Chemosphere 186 (2017) 209–217

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Establishment of a three-step method to evaluate effects of chemicals on development of zebrafish embryo/larvae

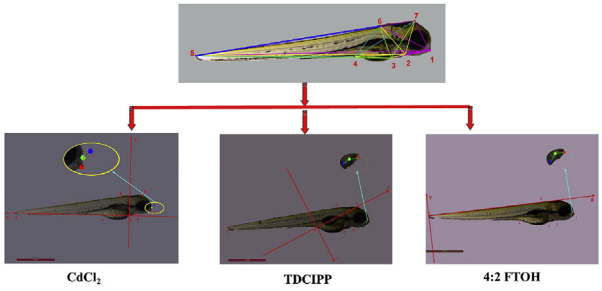
Guangyu Li [a], Huike Ye [a], Guanyong Su [c, d], Zhihua Han [e], Congxin Xie [a], Bingsheng Zhou [f], Robert J. Letcher [c], John P. Giesy [d, g, h], Hongxia Yu [d], Chunsheng Liu [a, b, *]

[a] College of Fisheries, Huazhong Agricultural University, Wuhan, 430070, China
[b] Collaborative Innovation Center for Efficient and Health Production of Fisheries in Hunan Province, China
[c] Department of Chemistry, Carleton University, Ottawa, Ontario, K1S 5B6, Canada
[d] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, 210023, China
[e] Nanjing Institute of Environmental Science, MEP, Nanjing, Jiangsu, 210042, China
[f] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Science, Wuhan, 430072, China
[g] Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B3, Canada
[h] Department of Zoology and Centre for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, United States

HIGHLIGHTS

- A method was developed to quantitatively determine effects of chemicals on phenotypes of zebrafish embryos.
- Seven points were selected, which led to acquisition of 21 lines and 105 angles.
- Exposure to chemicals changed lengths of some lines and magnitudes of some angles.
- Movement of the point describing the mouth was sensitive for chemical exposure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form
27 July 2017
Accepted 31 July 2017
Available online 2 August 2017

Handling Editor: David Volz

Keywords:
Chemical screening
$CdCl_2$
TDCIPP
FTOH
Developmental toxicity
Morphology

ABSTRACT

Core endpoints in zebrafish embryos are crucial indicators in screening harmful effects of chemicals. In this study, we established a three-step process to more quantitatively and less-subjective determine effects of chemicals on phenotypes of developing zebrafish embryos. Embryos were exposed to each of two concentrations of the representative chemicals cadmium chloride ($CdCl_2$), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) or 1H, 1H, 2H, 2H-nonafluoro-1-hexanol (4:2 FTOH) from 0.75 h post-fertilization (hpf) to 96 hpf. After exposure, larvae were imaged by use of a three-step method to describe morphology. Seven points were selected, which resulted in acquisition of 21 lines and 105 angles from images of larvae. Exposure to TDCIPP (0.1 or 0.2 mg/L), $CdCl_2$ (1 or 4 mg/L) or 4:2 FTOH (0.3 or 1 mg/L) significantly changed lengths of some lines and magnitudes of some angles, that resulted in differential scoring of points. Points were then prioritized and directions, distances and trajectories of movement were further described and standard reference values were developed. Movement of the point describing the mouth during embryonic development was found to be a sensitive parameter for

* Corresponding author. College of Fisheries, Huazhong Agricultural University, Wuhan, 430070, China.
E-mail address: liuchunshengidid@126.com (C. Liu).

http://dx.doi.org/10.1016/j.chemosphere.2017.07.163

assessment of adverse effects of chemicals. The present study provides a new strategy to characterize phenotypes of development of zebrafish embryo/larva following exposure to environmental toxins.

1. Introduction

It is estimated that global production of chemicals in 1930 was 1 million tones (t), and that by 2001 production had increased to 400 million t (EC, 2001). Portions of these chemicals are inevitably released into the environment during production, use, recycling or disposal and have potentials to cause adverse effects on wildlife or humans (Lammer et al., 2009a). To control possible risks caused by new or existing substances in the environment developed in some more developed and industrialized countries, programs to manage use of chemicals have been developed and regulations promulgated. These programs include the Organization for Economic Co-operation and Development (OECD) High Production Volume Program (HPV), the United States Environmental Protection Agency (USEPA) HPV Challenge Program, the Canadian categorization of the Domestic Substances List and the European Union REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) initiative (Lammer et al., 2009b). In these programs, a set of ecotoxicological tests are performed to develop thresholds or reference concentrations and relative potencies for use in assessing hazards and exposure risks of chemicals (Lammer et al., 2009a). Among those tests required, acute lethality of fish is a mandatory component of all the programs (OECD, 1992).

It has been hypothesized that fish would suffer severe distress and pain in acute tests when using lethality as an endpoint (Nagel, 2002; Chandroo et al., 2004; Braunbeck et al., 2005). Considering animal welfare and costs of testing as well as predictability, accuracy and precision of the tests, it seems feasible to replace acute lethality of juvenile or adult fishes with more sensitive predictors that can be applied to embryos, which in many countries are not defined as “living organisms” for which less rigorous approval processes are required (Lammer et al., 2009b). The embryonic stage of development is not regulated under current European Union legislation for protection of animals used for experimental and other scientific purposes, and thus is compatible with the 3R (Reduction, Refinement and Replace) principles. Furthermore, use of fish embryos is more cost-effective, less time-consuming, and requires less space and is more sensitive in most cases (Macova et al., 2008; Wu et al., 2016; Cheng et al., 2017). Besides reliability and reproducibility, results of tests using embryos of fishes are statistically correlated with lethality of older fishes ($R^2 = 0.854$) (Ratte and Hammers-Wirtz, 2003). Determination of lethality of fish embryos is already a mandatory component in routine whole effluent testing in Germany and has already been standardized internationally (DIN, 2001; Lammer et al., 2009a). Furthermore, beyond their use in acute lethality tests, fish embryos are excellent models for identifying mechanisms of toxic action and are useful indicators of longer-term effects (Scholz et al., 2008).

Core endpoints have been developed for use in assessments of hazards or risks of chemicals to zebrafish embryos (Lammer et al., 2009b). However, those endpoints are focused primarily on pre-set parameters observation under a microscope, such as malformations of the head or yolk sac and heartbeat and are thus subjective and can miss small changes in phenotype. In this study, a three-step, less-subjective method was established to quantitatively determine effects of chemicals on phenotypes of zebrafish embryos.

2. Materials and methods

2.1. Chemicals and reagents

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) was obtained from TCI Tokyo Chemical Industry Co., Ltd. (Japan). 1H, 1H, 2H, 2H-nonafluoro-1-hexanol (4:2 FTOH) was purchased from Tokyo Kasei Kogyo Co., Ltd (Japan). Cadmium chloride ($CdCl_2$) was obtained from Strem Chemicals Inc. (USA).

2.2. Exposure of embryos and imaging of larvae

All experimental protocols were approved by the Animal Care and Use Committee of Huazhong Agricultural University, and all experimental methods were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023). Maintenance of adult zebrafish and collection of embryos were performed as described previously (Lammer et al., 2009a; Liu et al., 2013). $CdCl_2$ was used as a model chemical since a previous study reported that exposure to the chemical caused altered axial curvature, ocular edema and submaxillary edema in zebrafish embryos (King-Heiden et al., 2009). TDCIPP and 4:2 FTOH were selected as test substances because these chemicals have frequently been detected in environmental media and/or tissues of wildlife and humans, and are considered to be (re)emerging environmental pollutants (Mahmoud et al., 2009; van der Veen and de Boer, 2012). Since zebrafish are susceptible to exposure to TDCIPP during early development, embryos were exposed not later than 0.75 h post-fertilization (hpf) (McGee et al., 2012). Briefly, embryos were randomly distributed in six-well culture plates where they were exposed to several concentrations of each chemical (TDCIPP, $CdCl_2$ or 4:2 FTOH) until 96 hpf. At 48 hpf, exposure solutions were renewed. Each well contained 10 embryos, with 10 mL of exposure solution, and each concentration was replicated in three wells. Control and exposure groups received 0.01% DMSO. Exposure concentrations (1 or 4 mg/L) of $CdCl_2$ were selected based on a previous study where exposure to 2 mg/L $CdCl_2$ caused altered axial curvature, ocular edema and submaxillary edema in zebrafish embryos (King-Heiden et al., 2009). For TDCIPP, 0.1 and 0.2 mg/L were selected as exposure concentrations since no significant effect on malformation based on examination under a microscope was observed in previous studies when exposure concentrations were $\leq$0.2 mg/L. For 4:2 FTOH, nominal exposure concentrations were 0.3 and 1 mg/L, which were selected based on a preliminary concentration-finding study. After exposure, effects of each chemical on hatching and mortality were quantified and larvae were used for imaging in methylcellulose solutions (3%, w/v) with a Leica M205FA microscope. Each larval fish was imaged manually under transmitted light. Setting parameters of microscope were: 100% aperture, 5 s exposure, 25 $\times$ magnification and 1.0 saturation. Good repeatability of our data from each batch of larvae suggested that morphologies of larvae were not changed during euthanized and imaging process.

