Zebrafish embryos and larvae as a complementary model for behavioural research

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Chapter 3

Effects of biocides and metals on zebrafish embryo development and larval locomotor activity

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Abstract

The zebrafish has been widely used as a predictive model in safety and toxicology. Low cost, high-throughput screening can be achieved with this model, and the zebrafish genome contains orthologues of the majority of human disease genes. However, previous studies indicate that the predictivity of the zebrafish model in toxicology varies between compound and compound class. We examined this issue by screening 24 compounds from two different compound classes, metals and biocides (pesticides and insecticides) for toxicity in the zebrafish model and looked at the effects on hatching, morphology and predictivity for mammalian toxicity. Wild-type zebrafish embryos were exposed to test compounds in 96-well plates for 96 hours starting at 24 hours post fertilization. Hatching was either delayed, accelerated or unaffected, depending on the compound. Three types of alteration in behavioural responses were noted: (i) hypoactivity; (ii) hyperactivity; and (iii) biphasic response (a dose-dependent shift between hypo- and hyperactivity). The LC_{50} of compounds was calculated and compared to published LD_{50} values in rodents. The zebrafish-rodent values were poorly correlated for both metals and biocides. We conclude that, although the zebrafish is a good model for some aspects of toxicology, its predictivity for mammalian toxicity needs to be determined per compound class.

Introduction

The zebrafish is a small, teleost fish of shallow, fresh-water habitat, which has emerged as a valuable model in the field of research especially in the last decade [28]. The advantages which have made it a popular model in research are manifold and include: external fertilisation and rapid development, easy maintenance at low cost, year-round spawning, rapid generation cycle (2-3 months), and suitability for high-throughput screening [43]. The zebrafish genome is nearly completely sequenced and contains orthologues of 70% of human disease genes [238,239]. The zebrafish is used in many fields of biological research including behavioural studies [60,208,240,241], chemical toxicity [59,98,242-245], drug discovery [66,246,247] and in human disease-modelling [44,248-250]. Forward and reverse genetic techniques can be used, as can large-scale, high-throughput screening.

Given the aforementioned advantages of the zebrafish, the effects of both short- and long-term exposure to a wide range of toxins can be studied with relative ease. A variety of compounds has been tested on zebrafish, including metals and organic compounds [251,252] and different drugs [253]. The main emphasis in these studies has been on lethality and malformations as general assay parameters, and has demonstrated that zebrafish larvae display dose-dependent toxicity phenotypes in response to a range of compounds and might therefore be potential animal model for toxicity screening [245,254,255].

The use of zebrafish in behavioural neuroscience is in its infancy compared to the use of rodents [256]. However, the availability of zebrafish lines, high-throughput screening and new bioassays for toxic and therapeutic endpoints in zebrafish are likely to increase its use in various fields of research which will result in greater insights into the mechanisms of toxicity of chemicals, as well as aiding in the discovery of new drugs for treating several human diseases [30,256,257]. Behaviour is one readout of toxicity [258]. Although the number of published studies on zebrafish behaviour is not large compared to comparable studies on rodents, many of the behaviours displayed by zebrafish are well-described. These include the open-field test [61,76], optomotor response [52], optokinetic response [50,51,139,140,259], photokinesis [60] and visual motor response test [49,55,260] among many others.

It has long been known that behavior of animals including zebrafish can be altered by drugs and chemicals [87,261,262]. These alterations are regarded as an observable

expression of effects on the nervous and locomotor systems [244]. Some environmental chemicals, such as pesticides, can cause developmental neurotoxicity resulting in neurodevelopmental disorders in humans [263,264]. This makes it important to determine the effects of these chemicals on living animal behaviour.

More information is needed on the predictivity of the zebrafish model in toxicity. In particular, we need to know the extent to which the toxicity of compounds tested on in the zebrafish model correlates with their toxicity in mammals (especially rodents and humans).

Several classes of compound have been tested on zebrafish and assessed for their ability to predict toxicity in rodents. The predicitivity was found to vary considerably according to compound or compound class [98,152,265]. In the current study, we have tested metals, pesticides and insecticides (and the latter two we shall collectively call 'biocides') on zebrafish embryos. We have compared the results with studies of toxicity of the same compounds in mammals. We chose these compounds because and because there is increasing awareness and concern regarding the environmental effects of these compounds [266,267].

Material and methods

Statement of ethics on animal use

All experimental procedures were conducted in accordance with The Netherlands Experiments on Animals Act that serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, and were performed only after a positive recommendation of the Animal Experiments Committee had been issued to the license holder.

Animal husbandry

Wild-type male and female adult zebrafish (*Danio rerio)* were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) who obtains stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). We limited our experiment to a single strain (AB) as different zebrafish strains have differences in their locomotor activity [268]. Fish were kept at a maximum density of 12 individuals in plastic 7.5 L tanks (1145, Tecniplast, Germany) containing a plastic plant as tank enrichment, in a zebrafish recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands) on a 14h light: 10h

dark cycle (lights on at 7h AM: lights off at 21h PM). Water and air temperature were maintained at 24 ^{o}C and 23 ^{o}C, respectively. Fish were purchased at the juvenile stage and were allowed to adapt to our facility for at least 2 months before being used as adult breeders. The fish were fed daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen Artemia (Dutch Select Food, Aquadistri BV, The Netherlands).

Zebrafish eggs were obtained by random mating between sexually mature individuals. Briefly, on the day (16h) before eggs were required, a meshed net allowing eggs to pass through but preventing adult fish from accessing/eating them, was introduced in the home tank of a group of 12 adult fish. Each breeding tank was only used once per month to avoid handling stress and ensure optimal eggs quantity and quality.

The eggs were harvested the next day (30 min after the onset of lights at 7h AM) and age was set as post fertilization day (dpf) 1 based on the staging system employed in *Zebrafish: a Practical Approach* [269]. They were placed in 9.2 cm Petri dishes containing 100 ml egg water (0,21 g/l Instant Ocean Sea Salt and 0,0005% (v/v) methylene blue). 50-60 eggs were place in one Petri dish in a climate room maintained at a temperature of 28 °C and 50% humidity and under a light-dark cycle of 14h:10h (lights on at 7h AM/lights off at 9h PM).

Zebrafish egg plating

We used 24h old embryos for exposure of chemicals after removing unfertilized eggs and refreshing the egg water. Thus, each larva was gently taken up into a plastic Pasteur pipette (VWR International B.V., The Netherlands) and directly transferred to a 96-well plate, one larva per well, and each well containing 250 µl egg water (control) or the relevant concentration of compound dissolved in egg water. Egg water was made from 0.21 g 'Instant Ocean®' salt in 1 L of Milli-Q water with a resistivity of 18.2 MΩ cm. Note that in order to eliminate further sources of disturbance or stress, the medium was not refreshed except on 2 dpf where it was completely replaced by fresh egg water and non-fertilized eggs were removed. At the end of the behavioural testing, the larvae were processed further as follows for morphological assessment.

