Effect of pesticides and metals on zebrafish embryo development and larval locomotor activity

Farooq Ahmad[1,2], Shaukat Ali[1,3] and Michael K. Richardson[1*]

1. Institute of Biology Leiden (IBL), Leiden University
   Sylvius Laboratory, Sylviusweg 72, 2333 BE, Leiden, The Netherlands
2. Present address: Department of Zoology, The Islamia University of Bahawalpur, Pakistan
3. Present address: Department of Zoology, Government College University, Lahore-54000, Lahore, Pakistan

*=Corresponding author: m.k.richardson@biology.leidenuniv.nl

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 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/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 or accelerated 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). 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, which has emerged as a valuable model in the field of research especially in the last decade (1). The advantages which have made it a popular model in research are manifold and include: external fertilisation and rapid development, low maintenance costs, easy, year-round spawning, rapid generation cycle (2-3 months), and ease of use for high-throughput screening (2). Its genome is also nearly completely sequenced and contains orthologues of 82% of human disease genes (3, 4). The zebrafish is used in many fields of biology research including behaviour (5-8), chemical toxicity (9-14), drug discovery (15-17) and in human disease modelling (18-21) by using forward and reverse genetic techniques together with large-scale, high-throughput screening. However, more information is needed on the predictivity of the zebrafish model in toxicity, that is, to what extent does the toxicity of compounds tested on zebrafish correlate with their toxicity in mammals (especially rodents and humans)?

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, and includes metals and organic compounds (22, 23) and mixtures of drugs (24). The main emphasis in these studies has been on lethality, embryo survival rate and organ malformation as general assay parameters, and demonstrated that zebrafish exhibit good dose-responsiveness to toxicity and are a suitable animal model for toxicity screening (14, 25, 26).

The use of zebrafish in behavioural neuroscience is in its infancy compared to the use of rodents (27). However, mutant zebrafish lines, morpholinos, high-throughput screening and new bioassays for toxic and therapeutic endpoints in zebrafish are likely to become more common. New technology is having a large impact on research, and this 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 (27-29). 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 (30, 31), optomotor response (32), optokinetic response (33-37), photokinesis (5) and visual motor response test (38-40) among many others.

It has long been known that behavioural patterns of animals including zebrafish can be altered by drugs and chemicals (41-43). These alterations are regarded as an observable expression of effects on nervous and locomotor systems (13). Some of the environmental chemicals, such as pesticides, can cause developmental neurotoxicity resulting in neurodevelopmental disorders in humans (44, 45). This makes it important to determine the effects of these chemicals on living animals and their behaviour.

Several classes of compound have been tested on zebrafish and assessed for their toxicity prediction in rodents. The predicitivity was found to vary considerably according to compound or compound class

(9, 46, 47). 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 they are very diverse chemically and because there is increasing awareness and concern regarding the environmental effects of these compounds (48, 49). For these reasons, the predictivity of the zebrafish in relation to the toxicity of these compounds in mammals is an important consideration, because it is a potential test model in environmental toxicology.

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 AB strain of zebrafish as different strains have differences in the locomotor activity (50). 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 artemias (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 the zebrafish text book

entitled *Zebrafish: a practical approach* (51). They were placed in 9.2 Petri dish containing 100 ml egg water (0,21 g/l Instant Ocean Sea Salt and 0,0005% (v/v) methyl 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

At 24 hours post fertilization (hpf), all embryos were checked for their natural spontaneous mortality as there are reports of an early natural death in zebrafish embryos cultured under certain conditions (9, 10). In order to avoid taking embryos during such a die-off, 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 96-well plate, one larva per well containing 250 µl egg water (control) or respective concentration of compound tested. Note that in order to eliminate further sources of disturbance/stress, the media was not refreshed except on 2dpf where the medium 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.

Morphological assessment

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline at 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. Embryos were subsequently differentiated in acid alcohol for 1 h and 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 dissecting stereo microscope. The phenotypes of malformations scored are defined in Table 1.

Table 1: Phenotypic endpoints scored in embryos at 5dpf. Some of these criteria have been described elsewhere (52).

Phenotype	Criteria
Yolksac	Yolksac enlarged by fluid accumulation
Heart	Pericardial sac enlarged by fluid accumulation
Meckel cartilage	Meckel cartilage grossly hypoplastic, missing or unfused in midline.
Melanocytes	Melanocytes aggregate and dispersed
Short body	Body was considered short if its total length was below 3.5mm at 6dpf
Tail	A bent on tail.

Body axis	**The trunk was bent on dorso-ventral axis**
Normal	**The larvae was considered with normal morphology if any of the above listed phenotypes were normal**

Egg water

Egg water was made from 0.21 g 'Instant Ocean®' salt in 1 L of Milli-Q water with resistivity of 18.2 MΩ cm.

Test COMPOUNDS

The compounds used in the present study are listed in the **Error! Reference source not found.**.

Table 2: List of compounds used in the study; all compounds were purchased from Sigma (Zwijndrecht, The Nethelrands).

	Compound	Compounds class	Molecular Formula	Molecular weight (g/mol)	Sigma Catalogue number
1	2,4-	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 test

A range-finding test was conducted using a logarithmic series to determine a suitable range of concentration (0, 1, 10, 100 and 1000 mg/L) as recommended in standard protocols (53). After 24hpf, zebrafish embryos were checked for the natural mortality and after removing dead embryos, healthy ones were transferred from Petri dish using a sterile plastic pipette into 96-well microtitre plates. A single embryo was placed in each well so that dead embryos 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. Each well contained 250 mL of either freshly prepared test compound; egg water (control) or vehicle (egg water with solvent where mentioned). 16 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 with concentrations lying in the range 0-100% mortality. The actual concentrations used are shown in Table S1. The concentrations were in a geometric series in which each was 50% greater than the next lowest value as recommended (53). 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.

Hatching and Mortality scoring

Hatching was monitored from 48-72 hpf which is the normal hatching period of zebrafish larvae (54). The hatching rate was recorded once all the embryos in any particular concentration were hatched.

Mortality rate (Table 3) 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 stopped beating and the change in appearance of tissues from a transparent to opaque.

Table 3: Cumulative % mortality recorded in 5d larvae after 96 h exposure

Cumulative % mortality											
Compounds		**Logarithmic series ‡ (mg/L)**					**Geometric series* (mg/L) ± SEM**				
		0	**1**	**10**	**100**	**1000**	**C0**	**C1**	**C2**	**C3**	**C4**	**C5**
	Compounds	**0**	**1**	**10**	**100**	**1000**	**C0**	**C1**	**C2**	**C3**	**C4**	**C5**
1	2,4-Dichlorophenoxyacetic	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 S1. N = 48 (3 replications x16) embryos

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 comprising of 14 minutes, called 'visual motor response test' was performed at 6 dpf as described elsewhere (55). All experiments were done at optimum temperature of 28 ± 0.5°C. The visual motor response test has been previously used as frequently alternating periods of light and dark for a very short duration (not more than 10 minutes). This test is used to check abrupt change of locomotor activity (also called as visual startle response) after sudden shift from light to dark (38, 55-57). The experimental recording protocol consisted of three phases. First two minutes were given in the ZebraLab to acclimatize in the new environment. This phase was necessary to make sure that basal locomotor activity of zebrafish larvae is without any bias resulting in handling of the plate or change of location and hence was not used in the further analysis. After this acclimatization, the basal phase started, and consisted of 4 minutes to measure the basal locomotor activity while light in the ZebraLab remained ON. Immediately after basal phase, the lights were suddenly turned off for 4 min to record sudden change of locomotor activity which is called as 'challenge phase'. Behavioural activity in the dark was also automatically recorded during this period with the help of infrared light. A third phase called 'recovery phase' was started immediately for 4 min after challenge phase to give zebrafish larvae time to recover from shock of darkness. All three phases consisted of 4-min to prevent habituation, and also to obtain more robust responses.

Endpoint

Total distance moved for each minute during the 14 minute period was recorded. Average distance moved was calculated in all 3 phases i.e. basal, challenge and recovery.

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

The hatching percentage was monitored from 48-72 hpf which is the normal hatching period (54). 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, 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] (Figure 1); (ii) compounds which delayed hatching, 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] (Figure 2); (iii) compounds which accelerated hatching, 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] (Figure 3).

Figure 1. Hatching percentage after exposure to compounds that caused dose-dependent delay in hatching (as indicated by percent survivors hatched at 72hpf).

Figure 2: Hatching percentage after exposure to compounds that caused dose-dependent acceleration of hatching (as indicated by percent survivors hatched at 48hpf)

Figure 3. Hatching percentage after exposure to compounds that had no effect on hatching at 72hpf.

Malformations

The malformations produced by the test compounds are summarized in Table 4. The features which were examined are yolk sac oedema, pericardial oedema, bent body, total length of the zebrafish larvae and pigmentation over the body. The compounds producing malformations in survivors were: Glyphosate, 2,4-Dichlorophenoxyacetic acid, diazinon, paraquat, methomyl and molinate (Table 4.) 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 1.

Table 4 : Malformations produced by varying concentrations (geometric series) of test compounds.

Compounds		C0	C1	C2	C3	C4	C5
1	2,4-Dichlorophenoxyacetic	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

Keys: 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.**

LC_{50} value calculation and correlation with LD_{50} values of rodents

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 5. No correlation was found between the zebrafish and rodent values. Thus, a correlation test produced spearman's rank correlation of -0.08498 (p=0.6999) and Pearson's correlation -0.1086 (p=0.6218) between zebrafish embryo LC_{50} and rodent LD_{50} values.

Table 5: 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	Rodent LD_{50} (mg/kg)	Rodent LD_{50} (mmol/kg)
1	2,4-Dichlorophenoxyacetic	65.3±0.41	0.29±0.030	370(#)	1.67
2	Acephate	5489.31±16.93	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±0.030	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±2.30	0.81±0.01	46(*)	0.18
22	Strontium Chloride	211.02±28.57	163.01±0.26	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

Keys:

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

The relative toxicity ([zebrafish LC_{50} mmol/L] ÷ [rodent LD_{50} mmol/kg]) of individual compounds is shown in Figure 4. Compounds which were less toxic in zebrafish than in rodents inlcude 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.

Figure 4: Relative toxicity of individual compounds tested in this study. Zebrafish embryo LC50 was determined based on cumulative mortality after 96 h exposure of compounds from three independent experiments and rodent LD50 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. On the basis of the visual motor response test, four distinct responses were found as follows:

Monotonic stimulation response

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 5). The compounds which showed monotonic stimulation response were: 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].

Figure 5: Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5dpf. 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. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

Monotonic suppression response

One-way ANOVA test followed by Dunnet's post hoc test for multiple comparisons showed that an increase in the concentration of the compounds caused suppression of locomotor activity (Figure 6). The locomotor activity of zebrafish larvae was significantly decreased in the challenge phase as compared to controls. The compounds with monotonic supression were: 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].