2.3. Development of a three-step procedure

Images of each larva were used to establish a three-step process for comprehensive identification of effects of chemicals on

phenotypes during development of embryo/larva.

(1) **Selection of points and measurements**. Parameters for identification of effects on phenotype were developed by use of seven points on each larva: 1) mouth; 2) frontal concave of pericardium; 3) posterior concave pericardium; 4) concave of yolk sac; 5) tail; 6) first fishbone point; 7) salient point of head (Fig. 1). Parameters included lengths of straight lines between each pair of points and angles among each set of three points. A total of 21 lines and 105 angles were obtained for each image. These parameters were measured by using open-source ImageJ software (available at http://rsb.info.nih.gov/ij/).

(2) **Statistical analyses and scoring and prioritizing of points**. Data for lengths and angles were used as continuous variables in statistical analyses. Statistical analyses were conducted by Kyplot Demo 3.0 software (Tokyo, Japan). Normality was evaluated by the Kolmogorov-Smirnow. Homogeneity of variances was checked by Levene's test. ANOVA followed by Tukey's multiple range test was adopted to determine significant differences between the control and each combination of chemical and concentration. A level of significance was set at P value <0.05. After statistical analyses, point's scoring and prioritizing was performed. A point would get one score when one length or angle including the point in one exposure group was significantly different compared with control group. Therefore, getting more scores for one point indicates that position of the point is more likely to be changed due to chemical exposure. Mean scores of each point for lines (AS_{line}) (Equation (1)), angles (AS_{angle}) (Equation (2)) or lines and angles ($AS_{line\ +\ angle}$) (Equation (3)).

$$AS_{line} = TS_{line}/21/2 \quad (1)$$

$$AS_{angle} = TS_{angle}/105/3 \quad (2)$$

$$AS_{line+angle} = AS_{line} + AS_{angle} \quad (3)$$

where TS_{line} and TS_{angle} are total scores of each point based on length and angle comparison, respectively. Values of AS_{line}, AS_{angle} and $AS_{line\ +\ angle}$ for each exposure concentration were calculated individually. Points were prioritized based on $AS_{line\ +\ angle}$ values. The entire data set consisted of nine treatment groups, twenty-four larva images each treatment group, twenty-one length data and 105 angle data each image, which resulted in more than 27,000 data points.

(3) **Calculation of direction, distance and trajectory of movement of points**. To identify direction, distance and trajectory of movement of points, a method based on coordinates was developed, where the point with the smallest sum of $AS_{line\ +\ angle}$ values in two exposure concentrations was set as the origin. When there were two points with the same least sum of $AS_{line\ +\ angle}$, the point with smaller marked number (from 1 to 7) was used as the origin. A straight line was drawn through the first point with the smallest sum of $AS_{line\ +\ angle}$ values among 7 points and the second point with the smallest sum of $AS_{line\ +\ angle}$ values among the rest of 6 points was set as the X-axis. The Y-axis was obtained by rotating the X-axis clockwise 90°. After the axes were established coordinates of each point were plotted for the image of each larva in the control and exposure groups, and direction and distance of movement of each point were calculated and trajectories determined. Here, to describe direction and distance of trajectories of points, the forward direction on the X-axis was set as “right”, and the reverse direction was set as “left”. Similarly, the forward direction of the Y-axis was set as “down”, and the reverse direction was set as “up”.

2.4. Examination of time-dependent profiles of parameters included in the three-step procedure during embryo/larva development

To further explore reasons for the observed trajectories of the mouth point, which was frequently changed due to exposure to chemicals compared with other points used in this study, time-dependent response profiles of lengths of lines, size of angles, AS_{line}, AS_{angle}, $AS_{line\ +\ angle}$ and coordinates of points during development at 72, 96 and 120 hpf were examined, and directions, distances and trajectories of movement of points were calculated. Since neither point 2 and 3 could be precisely identified in larvae after 72 or 120 hpf, only points 1, 4, 5, 6 and 7 were selected for use in that part of the study.

3. Results

3.1. Effects of chemical on length of lines and size of angles

While no significant effects on survival or hatching were observed due to exposure to either concentration of $CdCl_2$, TDCIPP or 4:2 FTOH from 0.75 hpf to 96 hpf (data not shown), significant effects were observed on the morphometry described by the three-step procedure.

$CdCl_2$. Length of $Line_{1-4}$, $Line_{1-6}$, $Line_{1-7}$, $Line_{2-6}$, $Line_{2-7}$, $Line_{3-4}$ and $Line_{4-6}$ were significantly decreased when exposed to 1 mg/L $CdCl_2$, while length of the other lines was not different (Table 1). Exposure to 4 mg/L $CdCl_2$ resulted in 11 lines being statistically significantly shorter or longer (Table 1). Exposure to 1 or 4 mg/L $CdCl_2$ caused significant alterations in partial angles.

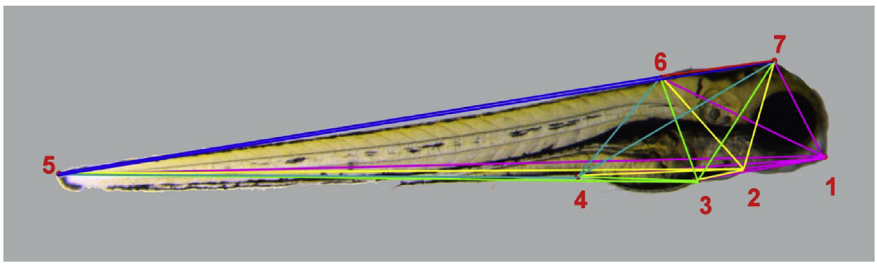


Fig. 1. Positions of seven points selected and lines and angles examined in this study. Points 1–7 represent mouth, frontal concave of pericardium, posterior concave pericardium, concave of yolk sac, tail, first fishbone point, and salient point of head, respectively.

Exposure to 1 mg/L $CdCl_2$ resulted in 38 angles being altered, while exposure to 4 mg/L $CdCl_2$ resulted in 64 angles being significantly altered (Table 1).

TDCIPP. Length of 3 lines in larvae exposed to 0.1 and 9 lines in those exposed to 0.2 mg/L TDCIPP were significantly different from those of controls. These lines included $Line_{1-2}$, $Line_{1-3}$, $Line_{1-4}$, $Line_{1-5}$, $Line_{2-3}$, $Line_{2-6}$, $Line_{2-7}$, $Line_{3-5}$, $Line_{3-6}$ and $Line_{6-7}$. Lengths of other lines were not different. For angles, of the 105 calculated, a total of 33 in larvae exposed to 0.1 and 32 exposed to 0.2 mg/L TDCIPP were significantly affected (Table 2).

4:2 FTOH. Exposure to 0.3 or 1 mg/L 4:2 FTOH caused dose-dependent alterations in length of lines and sizes of angles. Lengths of six lines, out of a total of 21 lines, were significantly greater due to exposure to 0.3 or 1 mg/L 4:2 FTOH. These lines included $Line_{1-2}$, $Line_{1-3}$, $Line_{1-4}$, $Line_{2-4}$, $Line_{3-4}$ and $Line_{4-7}$. Thirty-four and 48 angles out of 105 were significantly altered due to exposure to 0.3 or 1 mg/L 4:2 FTOH, respectively (Table 3).

3.2. Exposure to chemical caused differences in point scoring

Differences in scoring of points were observed due to exposure to both concentrations of $CdCl_2$, TDCIPP or 4:2 FTOH (Table 4). For the three chemicals tested, the point with the greatest $AS_{line\ +\ angle}$ was always the mouth point (Table 4).

