The Common Targeted Organ in Zebrafish Embryos Exposed to Eight Toxic Chemicals

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Research Article

Keywords: Tris(2,3-dibromopropyl) isocyanurate (TBC), Chlorinated paraffins(CPs), Hexabromocyclododecane(HBCD), Metals, Zebrafish(Danio rerio), Gas bladders

Posted Date: November 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1031984/v1

Abstract

Zebrafish (*Danio rerio*) embryos are widely used in toxicity tests, especially in investigations on chlorinated or brominated flame retardants (BFRs) and metals. A key challenge in environmental risk assessment (ERA) is how to clarify the same or different sites of toxic action in a species after exposure to the individual chemicals or chemical mixtures and further provide the common toxic sites or organs for risk assessment of chemical mixtures. In this study, zebrafish embryo was used to evaluate the sublethal toxicity (gas bladder damage) of tris(2,3-dibromopropyl) isocyanurate (TBC), Chlorinated paraffins (CPs), hexabromocyclododecane (HBCD), and Cu, Cd, Pb, Ag, Zn, and corresponding sublethal molecular levels (and inflammation-related enzymes [deiodinase (DIO) enzymes]) in fish through optical microscopy methods. The tested chemicals all caused failed inflation of the gas bladder, as indicated by activity inhibition of type 2 iodothyroxine deiodinase enzyme. We put up with the common targeted sites or organs for further studying the toxic mechanisms underlying the chemical mixtures.

1. Introduction

Zebrafish (*Danio rerio*) is worldwide used in a great number of studies on biological toxicology (Scholz 2013, Teixido et al. 2020). In the past ten years, zebrafish early life toxicity tests on chlorinated and brominated flame retardants (BFRs) got a lot of advances to reveal toxic action modes of EDCs on inflammation of cells both in vitro and in vivo (Li et al. 2011, Tokarz et al. 2013). Different chemicals initiated toxicity variation due to their different physio-chemical properties, which was reported in short-term exposure studieson single-species (Baird &Van den Brink 2007, Maltby et al. 2005). For some lipophilic compounds like polychlorobiphenyls (PCB), hexabromocyclododecanes (HBCD), and Tris(2,3-dibromopropyl) isocyanurate (TBC), only a small amount of them was metabolized and eliminated by test organisms, which made those compounds were concentrated in the various organs or tissues of the organisms from the surrounding water(Venquiaruti Escarrone et al. 2016). A key challenge in environmental risk assessment (ERA) is how to clarify the same or different sites of toxic action in a species after exposure to the individual chemicals or chemical mixtures To comprehensively explore the potential of zebrafish exposed different chemicals or chemical mixtures, it is necessary to find a common pathway to relate local toxic targeted sites or organs.

The zebrafish (*Danio rerio*) as toxicology model has many advantages, e.g., rapid development with some organs functioning already at 96 h post-fertilization, optical transparency in early life stages, and low-cost usage and has been widely used to explore toxic action mechanisms of many chemicals from molecular level to morphology changes. In many early developmental toxicity assays on fish, the gas bladder inflation obstacle is a key event in developmental toxicity(Stinckens et al. 2018) and the associated molecular effects include the activity of inflammation-related enzymes [deiodinase (DIO) enzymes] in the gas bladders. Moreover, it is well known that the gas bladder in teleosts is to maintain the normal swimming activities. In this study, we wanted to konw whether the gas bladder is a common targeted organ of most chemicals in fish and, if so, the failed gas bladder inflation can become the potential adverse outcome pathway of joint toxicity of chemical mixtures. We specifically investigated the effects

of chemicals on the gas bladder and molecular effects (DIO1 and DIO2 enzyme activity) in zebrafish larvae.