Figure 6. Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5dpf. These compounds showed 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. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

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 7). 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].

Figure 7: Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5dpf. 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. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

No effect

For some compounds, the locomotor activity of zebrafish larvae was unaffected, regardless of concentration tested (Figure 8). 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].

Figure 8. Distance moved during the challenge phase of the visual motor response test by zebrafish larvae at 5dpf. These compounds showed no significant difference in locomotor response as compared to control. Error bars represent ±SEM of N=48 control and survived embryos for each concentration of each compound from three independent experiments. Statistical icons: *=p<0.05, **=p<0.01 and ***=p<0.001

Discussion

Hatching

The first significant finding in the present study is that the differential hatching percentage 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 (58). We found that the time of hatching is influenced by compound type, and by concentration. Many compounds tested resulted in delayed hatching (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 concentration of paraquat did not have 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.

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 (59). The seconds step is the spontaneous movement of the embryo which starts around 19hpf until the hatching. 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. The other explanation lies in the presence of chorion around the zebrafish embryo. The 3.5 µm thick chorion (60) protects the zebrafish embryo against the toxic effects of compounds (61), and acclimation of different toxins (62). 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.

It remains to be elucidated how these chemicals can accelerate or inhibit the hatching process, and what the ecological consequences might be in the wild. However, 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; rather, individual outcomes such as pericardial oedema and yolk sac oedema are more reliable indicators of developmental toxicity (22). Thus, in order to see the teratogenic effects of compounds, we screened for malformations. It was found that 29% (7/24) of the compounds

339 produced none of the morphological abnormalities in the zebrafish embryos described in Table 1 at
340 any concentration. By contrast, 71% (17/24) compounds produced various malformations
341 summarized in Table 4. The most common abnormality in these larvae was dispersed pigmentation on
342 the body; this is considered an indication of stress (63). The compounds 2,4-Dichlorophenoxyacetic
343 acid, paraquat and barium chloride produced axial curvature and deformed or bent tail. It has been
344 suggested that these types of malformation might be due to delayed hatching (64), a conclusion
345 consistent with the results of the present study.

346 Acephate, amitrol, strontium chloride, stannic chloride, bromacil, dimethoate, MCPA and erbium
347 chloride caused no morphological deformities at any concentration. By contrast, diazinon, glyphosate,
348 hexazinone, methomyl, molinate, 2,4-Dichlorophenoxyacetic acid were among the most teratogenic
349 compounds tested resulting in multiple malformations. Benzophenone, gallium chloride and mercuric
350 chloride simply produced lethality.

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

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

356 The difference we find between LC_{50} of compounds in zebrafish and oral LD_{50} in rodents can be
357 explained by various factors. The first factor is that we are comparing the developmental toxicity of a
358 compound in the zebrafish embryo versus a rodent adult. Thus we are comparing different life stages.
359 Secondly, the route of exposure should also be taken to into account. In case of the zebrafish embryos,
360 we exposed chronically to compound for 96 h beginning at 24hpf. In the early part of this period, there
361 is a relatively impermeable chorion (3.5 µm thick, composed of three acellular layers) surrounding the
362 embryo (60). After hatching, the drug could, in principle, be absorbed through the skin, taken up by
363 the gills, or absorbed from the pharynx or gut. Little is known about the absorption of drugs by
364 zebrafish embryos. In the case of the rodent studies used here for comparison, compounds were
365 administered orally. An important issue for futures studies using the zebrafish embryo model is to
366 examine the route of absorption of compounds from the environment and to compare it with
367 absorption in rodents and other mammals from the digestive tract or other routes.

368 It has been reported that zebrafish LC_{50} values of a variety of compounds correlate well with the
369 corresponding LD_{50} values in rodents (9, 65) and birds (66). On this basis, it has been suggested that
370 zebrafish embryos/larvae are a good alternative method for developmental toxicity studies (67).
371 However, it has also been emphasised that special care should be taken in considering predictivity
372 because this parameter varies with the class of compounds (9). 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 (65) 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 (68) 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. They also tested 17 known chemicals which can disrupt the cell cycle in mammals, and found that 9 out of 17 compounds were positive. The other 8 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 (68) 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 5dpf. This test has previously proved effective as a simple locomotor behaviour test for assessing effects of compounds. (38, 40, 42, 55, 69). We chose larvae at 5dpf, a time point at which they display a wide range of behavioural repertoires, and at which many organs are differentiated (70).

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 supressed the locomotor activity in the zebrafish larvae in the present study, and also in rodents (71, 72). The effect of a few compounds on the locomotor activity of rodents and zebrafish larvae is shown in Table 6.

Table 6: 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	(71)

Methomyl	Decreased	Decreased	(72)
Dimethoate	Decreased	Decreased	(80)
Hydroquinone	Decreased	Decreased	(77)
2,4-Dichlorophenoxyacetic	Decreased	Decreased	(78)
Paraquat	Increased	Increased	(81)
Maneb	No effect	Decreased	(82)

399

400 On the other hand, some compounds increased the locomotor activity in the challenge phase as
401 compared to controls in our study. Specifically, zebrafish larvae treated with amitrol, stannic chloride
402 and paraquat showed hyperactivity in a concentration dependent manner. Paraquat-induced toxicity
403 has been linked to Parkinson's-like neurological degenerative mechanisms both in rats (73) and in
404 zebrafish (74). It is possible that the hyperactivity of zebrafish larvae recorded in this study in the
405 challenge phase was due to Parkinson-like tremors. Further work is required to examine this
406 possibility.

407 Some compounds in this study showed a biphasic effect, that is, either stimulation or suppression of
408 locomotor activity depending on the concentration. For example, erbium chloride, hydroquinone and
409 2,4-dichlorophenoxyacetic acid increased the locomotor activity in a concentration dependent manner
410 at lower concentrations, but suppressed it at higher concentrations. A biphasic response has also been
411 observed in rodents following exposure to toluene (75) and ethanol (76). Hydroquinone in rodents has
412 been known to decrease locomotor activity (77). Similarly, 2,4-dichlorophenoxyacetic acid is also
413 known to decrease the spontaneous locomotor activity in rats, contrary to our results where it was
414 increased initially before decreasing at higher dose (78). Possible explanations for the different
415 responses in zebrafish in this study, compared to the rodent literature, could include the different route
416 of exposure, as well as different concentrations in the tissues. Again, these findings emphasise the
417 need for comparative studies of absorption of compounds in the zebrafish embryo.

418 When exposing zebrafish embryos to toxicants, there are several possible mechanisms for the effect on
419 locomotor behaviour. For example, the toxicant could cause retarded development of the locomotor
420 and nervous systems, and the latter could include visual impairment. Visual impairment has been
421 implicated in the effects of ethanol on zebrafish because it causes abnormalities of eye development
422 (i.e. microphthalmia; see (55).

423 Hypoactivity can also be attributed to other malformations (79). However, the presence of
424 malformations cannot explain the hypoactivity seen in the present study after treatment with
425 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.

Conclusion

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 varying malformations during development at difference 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 LC_{50} values of compounds 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 wide scientific community as an alternative model for screening the developmental toxicity potential of compound, its predictivity for mammalian toxicity needs to be determined per compound class. More work is required to draw a general conclusion about predictive power of zebrafish model.

References

1. Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, Danio rerio. Biological Reviews. 2008;83(1):13-34.

2. Dahm R, Geisler R. Learning from small fry: The zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY). 2006;8(4):329-45.

3. Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci. 2005;86(1):6-19.

4. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498-503.

5. Ahmad F, Noldus LPJJ, Tegelenbosch RAJ, Richardson MK. Zebrafish embryos and larvae in behavioural assays. Behaviour. 2012;149(10-12):1241-81.

6. Fleisch VC, Neuhauss SC. Visual behavior in zebrafish. Zebrafish. 2006;3(2):191-201.

7. Haug MF, Biehlmaier O, Mueller KP, Neuhauss SC. Visual acuity in larval zebrafish: behavior and histology. Front Zool. 2010;7:8.

8. Richendrfer H, Pelkowski SD, Colwill RM, Creton R. On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res. 2012;228(1):99-106.

9. Ali S, van Mil HGJ, Richardson MK. Large-Scale Assessment of the Zebrafish Embryo as a Possible Predictive Model in Toxicity Testing. PLoS One. 2011;6(6).

10. Fraysse B, Mons R, Garric J. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotox Environ Safe. 2006;63(2):253-67.

11. McGrath P, Li CQ. Zebrafish: a predictive model for assessing drug-induced toxicity. Drug Discov Today. 2008;13(9-10):394-401.

12. Levin ED, Tanguay RL. Introduction to zebrafish: current discoveries and emerging technologies for neurobehavioral toxicology and teratology. Neurotoxicol Teratol. 2011;33(6):607.

13. Reiter L. An introduction to neurobehavioral toxicology. Environ Health Perspect. 1978;26:5-7.

14. McCollum CW, Ducharme NA, Bondesson M, Gustafsson JA. Developmental toxicity screening in zebrafish. Birth Defects Res C Embryo Today. 2011;93(2):67-114.

15. Barros TP, Alderton WK, Reynolds HM, Roach AG, Berghmans S. Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. Br J Pharmacol. 2008;154(7):1400-13.

16. Bowman TV, Zon LI. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. ACS Chem Biol. 2010;5(2):159-61.

17. Macrae CA. Cardiac Arrhythmia: In vivo screening in the zebrafish to overcome complexity in drug discovery. Expert Opin Drug Discov. 2010;5(7):619-32.

18. Ali S, Champagne DL, Spaink HP, Richardson MK. Zebrafish Embryos and Larvae: A New Generation of Disease Models and Drug Screens. Birth Defects Research Part C-Embryo Today-Reviews. 2011;93(2):115-33.

19. Guyon JR, Steffen LS, Howell MH, Pusack TJ, Lawrence C, Kunkel LM. Modeling human muscle disease in zebrafish. Biochimica Et Biophysica Acta-Molecular Basis of Disease. 2007;1772(2):205-15.

20. Newman M, Verdile G, Martins RN, Lardelli M. Zebrafish as a tool in Alzheimer's disease research. Biochim Biophys Acta. 2010.

21. Xia W. Exploring Alzheimer's disease in zebrafish. J Alzheimers Dis. 2010;20(4):981-90.

22. Ducharme NA, Peterson LE, Benfenati E, Reif D, McCollum CW, Gustafsson JA, et al. Meta-analysis of toxicity and teratogenicity of 133 chemicals from zebrafish developmental toxicity studies. Reprod Toxicol. 2013.

23. Vittozzi L, De Angelis G. A critical review of comparative acute toxicity data on freshwater fish. Aquat Toxicol. 1991;19(3):167-204.

