AN INTEGRATED STUDY ON TOXICITY OF DECHLORANE PLUS IN ZEBRAFISH

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Introduction

Dechlorane Plus (DP), a chlorinated flame retardant recently identified as a high production volume (HPV) chemical[1], has been observed persistent in the environment contaminant, bioaccumulative in organisms and susceptible to long-range atmospheric transport[2, 3]. Toxicity data for DP are very limited till now. Most of the toxicity data so far are provided by USEPA HPV Test Challenge report[1] and the manufacturer manual of Oxychem[4]. Besides these official reports, only five research reports have been published recently[5-9]. Most of the existing toxicity data of DP were concerned with the in-life parameters or clinical or anatomical pathology, but almost no significant toxic effects were observed at this level. Further studies on DP toxicity at molecular levels seem to be more powerful for revealing the subtle toxicological effects and mechanisms of DP. Moreover, animal species used in DP toxicity researches were very limited, mainly focused on mammalian and avian. Since DP were widely detected in water[2, 3] all over the world (0.0013-348 ng/L) and could be bioaccumulated through aquatic food webs (0.015-1971 ng/g lw), it is necessary to evaluate the risk of DP on aquatic biota.

In this study, acute and sub-acute toxicity of DP were firstly investigated in zebrafish (*Danio rerio*) embryos and adults, respectively. Then, proteomic profiles of zebrafish liver and brain after DP exposure were analyzed. Zebrafish, as a model organism, has been widely used for many years to assess the effects of chemical contaminants on development and toxicity. The goals of our study were to determine a) whether DP had any biological toxicity in zebrafish embryo or adult under the concentrations currently accumulated in aquatic organisms, and b) the possible toxicological mechanism of DP.

Materials and methods

Animal care and DP exposure. Wild type zebrafish, Tübingen strain, were maintained in an aquatic housing system (Beijing ESEN EnvironScience Ltd.) with the temperature of 28.0±0.5°C, conductivity of 744 μS/cm, pH of 7.5±0.25 and 14 h light/10 h dark photoperiod. DP was purchased from Anpon Electrochemical Co., Ltd in Jiangsu Province of China. Embryos at 8 hour past fertilization (hpf) were exposed to different doses of DP for up to 7 days. Stock solution (DP powder in solvent acetone) was diluted with Holt buffer (NaCl 3.5g, KCl 0.05g, $NaHCO_3$ 0.025g and $CaCl_2$ 0.1g in 1 liter deionized water) and the final concentrations of acetone in each exposure solutions were adjusted to 1%. All solutions were changed once a day and twenty embryos were used to evaluate daily for mortality and malformations. Six-month adult zebrafish were oral exposed to DP at the doses of 0.25, 2.5 and 7.5mg/g bw (body weight) per day for 7, 14 and 28 days, respectively. DP powder was mixed with 1g freshly hatched brine shrimp and 0.02g agar in 2ml distilled water. After microwave heating for 30 seconds, the mixtures were cooled and solidified. The solidified mixtures were cut into little pieces and fed as foods to the testing zebrafish within one day. Besides DP exposure group (TDP), two controls were set up: one was fed with freshly hatched brine shrimp only (Ctl1), the other was fed with mixtures of freshly hatched brine shrimp and agar (Ctl2). All zebrafish used were housed under the standard conditions.

DP analysis. Standards for individual *anti*- and *syn*-DP (CAS# 13560-89-9) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). All solvents used were of analytical grade (Sigma-aldrich, China). All samples were quantified at International Joint Research Center for Persistent Toxic Substances (IJRC-PTS), Dalian Maritime University, using an Agilent 6890GC coupled to an Agilent 5973N mass spectrometer detector (GC/MSD), according to the method of Jia *et al.*[10]. f_{syn} value was defined as the amount of *syn*-DP divided by the total amount of DP.

Morphological Observation. Zebrafish embryos were observed under Motic SMZ-168 Stereo Zoom Microscope, and imaged using Nikon E5400.

Superoxide dismutase (SOD)assay. Activity of SOD was measured using the assay kit of Nanjing Jiancheng Bioengineering Institute.

Apoptosis assay. Cell apoptosis was detected using *in situ* TUNNEL staining kit (TaKaRa Bio, Inc.).
Gene expression analysis. Gene (including *sod* and apoptosis-related gene *p53)* response to DP was detected using quantitative PCR (Q-PCR). Total RNA were isolated by TRizol reagent. Primers and probes were designed using Primer Express (Applied Biosystems) software (Table 1). Housekeeping gene *gapdh* was chosen as an internal control. Cycle threshold (Ct) data were normalized to *gapdh*, and the fold changes in target genes were calculated using the $2^{\Delta\Delta Ct}$ method. Q-PCR was carried out on an ABI7300 sequence detection system (Applied Biosystems), using the universal conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles (15 s at 95°C, 1 min at 60°C). Every sample was analyzed in triplicate.