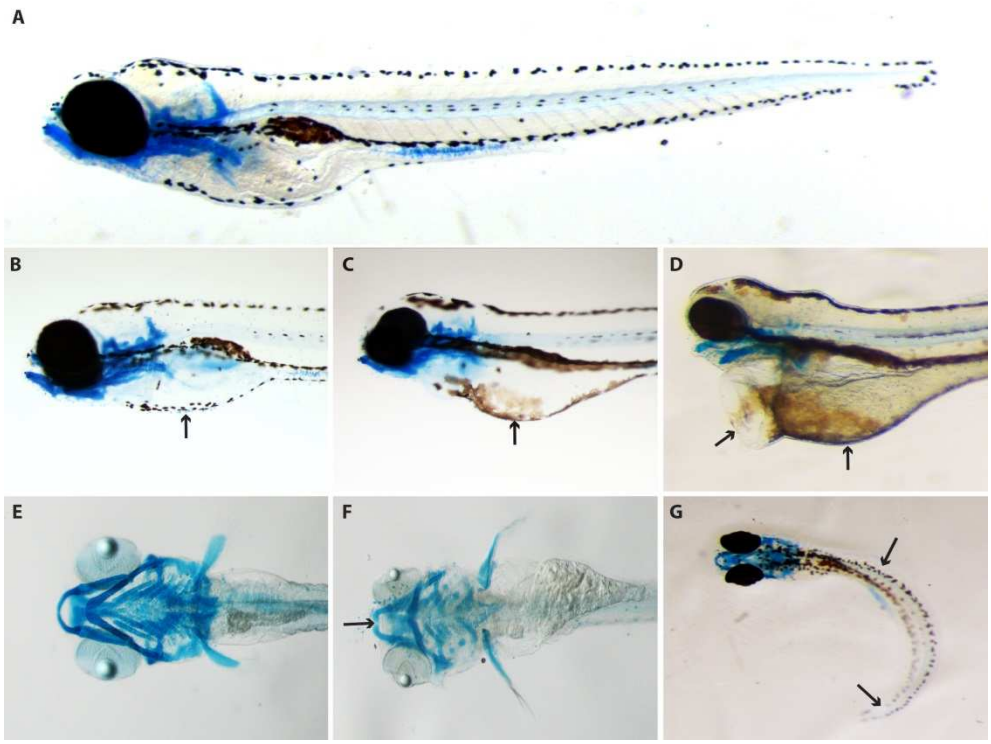

Figure 3.1. Phenotypes scored in the malformation assessment. (A) Normal zebrafish, left lateral view. (B) Normal phenotype of melanocytes with aggregated melanosomes giving a punctate appearance of the melanocytes (arrow); compare with (C) which shows melanocytes (e.g. arrow) with melanosomes dispersed in an expanded area of cytoplasm. (D) Zebrafish embryo showing pericardial oedema, yolk sac oedema and dispersed melanocytes (E) illustration of Meckel's cartilage and (F) shows a hypoplastic Meckel's cartilage while (G) shows the bent body axis and bent tail.

Test compounds

The compounds used in the present study are listed in the Table 3.1.

Table 3.1: List of compounds used in the study; all compounds were purchased from Sigma (Zwijndrecht, The Netherlands).

Compound		**Compound class**	**Molecular formula**	**Molecular weight (g/mol)**	**Sigma catalogue number**
1	2,4-dichlorophenoxyacetic acid (2,4-D)	Pesticide	$C_8H_6Cl_2O_3$	221.04	31518
2	Acephate	Insecticide	$C_4H_{10}NO_3PS$	183.2	45315
3	Amitrol	Pesticide	$C_2H_4N_4$	84.08	45324
4	Barium chloride	Metal	$BaCl_2$	208.23	202738
5	Benzophenone	Pesticide	$C_{13}H_{10}O$	182.22	427551
6	Bromacil	Pesticide	$C_9H_{13}BrN_2O_2$	261.15	45350
7	Diamethoate	Insecticide	$C_5H_{12}NO_3PS_2$	229.28	45449
8	Diazinon	Insecticide	$C_{12}H_{21}N_2O_3PS$	304.3	45428
9	Erbium chloride	Metal	$ErCl_3$	273.62	449792
10	Gallium chloride	Metal	$GaCl_3$	176.08	427128
11	Glyphosate	Pesticide	$C_3H_8NO_5P$	16.07	45521
12	Hydroquinone	Pesticide	$C_6H_6O_2$	110.11	H9003
13	Hexazinone	Pesticide	$C_{12}H_{20}N_4O_2$	252.32	36129
14	Maneb	Pesticide	$C_4H_6MnN_2S_4$	265.3	45554
15	2-methyl-4-chlorophenoxyacetic acid (MCPA)	Pesticide	$C_9H_9ClO_3$	200.62	45555
16	Mercuric chloride	Metal	$HgCl_2$	271.50	215465

17	Methomyl	Insecticide	$C_5H_{10}N_2O_2S$	162.21	36159
18	Molinate	Pesticide	$C_9H_{17}NOS$	187.3	36171
19	Paraquat dichloride	Pesticide	$C_{12}H_{14}Cl_2N_2$	257.16	36541
20	Pendimethalin	Pesticide	$C_{13}H_{19}N_3O_4$	281.31	36191
21	Stannic chloride	Metal	$SnCl_4$	189.62	204722
22	Strontium Chloride	Metal	$SrCl_2$	158.53	439665
23	Triclopyr	Pesticide	$C_7H_4Cl_3NO_3$	256.47	32016
24	Zinc chloride	Metal	$ZnCl_2$	136.28	229997

Range-finding

A range-finding test was conducted according to standard protocols [270] using a logarithmic concentration series (0, 1, 10, 100 and 1000 mg/L) to determine the effective concentration window. After 24hpf, living zebrafish embryos were transferred from the Petri dish into 96-well microtitre plates using a sterile plastic pipette. Dead embryos were recorded and discarded. A single embryo was placed in each well so that any embryos that subsequently died would not affect others, and also to allow individual embryos to be tracked for the whole duration of the experiment. We used a static non-replacement regime without any replacement or refreshment of egg water or test compound. Sixteen embryos for each concentration and 16 embryos as controls for each compound were used.

Geometric series and LC_{50} determination

A geometric series was selected based on the mortality rate of the range-finding series. The actual concentrations used are shown in Table 3.3. The concentrations were in a geometric series in which each was 50% greater than the next lowest value as recommended [270]. Each compound was tested in triplicate (48 embryos per concentration and 48 embryos for control and/or vehicle for each compound). LC_{50} (expressed in mg/L of egg water) was determined based on cumulative mortality obtained from three independent experiments at 120 hpf using Regression Probit analysis with SPSS Statistics for windows version 17.0 (SPSS Inc., Chicago, USA). The embryos were exposed to the compound for 96 h as in the

range finding test. The LC_{50} in mg/L was converted into LC_{50} mmol/L to make relative toxicity easier to examine.

Table 3.2: Phenotypic endpoints scored in zebrafish larvae at 5 dpf. Some of these criteria have been described elsewhere [271] and shown in Figure 3.1.

Phenotype	**Criteria**
Normal	The larva was considered to have normal morphology if any of the above listed phenotypes were normal
Yolksac	Yolksac enlarged by fluid accumulation
Heart	Pericardial sac enlarged by fluid accumulation
Meckel cartilage	Meckel's cartilage grossly hypoplastic, missing or unfused in midline
Melanocytes	Melanocytes *aggregated* (appearing as condensed, black spots with aggregated melanosomes); or *dispersed* (appearing as expanded, flat, grey cells with dispersed melanosomes)
Short body	Total body length below 3.5 mm at 5 dpf
Tail	A dorso-ventral bend in the tail
Body axis	The trunk was bent in the dorso-ventral plane

Hatching and mortality scoring

Hatching was monitored from 48-72 hpf which is the normal hatching period of zebrafish larvae [33]. The hatching rate was recorded once all the embryos in any particular concentration were hatched. The mortality rate (Table 3.4) was recorded at 48, 72, 96 and 120 hpf in both logarithmic series and geometric series using a dissecting stereomicroscope. Embryos were scored as 'dead' if there was no locomotor activity, the heart was not beating and the appearance of tissues had changed from transparent to opaque.

Automated behavioural recording

After 96h exposure to the compounds, each 96-well plate was placed in ZebraLab to automatically record the locomotor activity of larvae with the help of VideoTrack software (both from View Point, S.A., Lyon, France). A light-emitting diode (LED) panel illuminated the 96-well plate from below. Recording was done under infrared light which, like the LED panel, is a fixed component of the ZebraLab system. The white light intensity of the ZebraBox was 500 lux. Locomotor activity was assessed by a subtraction method used for detection of objects darker than background with a minimum object size. A threshold of 0.1 mm (minimum distance moved) was used for filtering all of the data to remove system noise. Locomotor endpoints were designed to express the changes in the general swimming activity in response to light-dark stimulus.