24. Alsop D, Wood CM. Metal and pharmaceutical mixtures: Is ion loss the mechanism underlying acute toxicity and widespread additive toxicity in zebrafish? Aquat Toxicol. 2013;140-141C:257-67.

25. Eimon PM, Rubinstein AL. The use of in vivo zebrafish assays in drug toxicity screening. Expert Opin Drug Metab Toxicol. 2009;5(4):393-401.

495 26. Rubinstein AL. Zebrafish assays for drug toxicity screening. Expert Opin Drug Metab Toxicol.
496 2006;2(2):231-40.

497 27. Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for
498 investigating chemical toxicity. Toxicol Sci. 2005;86(1):6-19.

499 28. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev
500 Genet. 2007;8(5):353-67.

501 29. Zon LI. Zebrafish: a new model for human disease. Genome Res. 1999;9(2):99-100.

502 30. Ahmad F, Richardson MK. Exploratory behaviour in the open field test adapted for larval
503 zebrafish: Impact of environmental complexity. Behav Processes. 2013;92:88-98.

504 31. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-
505 like behaviors: a review. Eur J Pharmacol. 2003;463(1-3):3-33.

506 32. Krauss A, Neumeyer C. Wavelength dependence of the optomotor response in zebrafish
507 (Danio rerio). Vision Res. 2003;43(11):1273-82.

508 33. Brockerhoff SE. Measuring the optokinetic response of zebrafish larvae. Nat Protoc.
509 2006;1(5):2448-51.

510 34. Brockerhoff SE, Hurley JB, Neuhauss S, Driever W, Dowling JE. Optokinetic Responses in Wild-
511 Type and Mutant Larval Zebrafish. Invest Ophthalmol Vis Sci. 1995;36(4):S918-S.

512 35. Huang YY, Neuhauss SCF. The optokinetic response in zebrafish and its applications. Frontiers
513 in Bioscience-Landmark. 2008;13:1899-916.

514 36. Qian H, Zhu Y, Ramsey DJ, Chappell RL, Dowling JE, Ripps H. The optokinetic response of
515 larval zebrafish. Invest Ophthalmol Vis Sci. 2005;46.

516 37. Zou SQ, Yin W, Zhang MJ, Hu CR, Huang YB, Hu B. Using the optokinetic response to study
517 visual function of zebrafish. J Vis Exp. 2010(36).

518 38. Ali S, Champagne DL, Richardson MK. Behavioral profiling of zebrafish embryos exposed to a
519 panel of 60 water-soluble compounds. Behav Brain Res. 2012;228(2):272-83.

520 39. Emran F, Rihel J, Adolph AR, Dowling JE. Zebrafish larvae lose vision at night. Proc Natl Acad
521 Sci U S A. 2010;107(13):6034-9.

522 40. Emran F, Rihel J, Dowling JE. A behavioral assay to measure responsiveness of zebrafish to
523 changes in light intensities. J Vis Exp. 2008(20).

524 41. Werboff J, Dembicki EL. Toxic Effects of Tranquilizers Administered to Gravid Rats. J
525 Neuropsychiatry. 1962;4(2):87-91.

526 42. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. Zebrafish behavioral
527 profiling links drugs to biological targets and rest/wake regulation. Science. 2010;327(5963):348-51.

528 43. Rihel J, Schier AF. Behavioral screening for neuroactive drugs in zebrafish. Dev Neurobiol.
529 2012;72(3):373-85.

530 44. Boulet SL, Boyle CA, Schieve LA. Health Care Use and Health and Functional Impact of
531 Developmental Disabilities Among US Children, 1997-2005. Arch Pediat Adol Med. 2009;163(1):19-
532 26.

533 45. Hass U. The need for developmental neurotoxicity studies in risk assessment for
534 developmental toxicity. Reprod Toxicol. 2006;22(2):148-56.

535 46. Ton C, Lin YX, Willett C. Zebrafish as a model for developmental neurotoxicity testing. Birth
536 Defects Research Part a-Clinical and Molecular Teratology. 2006;76(7):553-67.

537 47. Vaughan M, van Egmond R. The use of the zebrafish (Danio rerio) embryo for the acute
538 toxicity testing of surfactants, as a possible alternative to the acute fish test. Altern Lab Anim.
539 2010;38(3):231-8.

540 48. van der Werf HMG. Assessing the impact of pesticides on the environment. Agriculture,
541 Ecosystems & Environment. 1996;60(2–3):81-96.

542 49. Kamrin MA. Pesticide profiles : toxicity, environmental impact, and fate. Boca Raton, FL:
543 CRC/Lewis Publishers; 1997. 676 p. p.

544 50. Vignet C, Begout ML, Pean S, Lyphout L, Leguay D, Cousin X. Systematic screening of
545 behavioral responses in two zebrafish strains. Zebrafish. 2013;10(3):365-75.

546 51. Nusslein-Volhard C, Dahm R. Zebrafish: OUP Oxford; 2002.

547 52. Beker van Woudenberg A, Wolterbeek A, Te Brake L, Snel C, Menke A, Rubingh C, et al. A
548 category approach to predicting the developmental (neuro) toxicity of organotin compounds: the
549 value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reprod Toxicol. 2013;41:35-44.

550 53. Agency USEP. Ecological Effects Test Guidelines: OPPTS 850.1075: Fish Acute Toxicity Test,
551 Freshwater and Marine. 1996.

552 54. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of Embryonic-
553 Development of the Zebrafish. Dev Dynam. 1995;203(3):253-310.

554 55. Ali S, Champagne DL, Alia A, Richardson MK. Large-scale analysis of acute ethanol exposure in
555 zebrafish development: a critical time window and resilience. PLoS One. 2011;6(5):e20037.

556 56. Steenbergen PJ, Richardson MK, Champagne DL. The use of the zebrafish model in stress
557 research. Prog Neuropsychopharmacol Biol Psychiatry. 2011;35(6):1432-51.

558 57. Steenbergen PJ, Richardson MK, Champagne DL. Patterns of avoidance behaviours in the
559 light/dark preference test in young juvenile zebrafish: a pharmacological study. Behav Brain Res.
560 2011;222(1):15-25.

561 58. Ong KJ, Zhao X, Thistle ME, Maccormack TJ, Clark RJ, Ma G, et al. Mechanistic insights into
562 the effect of nanoparticles on zebrafish hatch. Nanotoxicology. 2013.

563 59. Sano K, Inohaya K, Kawaguchi M, Yoshizaki N, Iuchi I, Yasumasu S. Purification and
564 characterization of zebrafish hatching enzyme - an evolutionary aspect of the mechanism of egg
565 envelope digestion. Febs J. 2008;275(23):5934-46.

566 60. Bonsignorio D, Perego L, Del Giacco L, Cotelli F. Structure and macromolecular composition
567 of the zebrafish egg chorion. Zygote. 1996;4(2):101-8.

568 61. Ozoh PTE. Malformations and Inhibitory Tendencies Induced to Brachydanio-Rerio (Hamilton-
569 Buchanan) Eggs and Larvae Due to Exposures in Low Concentrations of Lead and Copper Ions. Bull
570 Environ Contam Toxicol. 1979;21(4-5):668-75.

571 62. Dixon DG, Sprague JB. Acclimation to Copper by Rainbow-Trout (Salmo-Gairdneri) - a
572 Modifying Factor in Toxicity. Can J Fish Aquat Sci. 1981;38(8):880-8.

573 63. Iwashita M, Watanabe M, Ishii M, Chen T, Johnson SL, Kurachi Y, et al. Pigment pattern in
574 jaguar/obelix zebrafish is caused by a Kir7.1 mutation: Implications for the regulation of melanosome
575 movement. PLoS Genet. 2006;2(11):1861-70.

576 64. Helvik JV, Walther BT. Development of Hatchability in Halibut (Hippoglossus-Hippoglossus)
577 Embryos. Int J Dev Biol. 1993;37(3):487-90.

578 65. Parng C, Seng WL, Semino C, McGrath P. Zebrafish: a preclinical model for drug screening.
579 Assay Drug Dev Technol. 2002;1(1 Pt 1):41-8.

580 66. Van Leeuwen CJ, Grootelaar EM, Niebeek G. Fish embryos as teratogenicity screens: a
581 comparison of embryotoxicity between fish and birds. Ecotox Environ Safe. 1990;20(1):42-52.

582 67. Selderslaghs IWT, Van Rompay AR, De Coen W, Witters HE. Development of a screening assay
583 to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod Toxicol.
584 2009;28(3):308-20.

585 68. Murphey RD, Stern HM, Straub CT, Zon LI. A chemical genetic screen for cell cycle inhibitors
586 in zebrafish embryos. Chem Biol Drug Des. 2006;68(4):213-9.

587 69. MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. Locomotion in larval
588 zebrafish: Influence of time of day, lighting and ethanol. Neurotoxicology. 2009;30(1):52-8.

589 70. Ahmad F, Tegelenbosch RAJ, Noldus LPJJ, Richardson MK. Zebrafish embryos and larvae in
590 behavioural assays. Behaviour. 2012;149:1241-81.

591 71. Garg SK, Thaker AM, Verma SP, Uppal RP, Garg BD. Neurotoxic effects of pendimethalin--a
592 weedicide. Indian J Exp Biol. 1987;25(7):463-6.

593 72. Bracy OL, Doyle RS, Kennedy M, Mcnally SM, Weed JD, Thorne BM. Effects of Methomyl and
594 Ethanol on Behavior in the Sprague-Dawley Rat. Pharmacol Biochem Be. 1979;10(1):21-5.

595 73. Ossowska K, Smialowska M, Kuter K, Wieronska J, Zieba B, Wardas J, et al. Degeneration of
596 dopaminergic mesocortical neurons and activation of compensatory processes induced by a long-
597 term paraquat administration in rats: Implications for Parkinson's disease. Neuroscience.
598 2006;141(4):2155-65.

599 74. Bretaud S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in
600 Parkinson's disease. Neurotoxicol Teratol. 2004;26(6):857-64.

601 75. Hinman DJ. Biphasic dose-response relationship for effects of toluene inhalation on
602 locomotor activity. Pharmacol Biochem Behav. 1987;26(1):65-9.

603 76. Gingras MA, Cools AR. Analysis of the biphasic locomotor response to ethanol in high and low
604 responders to novelty: a study in Nijmegen Wistar rats. Psychopharmacology (Berl). 1996;125(3):258-
605 64.

606 77. Addendum to the Final Report on the Safety Assessment of Hydroquinone. J Am Coll Toxicol.
607 1994;13(3):167-230.

608 78. Bernard PA, Toyoshima E, Eccles CU, Mayer RF, Johnson KP, Max SR. 2,4-
609 Dichlorophenoxyacetic Acid (2,4-D) Reduces Acetylcholinesterase Activity in Rat Muscle. Exp Neurol.
610 1985;87(3):544-56.

