Environmental Science and Pollution Research (2023) 30:27996–28009
https://doi.org/10.1007/s11356-022-24071-9

RESEARCH ARTICLE

Evaluation of the toxicity of di-iso-pentyl-phthalate (DiPeP) using the fish *Danio rerio* as an experimental model

Sheila Gabriel Santos[1,2] · Marília Cristina Oliveira Souza[3] · Fernando Barbosa-Junior[3] · Maritana Mela Prodocimo[4] · Fellip Rodrigues Marcondes[5] · William Almeida[5] · Marta Margarete Cestari[5] · Luciana Rodrigues Souza-Bastos[6] · Anderson Joel Martino-Andrade[7] · Izonete Cristina Guiloski[1,2]

Received: 13 April 2022 / Accepted: 3 November 2022 / Published online: 17 November 2022

Abstract

The presence of phthalates constitutes a risk to the health of aquatic environments and organisms. This work aimed to evaluate the toxic effects of di-iso-pentyl-phthalate (DiPeP) at environmentally relevant concentrations of 5, 25, and 125 µg/L in *Danio rerio* after subchronic exposure for 14 days. DiPeP altered the antioxidant system in the liver (125 µg/L), intestine (25 µg/L), brain, and gills in all concentrations tested. In animals exposed to 125 µg/L, DNA damage was identified in the gills. In addition, loss of cell boundary of hepatocytes, vascular congestion, necrosis in the liver, and presence of immune cells in the intestinal lumen were observed. Erythrocytic nuclear alterations in the blood occurred in animals exposed to 25 µg/L. DiPeP was quantified in muscle tissue at all exposure concentrations, appearing in a concentration-dependent manner. Contaminants such as DiPeP will still be used for a long time, mainly by industries, being a challenge for industry versus environmental health.

Keywords Biomarkers · Diisoamyl phthalate · Emerging contaminants · Toxicity · Zebrafish

Introduction

Plasticizers can easily contaminate the environment and induce adverse health effects on different organisms, including humans (Schug et al. 2011). Currently, phthalates are a great public health concern since are ubiquitous in the environment become human exposure a real possibility (Souza et al. 2022). Phthalates or phthalic acid esters are short-chain polymeric additives dispersed in a polymer matrix, causing a reduction in viscosity during processing and thus allowing greater flexibility to the product. This is possible due to the reduction of intermolecular forces of the polymeric chains, allowing a greater sliding between the chains (Koch et al. 2012). People use plasticizers all life (Luongo and Ostman 2016) and oral and dermal via becoming the most relevant exposition route (Wensing et al. 2005). This visible exposure, due to the increase in food packaging, indicates that food can be a great potential source of exposure since phthalates migrate from food and beverage packaging (Rudel et al. 2011).

In the environment, the persistence of phthalates is an emerging public health problem, due to the potential effects on reproduction, obesity, and development (Gutiérrez-García

Responsible Editor: Bruno Nunes

✉ Izonete Cristina Guiloski
izonete.cristina@pelepequenoprincipe.org.br;
iguiloski@gmail.com

1 Instituto de Pesquisa Pelé Pequeno Príncipe, Avenida Silva Jardim, Água Verde, 80.250-200, 1632 Curitiba, PR, Brasil

2 Faculdades Pequeno Príncipe, Curitiba, PR, Brasil

3 Laboratório de Toxicologia Analítica e de Sistemas (ASTox), Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brasil

4 Departamento de Biologia Celular e Molecular, Universidade Federal Do Paraná (UFPR), Setor de Ciências Biológicas, Curitiba, PR, Brasil

5 Departamento de Genética, Universidade Federal do Paraná (UFPR), Setor de Ciências Biológicas, Curitiba, PR, Brasil

6 Instituto de Tecnologia para o Desenvolvimento - Lactec, Curitiba, PR, Brasil

7 Departamento de Fisiologia, Universidade Federal do Paraná (UFPR), Setor de Ciências Biológicas, Curitiba, PR, Brasil

et al. 2019). Rocha et al. (2017) reported that one-quarter of Brazilian children had a hazard index of > 1 for phthalate exposures. The authors found a positive association between urinary phthalate concentrations and oxidative stress.

Phthalates can potentially affect different physiological processes in fish species such as reproductive health (Uren-Webster et al. 2010), embryonic development (Liu and Zhao 2009), and antioxidant system (Kang et al. 2010; Mankidy et al. 2013). The associated risk assessments are mainly directed toward certain phthalates, such as dimethyl phthalate (DMP), diethyl phthalate (DEP), and di-2-ethylhexyl phthalate (DEHP) (EFSA Panel on Food Contact, Materials 2019). The toxicological effects caused by other phthalates such as di-iso-pentyl phthalate (DiPeP) have not received considerable attention.

Di-iso-pentyl phthalate, also called diisoamyl phthalate, is the product of a reaction between phthalic anhydride and isoamyl alcohol, a compound that originates from the manufacture of sugarcane in the process to obtain ethanol. It is used in industrial applications as solvents and esters in general and it is found in commonly used products, such as shoes and hoses (Brisco 2011; Petrom 2016). There are already studies that detect this phthalate in landfills in Brazil (Do Nascimento Filho et al. 2003; Ferreira and Morita 2012). Although the toxicity of this phthalate is not well studied yet, the relevance of human exposure should be examined as metabolites have already been detected in the urine of pregnant women (Bertoncello Souza et al. 2018). High doses of antiandrogenic phthalates can lead to Phthalate Syndrome characterized by male reproductive tract abnormalities (reduced anogenital distance, cryptorchidism, hypospadias, and low sperm count Lioy et al. 2015; Bertoncello Souza et al. 2018). Changes in sexual behavior in rat males were also observed (Neubert da Silva et al. 2019). In humans, there is not a clear pattern of association between prenatal phthalate exposures and toxic effects (Radke et al. 2020).