A short test of 14 minutes (the 'visual motor response test') was performed at 6 dpf as described elsewhere [94]. This test is used to record any abrupt change in locomotor activity (visual startle response) after a sudden shift from light to dark [54,55,94,117]. All experiments were done at a temperature of 28 ± 0.5°C. The experimental recording protocol consisted of three phases. The first two minutes consisted of exposure to white light in the ZebraLab to allow the larvae to acclimatize to the new environment. This phase was necessary to ensure that changes in locomotor activity of the zebrafish larvae, due to handling of the plate or change of location, were not included in the main analysis.

After two minute **acclimatization phase**, the **basal phase** started. This consisted of four minutes in white light during which the basal locomotor activity of the zebrafish larvae was recorded. Immediately after the basal phase, the lights were suddenly turned off for four min. This is the **challenge phase**. Behavioural activity during the darkness of the challenge phase was recorded with the help of infrared light. A third phase, the **recovery phase** consisted of four min of white light exposure immediately after the challenge phase. Total distance moved for each minute during the 14 minute period was recorded. The average distance moved was calculated in all three phases (basal, challenge and recovery).

Morphological assessment

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (pH 7.2) at 4°C overnight. They were then rinsed five times in distilled water and dehydrated in a graded series of ethanol (25, 50, and 70%) for 5 min each. Embryos were rinsed in acid alcohol (1% concentrated hydrochloric acid in 70% ethanol) for 10 min. They were then

placed in filtered Alcian blue solution (0.03% Alcian blue in acid alcohol) overnight. The stain was differentiated in acid alcohol for 1 h and the embryos then washed 2x30 min in distilled water. All embryos remained in their original multiwall plates, so that each individual could be tracked throughout the entire experimental and analysis procedure. Analysis of embryo morphology was carried out using a stereo dissecting microscope. The phenotypes of malformations scored are defined in Table 3.2.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). One-way ANOVA was performed to analyse effect of various compounds on hatching rate and effect of compounds on locomotor activity. A Dunnett's post hoc test was used to analyse multiple comparisons.

Results

Hatching percentage

We divided the effects of compounds on hatching into three categories after doing one-way ANOVA followed by Dunnett's multiple comparison test:

(i) compounds which have no significant effect on hatching (Figure 3.2), namely 2,4-Dichlorophenoxyacetic acid [$F_{(4,10)}$=0.75, p=0.5801], MCPA [$F_{(3,8)}$=1.0, p=0.4411], barium chloride [$F_{(5,12)}$=0.8, p=0.5705], hexazinone [$F_{(5,12)}$=0.84, p=0.5464] and strontium chloride [$F_{(5,12)}$=2.4, p=0.0994].

(ii) compounds which delayed hatching (Figure 3.3), namely dimethoate [$F_{(5,12)}$=1029, p<0.0001], benzophenone [$F_{(2,6)}$=422.3, p<0.0001], triclopyr [$F_{(2,6)}$=558.3, p<0.0001], pendimethalin [$F_{(5,12)}$=406.2, p<0.0001], mercuric chloride [$F_{(2,6)}$=484, p<0.0001], stannic chloride [$F_{(5,12)}$=795, p<0.0001], maneb [$F_{(2,6)}$=993.5, p<0.0001], hydroquinone [$F_{(3,8)}$=400, p<0.0001], acephate [$F_{(4,10)}$=527.2, p<0.0001], gallium chloride [$F_{(2,6)}$=2257, p<0.0001], erbium chloride [$F_{(5,12)}$=253.2, p<0.0001], diazinon [$F_{(4,10)}$=475, p<0.0001], molinate [$F_{(5,12)}$=417.3, p<0.0001], zinc chloride [$F_{(5,12)}$=950.7, p<0.0001] and bromacil [$F_{(4,10)}$=975.8, p<0.0001].

Table 3.3 Geometric series concentrations used in the study after evaluation with range-finding series.

Compounds		**Geometric series concentrations mg/l**				
		C1	C2	C3	C4	C5
1	2,4-dichlorophenoxyacetic acid	10	20	40	80	160
2	Acephate	1000	2000	4000	8000	16000
3	Amitrol	1000	2000	4000	8000	16000
4	Barium chloride	10	20	40	80	160
5	Benzophenone	70	140	280	560	1120
6	Bromacil	100	200	400	800	1600
7	Diamethoate	100	200	400	800	1600
8	Diazinone	10	20	40	80	160
9	Erbium chloride	10	20	40	80	160
10	Gallium chloride	100	200	400	800	1600
11	Glyphosate	10	20	40	80	160
12	Hexazinone	100	200	400	800	1600
13	Hydroquinone	1	2	4	8	16
14	Maneb	8	16	32	64	128
15	MCPA	10	20	40	80	160
16	Mercuric chloride	0.1	0.2	0.4	0.8	1.6
17	Methomyl	10	20	40	80	160
18	Molinate	10	20	40	80	160
19	Paraquat	100	200	400	800	1600
20	Pendimethalin	100	200	400	800	1600
21	Stannic chloride	8	16	32	64	128
22	Strontium chloride	500	1000	2000	4000	8000
23	Triclopyr	70	140	280	560	1120
24	Zinc chloride	10	20	40	80	160

(iii) compounds which accelerated hatching (Figure 3.4), namely methomyl [$F_{(6,14)}$=484, p<0.0001], glyphosate [$F_{(2,6)}$=206, p<0.0001], paraquat [$F_{(4,10)}$=18.79, p<0.0001], and amitrol [$F_{(6,14)}$=205.9, p<0.0001].

Malformations

The malformations produced by the test compounds are summarized in Table 3.7. The compounds producing malformations in survivors were: glyphosate, 2,4-dichlorophenoxyacetic acid, diazinon, paraquat, methomyl and molinate (Table 3.7.) Mercuric chloride, gallium chloride and benzophenone produced lethality at all concentrations tested and hence malformations in survivors were not observed. The remaining compounds did not produce any of the malformations described in Table 3.2.

LC_{50} value and correlation with LD_{50} values of rodents from the literature

The LC_{50} values of zebrafish larvae determined after 96 h exposure to test compounds, and their corresponding LD_{50} values in rodents taken from the literature, are shown in Table 3.6.

The relative toxicity ([zebrafish LC_{50} mmol/L] ÷ [rodent LD_{50} mmol/kg]) of individual compounds is shown in Figure 3.5. Compounds which were less toxic in zebrafish than in rodents include bromacil, dimethoate, diazinon, glyphosate, haxezinone, MCPA, molinate, 2,4-Dichlorophenoxyacetic acid, acephate, barium chloride, benzophenone, erbium chloride, gallium chloride, hydroquinone, maneb, mercuric chloride, pendimethalin, triclopyr and zinc chloride. The compounds which were more toxic in zebrafish than in rodents were methomyl, paraquat, strontium chloride and stannic chloride.

Table 3.4: Cumulative percentage mortality recorded in 5d larvae after 96 h exposure.