611 79. Padilla S, Hunter DL, Padnos B, Frady S, MacPhail RC. Assessing locomotor activity in larval
612 zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicol Teratol. 2011;33(6):624-30.

613 80. Dell'Omo G, Pleskacheva MG, Wolfer DP, Lipp HP, Shore RF. Comparative effects of exposure
614 to an organophosphate pesticide on locomotor activity of laboratory mice and five species of wild
615 rodents. Bull Environ Contam Toxicol. 2003;70(1):138-45.

616 81. De Gori N, Froio F, Strongoli MC, De Francesco A, Calo M, Nistico G. Behavioural and
617 electrocortical changes induced by paraquat after injection in specific areas of the brain of the rat.
618 Neuropharmacology. 1988;27(2):201-7.

619 82. Morato GS, Lemos T, Takahashi RN. Acute exposure to maneb alters some behavioral
620 functions in the mouse. Neurotoxicol Teratol. 1989;11(5):421-5.

621

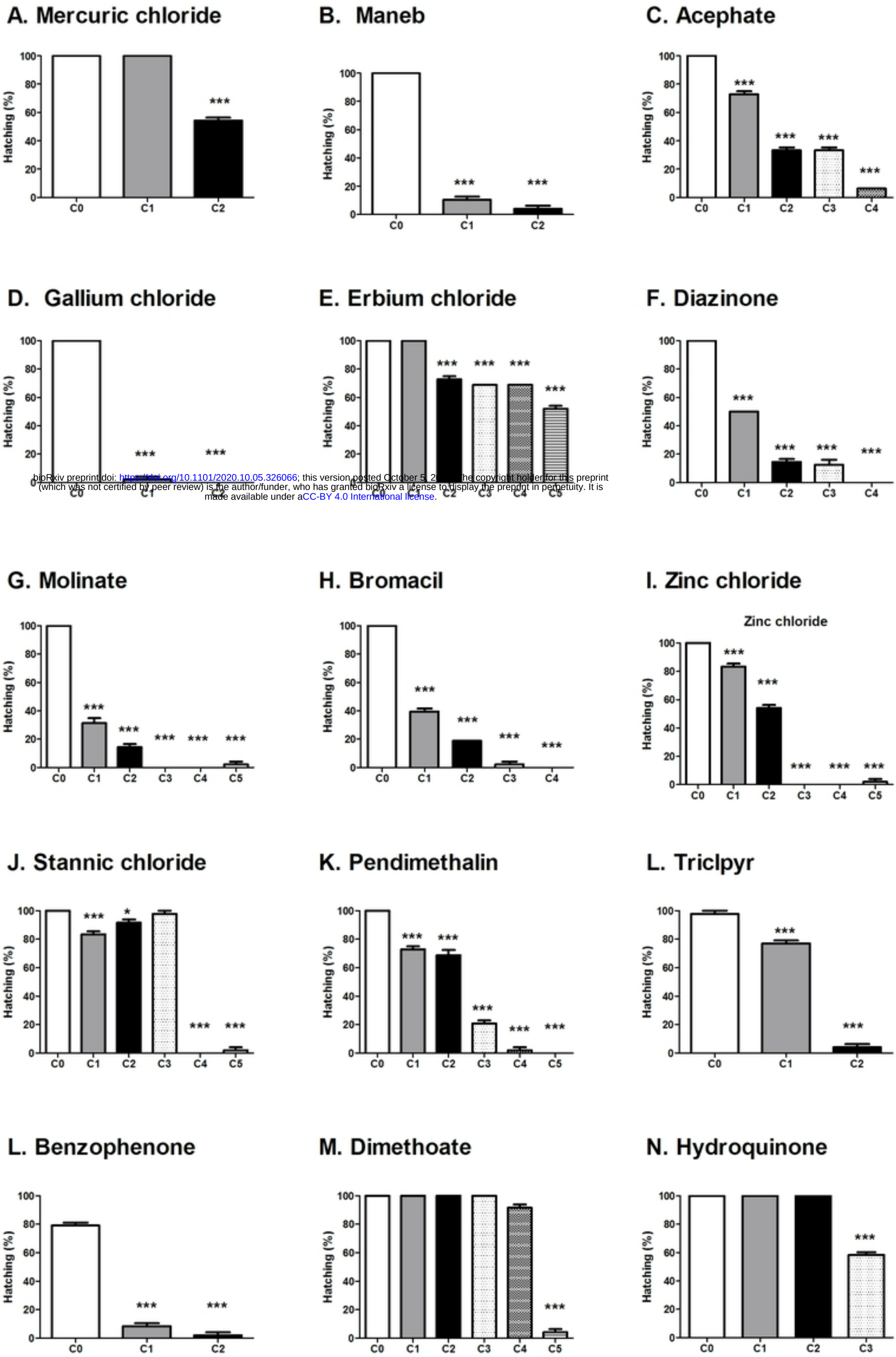


Figure 1

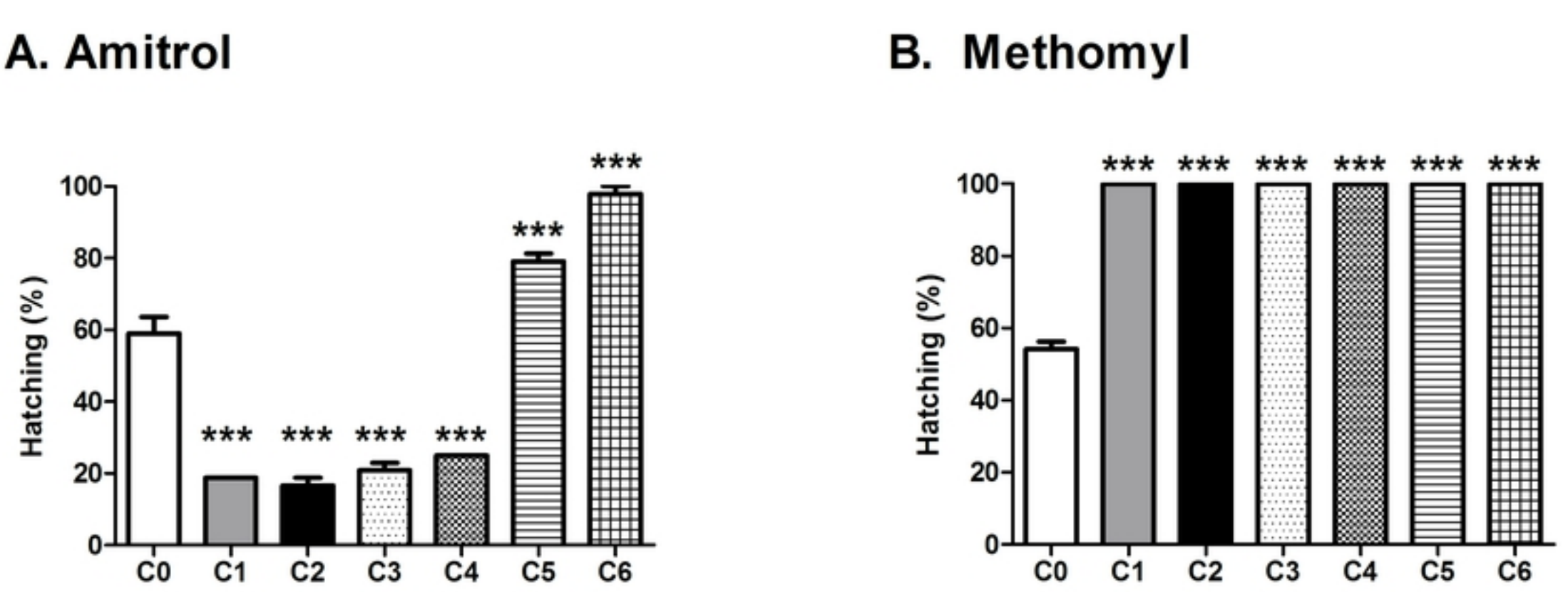
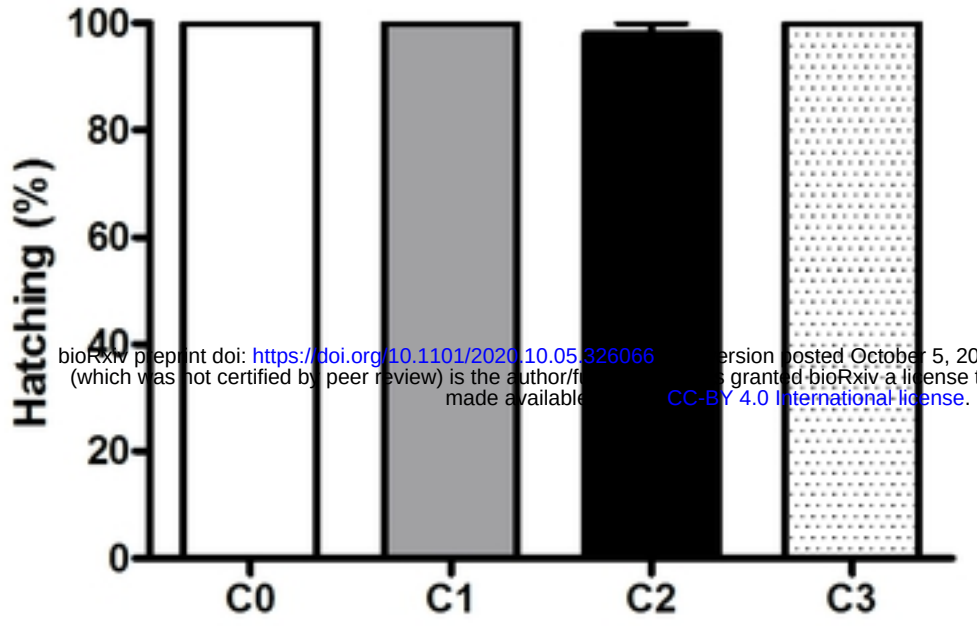
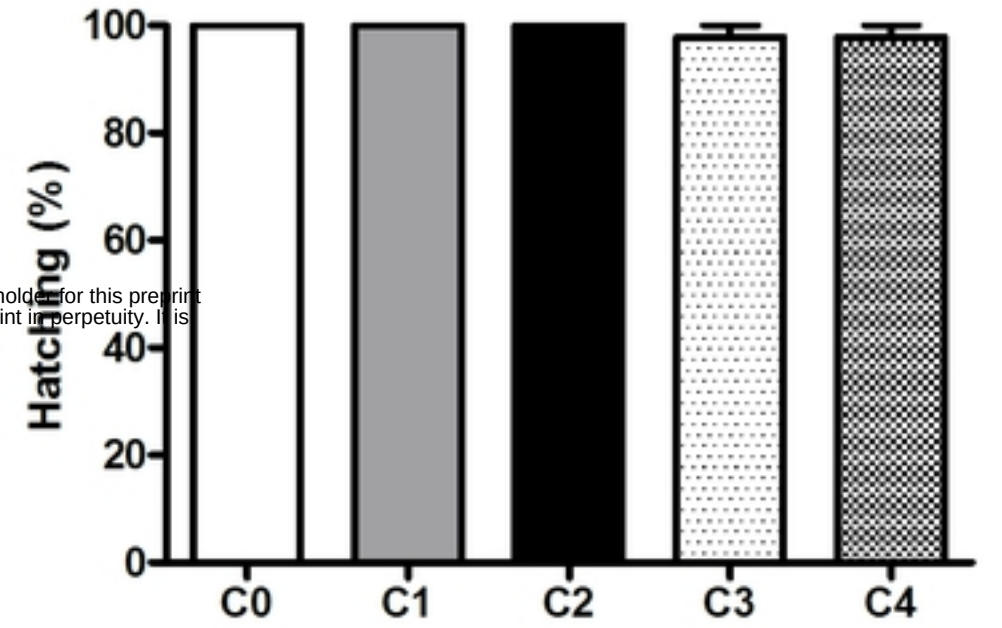
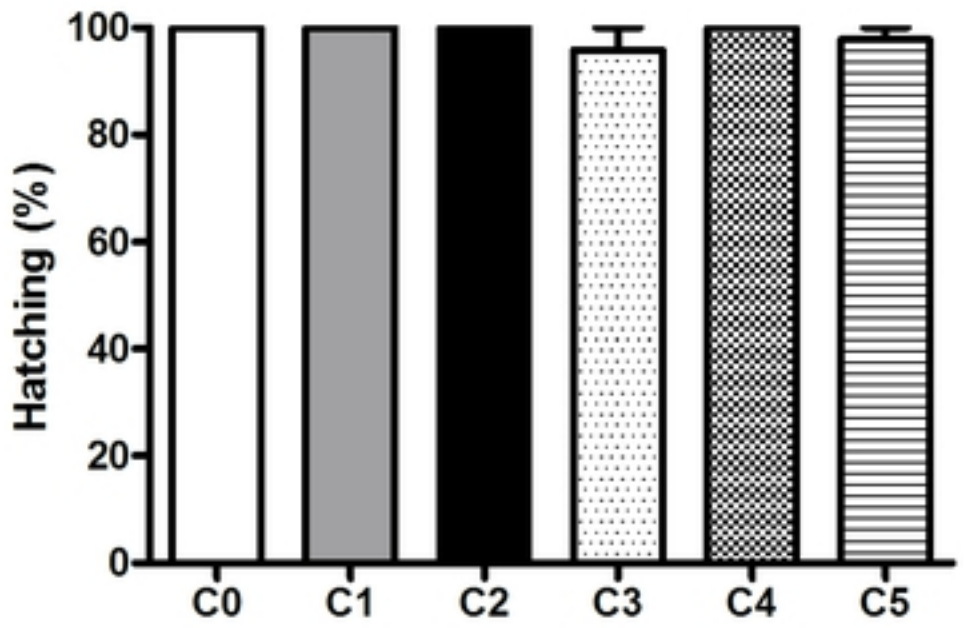
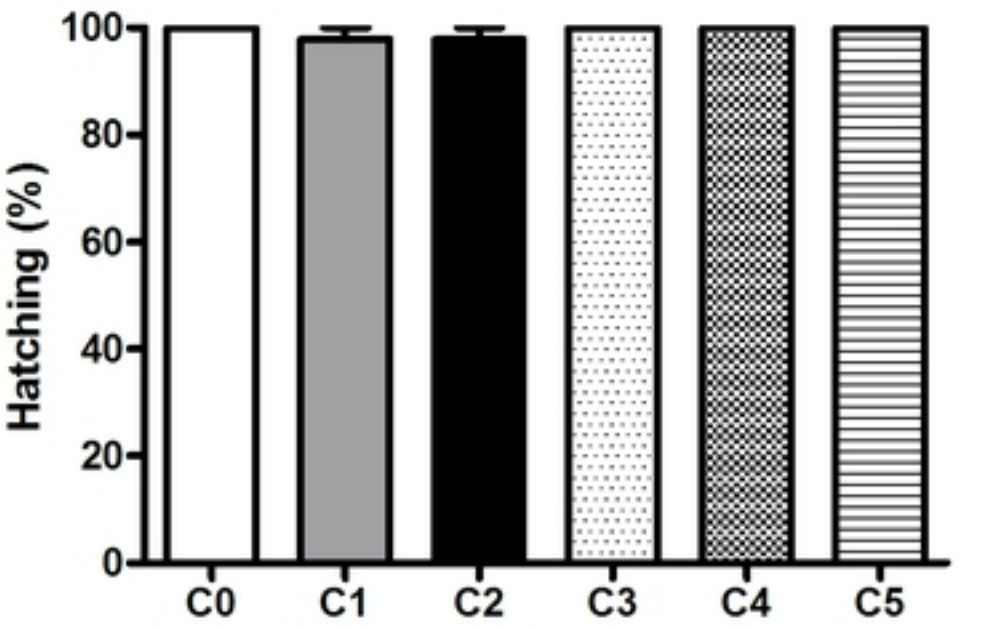