2. Materials And Methods

2.1 Organisms and chemicals

Zebrafish have been cultured in our laboratory for >8 years. For the maintenance and toxicity testing of these fish, we followed OECD guidelines (OECD 203 and 210). Zebrafish were cultured in 20-L aquariums filled with filtered tap water (filtered through a GF/C membrane with a 1.2-μm pore size; Millipore, Watford, UK). The zebrafish were fed with newly hatched brine shrimp (*Artemia salina*) three times a day and maintained with a 12:12 h light:dark photoperiod at a constant temperature of 28 ± 0.5°C. The embryos were used in the experiments because of their transparent bodies, which provide good visibility to various internal organs. This study was conducted in accordance with the national and institutional guidelines for the protection of human subjects and animal welfare.

Tris(2,3-dibromopropyl) isocyanurate (TBC, 97% purity) Hexabromocyclododecane (HBCD, 95% purity), and chlorinated paraffins (CPs, 95% purity) were purchased from Sigma-Aldrich (St Louis, MO). The TBC, HBCD, and CPs stock solutions were prepared by dissolving TBC, HBCD, and CPs powder in DMSO (dimethyl sulfoxide, Sigma), which were stored under 4°C. The Cu, Zn, Cd, Pb, and Ag stock solutions (1 g/L, >99%; Kermel Ultra Pure) were prepared by adding copper sulfate, zinc chloride, cadmium chloride, lead nitrate, and silver nitrate salts (>99%; Kermel Ultra Pure) into Ultrapure water (Milli-Q, R ≥18.2 MU cm), respectively.

2.2 Toxicity tests

For all control and Chemical-treated groups, larvae were maintained in an artificial climate box at 28°C ± 0.5°C and reared in sterile 6-well cell culture plates (Corning Inc., Corning, NY, USA) at a density of 30 embryos per well, with each well containing 10 mL of exposure solution. Each treatment was performed in triplicate. The larval fish were not fed during the 96-h test.

2.3 Chemical exposure

The above stock solutions were diluted into filtered tap water to create a series of chemical solutions. The normal embryos were respectively placed into TBC at 500, 1000, 2500, and 5000 μg/L, HBCD at 250, 500, 1000, and 2500 μg/L, CPs at 1000, 5000, 10000, and 20000 μg/L with the DMSO concentrations in exposure less than 0.4%, and, in the meantime, embryos in control groups were containing 0.4% DMSO. The metal groups were administered with 2, 4, 8, 10 μg L^{-1} of Cu, 100, 200, 500, 1000 μg L^{-1} of Zn, 5, 10, 20, 50 μg L^{-1} of Cd, 20, 40, 80, 100 μg L^{-1} of Pb, and 2, 4, 8, 10, and 20 μg L^{-1} of Ag^{+} dispersed in filtered tap water. After 96 h, all larvae were observed, collected, and impairment of gas bladders recorded in triplicate. Morphological observations of swim bladders were conducted under an optical microscope (10× objective lens).

2.4 In chemical screening for DIO enzyme inhibition

The DIO1 and DIO2 enzyme activity were determined by measuring the amount of free ^{125}I released through conversion of ^{125}iodine-labeled rT3 or T4 by DIO1 or DIO2, respectively(Cavallin et al. 2017, Vergauwen et al. 2018). All 96 hpf larvae collected in chemical groups were used to determine the activity of DIO1 and DIO2 enzymes, which was achieved using colorimetric assay kits from Shanghai JiangLai Bioengineering Institute (Shanghai, China) following the homogenization, labelling, washing, and chromogenic reaction instructions.

2.5 Statistical analyses

The obtained data are expressed as mean values ± standard deviation (SD). SPSS version 22.0 was used for all statistical analyses. The fold change of the area of the gas bladder was calculated by setting the area of the gas bladder in control groups to 1 and scaling the area of the gas bladder in chemical treated groups under an optical microscope (10×objective lens). The deformation rates of the swim bladders were compared statistically between the different chemical concentration treatments and the control group using an independent t-test. The differences among treated groups were compared by the independent t-test or one-way ANOVA followed by pairwise comparisons using a Student–Newman–Keuls post hoc test and considered statistically significant when p values were <0.05. All figures were plotted in Origin 9.1.