Cumulative percentage mortality												
Compounds		Logarithmic series ‡ (mg/L)					Geometric series* (mg/L) ± SEM					
		0	1	10	100	1000	C0	C1	C2	C3	C4	C5
1	2,4-dichlorophenoxyacetic acid (2,4-D)	0	0	0	81.25	100	0±0	0 ± 0	0±0	0±0	56.25±	100±0
2	Acephate	0	0	0	0	0	0±0	0±0	0±0	4±2	64±2	100±0
3	Amitrol	0	0	0	0	0	0±0	0±0	0±0	0±0	0±0	0±0
4	Barium chloride	0	0	0	87.5	93.75	0±0	0±0	0±0	0±0	58±2	100±0
5	Benzophenone	0	0	0	31.25	100	0±0	60±5	100±0	100±0	100±0	100±0
6	Bromacil	0	0	0	31.25	100	0±0	0±0	12.5±	94±	100±0	100±0
7	Diamethoate	0	0	0	6.25	100	0±0	0±0	0±0	0±0	88±	100±0
8	Diazinon	0	0	6.25	100	100	0±0	0±0	12.5±	100±0	100±0	100±0
9	Erbium chloride	0	0	6.25	100	100	0±0	0±0	0±0	0±0	96±2	100±0
10	Gallium chloride	0	0	0	0	100	0±0	0±0	100±0	100±0	100±0	100±0
11	Glyphosate	0	6.25	6.25	100	100	0±0	0±0	0±0	0±0	0±0	100±0

12	Hdroquinone	0	0	100	100	100	0±0	0±0	0±0	31±4	100±0	100±0
13	Hexazinone	0	0	0	0	100	0±0	0±0	6.25±	62.5±	100±0	100±0
14	Maneb	0	0	31.25	100	100	0±0	0±0	2±2	4±4	100±0	100±0
15	2-methyl-4-chlorophenoxyacetic acid (MCPA)	0	6.25	6.25	100	100	0±0	0±0	0±0	6.25±	100±0	100±0
16	Mercury chloride	0	100	100	100	100	0±0	0±0	60±2	100±0	100±0	100±0
17	Methomyl	0	0	12.5	87.5	100	0±0	43.75±	81.25±	81.25±	93.75±	87.5±
18	Molinate	0	0	6.25	100	100	0±0	0±0	6.25±	50±	100±0	100±0
19	Paraquat	0	0	0	0	87.5	0±0	0±0	0±0	6.25±	100±0	100±0
20	Pendimethalin	0	0	0	0	68.75	0±0	0±0	8±2	58±2	98±2	100±0
21	Strontium chloride	0	0	0	0	12.5	0±0	0±0	2±2	12±4	6±4	35±2
22	Tin chloride	0	0	0	100	100	0±0	0±0	0±0	0±0	14±2	100±0
23	Triclopyr	0	0	0	43.75	100	0±0	77±2	98±2	100±0	100±0	100±0
24	Zinc chloride	0	0	0	81.25	100	0±0	0±0	0±0	6±4	100±0	100±0

Key: (‡) This was a one-time range-finding experiment and hence there is no SEM.

(*) = Toxicity of each compound was different with the logarithmic range-finding so a different geometric scale was used for each compound. The values given are the mean percentage mortality; the geometric series concentrations are given for each compound in Table 3.3. n = 48 (3 replications x16) embryos

Table 3.5: Concentrations used in geometric series. For each compound, a geometric series of concentrations (C0 – C5) was used, based on the results of the logarithmic range-finding series.

	Compounds	**Concentrations in geometric series (mg/l)**					
		C0	C1	C2	C3	C4	C5
1	2,4-dichlorophenoxyacetic acid	0	10	20	40	80	160
2	Acephate	0	1000	2000	4000	8000	16000
3	Amitrol	0	1000	2000	4000	8000	16000
4	Barium chloride	0	10	20	40	80	160
5	Benzophenone	0	70	140	280	560	1120
6	Bromacil	0	100	200	400	800	1600
7	Diamethoate	0	100	200	400	800	1600
8	Diazinon	0	10	20	40	80	160
9	Erbium chloride	0	10	20	40	80	160
10	Gallium chloride	0	100	200	400	800	1600
11	Glyphosate	0	10	20	40	80	160
12	Hexazinone	0	100	200	400	800	1600
13	Hydroquinone	0	1	2	4	8	16
14	Maneb	0	8	16	32	64	128
15	MCPA	0	10	20	40	80	160
16	Mercury chloride	0	0.1	0.2	0.4	0.8	1.6
17	Methomyl	0	10	20	40	80	160
18	Molinate	0	10	20	40	80	160
19	Paraquat	0	100	200	400	800	1600
20	Pendimethalin	0	100	200	400	800	1600
21	Stannic chloride	0	8	16	32	64	128
22	Strontium chloride	0	500	1000	2000	4000	8000
23	Triclopyr	0	70	140	280	560	1120
24	Zinc chloride	0	10	20	40	80	160

Table 3.6: Zebrafish embryo LC_{50} values calculated in present study, and the corresponding rodent LD_{50} oral values based on the literature.

Compounds		**Zebrafish embryo LC_{50} (mg/l ±sem)**	**Zebrafish embryo LC_{50} (mmol/l ±sem)**	**Rodent LD_{50} (mg/kg)**	**Rodent LD_{50} (mmol/kg)**
1	2,4-dichlorophenoxyacetic acid	65.3±0.41	0.29±0.030	370(#)	1.67
2	Acephate	5489.31±16.9	29.97±0.09	233(*)	1.27
3	Amitrol	ND	ND	11000(*)	130.83
4	Barium chloride	135.35±3.26	0.65±0.03	132(*)	0.63
5	Benzophenone	89.28±18.74	0.49±0.12	2895(*)	15.89
6	Bromacil	270.2±0.67	1.03±0.071	5175(*)	19.82
7	Diamethoate	684.3±2.04	2.97±0.002	60(*)	0.26
8	Diazinon	27.5±0.38	0.09±0.024	96(*)	0.32
9	Erbium chloride	101.24±0.66	0.37±0	4417(♦)	16.14
10	Gallium chloride	334.55±1.17	1.90±0.01	4700(*)	26.69
11	Glyphosate	95.9±0.23	0.56±0.008	1568(*)	9.27
12	Hdroquinone	4.40±0.33	0.04±0	245(*)	2.23
13	Hexazinone	361.8±0.57	1.43±0.013	1690(*)	6.7
14	Maneb	42.45±0.35	0.16±0	2600(*)	9.80
15	2-methyl-4-chlorophenoxyacetic acid (MCPA)	47.2±0.3	0.23±0.010	550(*)	2.74
16	Mercuric chloride	0.27±0.00	0.001±0	6(⌘)	0.02
17	Methomyl	59.7±0.39	0.367±0.03	10(*)	0.06

18	Molinate	49.2±0.43	0.26±0.016	530(*)	2.83
19	Paraquat	500.8±0.70	1.94±0.034	120(*)	0.47
20	Pendimethalin	376.96±1.06	1.34±0.01	1340(*)	4.76
21	Stannic chloride	25841.98±2.3	0.81±0.01	46(*)	0.18
22	Strontium chloride	211.02±28.57	163.01±0.2	1874(☼)	11.82
23	Triclopyr	69.24±0.17	0.27±0	729(*)	2.84
24	Zinc chloride	89.96±2.02	0.66±0.02	350(*)	2.57

Key:

ND = Not determined

(*) = from Hazaradous Substances Data Bank at http://toxnet.nlm.nih.gov

(#) = from Extension Toxicology Network at http://extoxnet.orst.edu

(✞) = from ChemIDplus Advanced at http://chem.sis.nlm.nih.gov/chemidplus/cas/10138-41-7

(⌘) = from Material Safety Data Sheet at http://avogadro.chem.iastate.edu/MSDS/HgCl2.htm

