Effect of Benzophenone-3 to Acetylcholinesterase and Antioxidant System in Zebrafish (Danio Rerio) Embryos

Juan José Sandoval-Gío

Tecnológico Nacional de México: Tecnologico Nacional de Mexico

Elsa Noreña-Barroso

Universidad Nacional Autonoma de Mexico Facultad de Quimica

Karla Escalante-Herrera

Universidad Nacional Autonoma de Mexico Facultad de Ciencias

Gabriela Rodriguez-Fuentes (✉ grf@unam.mx)

Universidad Nacional Autonoma de Mexico Facultad de Quimica   https://orcid.org/0000-0003-4028-0775

Abstract

Benzophenone-3 (BP-3) is one of the most used UV filters. This study aimed to evaluate the toxic effects of BP-3 during embryo stages of zebrafish, four hours post-fertilization (4hpf). Embryos were exposed to 0, 1, and 10 µg L^{-1} of BP-3, for 72 hours. We investigated biochemical and molecular biomarkers of neurotoxicity (AChE) and the antioxidant system (gene expression of catalase, CAT, superoxide dismutase, SOD, glutathione peroxidase, GPX, the concentration of total glutathione, GSH, and lipid hydroperoxides, LPO). Results indicated that the acute exposure to BP-3 in zebrafish embryos did not show significant differences in survival, hatching rate, or in the biomarkers of antioxidant system. In contrast, there were significant differences associated with AChE gene expression and activity.

Introduction

Personal care products (PCPs) give hygiene, comfort, and protection. UV-filters are substances that absorb or reflect ultraviolet radiation from sunlight. UV-filters are used in a wide variety of PCP formulations to prevent skin damage, cancer, and other harmful effects. However, they are classified as emerging pollutants because they are lipophilic and persistent, posing a potential threat to the environment (Díaz-Cruz, and Barceló 2009; Tovar-Sánchez et al. 2013; Lei et al. 2015; Ramos et al. 2016).

Previous reports have documented environmental concentrations of UV-filters in several water sources like lakes, rivers, sediments, swamps, and wastewater treatment plants (Barón et al. 2013; Cunha et al. 2015). Organic UV-filters, including benzophenone derivatives, have been detected in riverine and marine sediments in concentrations ranging from hundreds to thousands ng g^{-1} dw (Juliano & Magrini, 2017). Moreover, Ruszkiewicz et al. (2017) reported that recent data indicate levels of BP-derivatives up to 0.3 mg L^{-1} in rivers and ng to µg L^{-1} in lakes. In the Mexican Caribbean, it was calculated that from 2007 to 2025 more than 4 thousand tons of these products would be used, which could represent a risk to aquatic life (Casas-Beltrán et al., 2020). The damage to the symbiotic physiology of coral reefs that results in coral bleaching at relatively low concentrations of UV-filters is an example of the potential threat posed by these PCPs to aquatic biota (Danovaro et al. 2008).

Globally, ecotoxicological investigations about effects of UV filters, including BP-3, in aquatic organisms have demonstrated adverse effects; the majority of these studies have been addressed to the endocrine disruption (Kunz et al. 2006; Coronado et al. 2008; Blüthgen et al. 2012; Krause et al. 2012; Rodríguez-Fuentes et al. 2012; Rodríguez-Fuentes et al. 2015a). Kinnberg et al. (2015) reported an adverse endocrine-disrupting effect of BP-3 in developing zebrafish by skewing phenotypic sex towards females, with a NOEC and LOEC of 191 and 388 µg L^{-1}, respectively. Other adverse effects like neurotoxicity and immunosuppression have been demonstrated for some UV-filters, although most of these investigations are still scarce and not conclusive (Frikeche et al. 2015; Sharifan et al. 2016; Ruszkiewicz et al 2017). It is debatable if UV-filters promote reactive oxygen species (ROS), altering the antioxidant system, and producing oxidative stress. Studies destined to elucidate these effects have not been conclusive (Liu et al. 2015; Rodríguez-Fuentes et al. 2015a; Asimakopoulos et al. 2016, He et al. 2019)