Danio rerio (zebrafish) is considered an excellent model for toxicology studies due to its sensitivity when exposed to chemicals and being able to quickly absorb compounds added to water (Guyon et al. 2007). The use of zebrafish in toxicology assays is recommended by international standards (Bertoletti 2009; OECD 2010).

Based on the above, this study aimed to evaluate the toxic effects of di-iso-pentyl phthalate at concentrations of 5, 25, and 125 µg/L in different organs of *Danio rerio* after subchronic exposure.

Material and methods

Reagents

Di-iso-pentyl-phthalate ($C_{18}C_{26}O_4$): available in liquid form (oil; CAS: 84777–06-0; 99% purity; Petrom®—Petrochemical Mogi das Cruzes-SP-Brasil).

Assessment of DiPeP absorption

To determine the degradation/absorption of DiPeP, two-liter aquariums were used, one with five fish (forming a pool of 0.300 g of total muscle tissue) and another without fish, with only the phthalate. The concentration used in this experiment was 125 µg/L of DIPeP, starting from 1 mg/mL of DiPeP (10 mg of DiPeP dissolved in 9 mL of acetone and 1 mL of Mili-Q water). Ten milliliters of water was collected, in triplicate, from both aquariums (with and without fish), at times: 0 (immediately after adding the contaminant to the tank water), 24, 48, 72, and 96 h. After the collection of water samples, these were analyzed employing gas chromatography coupled to mass spectrometry (GC–MS) from Thermo Fisher Scientific® (Waltham, MA, USA) to determine the amount of DiPeP that was absorbed by the fish at each time. After analysis in GC–MS, it was observed that fish absorbed 36% of DiPeP every 48 h.

Experimental model and ethical considerations

Danio rerio adult fish (0.246 ± 0.103 g; 3.1 ± 0.04 cm), of both sexes, were acquired from Planeta Aquários store, Curitiba-PR, and acclimatized for 2 weeks in two-liter tanks, four aquariums holding five fish ($n = 20$ per group). The aquariums were kept at a pH 7.00 ± 0.60, ammonia 0.04 ppm, nitrite 0 ppm, hardness: 3.00 ppm of $CaCO_3$, temperature 26 ± 1 °C, and photoperiod 14:10 h. The fish were fed once a day with commercial feed (Vipagran, 41.3% protein; Brazil). The leftover fish feed as well as the accumulation of excreta resulting from an excess of organic matter accumulated in the bottom of the aquarium were removed by siphoning every 48 h.

The experiment was conducted as provided in ARRIVE guidelines (Percie du Sert et al. 2020) and EU Directive 2010/63/EU (Council of European Union 2010) for animal experiments. The project was approved by the Ethics Committee on the Use of Animals of the Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba-PR, under the number 051–2020.

After the assessment of DiPeP absorption, phthalate exposure (DiPeP) was conducted under the controlled parameters. The choice of the concentrations of DIPeP used in this work was based on reported data concerning the occurrence of other phthalates (diethyl phthalate, di-2-ethylhexyl phthalate, dibutyl phthalate). In environmental matrices, including rivers, the total phthalates found in water samples ranged from 313 to 1640 ng/L (Selvaraj et al. 2014). In drinking water treatment plants, the range for phthalates was between 0.02 and 6.5 µg/L (Santana et al. 2014).In food, phthalates were found at concentrations between < 50 µg/kg and ≥ 300 µg/kg (Serrano et al. 2014).

The experimental groups were 5, 25, and 125 µg/L DiPeP, water control, and solvent control (acetone 0.0056%). According to the results of the absorption evaluation, every 48 h the amount of absorbed DiPeP (36%) was replaced.

The fish were exposed to DiPeP through water exposure for 14 days, changing half of the aquarium water every 48 h and replacing the contaminant to ensure homogeneity of concentrations throughout the bioassay. After 14 days of exposure, the fish were anesthetized with benzocaine (0.001%), euthanized, and the tissues were removed for evaluation of the antioxidant system, quantification of DiPeP, histopathology, genotoxicity, and carbonic anhydrase activity. Due to the small size of zebrafish organs, the bioassays were performed with 20 animals per group so that all analyses could be performed with an $n = 10$. However, some fish had very small organs, making it impossible to use them in the analyses, so we had $n = 7–10$.

Water quality parameters

The water used in the tests was monitored during the experiment, checking the parameters of pH, ammonia, nitrite, chlorine, and hardness (commercial kits from the Labcon Test brand: pH tropical, toxic ammonia, nitrite, chlorine test, carbonate hardness KH), and temperature.

Tissue preparation for the evaluation of antioxidant system and acetylcholinesterase

The brain, intestine, liver, and gill samples ($n = 7–10$ per group) were homogenized in 600 µL of sodium phosphate buffer (0.1 M, pH 7.0) and centrifuged at 4 °C, 12,000 g, for 15 min. Supernatants were aliquoted for the determination of superoxide dismutase (SOD; EC 1.15.1.1), total proteins (P), glutathione peroxidase (GPx; EC 1.11.1.9), glutathione S-transferase (GST; EC 2.5.1.18), and reduced glutathione or non-protein thiols (GSH). Acetylcholinesterase (AChE; EC 3.1.1.18) activity was performed only in the brain.

GSH concentration (non-protein thiols)

GSH concentration was performed by comparing it to the standard curve of GSH (0 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM). Trichloroacetic acid (50%) was used for protein precipitation in the samples, after which they were centrifuged at 4 °C, 12,000 g, for 10 min, and the supernatants were used for analysis. In microplate, 50 µL of the sample, 230 µL of Tris-base buffer (0.4 M; pH 8.9), and 20 µL of 2.5 mM 5.5′-dithio bis-2-nitrobenzoic (DTNB) dissolved in methanol and 0.4 M Tris-base buffer (pH 8.9) were added. The GSH concentration was expressed in µg·mg of protein^{-1} (Sedlak and Lindsay 1968).