(☼) = from http://www.guidechem.com/msds/10025-70-4.html

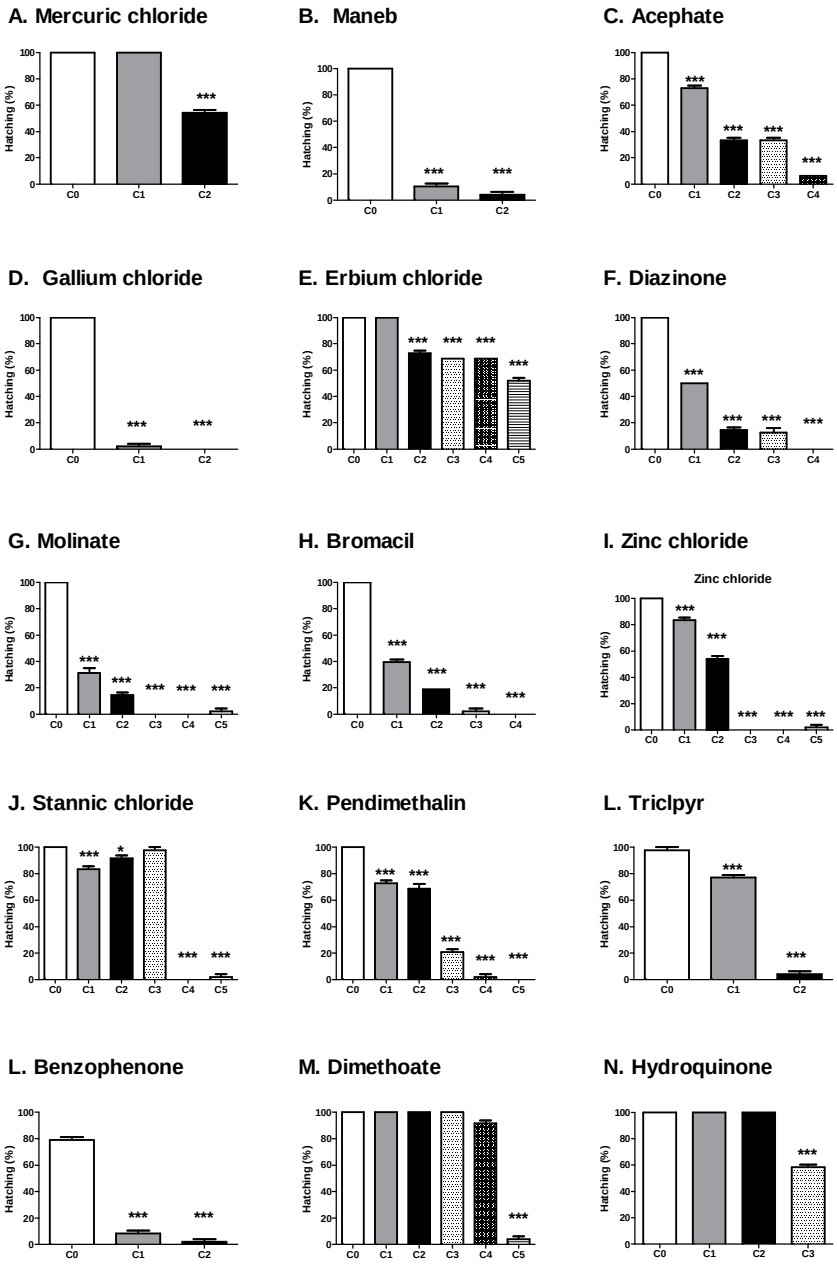

Figure 3.2. Hatching percentage after exposure to compounds that caused dose-dependent delay in hatching (as indicated by percent survivors hatched at 72 hpf). Data presented as ± SEM. * = <0.05

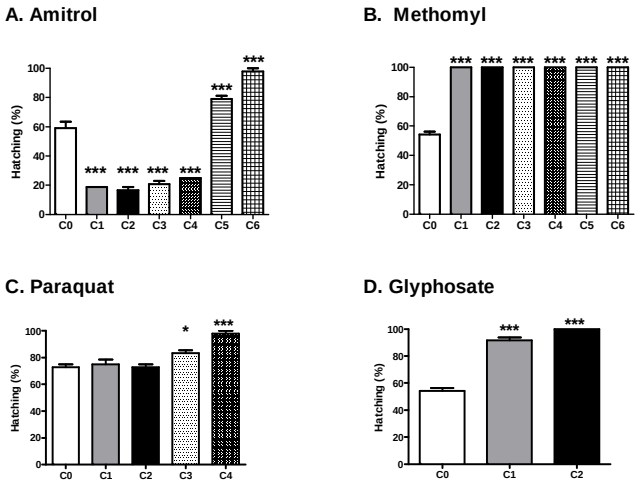

Figure 3.3: Hatching percentage after exposure to compounds that caused dose-dependent acceleration of hatching (as indicated by percent survivors hatched at 48 hpf). Data presented as ± SEM. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

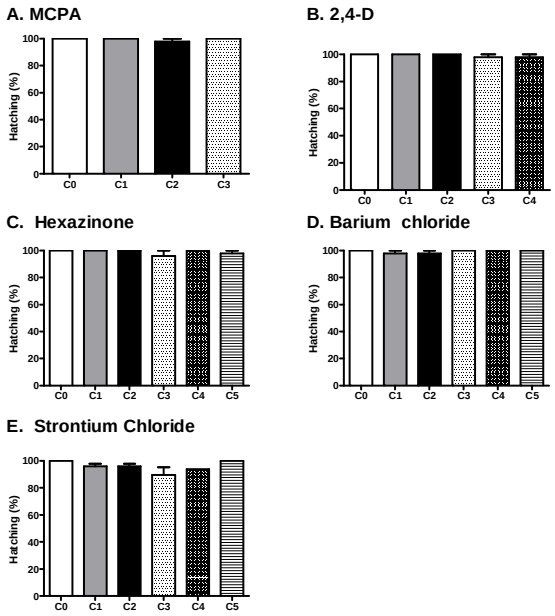

Figure 3.4. Hatching percentage after exposure to compounds that had no effect on hatching at 72 hpf. Data presented as ± SEM.

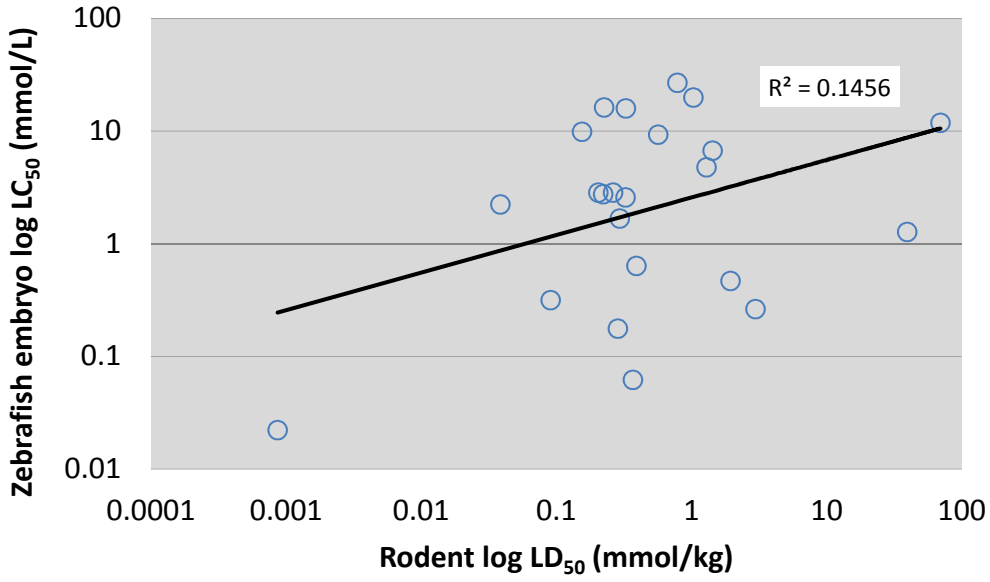

Figure 3.5: Relative toxicity of individual compounds tested in this study. Zebrafish embryo LC_{50} was determined based on cumulative mortality after 96 h exposure of compounds from three independent experiments and rodent LD_{50} was taked from the literature.

Locomotor activity

The visual motor response test was used to assess the integrity of the central and peripheral nervous system together with visual and musculoskeletal system development. Four distinct responses were found as follows:

Monotonic stimulation

One-way ANOVA followed by Dunnet's test for multiple comparisons showed that the locomotor activity of zebrafish larvae in the challenge phase was significantly increased (Figure 3.6) by: paraquat [$F_{(3,58)}$=7.439, p<0.001], stannic chloride [$F_{(3,58)}$=4.981, p=0.0038] and amitrol [$F_{(5,89)}$=4.155, p<0.001].