A. Amitrol
Hatching (%)
100
80
60
40
20
0
***
***
***
***
***
***
C0
C1
C2
C3
C4
C5
C6
B. Methomyl
Hatching (%)
100
80
60
40
20
0
***
***
***
***
***
***
C0
C1
C2
C3
C4
C5
C6
C. Paraquat
Hatching (%)
100
80
60
40
20
0
*
***
C0
C1
C2
C3
C4
D. Glyphosate
Hatching (%)
100
80
60
40
20
0
***
***
C0
C1
C2

Figure 2









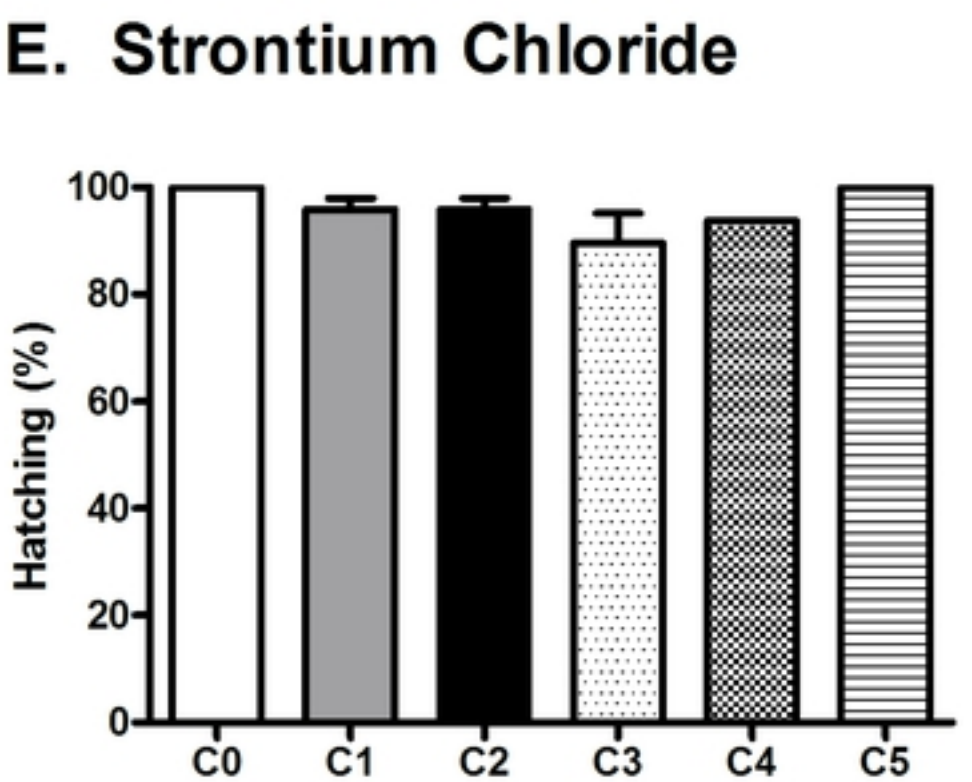


Figure 3

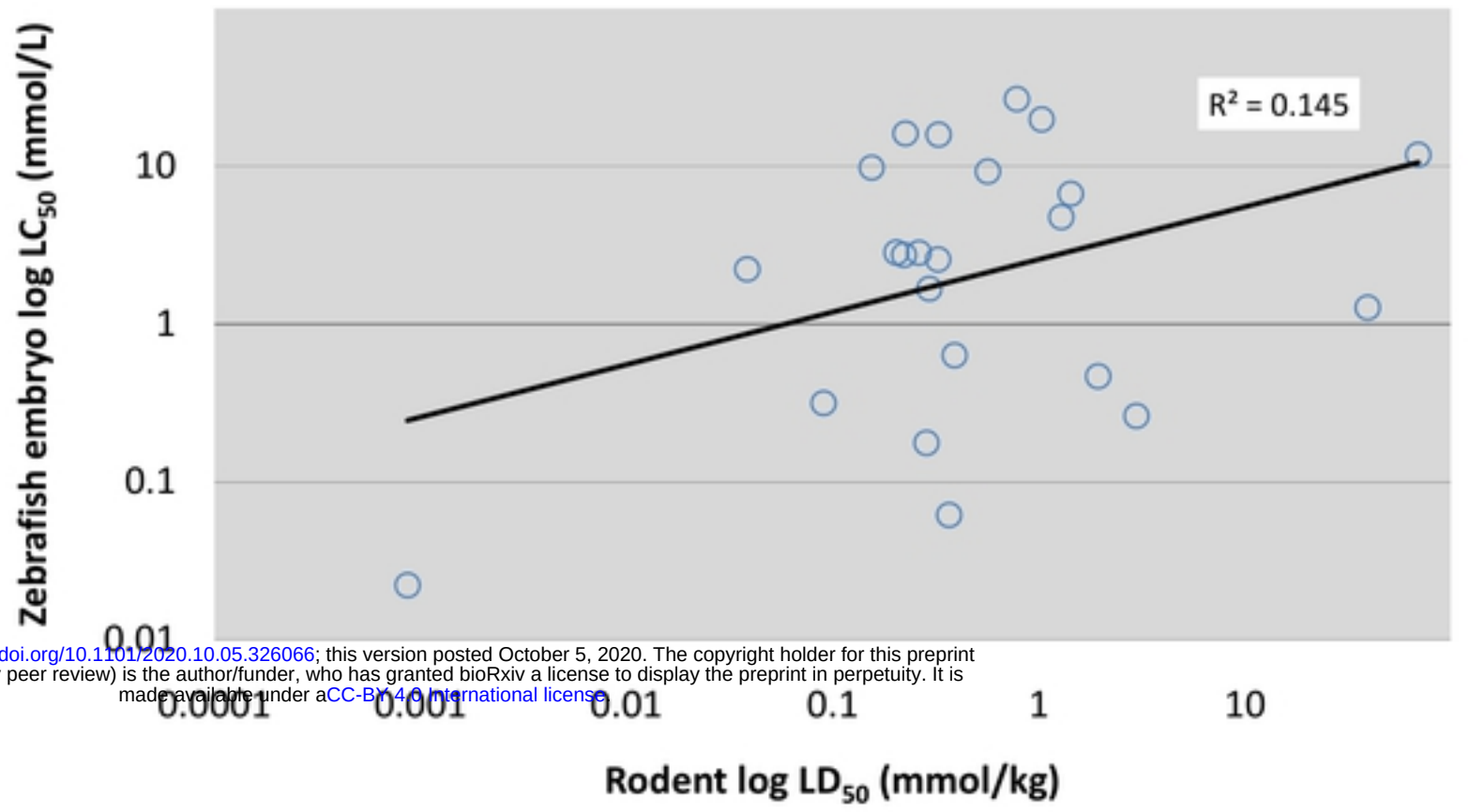
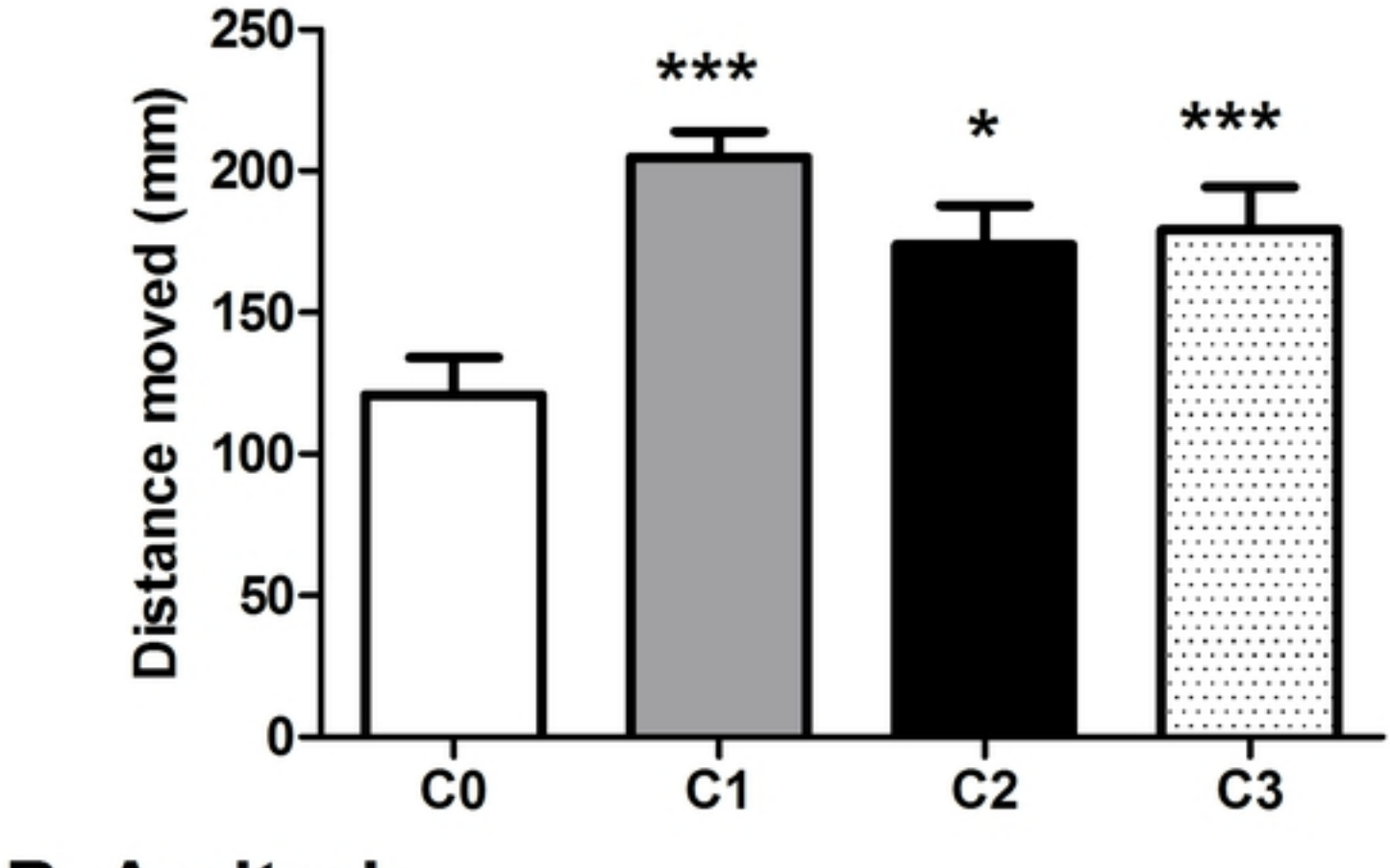
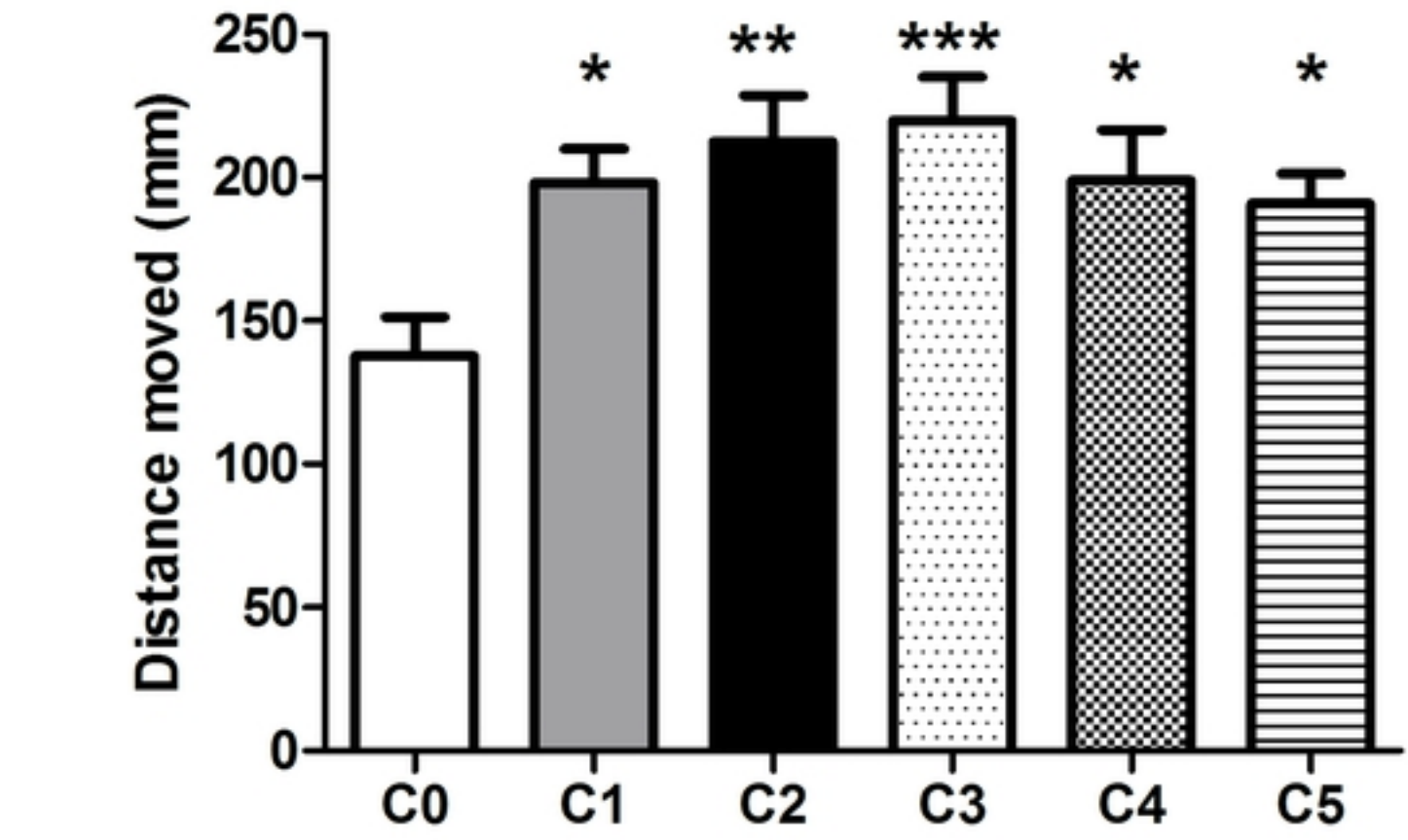


