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Supplementary Materials

Untargeted Metabolomics Associated with Behavioral and Toxicological Studies Yield Insights into the Impact of 2,6-Dichloro-3-hydroxy-1,4-benzoquinone Disinfection By-Product on Zebrafish Larvae

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1. Experimental

1.1. Zebrafish Toxicity Testing

The dechorionated embryos were placed in 24-well culture plates (2 embryos per well, 1.5 mL of solution per well) and each experiment was performed in triplicate. Preliminary tests were performed to evaluate the range of 0%-100% mortality. The concentration range was found to vary from 10 μg/L to 500μg/L. Five different concentrations of 2,6-DCBQ-OH were tested (10, 100, 200, 300, 400, 500 μg/L) and E3 medium embryo buffer was used as control (non-exposed).

Test media were renewed every 24h. Embryos were inspected for lethal and sublethal effects at 24, 48, 72, 96 h according to OECD TG 236 [1]. According to OECD TG203 (OECD, 2019) the validity of tests was ensured as the mortality of the control group was lower than 10% at the end of the exposure and dissolved oxygen concentration was ≥ 60 per cent of the air saturation value throughout the exposure.

Each experiment lasted 96 hours post-fertilization (hpf) and began at 24 hpf, approximately at 26-somite point, according to Kimmel et al. [2]. During the experiments, toxicity, morphological abnormalities including yolk-sac edema, spinal curvature, tail deformity, uninflated swim bladder, and cardiac defects were recorded every 24 h, using a stereoscope (Olympus BX43) equipped with a digital camera, and high-resolution pictures were taken to be processed with the Image-Pro Plus software (Image-Pro Plus 10, Media Cybernetics). The recordings were performed at the same time (10 am) every day and lasted until the end of the experiment. In total, 1318 embryos were studied, of which 229 belonged to the non-exposed group. 11 embryos of the non-exposed group were found to be dead (4.8%). This was attributed to the manipulations during the dechorionation. This percentage was further used to assess toxicity.

1.2. Heart Rate

The effect of 2,6-DCBQ-OH on the heart rate as a function of 2,6-DCBQ-OH concentration and the time post fertilization (h.p.f.) was studied, to evaluate the effect on developmental ontogeny. 60 embryos per concentration in addition to the 138 of non-exposed were studied. Of the concentration of 400μg/L, 24 specimens were studied, as many had an unassessable heart rate. Embryos were separately analyzed under microscope and stereoscope (Olympus SZX7 Stereo, Olympus KL300 LED light) and high-quality video recordings were obtained for 1 min using Basler MED Ace camera and Basler Microscopy software (Basler MED Ace 2.3 MP 164 color, Basler Microscopy Software V2.1, Basler AG 22926 Ahrensburg, Germany). The video recording frame rate

was set to 25 fps. To eliminate any effect of temperature, the measurements were taken place in a temperature-controlled room (28 °C) and the video recordings were performed using transmitted cold lighting (LED). The acclimatization period was set at 20 min before each recording. The heart rate of embryos, under the effect of various concentrations of 2,6-DCBQ-OH, was investigated on the second, third, and fourth day (48, 72, and 96 hpf, respectively) after the fertilization.

1.3. Locomotor Activity

The Visual Motor Response (VMR) test was employed to evaluate differences in basal locomotor activity (BLA) of zebrafish after exposure to various concentrations of 2,6-DCBQ-OH. Survived zebrafish embryos after the exposure to various concentrations of 2,6-DCBQ-OH were examined for morphological malformations. Embryos with obvious morphological deformities and those failed to Touching Motor Response (TMR) test were excluded from the following test. Larvae were exposed continuously for 6 dpf to the selected concentration using E3 culture medium, as dissolver. The experiments were conducted at a temperature of 28 °C and photoperiod of 14 h light:10 h dark. To achieve this aim, 100 larvae per concentration and 120 non-exposed, as control, were transferred to an isolated behavioral screening room to assess the larval swimming activity (i.e., distance moved, acceleration) and the ability to adapt to changing environmental conditions (dark/light cycling).

On the 6th day, normally developed larvae were individually placed into 48 -well plates, one larva per well. The well plate was paced into DanioVision Observation Chamber (Noldus Information Technology, Leesburg, VA). An infrared analog camera (25 frames/second) was used to track larval locomotor activity. The VMR test proceeded as per our established methods [3]. Data were analyzed independently for each trial, and total distance moved was used as an indicator of overall locomotor activity.