3. Results And Discussion

Failed inflation of the swim bladder and DIO enzyme inhibition

Exposure to individual chemicals for 96 h caused swim bladder inflation defects in zebrafish larvae, taking TBC as an example (Figure 1). Figure 1b showed failed inflation in the gas bladder of larvae exposed to 1000 μg/L TBC after 96 hpf. Figure 1c showed the decreased area in inflation of gas bladder of larvae exposed to 1000 μg/L TBC after 96 hpf.

Increasing chemical concentrations showed a significantly decreased relative area of the gas bladder(Figure 2). Increasing chemical concentrations showed significantly increased failed inflation of the gas bladder(Figure 3). There were no significant differences in the relative area of the larval gas bladder between the 250, 500 μg/L HBCD exposure group and the control group (Figure 2b). However, compared with the control group, there were significant differences in the relative area of the swim bladder in the 1000 and 2500 μg/L HBCD exposure group (Figure 2b). Also, there were no significant differences in the relative area of the larval gas bladder between the 2, 4 μg/L Cu exposure group, 100, 200 μg/L Zn exposure group, and the control group(Figure 2d and h).

HBCD could cause zebrafish larvae gas bladder defects, which led to the failure of gas bladder inflation, and the pathological phenomenon of losing the capacity of free swimming, which could ultimately lead to the death of zebrafish larvae. Following embryonic exposure to CPs, deformity effects of gas bladders

of zebrafish larvae were observed in CPs-treated groups and as the concentration of the CPs increased, deformity of gas bladders of zebrafish larvae increased, with an obvious dose-response relation (Figure 3c).

Increasing concentrations of TBC, HBCD, CPs, Cu, and Zn inhibited the enzyme activity of DIO1 in larvae after 96 h exposure, while increasing concentrations of Cd and Pb elevated the enzyme activity of DIO1(Figure 4). TBC, HBCD, CPs, Cu, Cd, Pb, Ag, and Zn all inhibited the enzyme activity of DIO2 in larvae after 96 h exposure(Figure 5). Most chemicals that were both strong DIO1 and DIO2 inhibitors had an adverse effect on posterior chamber inflation or gas bladder area. For example, PFOS exposure also caused spinal curvatures in zebrafish larvae and affected the gas bladder inflation(Hagenaars et al. 2014).

With an increase in Ag^+ concentration in the larval body, the enzyme activity of DIO1 did not vary (Figure 4), whereas the activity of DIO2 was inhibited and showed a credible logistic dose-response relationship (Figure 5). This result indicates that DIO2 enzymes might play a vital role in the aeration of the swim bladder. In support of this phenomenon, most compounds that strongly reflect DIO1 and DIO2 affect posterior chamber ventilation or surface area(Stinckens et al. 2018). Some compounds are only strong DIO2 inhibitors, whereas Ag^+, like tetrachlorobisphenol A, is a strong DIO1 inhibitor but not a DIO2 inhibitor(Stinckens et al. 2018). In a previous study, 4 days after fertilization, peaks of DIO1 and DIO2 mRNA expression were observed in normal developing zebrafish(Vergauwen et al. 2018), indicating that either isotype is dominant at this point. However, DIO2 is suggested to be the main contributor to TH activation in developing zebrafish embryos(Darras et al. 2015). Studies have shown that the knockout of DIO1 mRNA affects the embryonic development of zebrafish, whereas the knockout of DIO2 delays the development of ear capsule length, head-trunk angle, and pigmentation index(Houbrechts et al. 2016).

4. Conclusion

Although the accumulation amount of chemicals in organisms under joint exposure can be obtained in previous studies(Iwasaki &Gauthier 2016, Stankeviciute et al. 2018), the joint toxicity mechanism of toxicity is still unclear(Gao et al. 2016). The main reason is that the understanding of the possible interactions of chemicals in the process of toxicity is limited. In addition to paying attention to individual-level toxicological indicators (such as mortality), it is necessary to further study the impact of toxicological indicators (such as sub-lethal indicators) that have a stable dose-effect relationship at the molecular level. Eight chemical (TBC, HBCD, CPs, Cu, Cd, Pb, Ag, and Zn) toxicity tests in zebrafish embryos further proved that the gas bladder inflation obstacle is a key event in developmental toxicity(Stinckens et al. 2018) and the associated molecular effects [deiodinase (DIO) enzymes] in the gas bladders.