Table 1 Sequences of primers and probes used in the Q-PCR

Gene	GenBank Number	Primer	Probe
gapdh	NM_001115114	ACGGATTCGGTCGCATTG GGGTGGAGTCGTACTGGAACA	CCGTCTGGTGACCCGTGCTGCT
sod	AY195857	AGGATTGCAGCGTGTGCTAA AGATAGTACGCATGTTCCCAGACA	CAAGACCCTTTGCAAGGGACCACA
p53	BC095597	GAACAGCCTCAGCCATCCA GAACCTGAGCCTAAATCCATGATC	TCTCCCACCAACATCCACTGTTCCG

Proteomic analysis. Livers and brains from three biological replicates were dissected. Total protein of each sample was extracted for two-dimensional (2D) gel electrophoresis. Gel images obtained were analyzed by PDQuest software. Every 6 gels, 3 TDP and 3 Ctl1 at the same time point of check, were merged into one master gel. Proteins with great alterations (≥2.0) after DP exposure were selected for T-TEST analysis. Only proteins with statistical significance ($p \leq 0.05$) were excised from 2D gel and analyzed by MALDI-TOF/TOF mass. primary sequence obtained were searched using Mascot to identify the proteins.

Results and discussion:
Acute toxicity of DP on zebrafish embryonic development. Acute exposure of DP can induce observable spine side curve, cardiac edema and tail deformation in zebrafish embryos and larvae (Figure 1). Both dose-dependent and time-dependent effects were observed on total malformation rate in DP exposed zebrafish, using acetone as solvent (Figure 2).

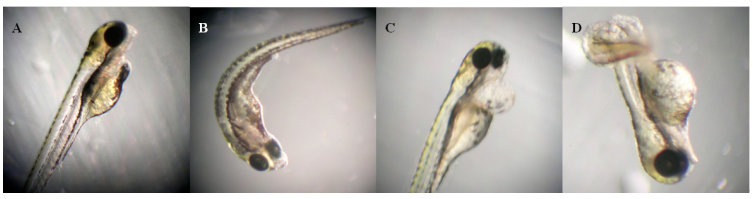

Figure 1. Acute exposure of DP can induce observable malformation. A. Normal; B. spine side curve; C. cardiac edema and D. tail deformation.

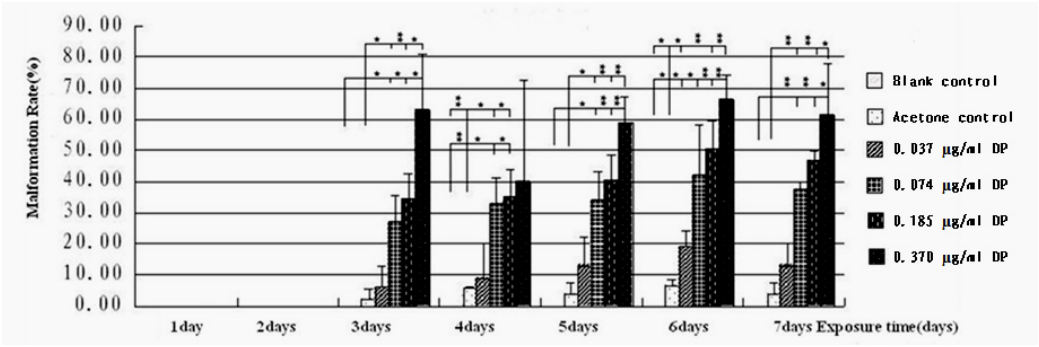

Figure 2. Acute exposure of DP on total malformation rate in zebrafish embryos

Accumulation of DP in zebrafish. DP could be bioaccumulated in zebrafish after 7-day oral exposure and the f_{syn} value of the accumulated DP in zebrafish were decreased (Table 2), suggesting *syn*-and *anti*-DP may have different metabolic pathways in zebrafish.

Table 2. DP accumulation in zebrafish after 7-day oral exposure

DP exposure (mg/g bw)	DP accumulated in zebrafish (ng/g)	f_{syn}*
0.25	618.4	0.49
0.50	587.2	0.41
2.50	762.8	0.3
7.50	1823.4	0.23
10.00	1878.5	0.36

*The isomer ratio (in the form of f_{syn}) of DP commercial products in China is 0.41.