GST activity

The methodology for analyzing the GST was described by Keen et al. (1976), with modifications. The samples were diluted 1:2 (v/v) in potassium phosphate buffer (0.1 M, pH 6.5). In 96-well microplates, 20 µL of the sample was added, in triplicate, followed by 180 µL of the reaction medium containing the solutions with 3 mM GSH dissolved in potassium phosphate buffer (0.1 M, pH 6.5) and 3 mM 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in ethanol. The reading was performed immediately at a wavelength of 340 nm with a total time of 5 min, with readings every minute. The gradual increase in absorbance was monitored, and activity was expressed in nmol of thioether formed·min^{-1}·mg of protein^{-1}.

GPx activity

The methodology was described by Paglia and Valentine (1967), using 10 µL of the sample and 130 µL of solution 1 (0.1 M sodium phosphate buffer, pH 7.0; sodium azide to 3.08 mM; NADPH at 0.308 mM; GSH at 3.08 mM; glutathione reductase at 1.54 U/mL). After 2 min, 60 µL of solution 2 (hydrogen peroxide — H_2O_2 to 5 mM; 0.1 M sodium phosphate buffer, pH 7.0) was added. The absorbance reading was carried out at 340 nm for 5 min with a one-minute interval between each reading. Enzyme activity was expressed in nmol·min^{-1}·mg protein^{-1}.

SOD activity

The analysis of SOD activity was based on the method proposed by Gao et al. (1998), which assesses the ability of SOD to inhibit the auto-oxidation of pyrogallol. In microtubes, 40 µL of the sample was added, followed by 885 µL of Tris 1 M/EDTA 5 mM buffer (pH 8.0) and 50 µL of pyrogallol (15 mM). The solution was incubated, and protected from light, for 30 min and the reaction was stopped with 25 µL of HCl (1 N). In the microplate, 200 µL of the reaction solution was added in triplicate and the reading was carried out in a spectrophotometer at 440 nm. Simultaneously, a control sample was performed with the addition of reagents but without incubation. Enzyme activity was expressed as U·mg protein^{-1}, where 1 U of SOD is responsible for inhibiting by 50% the auto-oxidation of pyrogallol.

AChE activity

The methodology for analyzing the AChE was described by Ellman et al. (1961), with modifications. The brain samples were diluted at 1:2 (v/v) and 25 µL was added to the microplate, followed by 200 µL of DTNB (5.5′-dithio

bis-2-nitrobenzoic; 0.75 mM), and 50 µL of acetylthiocholine (10 mM) was added. The reading was carried out in a spectrophotometer at 405 nm. Results were expressed in nmol·min^{-1}·mg of protein^{-1}.

Total protein quantification

The concentration of total protein was determined by the method of Bradford (1976) using a standard curve with bovine serum albumin (0, 125, 250, 500, 1000 µg/mL). For this analysis, brain and gill samples were diluted 1:2 (v/v), while liver and intestine samples did not need to be diluted. On a microplate, 10 µL of the sample was added, followed by 250 µL of Bradford's reagent (Sigma-Aldrich®), and the reading was performed at 595 nm. Results were expressed in mg of protein.

Histopathology

The liver and intestine ($n = 7$–10 per group) were fixed in ALFAC (80% ethanol, 10% formalin, and 5% acetic acid) for 16 h and kept in 70% ethanol until the dehydration phase. Samples were dehydrated in a graded series of alcohol concentrations (80%, 90%, 95%, and 100%) and inserted into Paraplast Plus® (Sigma-Aldrich®). Sections of 3 µm were obtained in a microtome (American Optical Manual Rotary Microtome—Model 820—Series 54,637) at an angle of 45 °C and stained with Eosin (3 min) and 2% Floxin (2 min), followed by a histological bath in water at the temperature of 50 °C. Then, the sections were adhered to a glass slide in 10% formalin and placed in an oven at 65 °C for 30 min. After this time, the slides were submerged in a 65 °C xylol solution for 5 min to completely remove the paraffin.

Based on histopathological findings, the lesion index was calculated according to Bernet et al. (1999) modified by Mela et al. (2013b, 2013a). Injuries and tissue changes were classified according to biological importance, such as 1 — minimal, easily reversible; 2 — moderate, reversible in most cases; and 3 — marked, usually irreversible, and severity establishing scores from 0 to 6. The injury index for each group of liver or bowel injuries was calculated using the formula: $I_{org.} = \sum_{rp} \sum_{alt}{}^{(a \times w)}$, where *org* represents the organ (constant), *rp* the reaction pattern, *alt* the change, *a* the score value, and *w* the injury importance factor (Bernet et al. 1999; Mela et al. 2013b, 2013a).

Genotoxicity

Comet assay

Comet assay was performed according to the method proposed by Singh et al. (1988) and modified by Ramsdorf et al. (2009). Gills were stored in microtubes containing 1 mL of fetal bovine serum (FBS) and subsequently homogenized at 15,000 rpm with the aid of a microhomogenizer. The preparation of slides was performed using 60 µL of suspension mixed with low melting point agarose. The suspension was placed on a microscope slide previously covered with 1.5% agarose. The slide was covered with a coverslip and placed in the refrigerator at 4 °C for 15 min. After this time, the coverslip was removed and the slides were placed in a lysis solution for 24 h at 4 °C. The slides were transferred to the electrophoresis tank, where they were immersed in electrophoresis buffer (0.3 M NaOH/0.001 M EDTA) with pH > 13 for 25 min. Electrophoresis was performed at 300 mA, 25 V for 25 min, and then, slides were neutralized in Tris buffer (0.4 M, pH 7.5) and fixed in absolute ethanol for 5 min. For the analysis, slides were stained with ethidium bromide (10 µg/mL) and analyzed under an epifluorescence microscope (Leica®) at 400 × magnification. Comets were visually classified according to the migration of DNA fragments into class 0 (no apparent damage), class 1 (small damage), class 2 (medium damage), class 3 (extensive damage), and class 4 (maximum damage) (Collins et al. 1997). Analysis was blinded with coded slides. For each fish, 100 nucleoids were analyzed.