Consequently, to note new evidence of adverse effects of BP-3 on aquatic biota, this research aimed to determine changes in the antioxidant system and neurotoxicity in embryos of *D. rerio.*

Material And Methods

The maintenance and reproduction of adult zebrafish used in this study have been previously described by Rodríguez-Fuentes et al. (2015a). After spawning, fertilized eggs of *D. rerio* were collected, washed, and transferred utilizing transfer pipettes to 5 L tanks and cultured for 4 h. Subsequently, embryos were transported to 6-well plates with 8 mL of treatment solution each one. Fifty embryos were placed in each well, and 3 replicates were evaluated per treatment per each of the tested biomarkers. Working solutions of BP-3 (CAS number 131-57-7, Fluka, USA) were prepared for the bioassays at nominal BP-3 concentrations of 1 and 10 µg L^{-1}. Control and solvent control treatments (ethanol 0.1%) were also tested (Rodríguez-Fuentes et al., 2015a).

At the beginning of the experiment, 50 mL of water were collected from every treatment and replicate to determine total BP-3 concentration by solid-phase microextraction and gas chromatography-mass spectrometry (SPME/GC-MS). A sample aliquot of 10 mL was placed in a 20 mL-SPME vial (Supelco, USA) with a screw cap with PTFE/silicone septum (Supelco, USA). Extraction of BP-3 was performed by direct immersion for 30 min at 55°C with a 65 µm PDMS/DVB SPME fiber (57310-U, Supelco, USA) and 700−900 rpm magnetic stirring. After the extraction, BP-3 was quantified using an Agilent Technologies 6850 Gas Chromatography System

equipped with a 5975B mass detector and a capillary column Zebron ZB-5MSi (30 m of length, 025 mm i.d. and 0.25 μm film thickness, Phenomenex, USA). Inlet temperature was 250°C, and samples were injected in a splitless mode (1 min purge time) and 10 min of desorption time. Oven temperature program started at 110°C for 2 min, ramp 1 was 20°C/min until 170°C, ramp 2 was 6°C/min until 260°C and held by 5 min; carrier gas was helium (ultra-pure grade). BP-3 was determined by selective ion monitoring (SIM) mode (target ions: 151, 227, 228 m/z), spectra generation frequency was 20 Hz, interface and ion source temperatures were 290°C, and 230°C, respectively. The MS ionization mode was electron ionization (EI). Calibration solutions were used to quantify BP-3 in the samples. The method detection limit was 0.005 μg L^{-1}. The bioassay lasted 96 h and was performed in agreement with institutional guidelines for the protection of animal welfare. The survival and hatching rate was evaluated in each well at the end of the exposure. Evaluations of apical endpoints were based on guidelines given by OECD 212 (1998) and Fraysse et al. (2006).

At the end of the bioassay, only embryos that were alive and hatched were placed in clean 1.5 mL microcentrifuge tubes. Samples that were used for gene expression were added 200 μL of RNA later® (Sigma, USA). All samples were snap-frozen in liquid nitrogen and were kept at -80°C until posterior analysis.

Tissue sample homogenization of zebrafish embryos for acetylcholinesterase (AChE) activity and GSH were performed in phosphate buffer pH 7.4. Samples for lipid peroxidation (LPO) analysis were homogenized with cold methanol. Homogenized embryos were evaluated for protein content by the Bradford method (Bradford et al. 1976).

The activity of AChE was measured with an adaptation of the method of Ellman et al. (1961) (Rodríguez-Fuentes et al. 2008). LPO was measured using a modification to a microplate reader using the FOX method (Hermes-Lima et al. 1995; Mhijalevic et al. 1996).