$CdCl_2$. Due to exposure to 1 mg/L $CdCl_2$, alterations in scores for lines for points 1 to 7 (TS_{line}) were 4, 3, 1, 3, 0, 3 and 2, respectively, and mean scores (AS_{line}) were 0.095, 0.071, 0.024, 0.071, 0.000, 0.071 and 0.048. Scores for alterations of angles for points 1 to 7 (TS_{angle}) were 22, 20, 9, 16, 9, 17 and 21, respectively, and the corresponding mean scores (AS_{angle}) were 0.070, 0.063, 0.029, 0.051, 0.029, 0.054 and 0.067. Mean scores for points 1 to 7 ($AS_{line} + AS_{angle}$) were 0.165, 0.135, 0.052, 0.122, 0.029, 0.125 and 0.114, respectively. After exposure to 4 mg $CdCl_2$/L, when considering only alterations in length of lines, mean scores (AS_{line}) for points 1 to 7 were 0.095, 0.095, 0.071, 0.048, 0.119, 0.048 and 0.048, respectively, and when considering only alterations in angles, mean scores (AS_{angle}) for points 1 to 7 were 0.076, 0.073, 0.073, 0.063, 0.067, 0.073 and 0.079, respectively. Mean scores ($AS_{line} + AS_{angle}$) for points 1 to 7 were 0.171, 0.168, 0.144, 0.111, 0.186, 0.121 and 0.127. The sum of mean scores for points 1 to 7 were 0.337, 0.303, 0.197, 0.233, 0.214, 0.246 and 0.241, respectively.

TDCIPP. When exposed to 0.1 mg/L TDCIPP, mean scores for alterations of lines (AS_{line}) were 0.048, 0.024, 0.024, 0.00, 0.00, 0.024 and 0.024, respectively. The greatest mean score when considering only alterations in angles was observed for point 1, with a mean score of 0.073. Mean scores for alterations of angles (AS_{angle}) for points 2 to 7 were 0.051, 0.041, 0.038, 0.041, 0.044 and 0.025, respectively. Mean scores for points 1 to 7 ($AS_{line} + AS_{angle}$) were 0.121, 0.075, 0.065, 0.038, 0.041, 0.068 and 0.049, respectively. Exposure to 0.2 mg/L TDCIPP resulted in mean scores, based on alterations of lines (AS_{line}) for point 1 to 7 of 0.095, 0.095, 0.095, 0.024, 0.048, 0.048 and 0.024, respectively. The greatest mean score when considering only alterations in angles was observed for point 1, with a score of 0.067. Mean scores for other points when considering only alterations in angles (AS_{angle}) were 0.044 (point 2), 0.041 (point 3), 0.044 (point 4), 0.032 (point 5), 0.035 (point 6) and 0.041 (point 7). Mean scores for points 1 to 7 ($AS_{line} + AS_{angle}$) were 0.162, 0.140, 0.137, 0.068, 0.079, 0.083 and 0.065. The sum of mean scores for points 1 to 7 were 0.283, 0.214, 0.202, 0.106, 0.121, 0.151 and 0.114, respectively.

4:2 FTOH. Scores for points 1 to 7 (TS_{line}) were 3, 2, 2, 4, 0, 0 and 1, respectively after exposure to 0.3 mg/L 4:2 FTOH when considering only alterations in lines. The corresponding mean scores (AS_{line}) were 0.071, 0.048, 0.048, 0.095, 0.00, 0.00 and 0.024, respectively. For alterations in angles, scores for points 1 to 7

Table 1
Effects on lengths of lines and sizes of angles in zebrafish larvae after exposure to different concentrations of $CdCl_2$ from 0.75 to 96 hpf[a,b,c].

Lines (mm)/Angles (degree)	Concentration (mg/L)		
	0	1	4
$Line_{1-2}$	0.43 ± 0.01	0.37 ± 0.01*	0.37 ± 0.01*
$Line_{1-4}$	1.37 ± 0.02	1.28 ± 0.02*	1.31 ± 0.03
$Line_{1-5}$	3.61 ± 0.03	3.52 ± 0.04	3.45 ± 0.06*
$Line_{1-6}$	0.87 ± 0.01	0.83 ± 0.01*	0.84 ± 0.01*
$Line_{1-7}$	0.49 ± 0	0.46 ± 0*	0.47 ± 0.01*
$Line_{2-3}$	0.2 ± 0.01	0.22 ± 0.01	0.25 ± 0.01*
$Line_{2-5}$	3.2 ± 0.03	3.15 ± 0.03	3.09 ± 0.05*
$Line_{2-6}$	0.6 ± 0.01	0.57 ± 0.01*	0.59 ± 0.01
$Line_{2-7}$	0.52 ± 0.01	0.48 ± 0*	0.48 ± 0.01*
$Line_{3-4}$	0.76 ± 0.01	0.7 ± 0.01*	0.72 ± 0.02*
$Line_{3-5}$	3.01 ± 0.03	2.95 ± 0.03	2.86 ± 0.06*
$Line_{4-5}$	2.24 ± 0.02	2.25 ± 0.02	2.15 ± 0.04*
$Line_{4-6}$	0.74 ± 0.01	0.7 ± 0.01*	0.73 ± 0.02
$Line_{5-6}$	2.84 ± 0.03	2.79 ± 0.03	2.74 ± 0.05*
∠213	1.85 ± 0.33	3.92 ± 0.63*	4.39 ± 0.8*
∠123	174.44 ± 0.85	169.61 ± 1.58*	169.31 ± 1.95*
∠132	3.7 ± 0.53	6.48 ± 0.99*	6.3 ± 1.19*
∠214	9.2 ± 0.66	5.89 ± 0.84*	6.14 ± 0.97*
∠124	166.68 ± 0.96	171.66 ± 1.2*	171.53 ± 1.28*
∠142	4.13 ± 0.31	2.45 ± 0.36*	2.34 ± 0.33*
∠215	13.83 ± 0.75	10.1 ± 1.01*	10.03 ± 1.11*
∠125	164.32 ± 0.85	168.7 ± 1.14*	168.78 ± 1.24*
∠152	1.84 ± 0.11	1.2 ± 0.13*	1.18 ± 0.13*
∠126	116.02 ± 0.94	121.49 ± 1.3*	121.07 ± 1.4*
∠162	26.1 ± 0.69	22.51 ± 0.86*	21.86 ± 0.59*
∠172	49.64 ± 1.06	46.37 ± 1.55	45.41 ± 1.27*
∠317	68.43 ± 0.89	72.74 ± 1.51*	72.42 ± 2.11*
∠137	46.04 ± 0.44	44.37 ± 0.88	43.41 ± 1.26*
∠416	28.68 ± 0.3	30.24 ± 0.39*	30.93 ± 0.54*
∠146	34.55 ± 0.55	36.93 ± 0.67*	36.21 ± 0.76
∠164	116.78 ± 0.8	112.84 ± 0.93*	112.86 ± 0.98*
∠417	58.8 ± 1	63.75 ± 1.33*	63.66 ± 1.56*
∠174	100.46 ± 1.15	95.05 ± 1.59*	95.77 ± 1.67*
∠516	24.04 ± 0.46	25.91 ± 0.62*	27.11 ± 0.8*
∠156	7.17 ± 0.13	7.47 ± 0.17	8.05 ± 0.27*
∠165	148.79 ± 0.58	146.62 ± 0.77	144.84 ± 1.05*
∠517	54.16 ± 1.09	59.43 ± 1.38*	59.84 ± 1.45*
∠175	118.97 ± 1.16	113.64 ± 1.46*	113.1 ± 1.51*
∠617	30.12 ± 0.8	33.52 ± 1.05*	32.74 ± 1.11*
∠176	120.71 ± 1.22	116.61 ± 1.76*	118.12 ± 1.71
∠243	3.75 ± 0.18	5.19 ± 0.48	5.43 ± 0.85*
∠253	1.1 ± 0.06	1.44 ± 0.13	1.53 ± 0.24*
∠236	95.12 ± 0.96	90.68 ± 1.41	90.2 ± 2.5*
∠263	19.47 ± 0.44	22.34 ± 0.82*	24.34 ± 1.17*
∠327	119.07 ± 1.48	124.34 ± 1.54*	121.75 ± 2.38
∠237	45.05 ± 0.98	39.14 ± 1.16*	39.49 ± 1.81*
∠273	15.89 ± 0.61	16.52 ± 0.61	18.76 ± 0.89*
∠427	104.31 ± 0.92	107.72 ± 0.89*	106.74 ± 0.92*
∠247	24.87 ± 0.27	23.6 ± 0.28*	22.9 ± 0.38*
∠274	50.82 ± 0.76	48.68 ± 0.75*	50.36 ± 0.83
∠257	8.71 ± 0.07	8.12 ± 0.08*	8.23 ± 0.11*
∠627	53.66 ± 0.75	57.37 ± 0.74*	56.29 ± 0.7*
∠267	55.28 ± 0.46	52.38 ± 0.45*	51 ± 0.8*
∠435	1.42 ± 0.25	2.33 ± 0.5	3.12 ± 0.6*
∠345	178.09 ± 0.34	176.95 ± 0.65	175.85 ± 0.81*
∠354	0.49 ± 0.09	0.72 ± 0.15	1.02 ± 0.2*
∠436	66.37 ± 0.44	67.5 ± 0.87	69.36 ± 0.99*
∠364	71.2 ± 0.73	67.98 ± 0.84*	66.66 ± 1.4*
∠437	116.45 ± 0.63	119.05 ± 0.87	120.07 ± 1.08*
∠374	34.93 ± 0.56	32.17 ± 0.53*	31.6 ± 0.7*
∠536	66.67 ± 0.48	68.14 ± 1.2	70.6 ± 1.43*
∠356	10.11 ± 0.1	10.1 ± 0.11	10.76 ± 0.4*
∠365	103.21 ± 0.5	101.76 ± 1.25	98.64 ± 1.72*
∠537	116.75 ± 0.59	119.68 ± 1.14	121.31 ± 1.34*
∠375	53.44 ± 0.54	50.76 ± 1.01	48.93 ± 1.25*
∠647	13.8 ± 0.34	15.73 ± 0.37*	15.65 ± 0.59*
∠467	145.95 ± 0.68	142.71 ± 0.7*	142 ± 1.07*
∠476	20.25 ± 0.37	21.56 ± 0.39*	22.35 ± 0.56*
∠657	0.36 ± 0.06	0.72 ± 0.11	1.26 ± 0.23*
∠567	177.62 ± 0.39	175.34 ± 0.71*	172.24 ± 1.16*
∠576	2.02 ± 0.34	3.94 ± 0.6*	6.5 ± 0.94*