Table 3.7 : Malformations produced by varying concentrations (geometric series) of test compounds.

Compounds		**C0**	**C1**	**C2**	**C3**	**C4**	**C5**
1	2,4-dichlorophenoxyacetic acid	0	DP	DP	DP, BT	DP, BT	X
2	Acephate	0	0	0	0	0	X
3	Amitrol	0	0	0	0	DP	DP
4	Barium chloride	0	0	0	BB	PE, BB	X
5	Benzophenone	0	YSE, DP	X	X	X	X
6	Bromacil	0	0	0	0	X	X
7	Diamethoate	0	0	0	0	0	X
8	Diazinon	0	SB, YSE, DP	SB, YSE, DP, PE	X	X	X
9	Erbium chloride	0	0	0	SB	X	X
10	Gallium chloride	0	NH, DP	X	X	X	X
11	Glyphosate	0	SB, YSE, DP	SB, YSE, DP	SB, YSE, DP	SB, YSE, DP	X
12	Hexazinone	0	0	DP, YSE	DP, YSE	X	X
13	Hydroquinone	0	DP	DP	DP	X	X
14	Maneb	0	0	PE	PE	X	X

15	2-methyl-4-chlorophenoxyacetic acid (MCPA)	0	0	0	0	X	X
16	Mercury chloride	0	PE	X	X	X	X
17	Methomyl	0	SB, DP, PE, *	SB, DP, *	SB, DP, *	SB, DP,*	SB, DP,*
18	Molinate	0	0	DP,YSE	DP,YSE	X	X
19	Paraquat	0	DP,*	SB, BT, YSE,*	SB, BT, YSE, DP,*	X	X
20	Pendimethalin	0	0	0	0	0	X
21	Stannic chloride	0	0	0	0	DP	X
22	Strontium chloride	0	0	0	0	0	0
23	Triclopyr	0	SB	SB, BT	X	X	X
24	Zinc chloride	0	0	YSE	YSE	X	X

Key: X = dead; 0 = No observed malformation; SB = short body; DP = dispersed pigmentation; YSE = Yolk sac oedema; PE = pericardial oedema; BT = bent tail; NH = not hatched

*= these embryos typically moved for a brief movement with signs of shivering on being touched.

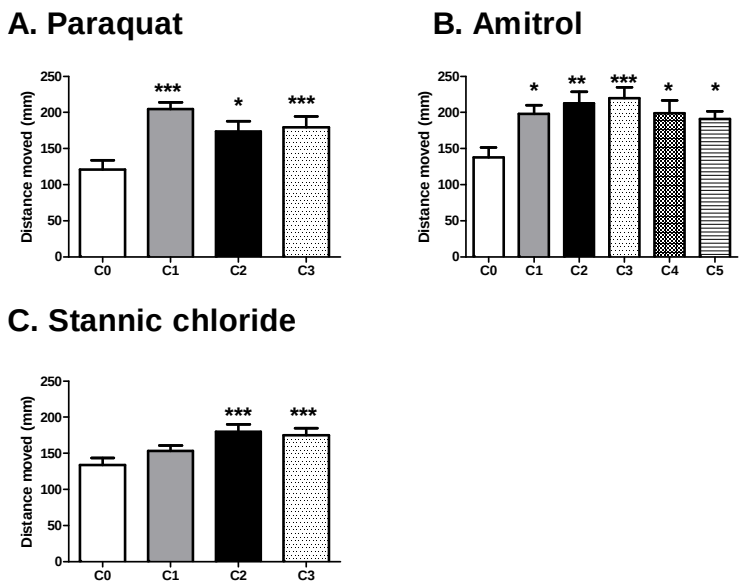

Figure 3.6: Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5 dpf. All these compounds displayed a significant concentration-dependent increase in distance moved. Error bars represent ±SEM of N=48 control and survived embryos for each concentration of each compound from three independent experiments. Data presented as ± SEM. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

Monotonic suppression

One-way ANOVA test followed by Dunnet's post hoc test for multiple comparisons showed that an increase in the concentration of compound caused significant suppression of locomotor activity in the challenge phase (Figure 3.7) with: strontium chloride [$F_{(5,78)}$=37.90, p<0.0001], zinc chloride [$F_{(3,53)}$=7.506, p<0.001], pendimethaline [$F_{(3,48)}$=28.13, p<0.0001], diazinon [$F_{(2,42)}$=5.267, p<0.001], hexazinone [$F_{(3,49)}$=16.08, p<0.0001], methomyl [$F_{(3,27)}$=25.75, p<0.0001], molinate [$F_{(3,51)}$=20.61, p<0.0001], dimethoate [$F_{(3,60)}$=13.31, p<0.0001] and barium chloride [$F_{(4,63)}$=10.80, p<0.0001].

Biphasic response (dose dependent stimulation and suppression)

One-way ANOVA test followed by Dunnet's post hoc test for multiple comparisons showed a significant difference in the locomotor activity of some compounds, at certain

concentrations tested, and controls (Figure 3.8). In these cases, the locomotor activity increased with increasing concentration, and then decreased at yet higher concentrations. The compounds with this biphasic response were erbium chloride [$F_{(3,58)}$=20.28, p<0.0001], 2,4-dichlorophenoxyacetic acid [$F_{(4,66)}$=9.143, p<0.0001] and hydroquinone [$F_{(3,56)}$=12.58, p<0.0001].

No effect

For some compounds, the locomotor activity of zebrafish larvae was unaffected, regardless of concentration tested (Figure 3.9). One-way ANOVA test followed by Dunnet's post hoc test for multiple comparisons showed no significant difference in the locomotor activity between the various concentrations of compounds. The compounds without any effect on the locomotor activity were: maneb [$F_{(3,55)}$=2.26, p=0.0908], glyphosate [$F_{(4,73)}$=0.5964, p=0.6664], MCPA [$F_{(3,59)}$=2.272, p=0.0895] and bromacil [$F_{(2,42)}$=2.154, p=0.1287].

Discussion

Hatching

The first significant finding in the present study is that the hatching depends on the compound tested. Hatching is an essential step in zebrafish development, and delayed hatching makes zebrafish more susceptible to predators; complete inhibition of hatching may also result in death [272]. We found that the time of hatching is influenced by compound type, and by concentration. Many compounds tested caused hatching to be delayed (compared to controls). However, four compounds were associated with accelerated hatching, namely: amitrol, methomyl, paraquat and glyphosate. With amitrol, lower concentrations delayed hatching, while higher concentrations accelerated it. By contrast, lower concentrations of paraquat had no effect on hatching while higher concentrations accelerated the hatching as compared to control larvae. The higher concentrations of methomyl and glyphosate also accelerated the hatching. Fourteen compounds out of the 24 tested had no significant effect on the hatching rate.