Relative gene expression of AChE, CAT, SOD, and GPx was determined by qRT-PCR using β-actin (BAC) as the housekeeping gene. Quantification primers for AChE, CAT, GPX, and BAC were designed from the reported GenBank sequences with accession numbers NM_131846.1, NM_130912, NM_131846.1, BC139513.1, and BC165823.1, respectively, to obtain amplicons of 100−200 base pairs (Rodríguez-Fuentes et al. 2015a; Rodríguez-Fuentes et al. 2015b). SOD quantification primers sequences were taken from Gonzalez et al. (2006). Total RNA was extracted using Gene Elute® Mammalian Total RNA Mike Prep Kit (Sigma, USA). Total RNA concentration was determined by evaluating fluorescence with the Quant-it® RNA Assay Kit (Invitrogen, USA). Two hundred nanograms of total RNA were used for the synthesis of cDNA with the iScript® Kit (Biorad, USA); 1 μL of cDNA was used for qPCR with iQ® SYBR Green Mix (Biorad, USA). The qPCR was carried out in an IQ5® thermocycler (Biorad, USA) with an initial denaturalization at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C and finally, a melting curve from 95°C to 50°C Δ+0.5°C/20 s. The relative expression of the RNA was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

For multivariate analysis of biomarkers, permutational MANOVA (PERMANOVA) and Principal Coordinate Analysis (PCO) were performed using Primer v 7.0 + PERMANOVA add on. Data were transformed using the function log(x + 1) and normalized, and the resemblance was calculated using the Euclidean distance of samples (Legendre & Legendre, 1998). The Monte Carlo test was applied to compare the different treatments in each data set for pair-wise tests if significant general differences were found.

Results And Discussion

BP-3 and its metabolites have been detected in the aquatic environment with consistency (Bae et al., 2016). BP-3 is commonly found in natural waters, with concentrations ranging between 0.8 and 8 μg L^{-1} (Balmer et al. 2005). To date, researchers have evidenced the toxic effects of this sunscreen on several species of fishes, including *D. rerio*, at concentrations ranging from the ones used in the present study (Coronado et al. 2008, Blüthgen et al. 2012).

For the present study, the mean (± one standard deviation) measured concentrations of BP-3 were 0.9 ± 0.1, 8.7 ± 2.9 μg L^{-1}, respectively, for nominal concentrations of 1 and 10, μg L^{-1}, respectively. BP-3 was not detected in the control and solvent control water samples.

Summary statistics of the tested biomarkers are presented in Table 1. The PERMANOVA of apical endpoints indicated no significant differences between treatments (Pseudo-F = 1.12, P(perm) = 0.34, Fig. 1). BP-3 concentrations used in this study did not influence survival or hatching rates. In discordance to our results, Cao et al. (2016) stated that many chemical substances could modify the hatching rate on *D. rerio*.

Table 1

Summary statistics of biomarkers in zebrafish embryos after 96h exposure to 3-Benzophenone. sd = standard deviation.

Biomarker	Control			Solvent Control			1 µg L^{-1} BP-3			10 µg L^{-1} BP-3		
	Mean	Median	sd	Mean	Median	sd	Mean	Median	sd	Mean	Median	sd
Survival %	82.56	90	18.11	73.22	72	20.12	78.11	86	23.25	71.33	77	21.89
Hatching rate %	83.67	88	18.7	77.44	83	16.58	74.22	78	21.67	82.22	84	10.76
AChE nmol/min/mg prot	93.06	83.07	18.03	72.37	72.1	6.15	70.96	70.57	3.47	64.99	62.61	6.65
AChEgene relative expresion	1	1	0	0.70	0.45	0.68	3.227	1.78	2.87	2.35	2.80	1.05
CATgene relative expresion	1	1	0	1.86	2.06	1.22	2.27	1.68	1.30	0.91	1.01	0.69
SODgene relative expresion	1	1	0	1.19	1.35	0.84	1.52	1.21	0.58	0.79	0.73	0.56
GPXgene relative expresion	1	1	0	0.76	0.38	0.76	2.89	2.64	1.80	2.07	2.24	0.88
LPO nmol/embryo	19.64	21.25	2.99	28.86	31.81	10.93	25.51	28.32	4.99	29.22	22.37	17.26
GSH nmol/mg prot	5.13	5.25	1.44	6.45	7.31	1.602	11.09	12.19	4.42	10.64	10.91	1.87