[a] Values represent mean ± SEM (n = 24).
[b] Significant differences from the control are indicated by $^*P < 0.05$.
[c] Size of the other lines and angles examined were not changed after exposure.

Table 2
Effects on lengths of lines and sizes of angles in zebrafish larvae after exposure to different concentrations of TDCIPP from 0.75 to 96 hpf[a,b,c].

Lines (mm)/Angles (degree)	Concentration (mg/L)		
	0	0.1	0.2
$Line_{1-2}$	0.39 ± 0.01	0.43 ± 0.01*	0.44 ± 0.01*
$Line_{1-3}$	0.61 ± 0.01	0.65 ± 0.01*	0.65 ± 0.01*
$Line_{1-4}$	1.29 ± 0.02	1.31 ± 0.02	1.35 ± 0.02*
$Line_{1-5}$	3.53 ± 0.03	3.57 ± 0.03	3.62 ± 0.03*
$Line_{2-3}$	0.22 ± 0.00	0.22 ± 0.00	0.21 ± 0.00*
$Line_{2-6}$	0.61 ± 0.01	0.61 ± 0.00	0.63 ± 0.01*
$Line_{2-7}$	0.51 ± 0.00	0.52 ± 0.00	0.53 ± 0.00*
$Line_{3-5}$	2.94 ± 0.02	2.96 ± 0.02	3.00 ± 0.02*
$Line_{3-6}$	0.54 ± 0.01	0.55 ± 0.00	0.56 ± 0.01*
$Line_{6-7}$	0.56 ± 0.01	0.54 ± 0.01*	0.55 ± 0.00
∠214	6.77 ± 0.56	11.28 ± 1.63*	10.21 ± 0.53*
∠124	170.19 ± 0.86	163.24 ± 2.48*	164.79 ± 0.85*
∠142	3.03 ± 0.30	5.48 ± 0.85*	5.00 ± 0.33*
∠215	10.45 ± 0.83	16.14 ± 2.22*	14.91 ± 0.62*
∠125	168.22 ± 0.95	161.73 ± 2.49*	163.03 ± 0.73*
∠152	1.33 ± 0.12	2.13 ± 0.27*	2.06 ± 0.12*
∠216	36.64 ± 0.40	40.60 ± 1.44*	38.59 ± 0.31
∠126	120.73 ± 1.04	112.36 ± 2.47*	115.42 ± 0.81*
∠162	22.62 ± 0.77	27.04 ± 1.11*	25.99 ± 0.67*
∠172	46.49 ± 1.36	51.16 ± 1.44*	51.32 ± 1.15*
∠314	7.89 ± 0.26	10.02 ± 0.87*	9.93 ± 0.33*
∠134	165.00 ± 0.51	160.59 ± 1.67*	161.07 ± 0.62*
∠143	7.10 ± 0.28	9.4 ± 0.80*	9.00 ± 0.31*
∠315	11.83 ± 0.48	14.87 ± 1.46*	14.64 ± 0.42
∠135	165.7 ± 0.59	161.97 ± 1.72*	162.22 ± 0.52*
∠153	2.46 ± 0.12	3.15 ± 0.26*	3.14 ± 0.11*
∠136	97.99 ± 0.69	92.83 ± 1.82*	96.39 ± 0.60
∠163	43.98 ± 0.71	47.84 ± 1.11*	45.29 ± 0.57
∠173	63.18 ± 1.28	66.97 ± 0.91*	67.25 ± 1.14*
∠416	30.13 ± 0.44	29.32 ± 0.58	28.39 ± 0.49*
∠146	39.72 ± 0.56	37.56 ± 0.94*	38.16 ± 0.37
∠164	110.15 ± 0.8	113.12 ± 1.30*	113.46 ± 0.68*
∠174	93.04 ± 1.42	96.20 ± 2.46	99.74 ± 1.41*
∠516	26.19 ± 0.59	24.46 ± 0.95	23.68 ± 0.56*
∠165	145.84 ± 0.69	148.13 ± 1.23	148.92 ± 0.67*
∠157	7.13 ± 0.09	6.64 ± 0.23*	6.67 ± 0.08
∠167	29.16 ± 0.65	26.93 ± 1.05*	26.65 ± 0.47*
∠263	21.36 ± 0.56	20.80 ± 0.56	19.3 ± 0.52*
∠427	109.01 ± 1.10	106.81 ± 1.01	106.03 ± 0.78*
∠247	24.45 ± 0.37	25.52 ± 0.37*	25.54 ± 0.31*
∠526	47.48 ± 0.67	49.37 ± 0.48*	47.61 ± 0.52
∠256	9.30 ± 0.09	9.54 ± 0.06*	9.46 ± 0.08
∠265	123.21 ± 0.73	121.09 ± 0.52*	122.93 ± 0.55
∠257	8.46 ± 0.10	8.66 ± 0.09	8.73 ± 0.08*
∠627	59.21 ± 0.85	55.94 ± 0.96*	56.66 ± 0.57*
∠267	51.78 ± 0.47	53.5 ± 0.51*	52.64 ± 0.43
∠435	1.11 ± 0.19	1.98 ± 0.32*	1.53 ± 0.28
∠345	178.56 ± 0.24	177.41 ± 0.42*	177.98 ± 0.37
∠354	0.34 ± 0.06	0.61 ± 0.10*	0.49 ± 0.09
∠436	67.01 ± 0.72	67.76 ± 0.87	64.68 ± 0.72*
∠437	121.55 ± 0.96	119.75 ± 0.92	117.96 ± 0.77*
∠374	29.86 ± 0.68	30.81 ± 0.66	32.50 ± 0.57*
∠537	122.25 ± 1.01	121.13 ± 0.87	119.12 ± 0.61*
∠375	48.16 ± 0.90	49.19 ± 0.78	51.07 ± 0.56*
∠637	54.54 ± 0.63	51.99 ± 0.90*	53.28 ± 0.44
∠376	52.32 ± 0.66	53.72 ± 0.52	54.77 ± 0.58*

[a] Values represent mean ± SEM (n = 24).
[b] Significant differences from the control are indicated by $^*P < 0.05$.
[c] Size of the other lines and angles examined were not changed after exposure.