Piscine Micronucleus Test

A blood extension was performed for the Piscine Micronucleus Test following the method proposed by Schmid (1976), Carrasco et al. (1990), and Fenech (2000). Slides were dried at room temperature for 24 h. Then, they were fixed with absolute alcohol for 15 min. Subsequently, the slides were stained with 10% Giemsa for 15 min, followed by washing with distilled water to remove excess dye and drying at room temperature for 24 h. Analysis was blinded with coded slides. For each fish, 2000 erythrocytes were counted per slide under an optical microscope at 1000 × magnification.

Carbonic anhydrase activity

Carbonic anhydrase (EC 4.2.1.1) activity was determined according to the method established by Henry (1996) and described by Vitale et al. (1999). The gills were weighed and homogenized at 10% (w/v) in phosphate buffer (0.01 M, pH 7.4). Then, the homogenate was centrifuged at approximately 2000 × g for 5 min at room temperature. The supernatant was divided into aliquots to measure the concentration of total protein and to quantify the enzyme activity. Distilled water saturated with CO_2 and a reaction medium with mannitol (0.225 M), sucrose (0.075 M), and Tris–phosphate buffer (0.01 M, pH 7.4) was added to the supernatant. The drop in pH resulting from the reaction was monitored every 4 s for 20 s. The calculation of carbonic anhydrase activity (CAA) was performed based on the descriptions by Burnett et al. (1981) and Vitale et al. (1999),

using the formula: CAA = [TC / (TNC − 1)]/mg of total protein, where TC is the rate of catalyzed reaction and TNC is the rate of the uncatalyzed reaction. Results were expressed as specific CAA·mg of protein^{-1}.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 statistical program. Data normality was evaluated using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) was applied for parametric data followed by Dunnett's test and, for non-parametric data, the Kruskal–Wallis test followed by Dunn's was used. The evaluation of outliers was performed using the Grubbs test. Results were expressed as mean ± standard error of mean or median and interquartile interval. A p-value < 0.05 was considered statistically significant. The bioassay was carried out with two control groups: water and solvent (acetone). As there was no statistical difference between the controls (Student's t-test; Figs. 1S, 2S, 3S, 4S, and 5S), the groups exposed to DiPeP were compared with the solvent control in which the phthalate was diluted.

Results

Experimental conditions

There was no mortality during the experiment. The water parameters were as follows: pH 7.00 ± 0.60, ammonia 0.04 ppm, nitrite 0 ppm, hardness: 3.00 ppm of $CaCO_3$, and temperature 26 ± 1 °C. All water parameters were determined in triplicate and values did not differ among groups.

Chemical analysis of DiPeP in water

DiPeP absorption experiment results

The DiPeP absorption demonstrated phthalate stability over 96 h. However, in the presence of fish, it was possible to observe a drop in phthalate concentration over time: 33.3% in 24 h, 35.8% in 48 h, 44.7% in 72 h, and 46.3% in 96 h (Table 1). Based on this result, it was decided to change the water with the replacement of phthalate (36%) every 48 h.

After 96 h, it was observed that there was a deposition of DiPeP in muscle tissue at a concentration of 37.96 µg/L.

Fourteen-day bioassay

Quantification of DiPeP in muscle tissue

DiPeP was quantified in *Danio rerio* muscle after 14 days of exposure. The values found were control group below the limit of detection (< LLOD), group 5 µg/L = 0.88 ± 0.02 ng/g, 25 µg/L = 4.76 ± 0.03 ng/g, and 125 µg/L = 22.68 ± 0.06 ng/g, demonstrating that there was deposition in muscle tissue.

Antioxidant system, osmoregulation, and neurotoxicity

In the liver, GPx enzyme activity and GSH concentration had a significant increase at 25 µg/L of DiPeP ($p < 0.05$) compared to the control group (Fig. 1 B and D, respectively). GST activity was reduced at the 5 and 125 µg/L of DiPeP ($p < 0.05$) (Fig. 1C). Statistical analysis did not show a difference in SOD activity in fish exposed to DiPeP (Fig. 1A).

In the intestine, GPx activity had a significant increase at 25 µg/L of DiPeP ($p < 0.05$) (Fig. 2B), and GST activity was reduced at the higher concentration of DiPeP ($p < 0.05$), compared to the control group (Fig. 2C). Statistical analysis did not show a difference in SOD activity and GSH concentration at different concentrations of DiPeP (Fig. 2 A and D).

In the gills, there was a significant increase in SOD enzyme activity at the concentrations of 25 and 125 µg/L of DiPeP ($p < 0.05$) (Fig. 3A). GPx and GST activity and GSH concentration did not show a significant difference in fish exposed to DiPeP (Fig. 3 B, C, and D).

Carbonic anhydrase activity showed no significant change in groups exposed to DIPeP when compared to the control group (Fig. 3E).

