiencies of eyes and otic vesicles”, providing no further information [83, 92]. Since the zebrafish eye contains fully differentiated cells connecting to the brain via the optic nerve by 28 hpf [93], its development can be impacted very early on, thus being of environmental importance. Further craniofacial malformations (Figs. 8i, j) can be less easily recorded, since observation is usually obstructed by active swimming from 120 hpf. Craniofacial deformations include the shortening or lengthening of the (lower) jaw, as well as general disorganisation of skeletal elements. Recent studies discovered a connection between histone deacetylase (HDAC) inhibition and craniofacial malformation [94–96] as well as neural tube defects [97–100]. These observations have already been implemented in the established AOPs (can be found under: <https://aopwiki.org/aops/274> and <https://aopwiki.org/aops/275>), thus presenting an important link between effects in mammals and zebrafish embryos. Again, however, the recent publications used a diverse array of nomenclature: “jaw malformation” [92]; “facial structure morphology”, “jaw and pharyngeal arch morphology” [69], and “lower jaw anomalies” describing pharyngeal arches’ shape [83]). All of the examples listed above illustrate the need for more differentiated assessment of deviations from normal zebrafish development as well as a harmonisation of nomenclature.

Additional sublethal morphological alterations in zebrafish embryos

Figure 9 illustrates the examples of less frequently described sublethal endpoints such as morphologically discernible yolk alterations, which can be identified from 48 hpf (Fig. 9a). Reduced yolk resorption (Figs. 9b, c) only becomes particularly evident from 96 hpf, when healthy embryos begin to deplete its nutrient resources. The present study identified reduced yolk resorption among valproic acid-related endpoints, which have been interpreted as symptoms of the embryonic malabsorption syndrome [101]. Again, however, numerous studies only used summary terms such as “yolk”, which might include yolk oedemata, yolk depletion, or yolk deformations, thus being less informative [69].

Reduced pigmentation can be seen at various degrees (Figs. 9d–f): either, the entire organism (Figs. 9d, e) or specific organs (Fig. 9f) may be affected. As an endpoint, pigmentation may be affected by a multitude of pathways, and there is only limited information about underlying mechanisms. As a final example of morphological effects, deformation or fragmentation of the notochord may occur at variable degrees of severity (Figs. 9g, h).

Notochord malformations may develop from an early stage (Fig. 9g) and are likely to persist (Fig. 9h), which, in severe cases, may lead to notochord fragmentation with multiple breaks in the notochord. Notochord malformation has been termed as “chorda malformation” or “larvae showing a bent spine” [91] or has simply been mentioned as “notochord morphology” without further differentiation [69]. It should be noted that notochord malformation is an entirely different pathology to, e.g., lordosis, and should be clearly differentiated. Overall, notochord malformation and fragmentation can be observed quite frequently (cf. Table 2).

Alterations in zebrafish embryo behaviour

Table 2 also includes behavioural changes such as tremor and modified spontaneous movement as additional “morphological” observations, which are, however, difficult to document without a special hardware and software. Such effects can often be linked to neurotoxic effects such as inhibition of acetylcholinesterase [102], which leads to minute erratic movements. Unfortunately, behavioural endpoints are frequently ignored in morphology-based teratogenicity experiments and have only rarely been discussed in this context. Occasionally, summarising terms such as “motility” and “locomotor activity” without further explanation (decrease or increase) can be found [91, 103, 104]). An example of a more informative term is “seizure liability” as “incidence of high-speed movements characteristic of seizure activity” [30]. Although the quantitative determination of behavioural effects requires video tracking and elaborate analysis [105–110], the presence or absence of behavioural changes can easily be recorded in a standard FET test and be used as a first indicator of, e.g., a neurotoxic potential of the test compound [111].

Conclusions

The present study on morphological effects by a wide array of test compounds with a wide spectrum of modes-of-action illustrates that toxic exposure of zebrafish embryos may lead to multiple developmental alterations beyond those listed in OECD TG 236 for the FET. The implementation of additional endpoints beyond the four core endpoints prescribed by OECD TG 236 adds significant value to the FET as a validated test system. However, the study also documents that the overall number of morphological alterations is limited—as holds true for other morphological criteria such as, e.g., histopathological and ultrastructural observations. As a consequence, morphological observations may serve as an important source of indicators for potential toxic mechanisms; additional (molecular) studies may usually be required to elucidate underlying pathways. The comparability and benefit of

FET studies could significantly be improved by a complete and more differentiated description of the observations and a harmonisation of the nomenclature used. Likewise, an at least approximate quantification of the observations (e.g., by morphological scoring) would certainly increase the reliability and robustness of the studies and, thus, the overall value of FET studies for regulatory (eco)toxicology. Given such improvements, FET data could make an important contribution to modern hazard and risk assessment compatible with the 3R principles.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12302-020-00398-3>.

Additional file 1. Catalogue of adverse effects in the fish embryo acute toxicity (FET) test.

Abbreviations

AFT: Acute fish toxicity test; AOP: Adverse outcome pathway; DMSO: Dimethyl sulfoxide; EC: Effect concentration; ECHA: European Chemical Agency; FET: Fish embryo acute toxicity test; GHS: Globally Harmonised System of Classification, Labelling and Packaging of Chemicals; HPF: Hours post-fertilisation; LC: Lethal concentration; MPP+: 1-Methyl-4-pyridinium iodide; OECD: Organization for economic co-operation and development; PBT: Persistence, bioaccumulation and toxicity assessment; PCB 180: Polychlorinated biphenyl 180; PE: Pericardial oedemata; PNEC: Predicted no-effect concentration; REACH: Registration, evaluation, authorization, and restriction of chemicals; STS: Sodium tetradecyl sulphate; TG: Test guideline; YE: Yolk oedemata.

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Authors' contributions

KB tested 2,2-dimethyl valeric acid, 2-ethylbutyric acid, 2-ethylhexanoic acid, 2-methylhexanoic acid, 2-methylpentanoic acid, 2-propylheptanoic acid, 4-ene-valproic acid, 4-pentenoic acid, hexanoic acid and valproic acid and was a contributor in writing the manuscript. RS tested 1-octanol, 2,3,6-trimethyl phenol, 3,4-dichloroaniline, 2,4-dinitrophenol, dinitro-*o*-cresol 6-Methyl-5-heptene-2-one, carbamazepine, copper (II) sulphate pentahydrate, DMSO, ethanol, luviquat, malathion, merquat, methylmercury (II) chloride, prochloraz, sodium chloride, STS, triclosan and triethylene glycol. RH tested acrylamide, carbaryl, clofibrate, colchicine, hexachlorophene, ibuprofen, MPP+, paracetamol, paraquat, PCB 180, rifampicin, rotenone, sulfisoxazole, taxol, tebuconazole, tolbutamide, and triphenylphosphate, and was a major contributor in writing the manuscript. LB was a contributor in writing the manuscript and aided in the data analysis. TB was a contributor in the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The work was conducted in strict accordance with governmental legislations in the aquatic toxicology research group of the University of Heidelberg (Licence number: 35-9185.64/BH).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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