Table 3
Effects on lengths of lines and sizes of angles in zebrafish larvae after exposure to different concentrations of 4:2 FTOH from 0.75 to 96 hpf[a,b,c].

Lines (mm)/Angles (degree)	Concentration (mg/L)		
	0	0.3	1
$Line_{1-2}$	0.41 ± 0.01	0.48 ± 0.01*	0.49 ± 0.01*
$Line_{1-3}$	0.60 ± 0.01	0.68 ± 0.01*	0.70 ± 0.01*
$Line_{1-4}$	1.30 ± 0.02	1.43 ± 0.02*	1.45 ± 0.01*
$Line_{2-4}$	0.90 ± 0.02	0.96 ± 0.02*	0.99 ± 0.02*
$Line_{3-4}$	0.71 ± 0.02	0.76 ± 0.02*	0.78 ± 0.01*
$Line_{4-7}$	1.14 ± 0.01	1.20 ± 0.02*	1.21 ± 0.01*
∠214	7.59 ± 0.75	10.7 ± 0.73*	12.69 ± 0.46*
∠124	168.89 ± 1.12	164.07 ± 1.08*	161.13 ± 0.6*
∠142	3.52 ± 0.39	5.23 ± 0.38*	6.18 ± 0.20*
∠215	10.82 ± 0.77	13.96 ± 1.18*	15.84 ± 0.61*
∠125	167.82 ± 0.88	164.06 ± 1.35*	161.86 ± 0.69*
∠152	1.36 ± 0.11	1.98 ± 0.18*	2.30 ± 0.09*
∠216	35.16 ± 0.88	35.71 ± 0.93	37.44 ± 0.68*
∠126	122.53 ± 1.59	118.26 ± 1.68	115.65 ± 1.70*
∠162	22.31 ± 0.89	26.03 ± 0.98*	26.91 ± 1.18*
∠127	65.47 ± 1.13	61.25 ± 1.04*	59.79 ± 0.65*
∠172	47.39 ± 1.07	54.43 ± 1.30*	55.6 ± 0.82*
∠134	162.90 ± 1.05	161.20 ± 0.74	160.08 ± 0.69*
∠143	7.85 ± 0.50	8.88 ± 0.33	9.39 ± 0.30*
∠153	2.43 ± 0.14	2.89 ± 0.20*	3.07 ± 0.11*
∠316	37.32 ± 0.61	34.93 ± 0.63*	35.28 ± 0.76*
∠163	40.45 ± 1.06	45.11 ± 1.41*	45.35 ± 1.96*
∠317	69.30 ± 1.43	63.54 ± 1.01*	62.45 ± 0.56*
∠137	48.12 ± 0.97	45.11 ± 0.82*	44.17 ± 0.57*
∠173	62.58 ± 0.87	71.35 ± 0.92*	73.38 ± 0.76*
∠416	28.07 ± 0.50	25.01 ± 0.43*	24.75 ± 0.63*
∠164	110.79 ± 1.30	119.06 ± 1.12*	118.12 ± 2.38*
∠417	60.05 ± 1.37	53.62 ± 1.13*	51.92 ± 0.57*
∠147	22.62 ± 0.37	20.1 ± 0.40*	19.27 ± 0.21*
∠174	97.33 ± 1.37	106.28 ± 1.29*	108.81 ± 0.61*
∠516	24.75 ± 0.81	21.75 ± 0.61*	21.60 ± 0.68*
∠156	7.61 ± 0.18	6.99 ± 0.22*	7.18 ± 0.18
∠165	147.65 ± 0.96	151.25 ± 0.80*	151.21 ± 0.66*
∠517	56.73 ± 1.24	50.36 ± 1.33*	48.77 ± 0.61*
∠157	7.07 ± 0.11	6.51 ± 0.13*	6.37 ± 0.08*
∠175	116.19 ± 1.25	123.13 ± 1.44*	124.86 ± 0.65*
∠617	31.99 ± 1.24	28.61 ± 0.93*	27.17 ± 0.69*
∠167	28.95 ± 0.57	25.87 ± 0.59*	24.11 ± 0.66*
∠176	119.07 ± 1.33	125.52 ± 1.32*	128.72 ± 0.66*
∠324	16.45 ± 1.53	13.77 ± 1.43	11.87 ± 1.12*
∠234	159.03 ± 1.97	162.59 ± 1.68	164.92 ± 1.39*
∠243	4.52 ± 0.49	3.65 ± 0.29	3.21 ± 0.29*
∠325	17.76 ± 1.91	13.77 ± 1.54	11.14 ± 1.11*
∠235	161.13 ± 2.04	165.32 ± 1.62	168.09 ± 1.18*
∠253	1.11 ± 0.13	0.91 ± 0.09	0.77 ± 0.08*
∠326	63.50 ± 1.58	59.58 ± 1.74	57.35 ± 2.19*
∠327	120.57 ± 2.46	116.58 ± 1.64	113.21 ± 1.26*
∠237	44.24 ± 2.17	46.50 ± 1.22	49.01 ± 1.01*
∠273	15.19 ± 0.63	16.92 ± 0.82	17.78 ± 0.77*
∠264	88.47 ± 1.14	93.03 ± 0.81*	91.20 ± 1.68
∠274	49.94 ± 1.11	51.85 ± 0.76	53.21 ± 1.05*
∠256	8.93 ± 0.20	8.97 ± 0.11	9.49 ± 0.16*
∠364	70.34 ± 1.36	73.95 ± 0.92*	72.77 ± 0.96
∠347	30.46 ± 0.59	28.98 ± 0.33	28.66 ± 0.33*
∠376	56.49 ± 0.97	54.17 ± 0.86*	55.34 ± 0.75
∠547	152.28 ± 0.71	154.63 ± 0.65	155.6 ± 0.60*
∠475	18.86 ± 0.55	16.85 ± 0.44*	16.05 ± 0.44*
∠476	21.74 ± 0.47	19.24 ± 0.31*	19.91 ± 0.34*

[a] Values represent mean ± SEM (n = 24).
[b] Significant differences from the control are indicated by $^*P < 0.05$.
[c] Size of the other lines and angles examined were not changed after exposure.

(TS_{angle}) were 29, 10, 8, 12, 11, 15 and 17, respectively, with corresponding mean scores (AS_{angle}) of 0.092, 0.032, 0.025, 0.038, 0.035, 0.048 and 0.054. Mean scores for points 1 to point 7 ($AS_{line} + AS_{angle}$) were 0.163, 0.079, 0.073, 0.133, 0.035, 0.048 and 0.078, respectively. Due to exposure to 1 mg/L 4:2 FTOH, scores (TS_{line}) of 3, 2, 2, 4, 0, 0 and 1 were observed for points 1 to 7 when considering only alterations in lines. The corresponding mean scores (AS_{line}) were 0.071, 0.048, 0.048, 0.095, 0.00, 0.00 and 0.024. When only considering alterations in angles, scores for point 1 to 7 (TS_{angle}) were 32, 23, 19, 18, 15, 15 and 22, respectively, while corresponding mean scores (AS_{angle}) were 0.102, 0.073, 0.060, 0.057, 0.048, 0.048 and 0.070. Mean scores ($AS_{line} + AS_{angle}$) for point 1 to point 7 were 0.173, 0.121, 0.108, 0.152, 0.048, 0.048 and 0.094, respectively. The sum of mean scores for points 1 to 7 were 0.337, 0.200, 0.181, 0.286, 0.083, 0.095 and 0.171, respectively.

Table 4
Exposure to $CdCl_2$, TDCIPP or 4:2 FTOH from 0.75 to 96 hpf caused differentiations in scoring of points.