AChE catalyzes the rupture of the neurotransmitter acetylcholine. Organophosphate pesticides and carbamates are the typical AChE inhibitors, but it has been reported many other compounds that have anti-AChE properties (Colović et al., 2013). The PERMANOVA of AChE data indicated that there were significant differences (Pseudo-F = 3.66, P(perm) = 0.02). Pair-wise tests show significant differences between control and 10 µg L^{-1} BP-3 (P(MC) = 0.037, Fig. 2). There is an increase in AChE gene expression and a diminution of AChE activity as BP-3 concentration augments. Our results are different from previous studies with other animal models that have concluded that AChE is not inhibited by BP-3 (Campos et al. 2017a,b; Muñiz-González and Martínez-Guitarte, 2018). It is essential to note that in these previous studies, the gene expression of AChE was not evaluated. On the other hand, neurotoxicity of BP-3 has been previously reported; Feduik et al. (2010) found that BP-3 decreased cell viability in rat neurons. In accordance, Broniowska et al. (2019) noted in neuroblastoma cell lines reduced cell viability and increased caspase-3 activity. Interestingly, there are also reports of benzophenone-based derivatives that have been proposed as potent and selective AChE inhibitors (Belluti et al., 2011).

Zebrafish embryos exposed to BP-3 did not show significant differences in antioxidant system biomarkers. PERMANOVA indicated no significant differences between treatments (Pseudo-F = 1.60, P(perm) = 0.12). The PCO results for the biomarkers of the antioxidant system indicated that the first two axes were able to explain 69.13% of the total variation. Gene expression of CAT and SOD were strongly associated with PCO1, whereas expression of GPX and LPO and GSH concentrations were associated with PCO2 (Fig. 3).

Records about the effect of antioxidant enzymes formed in response to the production of ROS during exposure to pollutants are diverse (Richetti et al. 2011; Blahová et al. 2013; Praskova et al. 2014). In the specific case of BP-3, data are very inconclusive. Rodríguez-Fuentes et al. (2015a) observed a trend toward an increase in GPx gene expression as a significant positive correlation in eleuthero-embryos exposed to BP-3. Still, no significant differences in SOD, CAT, GPx transcription were found. Campos et al. (2017) did not see significant differences in lipid peroxidation or CAT and GST activities; however, they found significant differences in total glutathione levels when they exposed the aquatic insect *Sericostoma vittatum* to various concentrations of BP-3. Li et al. (2018) report

enhanced CAT activity in zebrafish embryos exposed to a mix of UV-filters, including BP-3, that suggest an adaptive response and a compensatory mechanism to avoid oxidative stress.

Differences between studies could be due to several factors, like the life stage and the organisms' biotransformation capacities. Le Fol et al. (2017) mention that biotransformation processes are a critical factor influencing toxic response, but there are significant gaps of information regarding the characterization of functional metabolic capacities expressed in embryo and adult zebrafish. Moreover, Blüthgen et al. (2012) reported that unlike that observed in adults, BP-3 is not metabolized to benzophenone-1 (BP-1) in zebrafish eleuthero-embryos (up to 5 dpf), probably because the required enzymes are not fully active at this stage of fish development. These metabolic differences may influence the biomarker response since an increase in the toxicity of BP-1 compared to BP-3 has been reported due to BP-1 higher hydroxylation (He et al., 2019). It has also been documented a low gene expression of antioxidant enzymes in *D. rerio* in response to prooxidants because of immature antioxidant and detoxification systems (Rodríguez-Fuentes et al. 2015a). The chorion also plays an essential protective role (Embry et al., 2010; Negro et al., 2014). The chorion is present in the embryo of *D. rerio* until 48 hpf (Kimmel et al. 1995); therefore, the organisms are directly exposed to the contaminants for a shorter time. In supporting this evidence, Mu et al. (2013) found higher sensitivity to difenoconazole in the larvae of zebrafish than the embryo.

In conclusion, acute exposure to BP-3 in zebrafish embryos did not show differences in survival, hatching rate, or antioxidant system. In contrast, there were significant differences associated with AChE gene expression and activity. It is crucial to expand the information on the effects of organic UV-filters (individual compounds and mixtures) on fish and other organisms at different stages of development, given the increase in the use of these substances worldwide due to their presence in a large number of products like PCPs, textiles, plastics, and paints.