In summary, the combined toxicity of chemicals is widespread in the actual environment. The current research mainly focuses on the dose-effect relationship between exposure concentration and the mortality endpoint, ignoring the combined effects that may exist at the biological molecular level, which leads to the uncertainty of chemical environmental risk assessment and the benchmark value. In this

study, we select molecular level (such as DIO enzyme) and individual toxic indicator (gas bladder malformation) to study the toxic effect of chemicals in organisms and propose potential effect indicators for the combined effects of chemicals in organisms. The research results are of great significance for revealing the joint toxic mechanism of chemicals, scientifically predicting the joint toxicity of chemicals in the water environment, and accurately evaluating the joint toxicity and risk of chemicals.

Declarations

Acknowledgements:

This study was supported by the National Natural Science Foundation of China (41877498), China Postdoctoral Science Foundation-funded project (2019M651017) and Tianjin Natural Science Foundation (19JCQNJC08100).

Author Contribution

Yongfei Gao- responsible for the overaching research goals, data analyses and writting of the manuscript.

Pengyuan Yang- Coordination of experiment and preparation.

Ethics approval and consent to participate In this study, none of the authors used human beings as research subjects. International, national, and institutional guidelines for the care and use of animals were followed.

Consent to publish In the present study, no personal information is used (including any individual's personal details, images or videos).

Conflict of interest The authors declare no competing financial interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Data Availability

All data, associated metadata, and calculation tools are available through figshare, are available from the authors. All data (including data that are not publicly available) must be made available to the editor and reviewers during the review process, if requested.

Consent to Publish

The work described has not been published previously and is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language.

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Figures

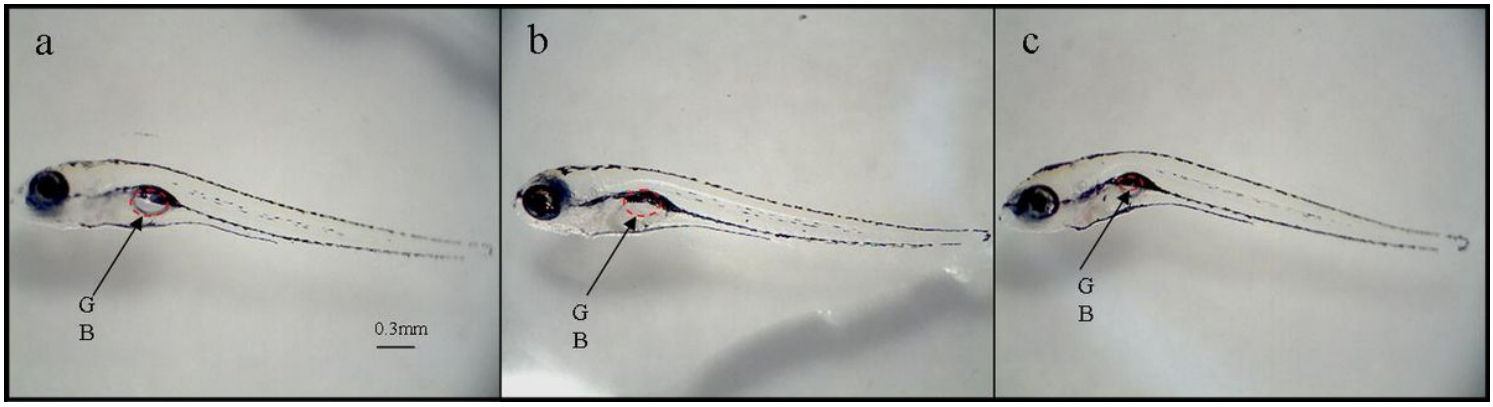

Figure 1

The 96 hpf treatment with individual chemicals (e.g., TBC) leads to defects in the gas bladder in larvae development. (a)The profile picture of zebrafish in the control group, arrow points to the normal gas bladder(GB);(b)1000 μg/L TBC exposed larvae, showing defects in inflation of gas bladder(failed inflation□(c)1000 μg/L TBC exposed larvae, showing defects in inflation of gas bladder(decreased area).