The movement of the larvae within each well was recorded with the aim of a 2-d track plot system. The preferences of the larvae moving in the inner or outermost area of each well were inspected in relation to 2,6-DCBQ-OH concentration and light or dark condition as a mean to evaluate possible behavioral alteration and anxiolytic /anti-anxiolitc effects.

Moreover, the locomotor activity of non-exposed and exposed to each 2,6-DCBQ-OH concentration was tracked in a 30-min trial period as follows: 10 min dark, 10 min light, and 10 min dark. This tracking circle was followed to analyze the ability of the embryos to adapt to changing environmental stimuli (i.e., alternating phases of light and dark) [25].

By testing this adaptation, the quantification of the behavior in two distinct environments is possible. The larvae were placed in 48-well plates with 1,5 mL of E3 embryo buffer in each well. Then, the plates were transferred to a behavioral testing chamber with a temperature control unit (DanioVision, Noldus Inc., Wageningen, The Netherlands) and kept in dark, at 28 °C, for 1 h, for acclimatization. The position of each larva was recorded by an IR digital video camera Basler acA1300-60gm (Basler Inc. Exton, PA). Any background noise was removed by setting a minimum distance input with a filter of 10% of the larva body, equivalent to 0.4 mm.

The video recordings were held between 10 am and 12 pm to stabilize zebrafish basal activities related to circadian rhythms.

1.4. Vibrational Startle Response (VSR)

Tapping stimulus is provoked by a piston installed in the behavior recording system (DanioVision Tapping Device DVOC-004x / T, Noldus, Wageningen, The Netherlands). Larvae were transferred on the day of the recording at least one hour before the start of the experiment to the behavioral measurement chamber to achieve acclimatization. The experiments were performed on 24 well plates where one larva was placed in each well containing 1.5 ml of E3 buffer. After one hour of acclimatization, 2-minute video recordings were made at 60 frames per second, in full dark. The application of the tapping

stimulus was performed when the piston hit on the plate bearing. The response to the tapping stimulus was analyzed for each individual larva by measuring the distance (cm) it travelled for a period of 10 seconds after the stimuli and the reaction time (msec). Noldus Inc. software, EthoVision, was used for statistical analysis. The results were considered statistically significant where $p < 0.001$ and marginally statistically significant where $p < 0.05$.

1.5. Instrumentation for Metabolomic Study

^{1}H-NMR experiments were carried out using a Bruker AV-500 spectrometer equipped with a TXI cryoprobe (Bruker BioSpin, Rheinstetten, Germany). System control and spectra processing were carried out using the TopSpin 2.1 software. Spectra were recorded at 298 K. Chemical shifts were reported using TSP-d4 (0.02 mmol/L) as reference. Acquisition time was set at 4.1 sec and relaxation delay 5 sec, using 90° pulse length. In addition, 64 K points were used and 256 scans were recorded. After Fourier transform, phase and baseline were manually corrected. For the chromatographic separation of the metabolites, a UHPLC Accela LC system (Thermo Fisher Scientific, Inc. GmbH, Bremen, Germany) was used. The chromatographic column was a Hypersil GOLD 1.9 μm particle size (100 mm × 2.1 mm I.D), kept at 30°C. Water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid were used for the gradient elution. The flow rate was set at 300 μL min^{-1}. The elution program was as follows: 0-13.78 min, 20-90% B, 13.78-15.28 min, 90% B, 15.28-18.06 min, 90-20% B, followed by a 2-min re-equilibration time of the column. A hybrid linear trap quadrupole (LTQ) Orbitrap mass spectrometer (LTQ-Orbitrap XL 2.5.5 SP1, Thermo Fisher Scientific, Inc. GmbH, Bremen, Germany), equipped with an Electrospray Ionization (ESI) source, was used for metabolite detection. Nitrogen was used as the sheath and auxiliary gas to deliver effluents to the ion source. Both ionization modes were employed. When positive ionization mode was employed, 2.5 μL of the sample was injected, and source voltage, tube lens, heated capillary voltage, and temperature were set at 3.40 kV, 110 V, 40.00 V, and 320 °C, respectively. In the negative ionization mode, 10 μL of the sample was injected and source voltage, tube lens, heated capillary voltage, and temperature were set at 3.70 kV, 120 V, -30.00 V, and 320 °C, respectively. In both cases, a full scan mode (60000 resolution and m/z range: 50-1000) and a most-intense-ion scan mode (MS/MS fragmentation of the most abundant ion) (7500 resolution) were used.

2. Results

2.1. LC_{50} Estimation

Exposure to 2,6-DCBQ-OH showed a dose-dependent increase in toxicity. Toxicity increased as exposure concentration increased. Toxicity was observed from the lowest concentration (10μg/L), then showed a sharp increase after 100μg/L and peaked at the highest concentration (500μg/L). Figure S1 present the estimated toxicity curve and lethal concentrations as calculated respectively.