SOD activity. Within the 28-day exposure of DP, significant increases of SOD activity in DP exposed groups were observed, compared with the controls (Figure 3). This obvious dose-dependent effect on SOD activity illustrated DP might induce the accumulation of superoxide radicals and thus result an increasing of SOD activity to scavenge these extra superoxide radicals. Additionally, at the dose of 7.5 mg DP/g bw exposure, there was a significant decrease of SOD activity after 28-day exposure, compared with 14-day exposure. It is assumed that high DP concentration and long exposure time may lead to cell damage or apoptosis, and thus affected the activity of SOD.

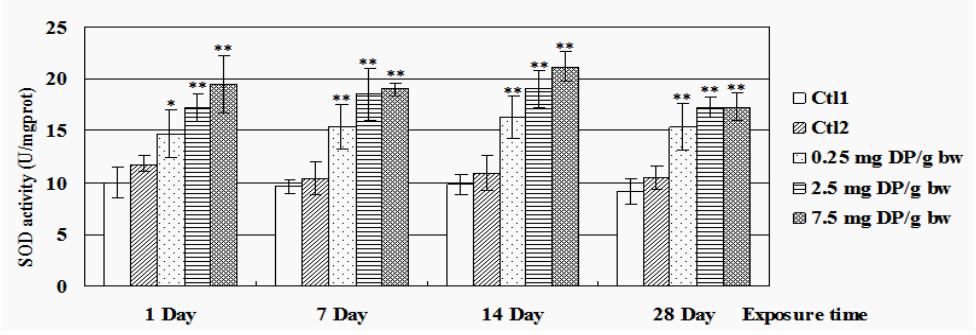

Figure 3. SOD activity after DP exposure in zebrafish. (*p≤0.05; **p≤0.01; compared with Ctl1)

Cell apoptosis. Intestine tissues of three zebrafish were chosen from each exposure for serial sections and totally 140 sections were obtained for analysis. Obvious cell apoptosis was detected in zebrafish intestine after 7, 14 and 28-day exposure of 7.5 mg DP/g bw (Figure 4). No detectable apoptosis was found till 28 days in 0.25 and 2.5 mg DP/g bw exposure group.

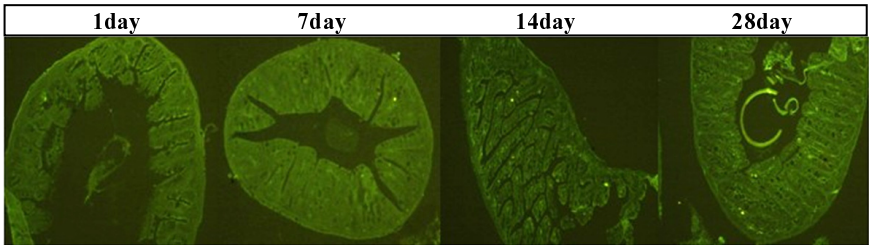

Figure 4. Apoptosis in zebrafish intestine after 7.5 mg DP/g bw exposure

Gene expression. The mRNA expression levels of *sod* (Superoxide dismutase) and *p53* (apoptosis pathway-related) genes were found significantly changed after DP exposure (Figure 5). The expression of *sod* were down-regulated when zebrafish were exposed to low concentrations of DP for relative short time, but up-regulated when zebrafish were exposed to high concentrations of DP for relative long time. We presume it is the DP-induced superoxide radicals accumulated in zebrafish stimulate *sod* gene expression to synthesize more superoxide dismutase to scavenge the radicals. Protein p53 is a well-known tumor suppressor protein that plays an important role in DNA damage and several cellular responses to stress in animals, such as membrane damage, oxidative stress, osmotic shock, heat shock, *etc.*. Interestingly, our results demonstrated the expression of *p53*

decreased significantly after 28 day exposure to 7.5 mg DP/g bw, but obvious cell apoptosis was detected in zebrafish intestine after 7, 14 and 28-day exposure of 7.5 mg DP/g bw (Figure 4). The reason may be two different tissues for analysis. Oral exposure of high concentration of DP may lead to apoptosis in intestine easily. Liver, as a vital organ for detoxification, may have more tolerance to toxic substances.