Declarations

Ethics approval

This study was conducted in accordance with Facultad de Química- UNAM institutional guidelines for the protection of animal welfare.

Consent for publication

This paper does not contain any individual person's data of any form.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

JJSG performed the assay, KSEH did the qPCR analysis, ENB did the analysis of the real BP-3 exposure concentrations used in this study, GRF did the multivariate analysis of results . All the listed authors helped in the elaboration of the manuscript and the discussion of the results.

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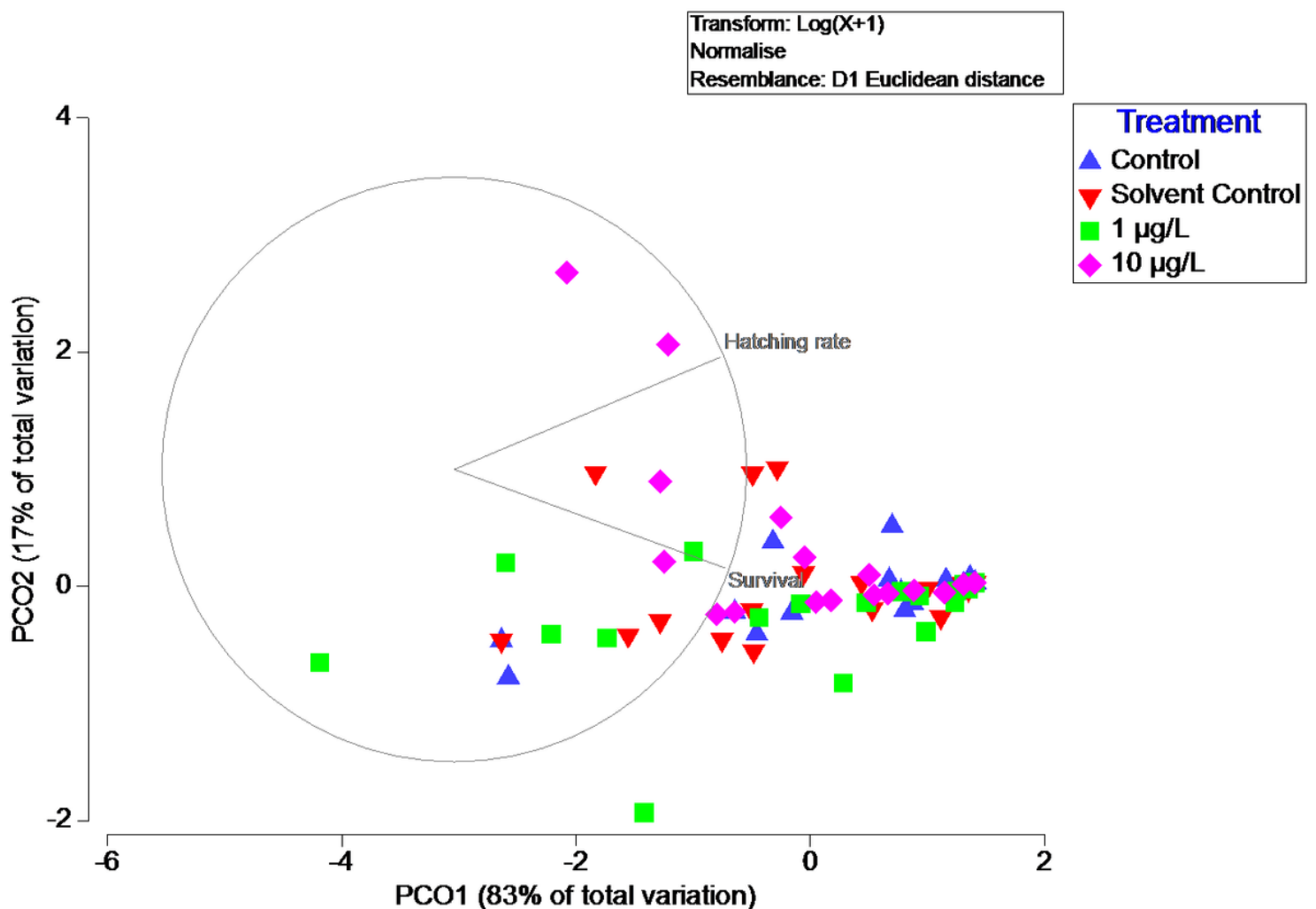
Figures