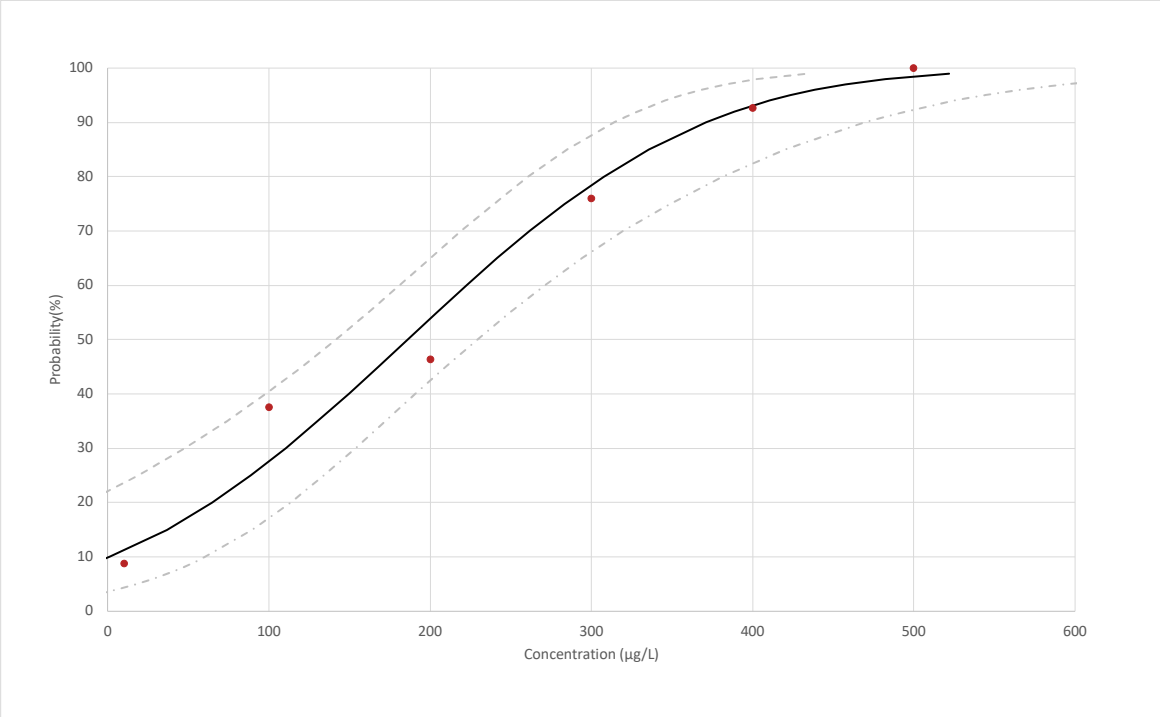

Figure S1. Mortality pattern of zebrafish larvae exposed to different concentrations of 2,6-DCBQ-OH.