In the brain, SOD activity had a significant increase at concentrations of 5 and 25 µg/L of DiPeP ($p < 0.05$) compared to the control group (Fig. 4A), as well as an increase in GPx activity in the group exposed to 25 µg/L of DiPeP ($p < 0.05$) (Fig. 4B). GST activity was reduced in all groups exposed to DiPeP ($p < 0.05$) (Fig. 4C). GSH concentration did not show a statistical difference in the DiPeP-exposed groups (Fig. 4D).

AChE enzyme activity showed no significant difference in DiPeP-exposed groups when compared to the control group (Fig. 4E).

Histopathology

Histologically the intestine of *Danio rerio* is consisting of four layers: the mucosa, submucosa, muscular, and serosa. The mucosa was folded into long and relatively narrow branched villi. Two cell types were identified in the epithelial layer: the absorptive or columnar and the goblet cells. In fish exposed to 125 µg/L of DiPeP we observed the presence of cells of the immune system; in the lumen of the organ, organ could cause inflammation of the intestinal wall. There was an increase in the injury index in animals exposed to

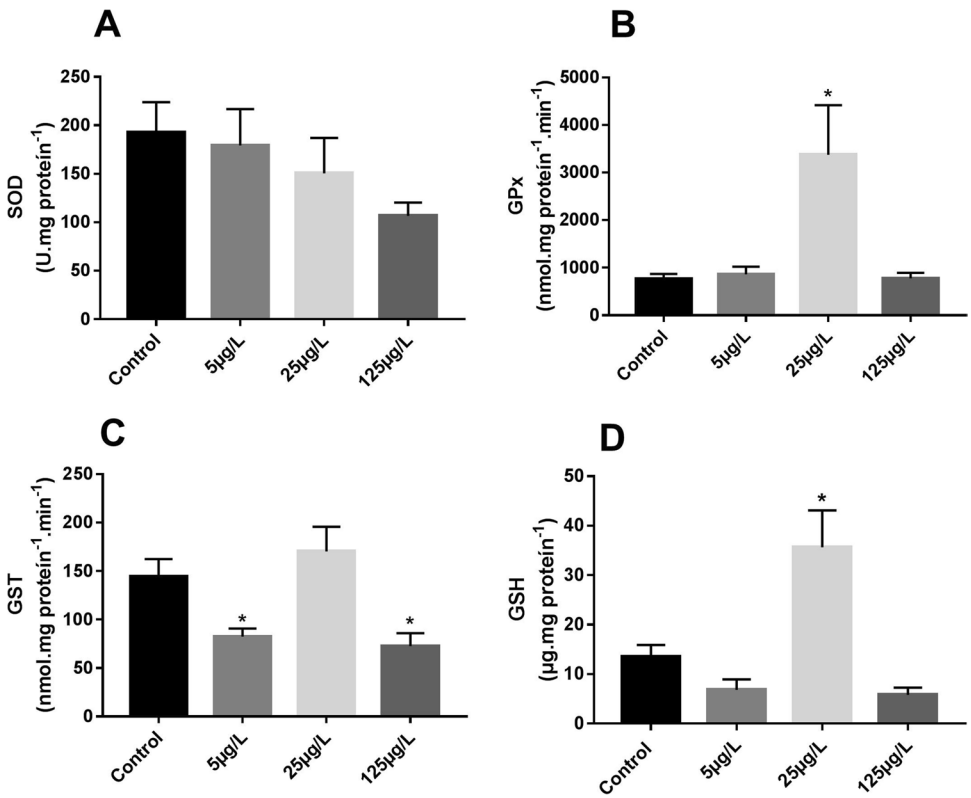

Fig. 1 Antioxidant system in the liver of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). **A** Superoxide dismutase activity, **B** glutathione peroxidase activity, **C** glutathione S-transferase activity, and **D** reduced glutathione concentration or non-protein thiols. Results are expressed as mean ± standard error. One-way ANOVA was followed by Dunnett's test. $n = 7–10$. Asterisk indicates statistical difference compared to the control group ($p < 0.05$)