Figure 1

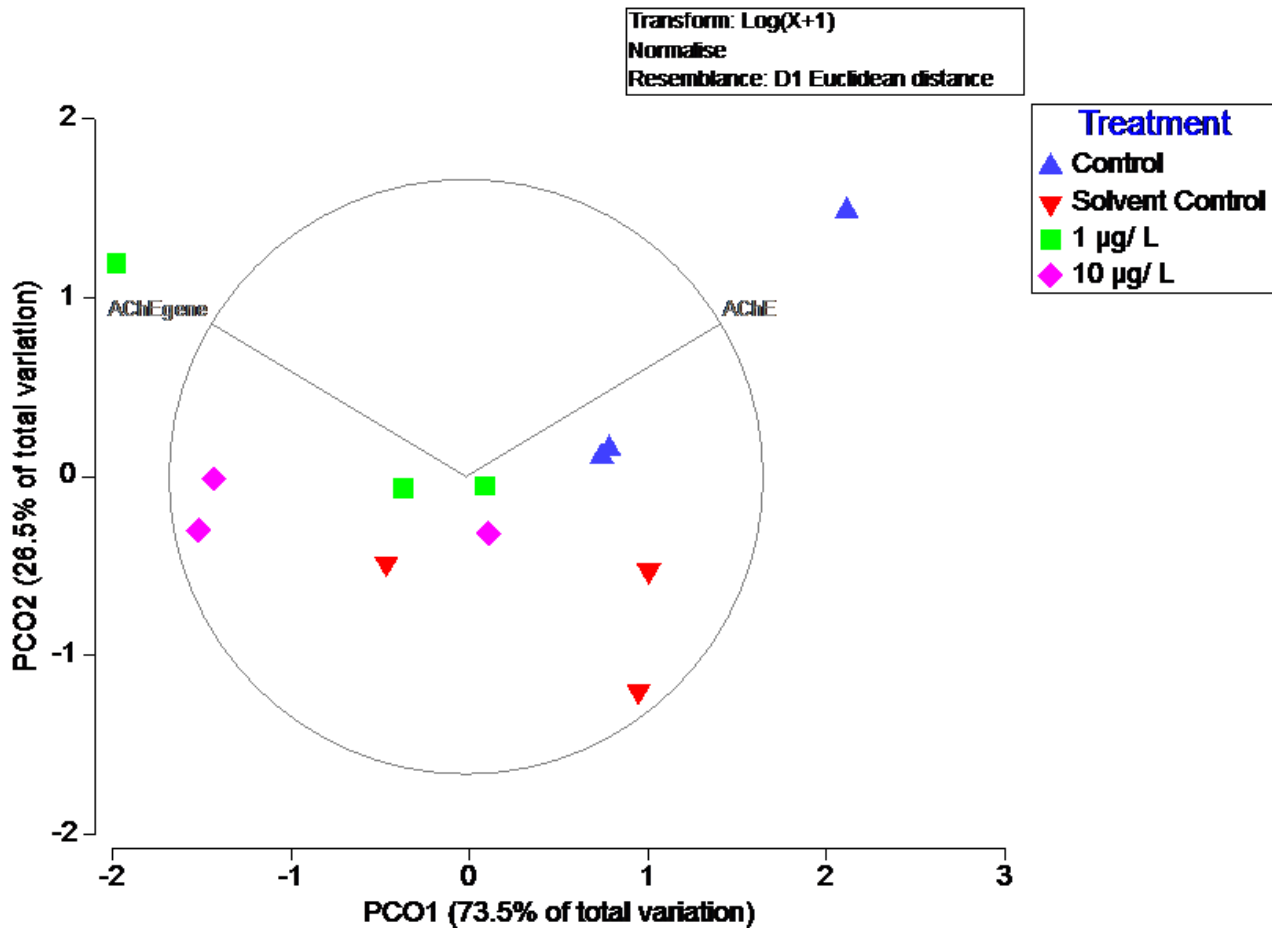Principal coordinate analysis (PCO) of apical endpoints in zebrafish embryos during acute BP-3 exposure