2.2. Metabolomic Study

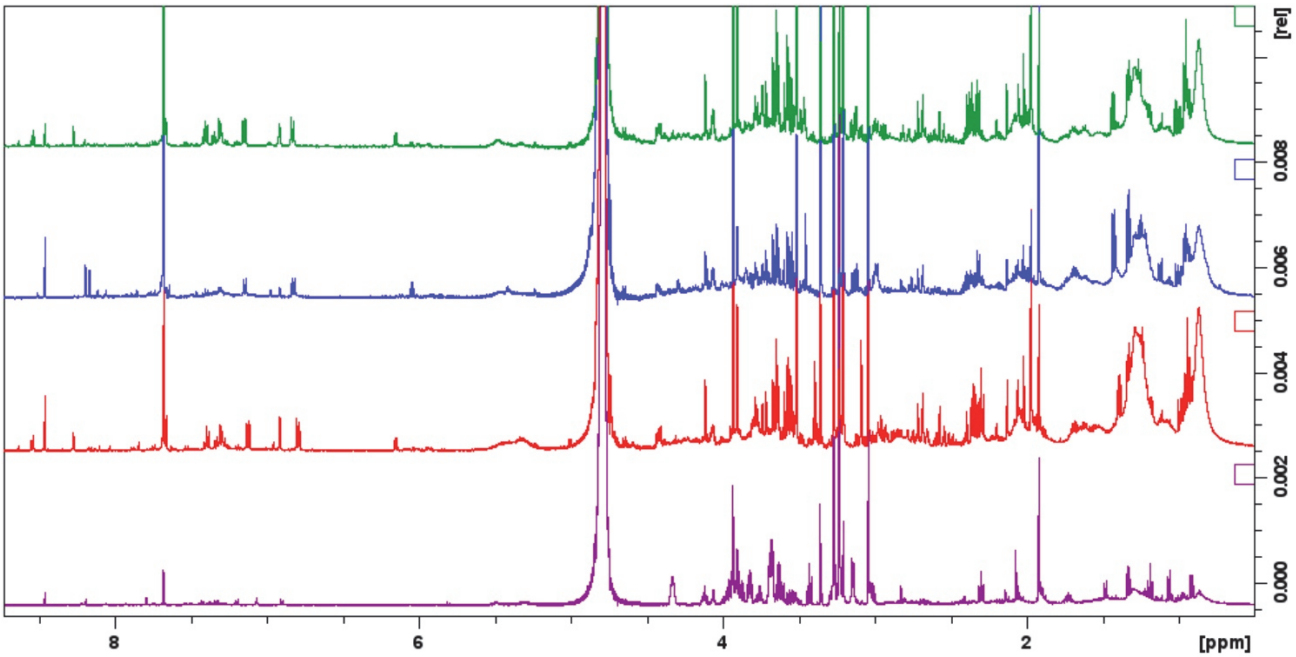

Figure S2. NMR spectra of metabolites of control samples (purple spectrum) and samples exposed to 200 µg/L (red spectrum), 300 µg/L (blue spectrum) and 500 µg/L (green spectrum) of 2,6-DCBQ-OH.

Table S1. List of metabolites found in the control and exposed to 2,6-DCBQ-OH individuals.

Metabolites	**Control**	**200 µg/L**	**300 µg/L**	**500 µg/L**
5(R)-Lactate	✓	✓		
(S)-3-Methyl-2-oxopentanoic acid	✓			
11-Deoxycorticosterone	✓			
11-Deoxycortisol	✓			
2-Oxobutanoate		✓		✓
3-Methoxytyramine			✓	
3-Methyl-2-oxobutanoic acid	✓	✓	✓	✓
3,4-Dihydroxy-L-phenylalanine			✓	
3,5-Diiodo-L-tyrosine		✓		
4-Hydroxyphenylacetate	✓		✓	
5-Hydroxy-L-tryptophan			✓	
6-Deoxy-L-galactose		✓		✓
Adenosine				✓
Adrenosterone	✓			
Agmatine			✓	
beta-Alanyl-N(pi)-methyl-L-histidine		✓	✓	✓
beta-Carotene	✓	✓	✓	✓
beta-Sitosterol				✓
Bilirubin			✓	✓
Butanoic acid	✓			
Cadaverine	✓			
Chenodeoxycholate			✓	✓
Cholesterol sulfate	✓			
Corticosterone				✓
Cortisone				✓
CTP	✓			
Cytidine			✓	
D-Ribose 5-phosphate			✓	
dADP	✓			
Decanoic acid	✓	✓	✓	
Deoxyinosine		✓		
Deoxyuridine		✓		
Dodecanoic acid	✓			
Estrone	✓	✓	✓	
Ethanolamine				✓
Folate	✓	✓		
Guanosine		✓		
Hexadecanoic acid	✓			
Histamine				✓
Homovanillate				✓
Indole-3-acetate		✓		
Inosine			✓	

L-Adrenaline		✓		
L-Alanine	✓			✓
L-Arginine				✓
L-Citrulline	✓			
L-Histidine	✓			
L-Leucine			✓	
L-Methionine				✓
L-Noradrenaline		✓		
L-Phenylalanine				✓
L-Selenomethionine	✓	✓	✓	✓
L-Threonine		✓		✓
L-Tryptophan		✓	✓	
L-Valine	✓		✓	
Linoleic acid	✓			
Maltose				✓
Methylmalonate		✓	✓	✓
N-Acetyl-L-glutamate	✓	✓	✓	
NAD+				✓
Octadecanoic acid	✓			
Pantothenate	✓		✓	
Phenethylamine	✓		✓	✓
Phenylacetic acid			✓	✓
Propanoate			✓	
Pyridoxal		✓		
Pyridoxine		✓		
Retinoate		✓		✓
Retinoic acid	✓			
Serotonin		✓	✓	
sn-Glycerol 3-phosphate				✓
Sphingomyelin	✓			
Squalene				✓
Taurine				✓
Tetradecanoic acid	✓			
Thiamine			✓	
Thymidine	✓		✓	
Thyroxine		✓		✓
Tryptamine		✓		
Tyramine				✓
Valine	✓			

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