Chemicals	Concentrations (mg/L)	Scores	Points						
			1	2	3	4	5	6	7
CdCl2	1	TS_{line}	4	3	1	3	0	3	2
		AS_{line}	0.095	0.071	0.024	0.071	0.000	0.071	0.048
		TS_{angle}	22	20	9	16	9	17	21
		AS_{angle}	0.070	0.063	0.029	0.051	0.029	0.054	0.067
		$AS_{line + angle}$	0.165	0.135	0.052	0.122	0.029	0.125	0.114
	4	TS_{line}	4	4	3	2	5	2	2
		AS_{line}	0.095	0.095	0.071	0.048	0.119	0.048	0.048
		TS_{angle}	24	23	23	20	21	23	25
		AS_{angle}	0.076	0.073	0.073	0.063	0.067	0.073	0.079
		$AS_{line + angle}$	0.171	0.168	0.144	0.111	0.186	0.121	0.127
Sum			0.337	0.303	0.197	0.233	0.214	0.246	0.241
TDCIPP	0.1	TS_{line}	2	1	1	0	0	1	1
		AS_{line}	0.048	0.024	0.024	0	0	0.024	0.024
		TS_{angle}	23	16	13	12	13	14	8
		AS_{angle}	0.073	0.051	0.041	0.038	0.041	0.044	0.025
		$AS_{line + angle}$	0.121	0.075	0.065	0.038	0.041	0.068	0.049
	0.2	TS_{line}	4	4	4	1	2	2	1
		AS_{line}	0.095	0.095	0.095	0.024	0.048	0.048	0.024
		TS_{angle}	21	14	13	14	10	11	13
		AS_{angle}	0.067	0.044	0.041	0.044	0.032	0.035	0.041
		$AS_{line + angle}$	0.162	0.140	0.137	0.068	0.079	0.083	0.065
Sum			0.283	0.214	0.202	0.106	0.121	0.151	0.114
4:2FTOH	0.3	TS_{line}	3	2	2	4	0	0	1
		AS_{line}	0.071	0.048	0.048	0.095	0	0	0.024
		TS_{angle}	29	10	8	12	11	15	17
		AS_{angle}	0.092	0.032	0.025	0.038	0.035	0.048	0.054
		$AS_{line + angle}$	0.163	0.079	0.073	0.133	0.035	0.048	0.078
	1	TS_{line}	3	2	2	4	0	0	1
		AS_{line}	0.071	0.048	0.048	0.095	0	0	0.024
		TS_{angle}	32	23	19	18	15	15	22
		AS_{angle}	0.102	0.073	0.060	0.057	0.048	0.048	0.070
		$AS_{line + angle}$	0.173	0.121	0.108	0.152	0.048	0.048	0.094
Sum			0.337	0.200	0.181	0.286	0.083	0.095	0.171

TS_{line}: total scores of each point based on length comparison; TS_{angle}: total scores of each point based on angle comparison; AS_{line}: mean scores of each point based on length comparison; AS_{angle}: mean scores of each point based on angle comparison; Sum: $AS_{line + angle}$ in the first exposure concentration + $AS_{line + angle}$ in the second exposure concentration.

3.3. *Exposure altered coordinates of points and direction, distance and trajectory of movement*

Exposure to each chemical caused alterations in phenotypes, evidenced by movement of coordinates of points. Since the point with the greatest $AS_{line + angle}$ was always associated with the mouth (point 1), details for changes in that point due to exposure to chemicals are given (Fig. 2A–C and Table 5).

$CdCl_2$. Point 3 was used as the origin for $CdCl_2$. Point 1 was moved to the right by 0.032 and 0.010 and up by 0.023 and 0.028 mm after exposure to 1 and 4 mg/L $CdCl_2$, respectively (Fig. 2A). For the X-axis, points 2, 4, 5, 6 and 7 were moved to the left after exposure to 1 or 4 mg/L $CdCl_2$. For the Y-axis, points 2 and 4 were moved down, and point 7 was moved up after exposure to 1 or 4 mg/L $CdCl_2$, respectively.

TDCIPP. Point 4 was used as the origin for fish exposed to TDCIPP. Point 1 was moved to the right by 0.026 and up by 0.021 mm in 0.3 mg/L exposure group, and moved to the right by 0.062 and down by 0.001 mm in 1 mg/L exposure group, respectively (Fig. 2B). For point 2, a 0.008 mm left movement, and a 0.007 mm right movement were observed after exposure to 0.1 mg/L TDCIPP, and a 0.015 mm and a 0.023 mm down movement were observed after exposure to 1 mg/L TDCIPP. Point 5 was moved to the left by 0.021 and 0.024 mm and up by 0.008 and 0.028 mm after 0.1 or 0.2 mg/L TDCIPP exposure, respectively. Point 6 was moved to the right by 0.012 and 0.009 mm and down by 0.005 and 0.004 mm after 0.1 or 0.2 mg/L TDCIPP exposure, respectively.

4:2 FTOH. Point 5 was used as the origin for assessing exposure to 4:2 FTOH. Point 1 was moved to the left by 0.064 and down by 0.029 mm after 0.3 mg/L 4:2 FTOH exposure. Exposure to 1 mg/L 4:2 FTOH caused point 1 to be moved to the left by 0.053 and down by 0.026 mm (Fig. 2C). For the X-axis, point 2 was moved to the left after exposure to 0.3 mg/L 4:2 FTOH, but moved to the right after exposure to 1 mg/L 4:2 FTOH. For the Y-axis, the point was moved up after exposure to either 0.3 or 1 mg/L 4:2 FTOH. Points 3 and 4 were moved to the right and down after exposure to 0.3 mg/L 4:2 FTOH.

3.4. *Position of mouth point was changed during larva development*

Time-dependent response profiles of lengths of lines, sizes of angles, AS_{line}, AS_{angle}, $AS_{line + angle}$ and coordinates of points during development of larvae at 72, 96 and 120 hpf are shown (Table S1–S3). Lengths of 9 lines and sizes of 23 angles were significantly altered at 96 hpf compared with that at 72 hpf. At 120 hpf, lengths of 9 lines and sizes of 24 angles were significantly altered compared with that at 72 hpf. $AS_{line + angle}$ for point 1, 4, 5, 6 and 7 were 0.36, 0.33, 0.33, 0.33, and 0.32 at 96 hpf and were 0.37, 0.33, 0.33, 0.35 and 0.32 at 120 hpf, respectively. Point 7 was used as the origin. Point 1 was moved to the left by 0.259 and 0.389 mm and down by 0.169 and 0.159 mm at 96 and 120 hpf compared with that at 72 hpf. Point 5 was moved to right by 0.330 and 0.550 mm and up by 0.300 and 0.113 mm at 96 and 120 hpf compared with that at 72 hpf (Fig. 3).