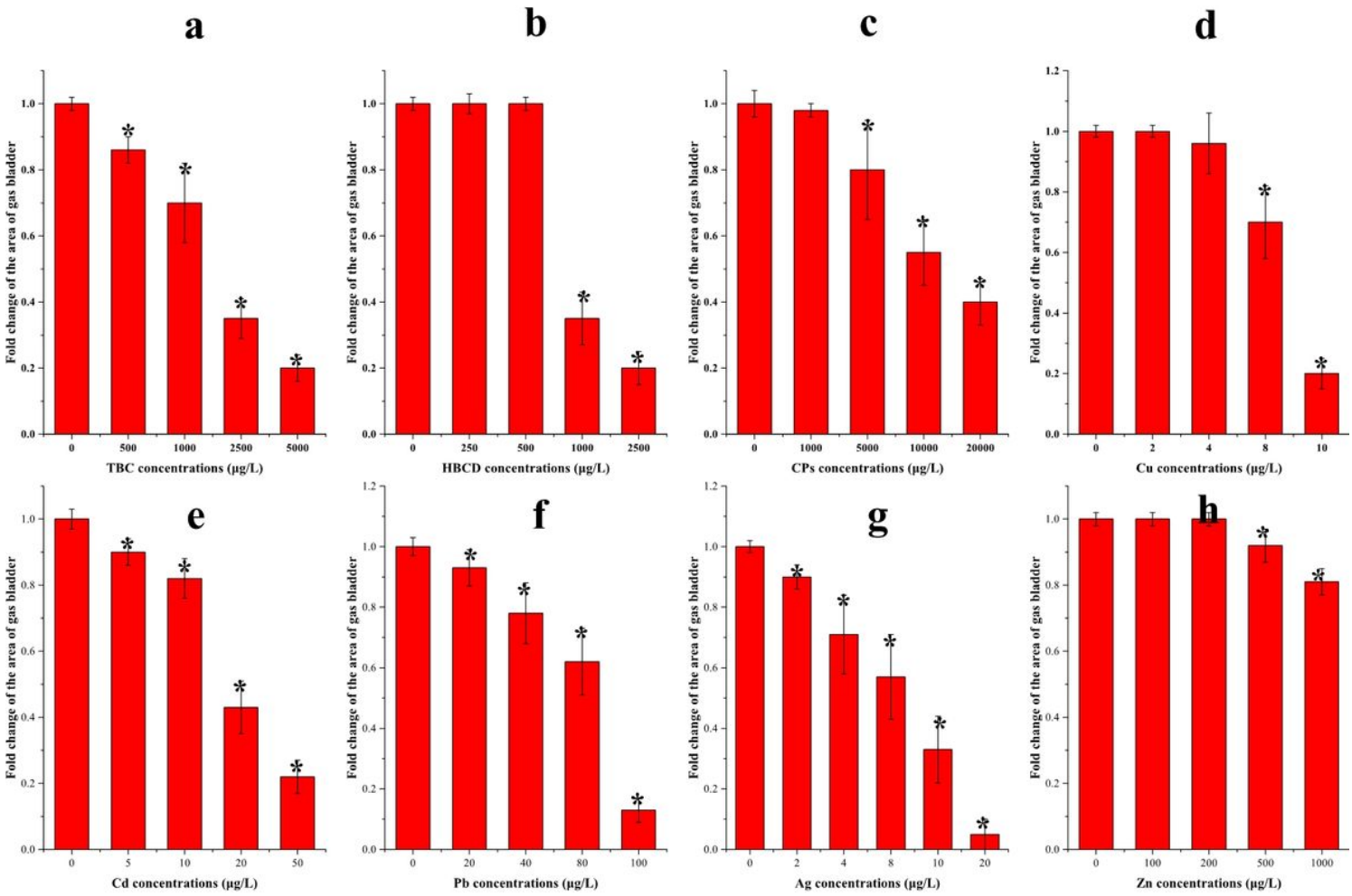

Figure 2

Relative areas of the gas bladder of zebrafish larvae exposed to chemicals at 96 hpf. Asterisk means significant difference with control group($p \leq 0.05$).