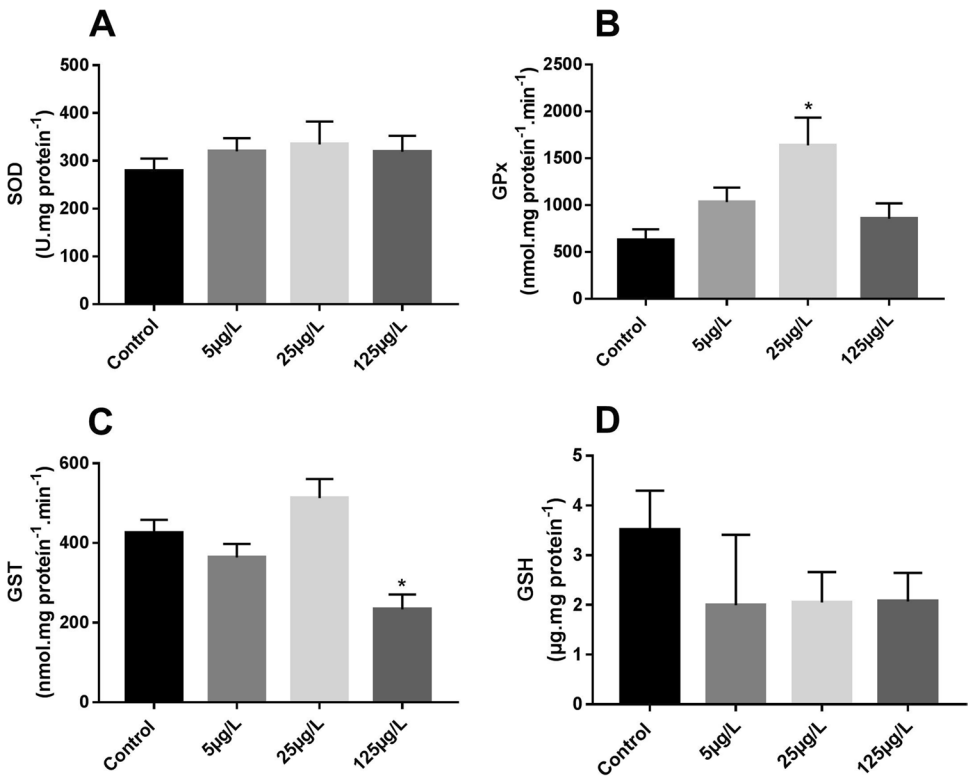

Fig. 2 Antioxidant system in the intestine of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). **A** Superoxide dismutase activity, **B** glutathione peroxidase activity, **C** glutathione S-transferase activity, **D** and reduced glutathione concentration or non-protein thiols. Results are expressed as mean ± standard error. One-way ANOVA was followed by Dunnett's test. $n = 7–10$. Asterisk indicates statistical difference compared to the control group ($p < 0.05$)

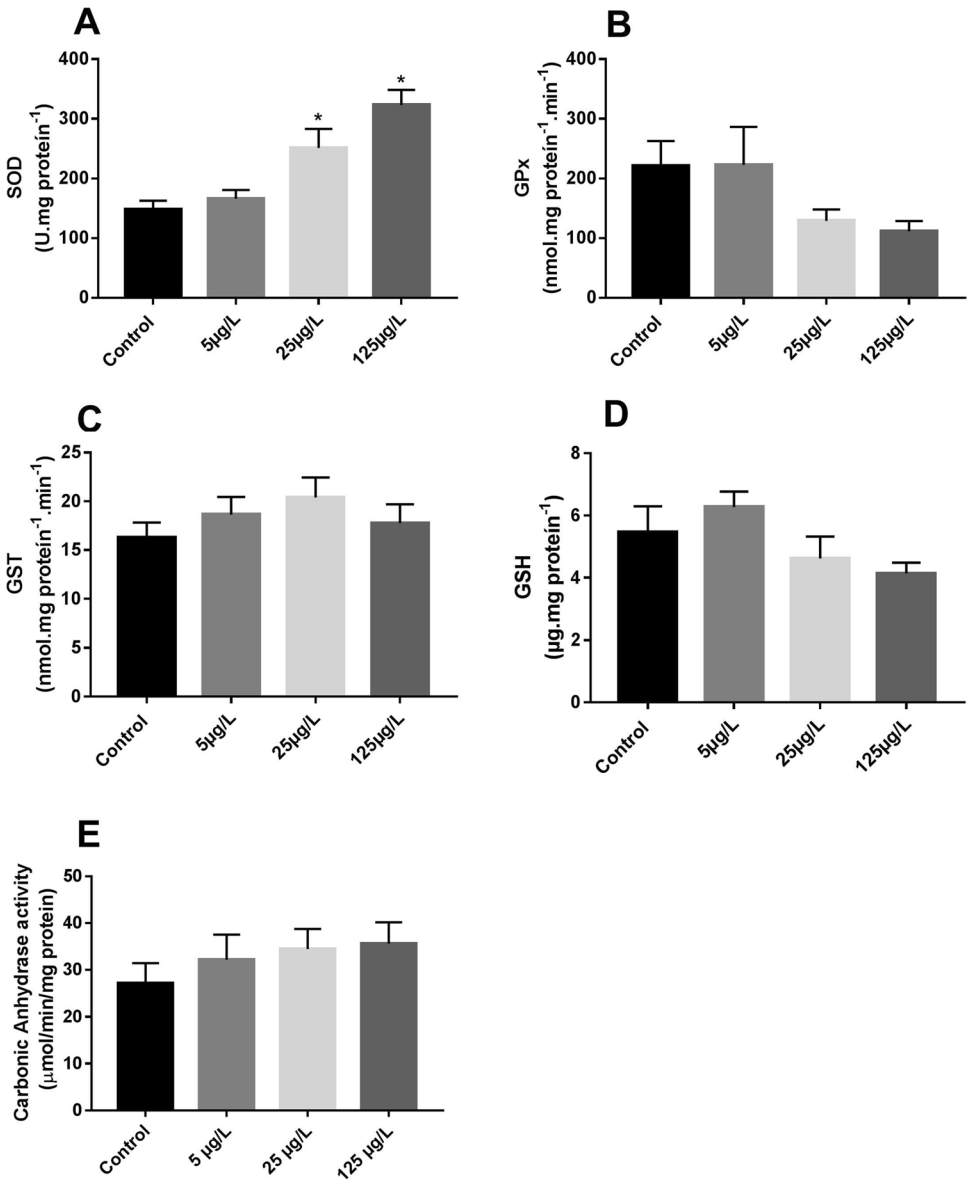

Fig. 3 Antioxidant system and osmoregulation in the gills of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). **A** Superoxide dismutase activity, **B** glutathione peroxidase activity, **C** glutathione S-transferase activity, **D** reduced glutathione concentration or non-protein thiols, and **E** carbonic anhydrase activity. Results are expressed as mean ± standard error. One-way ANOVA was followed by Dunnett's test. $n = 7–10$. Asterisk indicates a statistical difference from the control group ($p < 0.05$)

DiPeP, and at the highest concentration, this increase was statistically significant ($p < 0.05$) (Fig. 5).

The histopathological evaluation of the liver showed hepatocytes with dense and compactly organized cytoplasm and a nucleus located centrally between sinusoids (Fig. 6A). In the DiPeP-exposed groups the observed changes were vascular congestion (Fig. 6B), necrosis (Fig. 6C), and loss of cell limits (Fig. 6D). The higher concentration of DiPeP (125 µg/L) caused morphological changes statistically significant ($p < 0.05$) (Fig. 7).