Figure 4





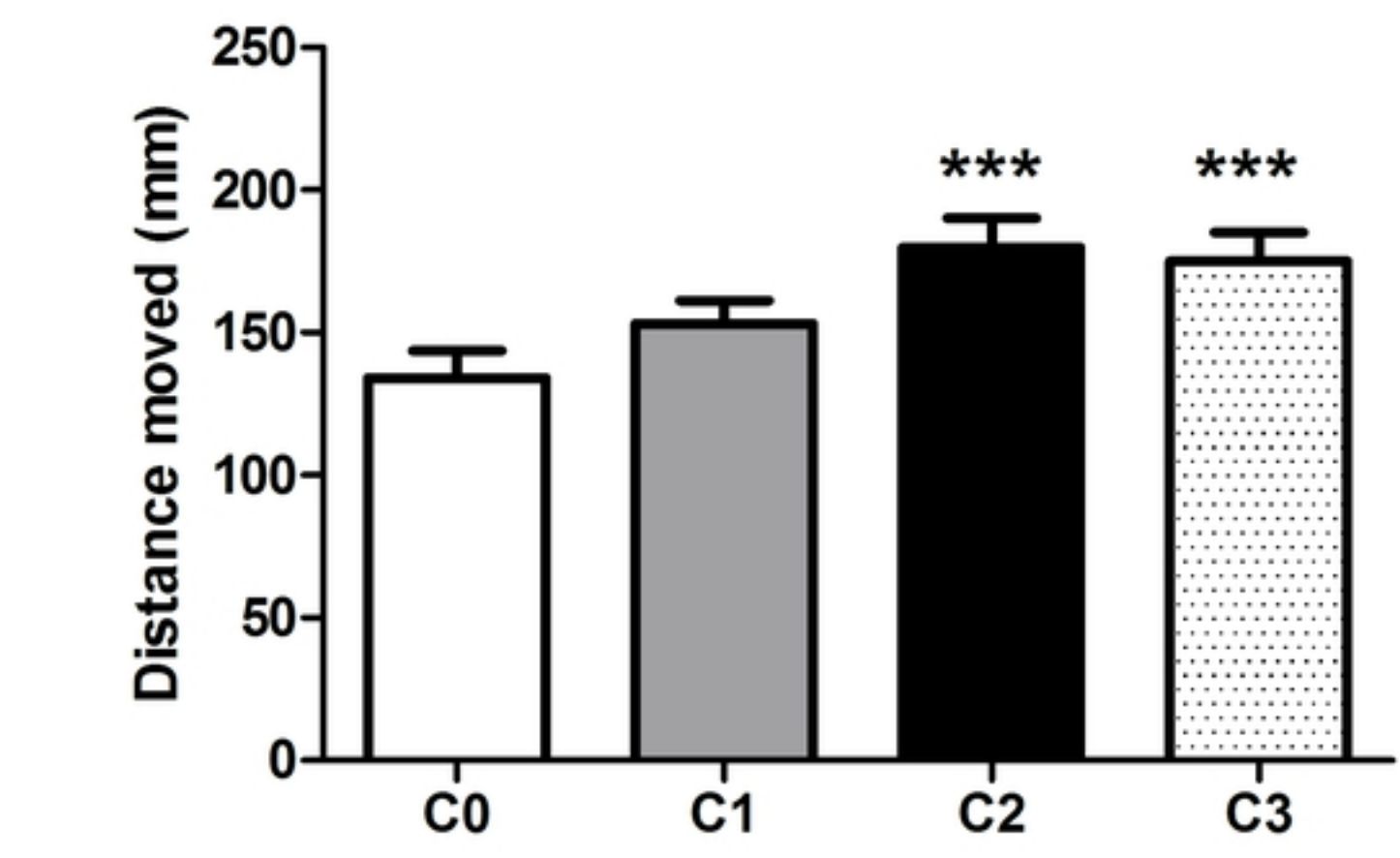


Figure 5

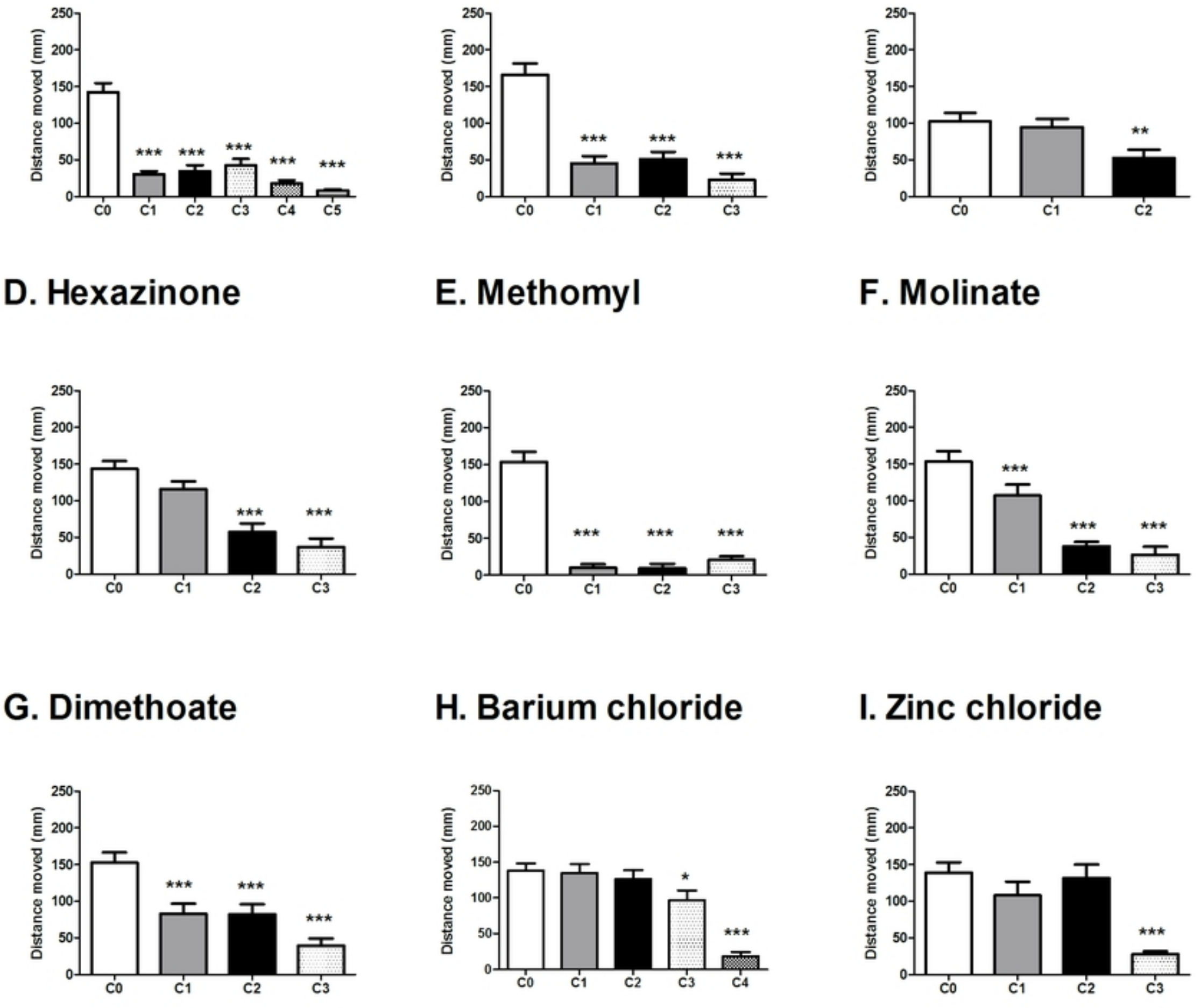
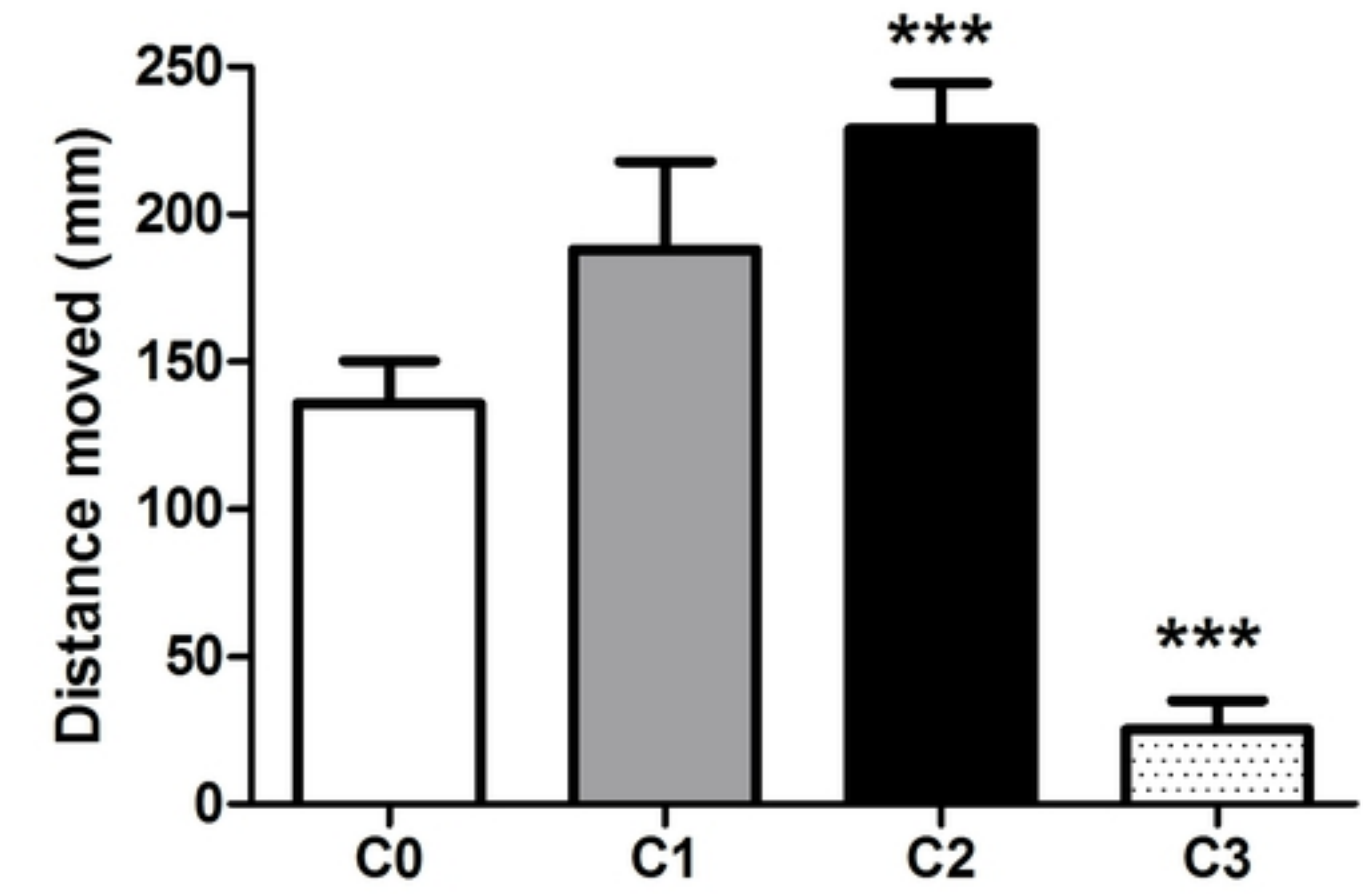
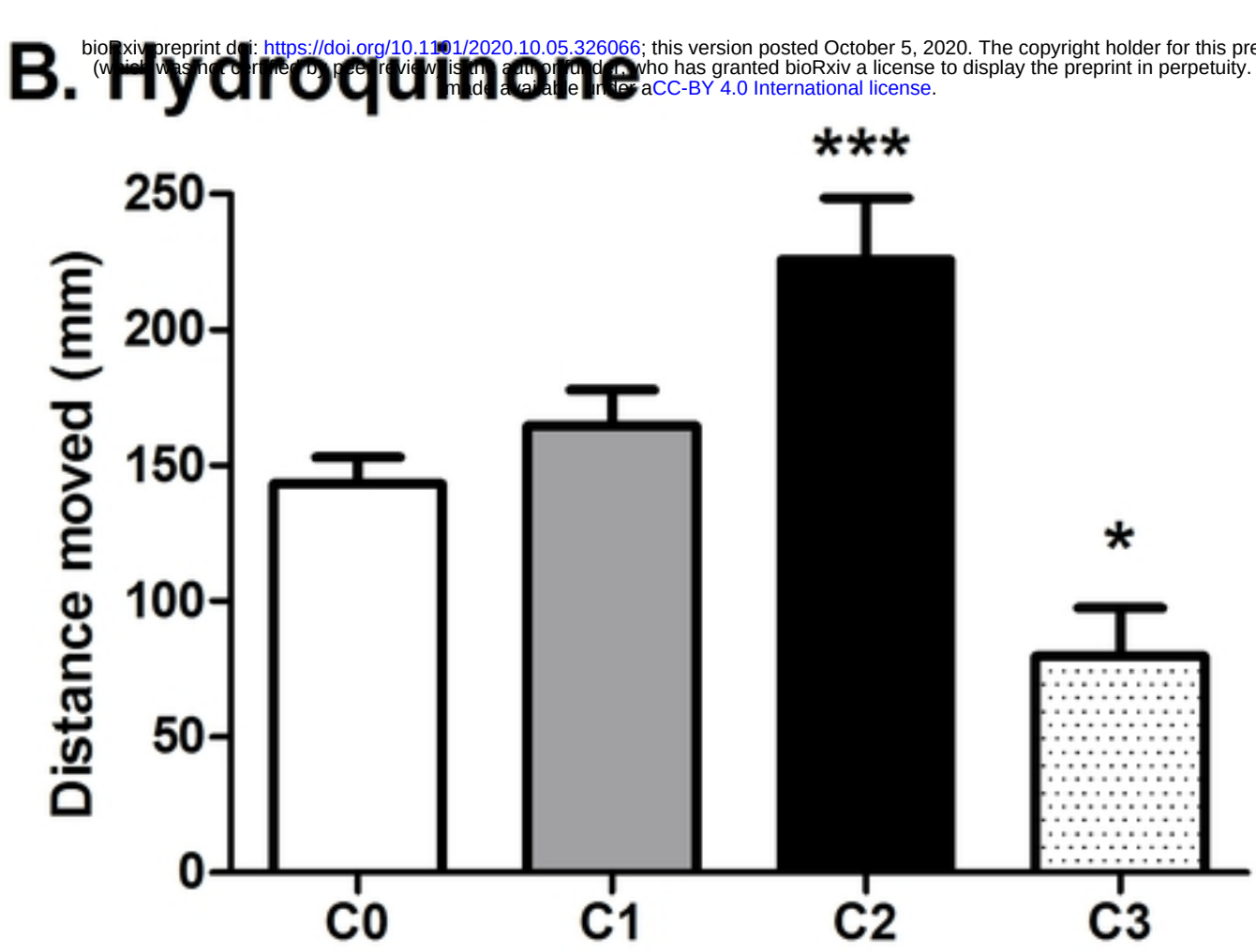