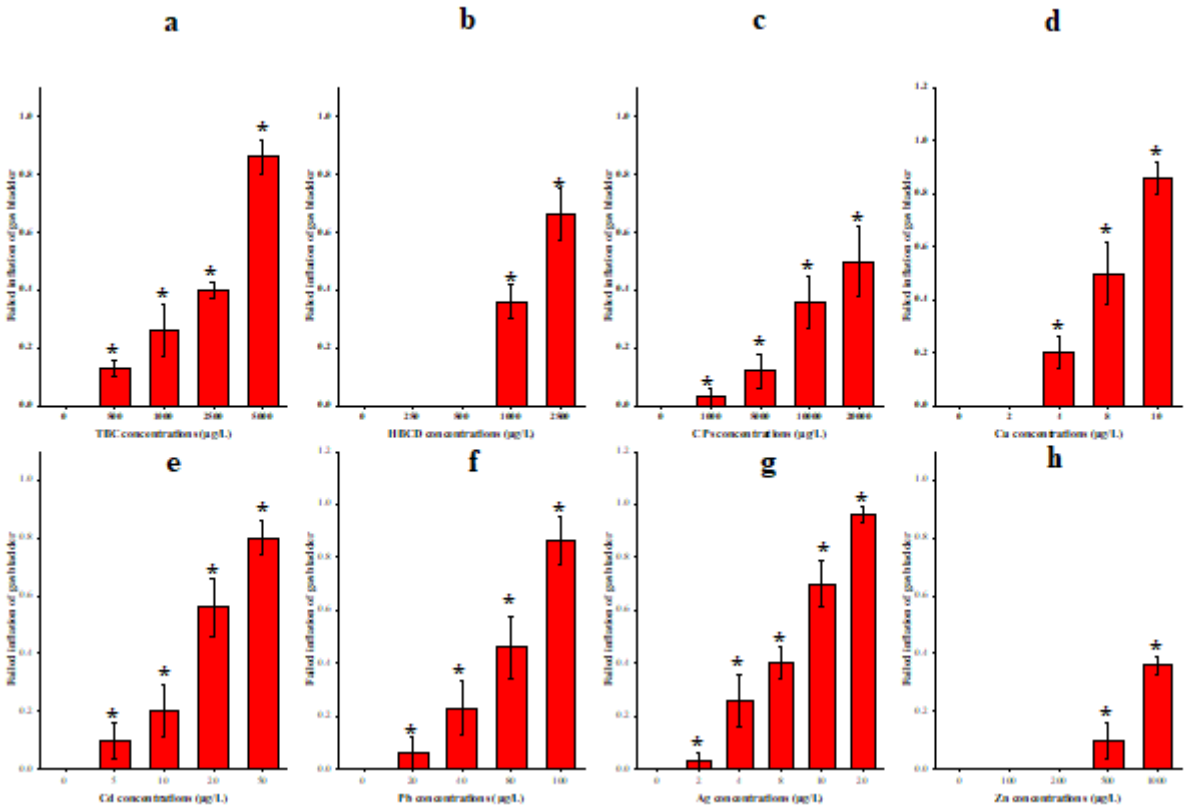

Figure 3

Deformity of the gas bladder of zebrafish larvae following embryonic exposure to different concentrations of individual chemicals during 96 hpf. Each bar represents the mean ± SE （n=3,30 embryos in each replicate）. *: means the significant difference from the control group. p≤0.05.