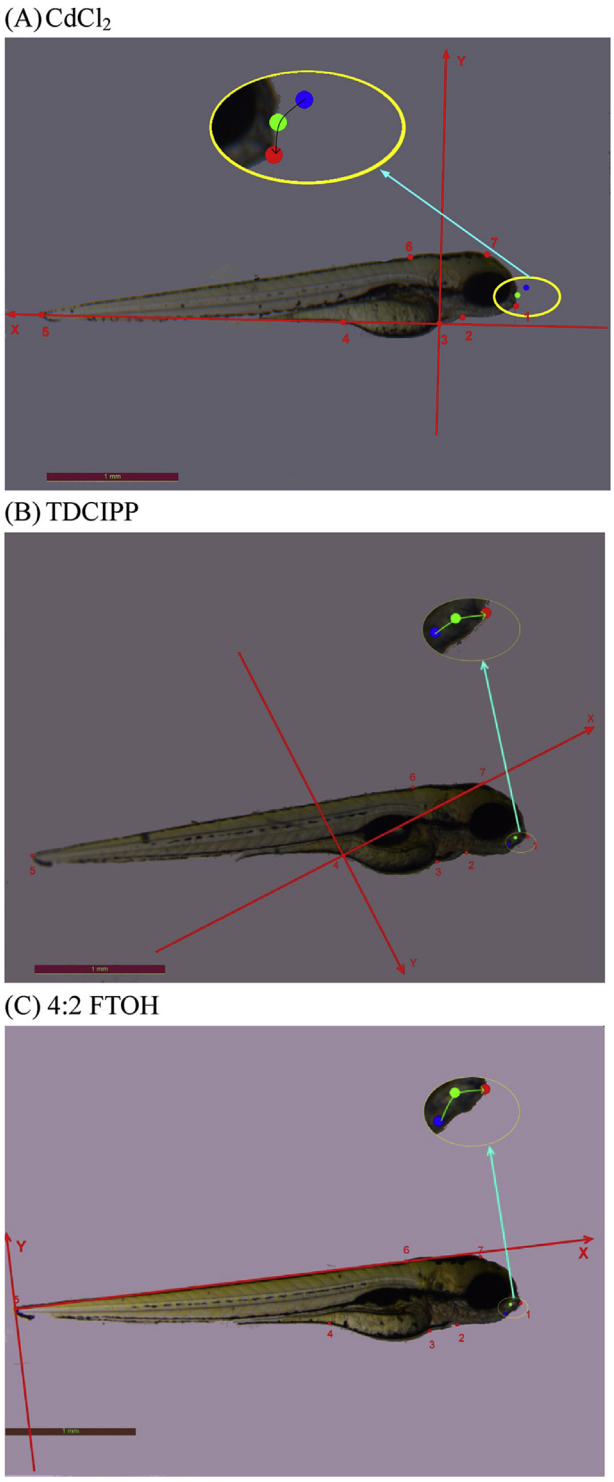


Fig. 2. Trajectory of Movement of mouth point in 96 hpf zebrafish larvae after exposure to (A) $CdCl_2$, (B) TDCIPP or (C) 4:2 FTOH from 0.75 to 96 hpf. Red point: position of mouth point in high concentration group; Green point: position of mouth point in low concentration group; Blue point: position of mouth point in control group. Arrow indicates direction of movement, and the figure is visualized with zebrafish larvae of high concentration group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

As a prominent small fish model, zebrafish has been used in various research fields for many decades, including aquatic toxicology (Gerhard, 2007; Eimon and Rubinstein, 2009; Embry et al., 2010). Besides its small size, acceptable experimental cost, diverse adaptability and short breeding cycle, one of the greatest advantages is that the fish has high fecundity and can produce transparent embryos (Dai et al., 2014). Therefore, embryos of zebrafish have frequently been used for screening for toxic potencies of chemicals, where molecular responses have been used to understand mechanisms of toxic and indication of possible adverse and long-term effects and changes in morphology during development have been observed to be correlated with longer-term survival and fecundity (Rubinstein, 2006; Thienpont et al., 2011). Recently, tests to determine toxic potencies to embryos have been a mandatory component in routine testing of whole effluents in Germany and have already been standardized internationally (DIN, 2001; Lammer et al., 2009a), and core endpoints in zebrafish embryos have been developed.

Environmental stress caused by pollution with chemicals often has direct effects on physiological states of individuals. Even when short-term lethality is not observed when animals are exposed to most concentrations of toxicants in aquatic environments, continuous, chronic exposure can result in lesser fitness, which could subsequently lead to adverse effects on survival, tolerance of other stresses, resistance to disease growth and reproductive effects such as fertility or fecundity (Polak et al., 2002). Therefore, there was a need to develop a series of sub-lethal parameters for evaluating the risks of chemicals. In previous studies, some indicators of stress within populations (e.g., body length, malformation of head or yolk sac) and heartbeat have been applied to assessments of effects of chemicals on fishes (Lammer et al., 2009b). In addition, fluctuating asymmetry (FA) has been used as a morphological assessment of effects of stressors on aquatic organisms (Van Valen, 1962; Valentine and Soulé, 1973; Wilkins et al., 1995). FA has been found to be a promising indicator for monitoring exposure of chemical and predicting population-level effects (Van Valen, 1962; Valentine and Soulé, 1973; Wilkins et al., 1995; Polak et al., 2002). However, these parameters are preset and limited and could not represent all morphological changes. Thus, it is important to develop a less-subjective and accurate method to quantitatively determine the effects of chemicals on phenotypes of zebrafish embryo/larva. In the study results of which are reported here, a three-step assay was established to comprehensively and quantitatively determine phenotypic effects of chemicals on development of embryo/larva of zebrafish.

The three-step procedure developed in this study allows a comprehensively and quantitatively determination of effects of chemicals on phenotypes of developing embryos and larvae of zebrafish. Although lethality and teratogenicity have been used as endpoints in tests with embryos of zebrafish (Lammer et al., 2009a), each of these endpoints is limited and their alterations due to exposure to chemicals were subjective and not quantifiable. Therefore, some unpredictable and small changes in phenotype might have been ignored. In this study, seven points were selected on images of larvae, which were then used to generate 21 lines, lengths of which could be calculated and 105 angles, which could be quantified, were determined by their intersections. These data more comprehensively and quantitatively described changes in morphology during development of larvae exposed to chemicals. In this study, it was determined that exposure to 0.1 or 0.2 mg/L TDCIPP significantly altered lengths of more than 50 lines or angles, while studies using the traditional methods of microscopic examination found no significant malformations due to exposure of zebrafish to concentrations of TDCIPP $\leq$0.2 mg/L (McGee et al., 2012; Fu et al., 2013). These results suggest that the three-step method developed during this study is more sensitive than

Table 5
Directions and distances of movement of points after exposure to different concentrations of $CdCl_2$, TDCIPP or 4:2 FTOH from 0.75 to 96 hpf.

Chemicals	Concentrations (mg/L)	Point 1		Point 2		Point 3		Point 4		Point 5		Point 6		Point 7	
		X axis	Y axis	X axis	Y axis	X axis	Y axis	X axis	Y axis	X axis	Y axis	X axis	Y axis	X axis	Y axis
$CdCl_2$	1	0.032	0.023	0.018	0.021	0	0	0.065	0.004	0.078	0	0.021	0.006	0.029	0.020
	4	0.010	0.028	0.039	0.017	0	0	0.046	0.009	0.142	0	0.039	0.006	0.045	0.024
TDCPP	0.1	0.026	0.021	0.008	0.015	0.003	0.010	0	0	0.021	0.008	0.012	0.005	0.009	0
	0.2	0.062	0.001	0.007	0.023	0.021	0.026	0	0	0.024	0.028	0.009	0.004	0.004	0
4:2 FTOH	0.3	0.064	0.029	0.004	0.008	0.013	0.005	0.060	0.021	0	0	0.029	0	0.009	0.002
	1	0.053	0.026	0.014	0.026	0.037	0.004	0.094	0.025	0	0	0.026	0	0.009	0.011

Numbers indicate distances of movement of points; Different colors indicate directions of movement. White: origin or axis-self; yellow: left; green: down; red: right; blue: up.

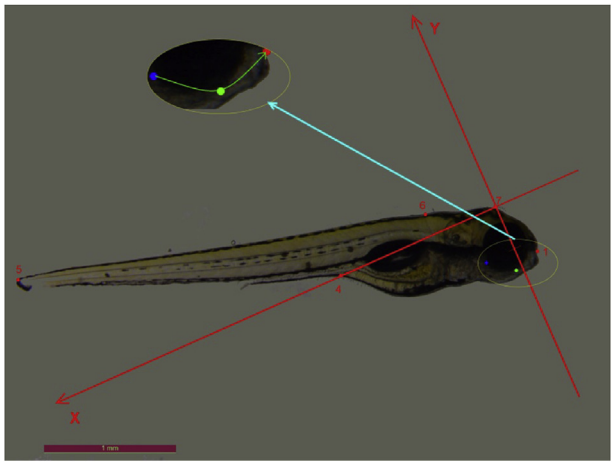


Fig. 3. Trajectory of Movement of mouth point in 72, 96 and 120 hpf zebrafish larvae. Red point: position of mouth point in 120 hpf larvae; Green point: position of mouth point in 96 hpf larvae; Blue point: position of mouth point in 72 hpf larvae. Arrow indicates direction of movement, and the figure is visualized with 120 hpf zebrafish larvae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previous methods based on more limited observations through the microscope. Similarly, no significant effects on survival or hatching were observed after exposure to 4:2 FTOH, but significant changes in more than 50 lines or angles further demonstrated the greater sensitivity of parameters developed in this study.