Figure 2

Principal coordinate analysis (PCO) of AChE activity and gene expression in zebrafish embryos during acute BP-3 exposure
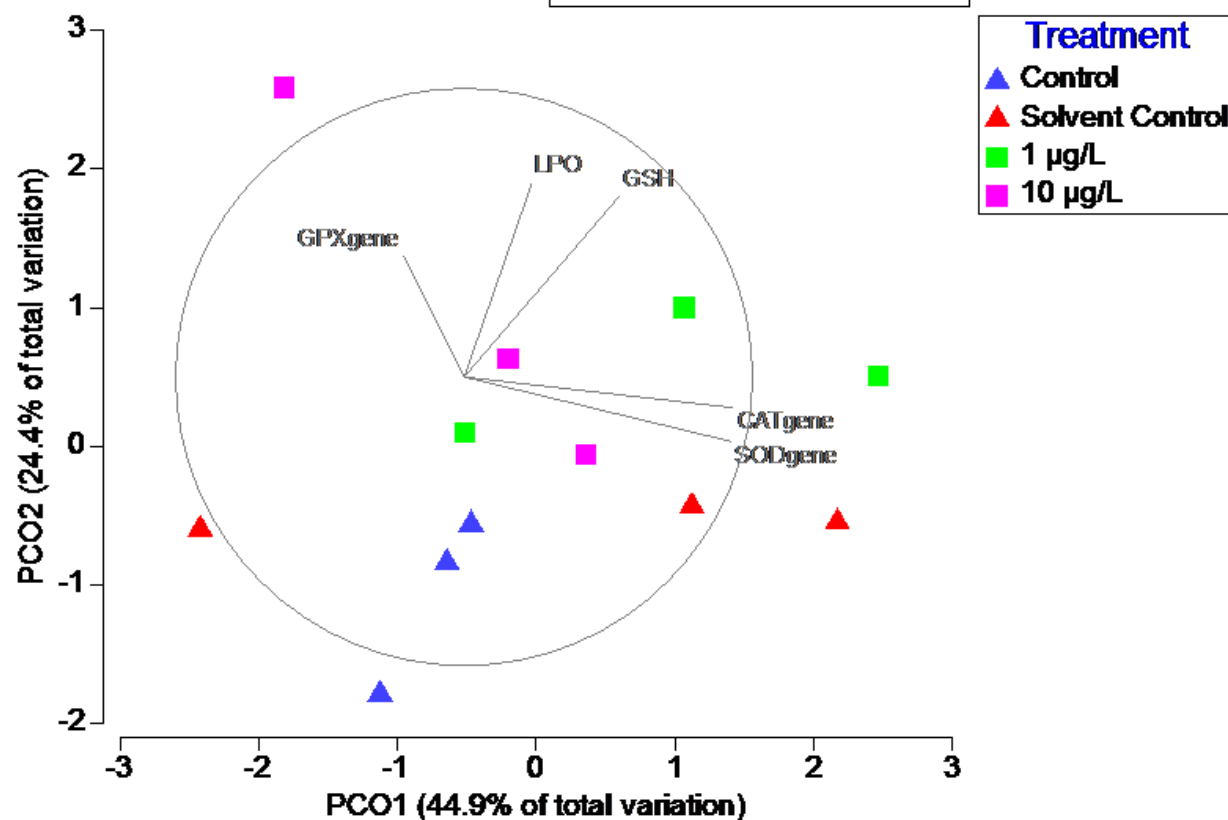
Figure 3

Principal coordinate analysis (PCO) of antioxidant system in embryos during BP-3 exposure