Genotoxicity: comet assay

The comet assay was used to determine DNA damage in the gills of *Danio rerio* at three different concentrations of DiPeP and the control (Fig. 8). The data showed that, compared to the control group, the concentration of 125 µg/L of DiPeP caused a significant increase in the score of DNA damage in the gills ($p < 0.05$).

Genotoxicity: Piscine Micronucleus Test

In waterborne exposure to DiPeP, no micronuclei occurred in any of the groups. The erythrocytic nuclear alterations (ENAs) observed were Binuclei (double nucleus), Blebbed (evagination), Notched (invagination), and Vacuolated (presence of vacuole). The least observed alteration was binucleus and the most observed was Blebbed. The group exposed to 25 µg/L of DiPeP showed a significant increase in Blebbed, Vacuolated, and Total AEN changes ($p < 0.05$) (Table 2).

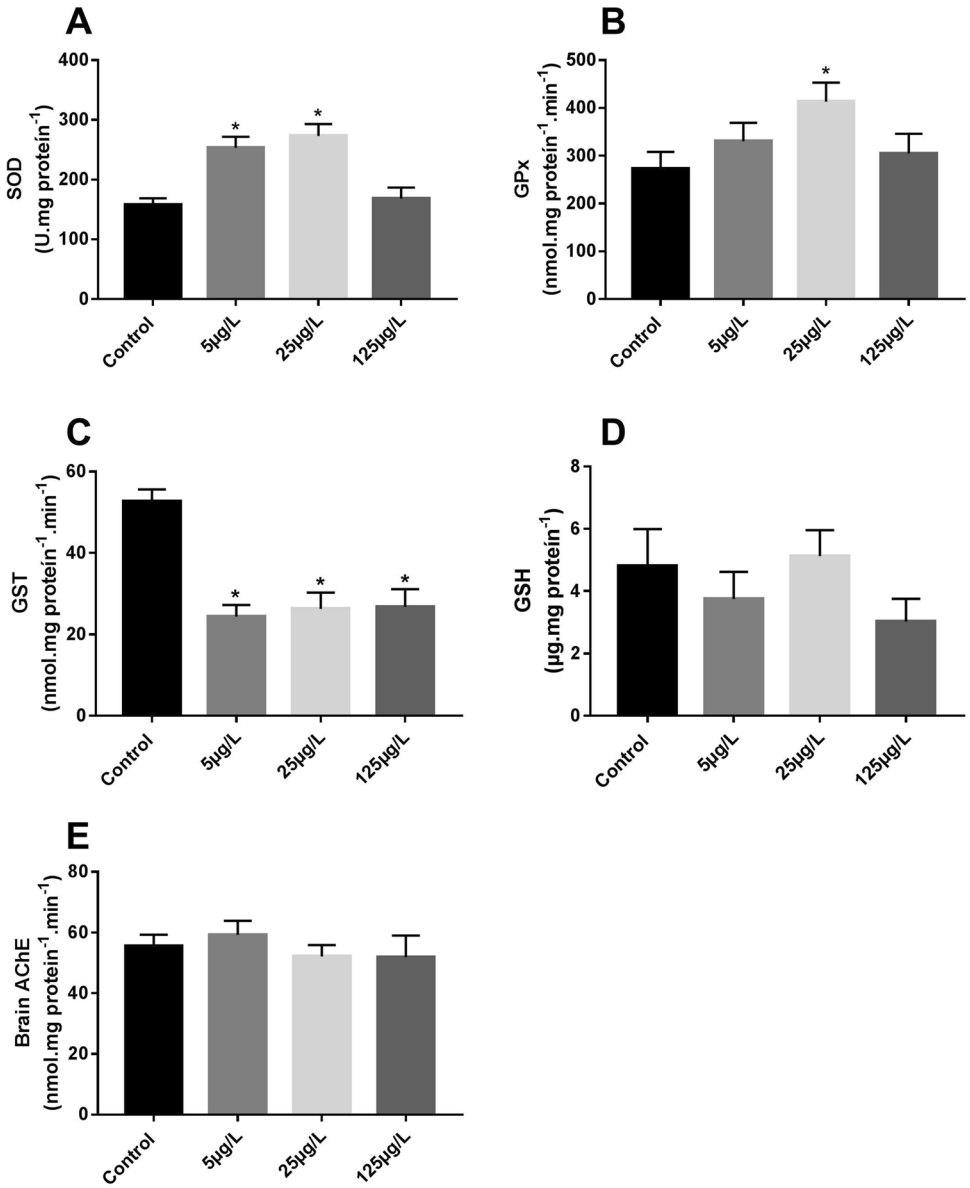

Fig. 4 Antioxidant system and neurotoxicity in the brain of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). **A** Superoxide dismutase activity, **B** glutathione peroxidase activity, **C** glutathione S-transferase activity, **D** reduced glutathione concentration or non-protein thiols, and **E** acetylcholinesterase activity. Results are expressed as mean ± standard error. One-way ANOVA was followed by Dunnett's test. $n = 7–10$. Asterisk indicates a statistical difference from the control group ($p < 0.05$)

Discussion

Our study provides important information on the deposition of DiPeP in fish tissue, not yet reported in the literature, which may contribute to establishing safe threshold levels concerning animal and human health. Lu et al. (2021) evaluated the presence of phthalates and other endocrine disruptors in fish consumed by the Taiwanese population and the predominant compounds were diethyl phthalate, di-2-ethylhexyl phthalate, and di-iso-nonyl phthalate with mean concentrations of 42.5–52.9 ng/g dry weight. These results corroborate the findings in this paper that show the ability to accumulate phthalates, which, in the long term, can cause risks of intoxication for humans through biomagnification processes, that is, a progressive accumulation of DIPeP along with trophic levels.