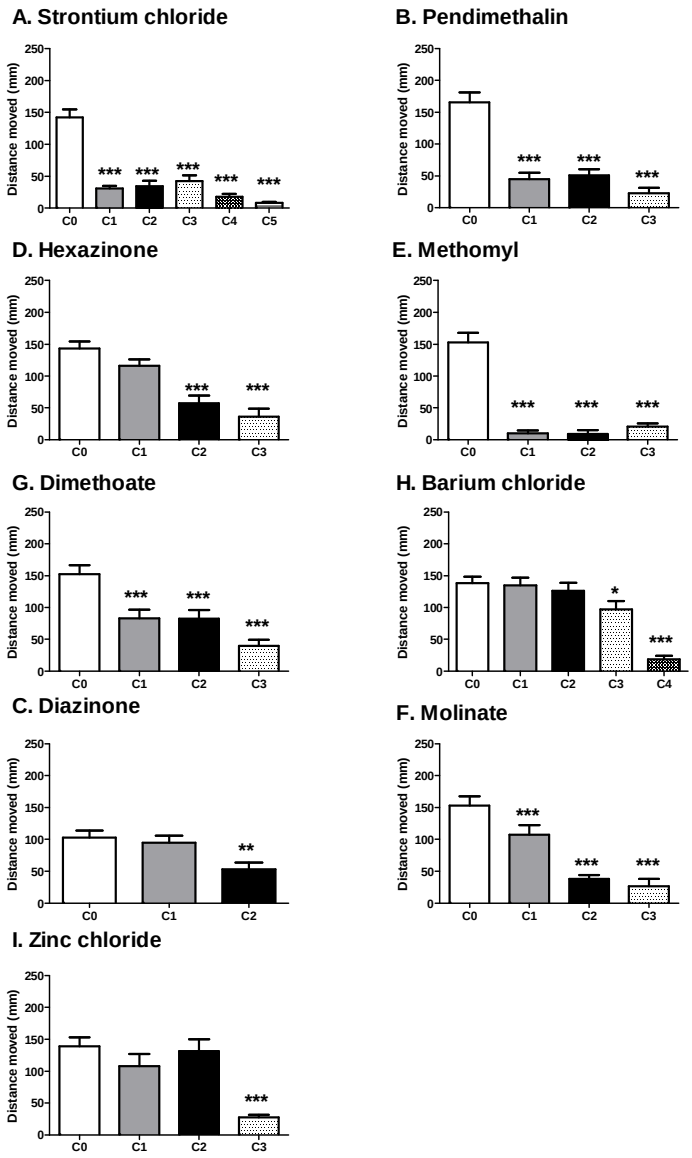

Figure 3.7. Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5 dpf. These compounds produced a significant concentration-dependent decrease in the locomotor activity. Error bars represent ±SEM of N=48 control and survived embryos for each concentration of each compound from three independent experiments. Data presented as ± SEM. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

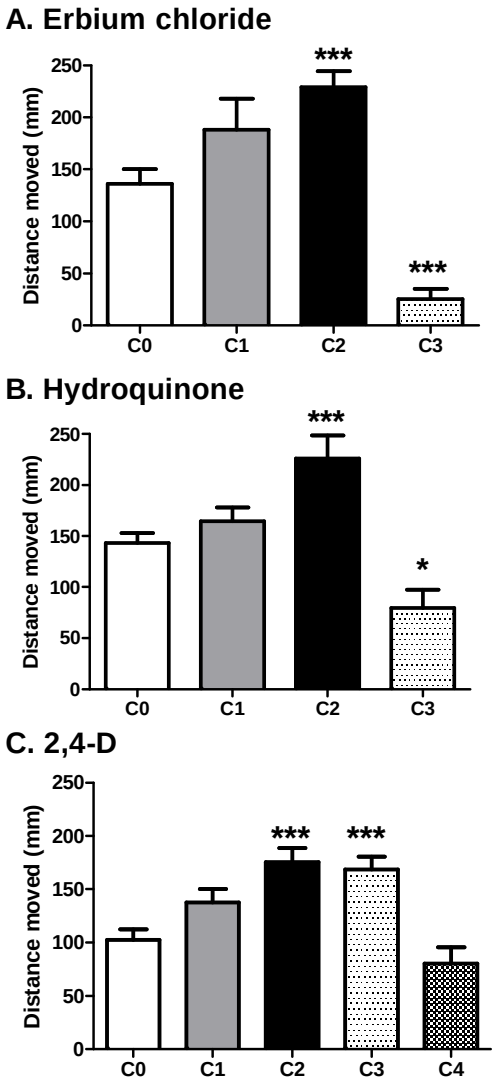

Figure 3.8: Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5 dpf. These compounds showed a significant concentration-dependent increase and then a decrease at a high concentration in the locomotor activity. Error bars represent ±SEM of N=48 control and survived embryos for each concentration of each compound from three independent experiments. Data presented as ± SEM. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

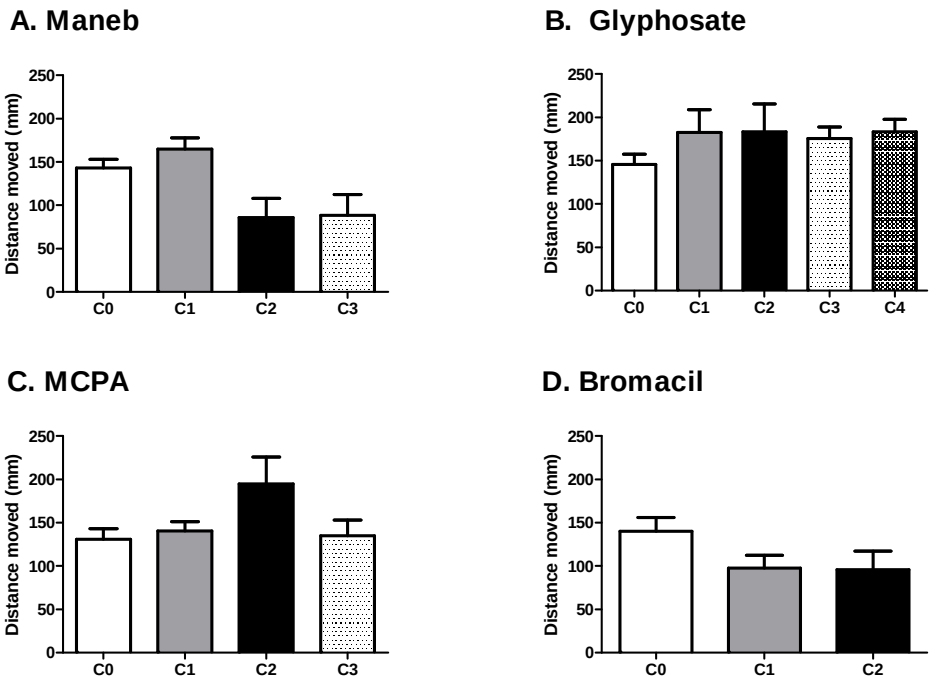

Figure 3.9. Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5 dpf. These compounds showed no significant difference in locomotor response as compared to control. Error bars represent ± SEM of n=48 controls and survivors for each concentration of each compound from three independent experiments.

Hatching in zebrafish takes place in two steps. The first step is the release of hatching enzyme by the hatching gland which breaks down the inner vitelline envelope of the acellular chorion [273]. The second step is the spontaneous movement of the embryo which starts around 19 hpf. The delayed hatching in the present case might be due to delay in the release of hatching enzyme or a delay in the spontaneous movement activity. Another explanation could lie in the presence of chorion around the zebrafish embryo. The 3.5 µm thick chorion [274] protects the zebrafish embryo against the toxic effects of compounds [275], and acclimation to different toxins [276]. The chorion is relatively impermeable, even to small molecules such as ethanol [94]. It is even possible that delayed hatching might allow the embryo to survive short-term exposure of compounds, which would have killed the hatched (non-chorion-protected) larvae.

The mechanisms by which the toxicants studied here can accelerate or inhibit the hatching process remains to be determined. It will also be interesting to know what ecological or evolutionary consequences might be for a change in hatching time in response

to toxicant exposure. In any case, this phenomenon shows that the embryo can react to chemicals at concentrations at which larval survival is not affected. Although the mechanism and consequences of delayed or accelerated hatching are unknown, it is possible that hatching time may serve as a sublethal response variable for embryonic development in toxicity tests. Further work is required to examine these issues.

Morphological malformations

It has been found that the physical properties of chemicals did not fully predict lethality or developmental outcomes and individual outcomes such as pericardial oedema and yolk sac oedema might be reliable indicators of developmental toxicity for a range of compounds [251]. Thus, in order to see the teratogenic effects of compounds, we screened for malformations. We found that 7/24 compounds produced none of the morphological abnormalities in the zebrafish embryos described in Table 3.2 at any concentration. By contrast, 17/24 compounds produced one or more of the malformations described in Table 3.7. The most common abnormality in these larvae was dispersed melanocyte pigmentation on the body; this is considered an indication of stress [277]. The compounds 2,4-dichlorophenoxyacetic acid, paraquat and barium chloride produced axial curvature and deformed or bent tail. It has been suggested that these types of malformation might be due to delayed hatching [278], a conclusion consistent with the results of the present study.