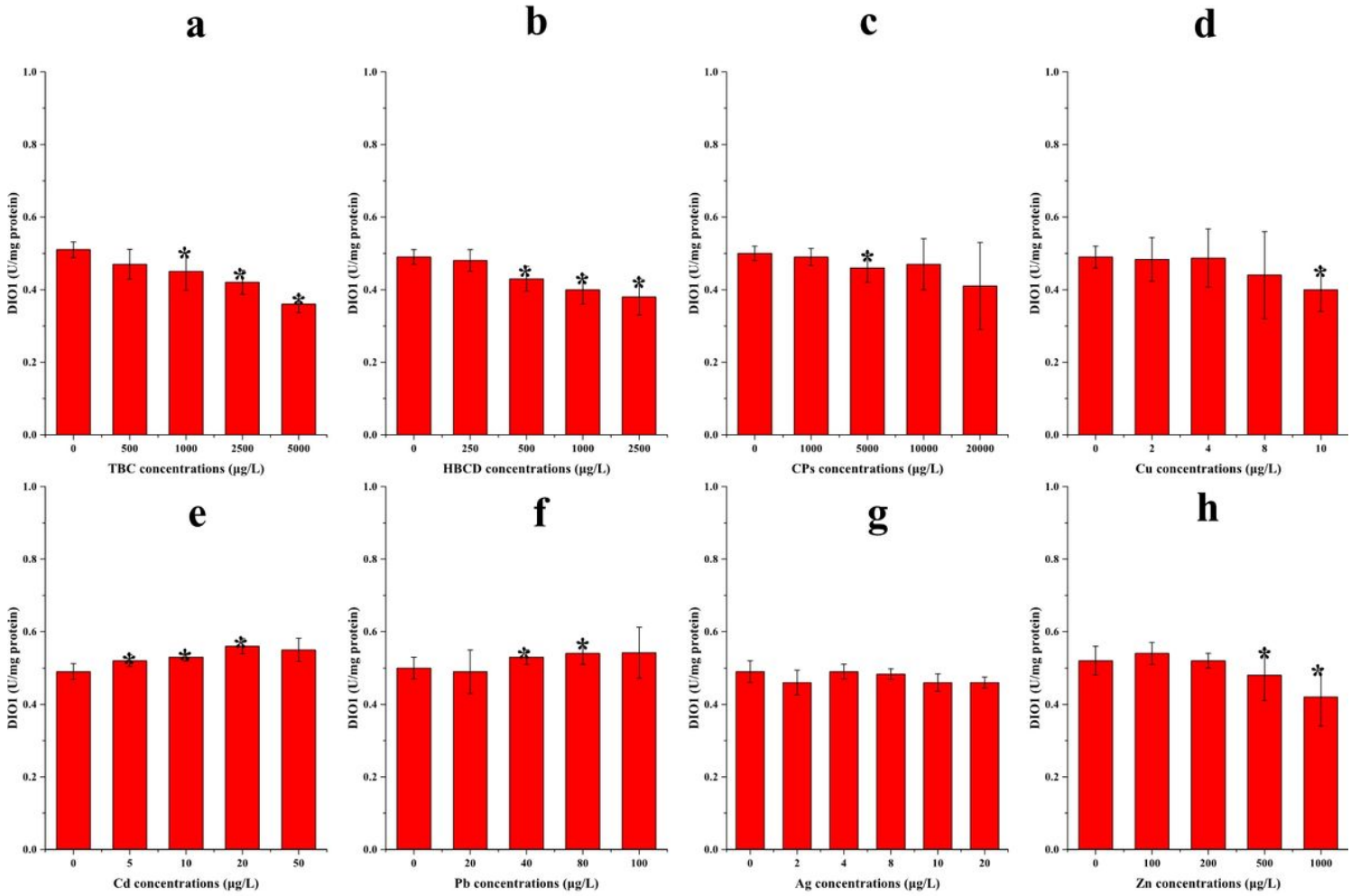

Figure 4

The relationships between individual chemical concentration (μg L-1) and DIO1 activity (U mg protein-1). Each bar represents the mean ± SE （n=3,30 embryos in each replicate）. *: means significantly different from the control group. $p \leq 0.05$.