A scoring method was developed to prioritize points, positions of which changed due to exposure to chemicals. Observed significant alterations in lengths of lines or sizes of angles due to exposures to chemicals resulted from changes in relative positions of points. Since changes of one parameter, such as length of a line or size of an angle, might be caused by change(s) in positions of one or several points, it was deemed important to prioritize points and find whose positions that were responsible for changes in scores based on observed lengths and angles. In this study, using the method of scoring developed and results for seven points on images were successfully prioritized and reordered by comparing $AS_{line\ +\ angle}$ after exposure to $CdCl_2$, TDCIPP or 4:2 FTOH. Especially, results demonstrated that greater scores were obtained for points 1, 2 and 6 after exposure to $CdCl_2$. A previous study reported that exposure to $CdCl_2$ caused altered axial curvature, ocular edema and submaxillary edema in zebrafish embryos (King-Heiden et al., 2009), and thus our results were comparative with the previous data. Additionally, the results demonstrated that, $AS_{line\ +\ angle}$ of the mouth point was greatest after exposure to each of the chemicals tested. Results of further time-dependent studies also suggested that movement of the mouth point was the most sensitive change in morphology during development of zebrafish. Therefore, results of this study suggested that movement of the mouth point of zebrafish larvae might be a sensitive parameter for monitoring chemical exposure that might have been ignored in previous studies due to limitations of study methods.

The coordinate method was developed to identify direction, distance and trajectory of movement of points. After prioritization of points, it is important to identify their moving direction and distance. In this study, after determining the origins of the X- and Y-axes, coordinates of all points were calculated and relative direction and distance of movement compared with those of unexposed controls. Furthermore, for some prioritized points, such as the mouth point, trajectories can be compared after exposure to various chemicals and concentrations with corresponding positions for the control group. Information obtained in this part of the study is not only key to express phenotypic changes due to chemical exposure, but is also useful for describing trajectory of moving or prediction of toxicity and might even be diagnostic of mechanisms of toxicity. By integrating scoring and coordinate methods, morphological changes can be precisely expressed and altered trajectories can be described. Also, movement of the point defining the mouth was found to be a sensitive parameter to monitor during exposure to chemicals. The method described is more sensitive and quantitative than simple microscopic examination for deformities.

Contributions

C. Liu, G. Li, C. Xie, B. Zhou, Z. Han and H. Yu conceived and designed the work. H. Ye, C. Liu and G. Li performed the experiments. C. Liu and G. Li draft the manuscript. G. Su, R.J. Letcher and J.P. Giesy modify the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This work was supported by Fundamental Research Funds for the Central Universities (2016PY029, 2662015PY036), Huazhong Agricultural University Scientific & Technological Self-Innovation

Foundation (2014RC001), the National Nature Science Foundation of China (NSFC) (Grant No. 31570510) and Key Projects in Fok Ying Tung Education Foundation (Grant No. 141078). Dr. Zhihua Han was supported by National Science Foundation of Jiangsu Province (BK20130100). Prof. Giesy was supported by the Canada Research Chair program, and as a concurrent professor of the University of Nanjing as well as the 2012 "High Level Foreign Experts" (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. The research was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (Project # 326415-07) and a grant from the Western Economic Diversification Canada (Project # 6578, 6807 and 000012711). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.07.163.

References

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC50 test in chemical assessment: the fish embryo toxicity test goes multi-species—an update. Altex 22, 87–102.

Chandroo, K.P., Duncan, I.J.H., Moccia, R.D., 2004. Can fish suffer? Perspectives on sentience, pain, fear and stress. Appl. Anim. Behav. Sci. 86, 225–250.

Cheng, H.C., Yan, W., Wu, Q., Liu, C.S., Gong, X.Y., Hung, T.C., Li, G.Y., 2017. Parental exposure to microcystin-LR induced thyroid endocrine disruption in zebrafish offspring, a transgenerational toxicity. Environ. Pollut. 230, 981–988.

Dai, Y.J., Jia, Y.F., Chen, N., Bian, W.P., Li, Q.K., Ma, Y.B., Chen, Y.L., Pei, D.S., 2014. Zebrafish as a model system to study toxicology. Environ. Toxicol. Chem. 33, 11–17.

DIN, 2001. German Standard Methods for the Examination of Water, Waste Water and Sludge—Subanimal Testing (Group T)—Part 6: Toxicity to Fish. Determination of the Non-acute-poisonous Effect of Waste Water to Fish Eggs by Dilution Limits (T 6). German Standardization Organization. DIN 38415–38416.

EC (European Commission), 2001. White Paper—Strategy for a Future Chemicals Policy. European Commission, Brussels.

Eimon, P.M., Rubinstein, A.L., 2009. The use of in vivo zebrafish assays in drug toxicity screening. Expert. Opin. Drug Metabol. 5, 393–401.

Embry, M.R., Belanger, S.E., Braunbeck, T.A., Galay-Burgos, M., Halder, M., Hinton, D.E., Lcbonard, M.A., Lillicrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat. Toxicol. 97, 79–87.

Fu, J., Han, J., Zhou, B., Gong, Z., Santos, E.M., Huo, X., Zheng, W., Liu, H., Yu, H., Liu, C., 2013. Toxicogenomic responses of zebrafish embryos/larvae to tris (1, 3-dichloro-2-propyl) phosphate (TDCPP) reveal possible molecular mechanisms of developmental toxicity. Environ. Sci. Technol. 47, 10574–10582.

Gerhard, G.S., 2007. Small laboratory fish as models for aging research. Ageing Res. Rev. 6, 64–72.

King-Heiden, T.C., Wiecinski, P.N., Mangham, A.N., Metz, K.M., Nesbit, D., Pedersen, J.A., Hamers, R.J., Heideman, W., Peterson, R.E., 2009. Quantum dot nanotoxicity assessment using the zebrafish embryo. Environ. Sci. Technol. 43, 1605–1611.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009b. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. 149C, 196–209.

Lammer, E., Kamp, H.G., Hisgen, V., Koch, M., Reinhard, D., Salinas, E.R., Wendler, K., Zok, S., Braunbeck, T., 2009a. Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*). Toxicol. Vitro 23, 1436–1442.

Liu, C., Wang, Q., Liang, K., Liu, J., Zhou, B., Zhang, X., Liu, H., Giesy, J.P., Yu, H., 2013. Effects of tris(1,3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. Aquat. Toxicol. 128, 147–157.

Macova, S., Dolezelova, P., Pistekova, V., Svobodova, Z., Bedanova, I., Voslarova, E., 2008. Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*. Neuro. Endocrinol. Lett. 29, 680–684.

Mahmoud, M.A., Kärrman, A., Oono, S., Harada, K.H., Koizumi, A., 2009. Polyfluorinated telomers in precipitation and surface water in an urban area of Japan. Chemosphere 74, 467–472.

McGee, S.P., Cooper, E.M., Stapleton, H.M., Volz, D.C., 2012. Early zebrafish embryogenesis is susceptible to developmental TDCPP exposure. Environ. Health Perspect. 120, 1585.

Nagel, R., 2002. DarT: the embryo test with the Zebrafish *Danio rerio*—A general model in ecotoxicology and toxicology. Altex 19, 38–48.

OECD, 1992. OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 203: Acute Toxicity for Fish. Organization for Economic Cooperation and Development, Paris, France.

Polak, M., Opoka, R., Cartwright, I.L., 2002. Response of fluctuating asymmetry to arsenic toxicity: support for the developmental selection hypothesis. Environ. Pollut. 118, 19–28.

Ratte, H.T., Hammers-Wirtz, M., 2003. Evaluation of the Existing Data Base from the Fish Embryo Test. UBA (German Environmental Agency, Dessau) Report under contract No. 363 01 062, p. 27.

Rubinstein, A.L., 2006. Zebrafish assays for drug toxicity screening. Expert. Opin. Drug Metabol. 2, 231–240.

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. 15, 394–404.

Thienpont, B., Tingaud-Sequeira, A., Prats, E., Barata, C., Babin, P.J., Raldúa, D., 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environ. Sci. Technol. 45, 7525–7532.

van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88, 1119–1153.

Valentine, D.W., Soulé, M.E., 1973. Effect of p,p'-DDT on developmental stability of pectoral fin rays in the grunion, *Leuresthes tenius*. Fish. Bull. 71, 920–921.

Van Valen, L., 1962. A study of fluctuating asymmetry. Evolution 125–142.

Wilkins, N.P., Gosling, E., Curatolo, A., Linnane, A., Jordan, C., Courntney, H.P., 1995. Fluctuating asymmetry in Atlantic salmon, European trout and their hybrids, including triploids. Aquaculture 137, 77–85.

Wu, Q., Yan, W., Liu, C.S., Li, Li, Yu, L.Q., Zhao, S.J., Li, G.Y., 2016. Microcystin-LR exposure induces developmental neurotoxicity in zebrafish embryo. Environ. Pollut. 213, 793–800.