Acephate, amitrol, strontium chloride, stannic chloride, bromacil, dimethoate, MCPA and erbium chloride caused no morphological deformities at any concentration. By contrast, diazinon, glyphosate, hexazinone, methomyl, molinate, 2,4-Dichlorophenoxyacetic acid were among the most teratogenic compounds tested resulting in multiple malformations. Benzophenone, gallium chloride and mercuric chloride killed the embryos without, at least in the range of concentrations studied here, producing malformations.

LC_{50} of zebrafish vs. LD_{50} of rodents

In the present study, the correlation between LC_{50} of zebrafish and LD_{50} of rodents was very weak for metals and biocides considered together (R2=0.1456). We compared the LC_{50} of zebrafish with oral LD_{50} in rodents taken from the literature. Where data were available from more than rodent species, we did not take the average, but used a single value from one study.

Table 3.8: Comparison of effects of selected compounds on zebrafish and rodents locomotor activity. The effect on zebrafish larvae are derived from the present study while the effect on rodent is derived from the literature.

Compound	**Effect of compound on zebrafish locomotor activity in present study**	**Effect of compound on rodent locomotor activity (from literature)**	**References**
Pendimethalin	Decreased	Decreased	[279]
Methomyl	Decreased	Decreased	[280]
Dimethoate	Decreased	Decreased	[281]
Hydroquinone	Decreased	Decreased	[282]
2,4-dichlorophenoxyacetic acid	Decreased	Decreased	[283]
Paraquat	Increased	Increased	[284]
Maneb	No effect	Decreased	[285]

The difference we find between LC_{50} of compounds in zebrafish and oral LD_{50} in rodents might be explained by various factors. The first is that we are comparing the developmental toxicity of a compound in the zebrafish embryo versus a rodent adult. Thus we are comparing different life stages. Secondly, the route of exposure should also be taken to into account. In case of the zebrafish embryos, we exposed chronically to compound for 96 h beginning at 24 hpf. In the early part of this period, there is a relatively impermeable chorion surrounding the embryo [274]. After hatching, the toxicant could, in principle, be absorbed through the skin, taken up by the gills, or absorbed from the pharynx or gut. Unfortunately, little is known about the absorption of drugs by zebrafish embryos. In the case of the rodent studies used here for comparison, compounds were administered orally. An important issue for futures studies using the zebrafish embryo model is to examine the route of absorption of compounds from the environment and to compare it with absorption in rodents and other mammals from the digestive tract or other routes.

It has been reported that zebrafish LC_{50} values of a variety of compounds correlate well with the corresponding LD_{50} values in rodents [98,286] and birds [287]. On this basis, it has been suggested that zebrafish embryos/larvae are a good alternative method for developmental toxicity studies [288]. However, it has also been emphasized that special care should be taken in considering predictivity because this parameter varies with the class of compounds [98]. The authors showed that the slope of the regression line (zebrafish LC_{50} *vs.* rodent LD_{50}) varied from 0.36 to 1.27 depending on the compound class. In another study, Parng and colleagues [286] showed that LC_{50} values of 11 out of 18 compounds were correlated with the LD_{50} values of those compounds in mice. Together, these studies suggest that the predictivity of the zebrafish embryo model is critically dependent on compound class.

Another example of comparative toxicology in the zebrafish model [289] used multiple approaches to study cell cycle inhibition of various compounds. Zebrafish embryos were tested to screen 16,320 compounds to assess the level of serine-10-phosphorylated-histone 3 (marker for mitotic cells). They also tested 17 known chemicals which can disrupt the cell cycle in mammals, and found that nine out of 17 compounds were positive. The other eight chemicals were active in the *in vitro* AB9 zebrafish fibroblast culture preparation making a total of 94% of tested compounds that were active in zebrafish assays. Thus, the authors concluded [289] that the drug target conservation between zebrafish and mammals is very high.

In summary, our results, together with other studies, suggest that although the zebrafish embryo is a valid alternative/complimentary model in toxicity studies, its use as a surrogate to predict rodent and human acute toxicity can depend strongly on the compound type.

Locomotor activity

In order to see the effect of compound type on locomotor activity, we used the visual motor response test at 5 dpf. This test has previously proved effective as a simple test for assessing effects of compounds. [49,55,87,94,122]. We chose larvae at 5 dpf, a time point at which they display a wide range of behavioural repertoires, and at which many organs are differentiated [33,290].

A number of compounds that we tested showed a significant concentration-dependent suppression of locomotor activity in the visual motor response test. These include agents that have a comparable effect in rodents. Pendimethalin and methomyl suppressed the

locomotor activity in the zebrafish larvae in the present study, and also in rodents [279,280]. The effect few compounds on the locomotor activity of rodents and zebrafish larvae is shown in Table 3.8.

On the other hand, some compounds increased the locomotor activity in the challenge phase as compared to controls in our study. Specifically, zebrafish larvae treated with amitrol, stannic chloride and paraquat showed hyperactivity in a concentration dependent manner. Paraquat-induced toxicity has been linked to Parkinson's-like neurological degenerative mechanisms both in rats [291] and in zebrafish [292]. It is possible that the hyperactivity of zebrafish larvae recorded in this study in the challenge phase was due to Parkinson-like tremors. Further work is required to examine this possibility.

Some compounds in this study showed a biphasic effect, that is, either stimulation or suppression of locomotor activity depending on the concentration. For example, erbium chloride, hydroquinone and 2,4-dichlorophenoxyacetic acid increased the locomotor activity in a concentration dependent manner at lower concentrations, but suppressed it at higher concentrations. A biphasic response has also been observed in rodents following exposure to toluene [293] and ethanol [294]. Hydroquinone in rodents has been known to decrease locomotor activity [282]. Similarly, 2,4-dichlorophenoxyacetic acid is also known to decrease the spontaneous locomotor activity in rats, contrary to our results where it was increased initially before decreasing at higher dose [283]. Possible explanations for the different responses in zebrafish in this study, compared to the rodent literature, could include the different route of exposure, as well as different concentrations in the tissues. Again, these findings emphasize the need for comparative studies of absorption of compounds in the zebrafish embryo.

When exposing zebrafish embryos to toxicants, there are several possible mechanisms for the effect on locomotor behaviour. For example, the toxicant could cause retarded development of the locomotor and nervous systems, and the latter could include visual impairment. Visual impairment has been implicated in the effects of ethanol on zebrafish because it causes abnormalities of eye development (i.e. microphthalmia; see [94]).

Hypoactivity can also be attributed to other malformations [129]. However, the presence of malformations cannot explain the hypoactivity seen in the present study after treatment with pendimethalin, strontium chloride and dimethoate, in which no malformations were present. In contrast, we found that larvae exposed to glyphosate were severely malformed but showed no difference in locomotor activity. In conclusion, there

are multiple factors which can contribute to the hyper- or hypoactivity in the zebrafish larvae and a single factor cannot explain all the variations in locomotion.

Conclusions

We have shown that different classes, and even different compounds within the same class, produce a range of different effects on zebrafish. Hatching was either delayed or accelerated depending on the compound, and the compounds produced a varying spectrum of malformations during development at different concentrations. Zebrafish larvae showed three types of behavioural responses: (i) hypoactivity; (ii) hyperactivity; and (iii) biphasic response (a dose-dependent shift between hypo- and hyperactivity). When the zebrafish LC_{50} values of compounds, derived in this study, were compared to published LD_{50} values in rodents, they showed poor correlation. It can be suggested that although the zebrafish embryo model has been embraced by the scientific community as an alternative model for screening the developmental toxicity potential of compounds, its predictivity for mammalian toxicity needs to be determined per compound class. More work is required to draw a general conclusion about the predictive power of the zebrafish model.

Acknowledgments

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