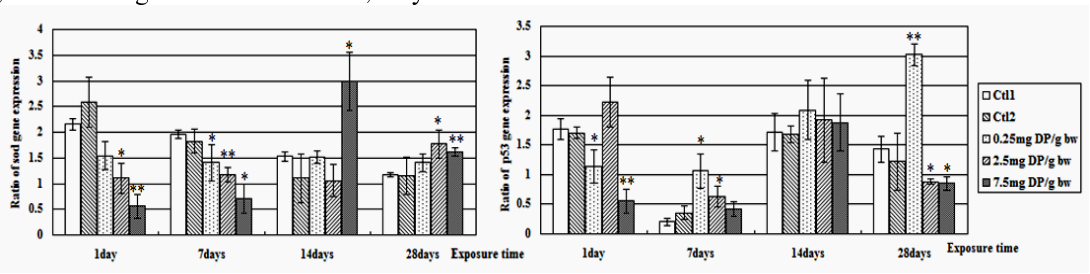

Figure 5. Relative expression level of *sod* and *p53* genes after DP exposure（*p≤0.05; **p≤0.01; compared with Ctl1）

Proteomic profiles. In preliminary experiment, no significant alteration was found between gels of two controls (Ctl_1 and Ctl_2), which means uptake of agar has no effect on zebrafish proteomic profiles. In the subsequent experiments, only Ctl_2 was used for analysis. Commonly, more than five hundred protein spots can be obtained stably on each gel of zebrafish brain/liver. There were 25 proteins showing significant differential expression in brain, 13 up-regulated and 12 down-regulated. In liver, more proteins (*i.e.* 20) were found up-regulated and only 5 proteins down-regulated. Six protein spots were randomly excised for MS sequencing. The identified proteins are related with DNA damage, protein synthesis, immunity response, cell apoptosis and cytoskeleton *etc.*. Our results demonstrated proteomic profiles of zebrafish liver and brain do have significant alternations after DP exposure and proteomic analysis will help to reveal the possible mechanisms on toxic effects of DP.

Table 3. Proteins with significant alterations in liver and brain (fold≥2.0; p≤0.05)

Tissue	time (day)	Protein name	Accession Number	ratio	*p* value
liver	7	Apolipoprotein A1/A4/E domain	AAH76032	0.40	0.023
		60S acidic ribosomal protein P0	Q9PV90	3.13	0.006
	14	60S acidic ribosomal protein P2	NP_001093906	2.31	0.029
	28	c-type lectin	ABD16187	2.03	0.034
brain	28	14-3-3 protein	AAQ18147	0.36	0.005
		F-actin capping protein	AAH67631	2.12	0.009

Conclusion

The concentrations of DP accumulated in zebrafish after 7 day exposure of 7.5 mg DP/g bw are almost at the same levels reported in aquatic organisms from the reservoir near e-waste recycling sites, south China. Our results presented in this study undoubtedly revealed the biological toxicity of DP to aquatic animals.

Acknowledgements

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References

1. U.S. EPA. High Production Volume (HPV) Challenge. (available online at: http://www.epa.gov/chemrtk/pubs/summaries/dechlorp/c15635tc.htm.)
2. Sverko E, Tomy GT, Reiner EJ, Li YF, McCarry BE, Arnot JA, Law RJ, Hites RA. (2011); *Environ Sci Technol.* 45 (12): 5088-98
3. Xian QM, Siddique S, Li T, Feng YL, Takser L, Zhu J P. (2011); *Environ Int.* 37 (7): 1273-84.
4. Dechlorane Plus® Manual, provided by OxyChem®, 2007. (available online at: http://www.oxy.com/Our_Businesses/chemicals/Documents/dechlorane_plus/dechlorane_plus.pdf)
5. Crump D, Chiu S, Gauthier L T, Hickey N J, Letcher R J, Kennedy S W. (2011); *Comp Biochem Phys C.*154 (2):129-34
6. Brock W J, Schroeder RE, McKnight CA, VanSteenhouse JL, Nyberg JM. (2010); *Int J Toxicol.* 29 (6): 582-93.
7. Li Y, Yu L, Wang J, Wu J, Mai B, Dai J. (2013); *Chemosphere.* 90 (7): 2149-56

8. Wu B, Liu S, Guo X, Zhang Y, Zhang X, Li M, Cheng S. (2012); *Environ Sci Technol.* 46 (19):10758-64.
9. Li Y, Yu L, Zhu Z, Dai J, Mai B, Wu J, Wang J. (2013); *Environ Toxicol Chem.* [Epub ahead of print]
10. Jia HL, Sun YQ, Liu XJ, Yang M, Wang DG, Qi H, Li S, Sverko E, Reiner EJ, Li YF. (2011); *Environ Sci Technol.* 45 (7):2613-8