Figure 6





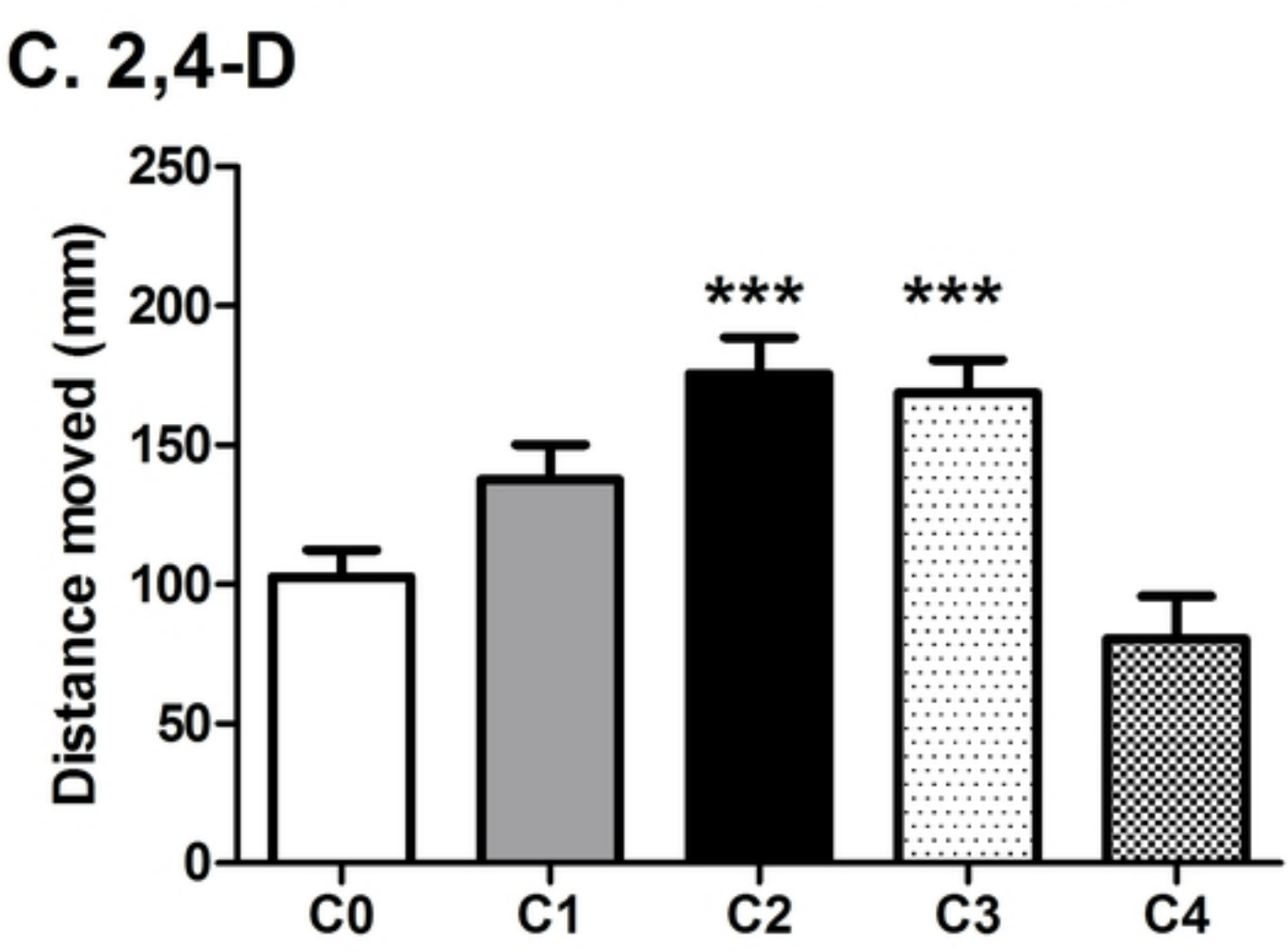


Figure 7

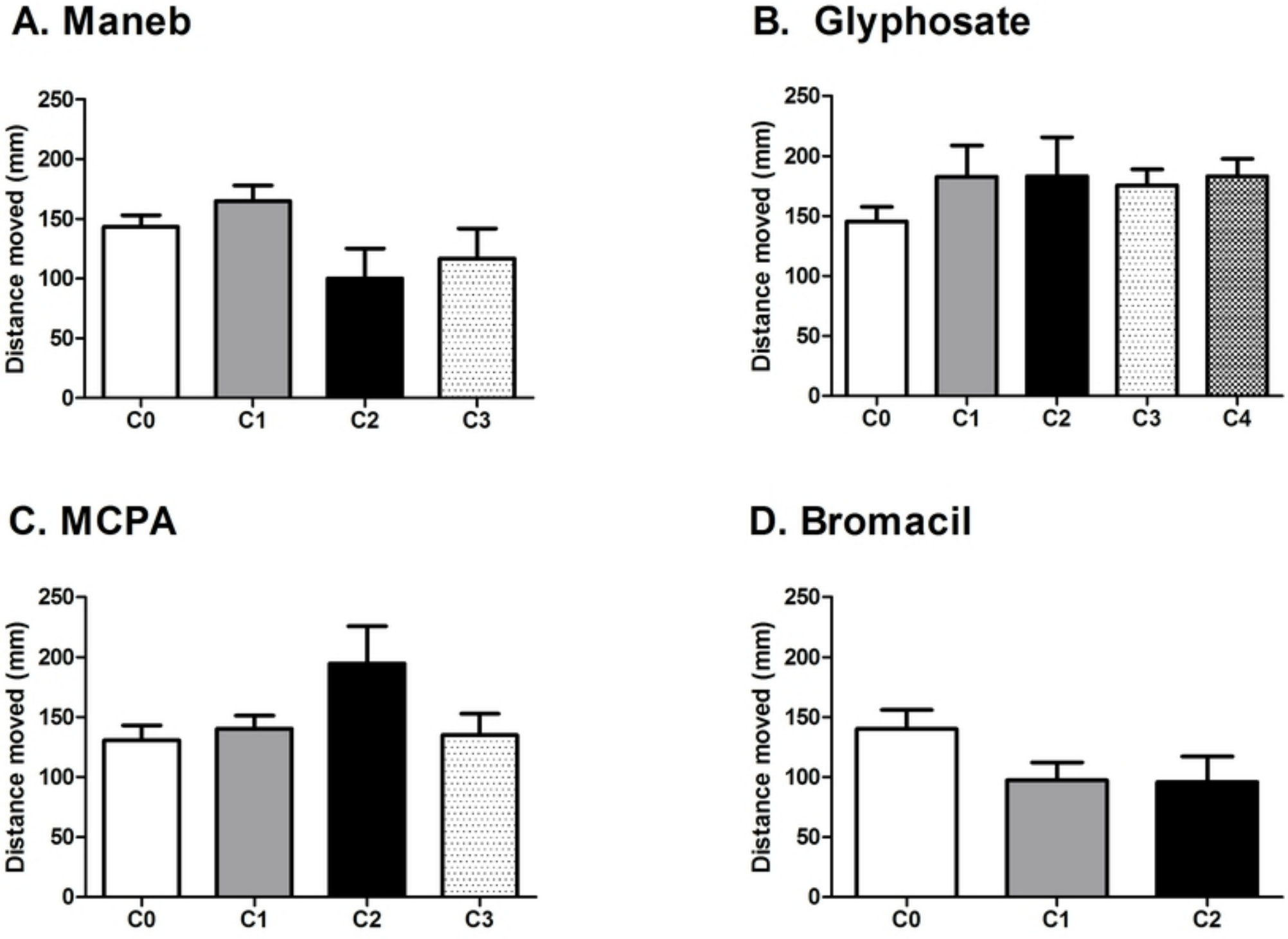

A. Maneb
Distance moved (mm)
250
200
150
100
50
0
C0
C1
C2
C3
B. Glyphosate
Distance moved (mm)
250
200
150
100
50
0
C0
C1
C2
C3
C4
C. MCPA
Distance moved (mm)
250
200
150
100
50
0
C0
C1
C2
C3
D. Bromacil
Distance moved (mm)
250
200
150
100
50
0
C0
C1
C2

Figure 8