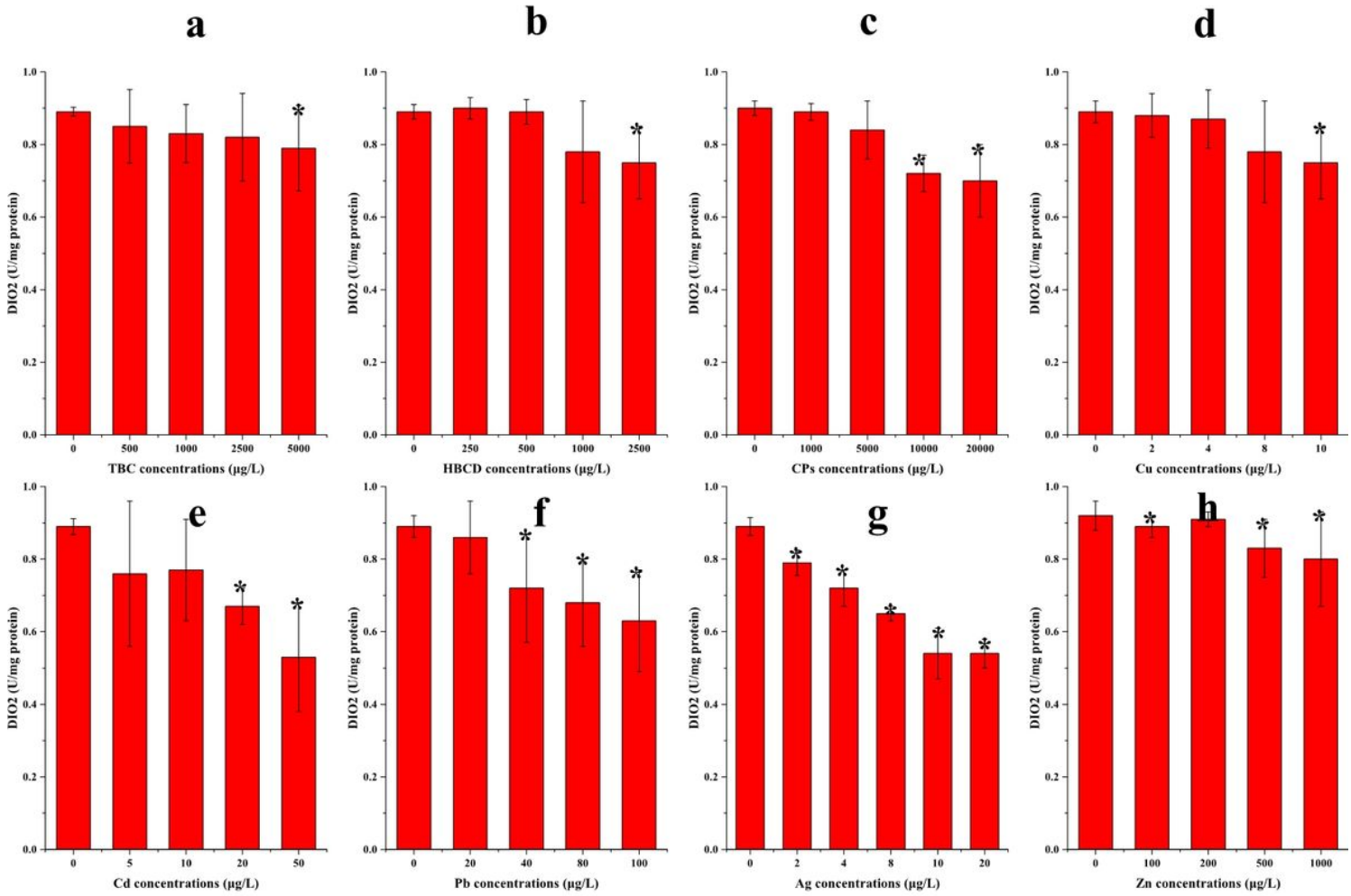

Figure 5

The relationships between individual chemical concentration (μg L-1) and DIO2 activity (U mg protein-1). Each bar represents the mean ± SE （n=3,30 embryos in each replicate）. *: means significantly different from the control group. $p \leq 0.05$.