It is already known that the bioaccumulation of toxic substances can generate free radicals, especially intracellular ROS. In the intracellular environment, a dynamic balance is

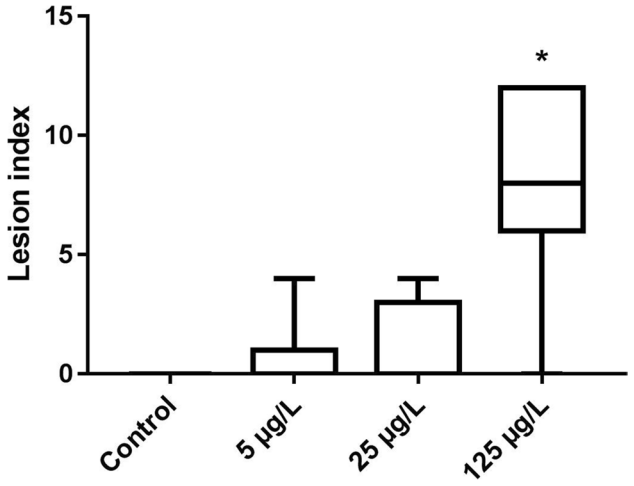

Fig. 5 Intestine Lesion Index of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). Results are expressed as median ± interquartile range. Kruskal–Wallis followed by Dunn's test. $n = 7–10$. Asterisk indicates statistical difference compared to the control group ($p < 0.05$)

Table 1 DiPeP concentration values determined in water samples (0 h, 24 h, 48 h, 72 h, and 96 h)

Time	DiPeP concentration (µg/L)
0 h — presence of fish	120.69 ± 0.03
24 h — presence of fish	80.53 ± 0.02
48 h — presence of fish	77.47 ± 0.02
72 h — presence of fish	66.83 ± 0.001
96 h — presence of fish	64.82 ± 0.04
0 h — absence of fish	121.88 ± 0.04
24 h — absence of fish	122.59 ± 0.04
48 h — absence of fish	113.26 ± 0.01
72 h — absence of fish	112.89 ± 0.01
96 h — absence of fish	128.42 ± 0.02

Values are expressed in micrograms per liter

maintained between the production and elimination of ROS, and these can become reactive, attacking cellular targets via oxidative damage and thus causing cell death (Van der Oost et al. 2003; Lushachak 2011).

Therefore, the antioxidant system acts to prevent such oxidative damage (Velisek et al. 2012). As an antioxidant enzyme of great importance, GPx acts by catalyzing the reduction of hydroperoxides and consequently assisting in the maintenance of antioxidants in aquatic organisms (Zhou et al. 2011). In this study, there was an increase in GPx activity and GSH concentration in liver tissue when exposed to a concentration of 25 µg/L of DiPeP. GSH is a non-enzymatic antioxidant that is also used by other enzymes of the glutathione group such as GPx and GST. GPx catalyzes the reaction of GSH with hydrogen peroxide or lipid peroxides performing a cellular detoxification function (Sarikaya and Doğan 2020). Therefore, this increase in GPx activity to combat reactive species may have caused an augmentation in GSH consumption, which led to the elevated synthesis of the molecule and, consequently, a higher concentration compared to the control group.

In the intestine and brain of *Danio rerio* exposed to DiPeP, there was also an increase in GPx at the concentration of 25 µg/L; thus, a pattern of increase in the response of GPx against a concentration of 25 µg/L of DiPeP was observed. DEP phthalate at a dose of 900 mg/kg also induced GPx activity in the liver of *Paralichthys olivaceus* fish after parenteral exposure for 3 days (Kang et al. 2010).

DiPeP was responsible for reducing the activity of GST in the liver and intestine, and there was also a reduction in the activity of the enzyme in the brain after exposure to all concentrations of DiPeP. This demonstrates similar effects on the antioxidant system of these organs, with the brain being the most affected. No data were found about the effects of phthalates on the antioxidant system of fish intestines. GST is considered an important phase II enzyme in organisms, acting to facilitate the excretion of xenobiotics in addition to its antioxidant capacity (Regoli and Giuliani 2014; Allocatti et al. 2018). Therefore, a reduction in GST can increase the toxicity of compounds such as phthalates since their metabolism will be reduced. In a study carried out with annelids of the *Eisenia fetida* species exposed to doses (0.1, 1, 10, and 50 mg/kg) of butyl benzyl phthalate (BBP) at different times (7, 14, 21, and 28 days), a reduction in GST activity was also observed after 28 days of exposure (Song et al. 2018).

Zhang et al. (2014) described in their study that GST activity also decreased in the liver of carp exposed to a concentration of 125 µg/L of DEP for 20 days, demonstrating a similar effect to that found in our study. This reduction can impair the protection of cells against antioxidant substances and thus cause cellular damage (Aikten and Roman 2008; Liu et al. 2014). Many enzymes involved in antioxidant defenses can be inactivated by the excess of oxidants (Modesto and Martinez 2010). The reduction of enzymes such as GST was verified in *Chironomus riparius* larvae exposed to BBP at concentrations from 1 to 1000 µg/L (Santos Morais et al. 2020).

SOD is considered the main defense mechanism against oxidative damage derived from the increase in ROS being responsible for catalyzing the dismutation of the superoxide anion (Velisek et al. 2011) in hydrogen peroxide. In our study, a significant increase in brain SOD activity at concentrations of 5 and 25 µg/L of DiPeP was observed. Other studies reported similar results in the brain of exposed fish to synthetic organic pollutants and biotoxins (da Silva et al. 2011; Xing et al. 2012). In the present study, the increase in SOD activity suggests that it is an organism's response to

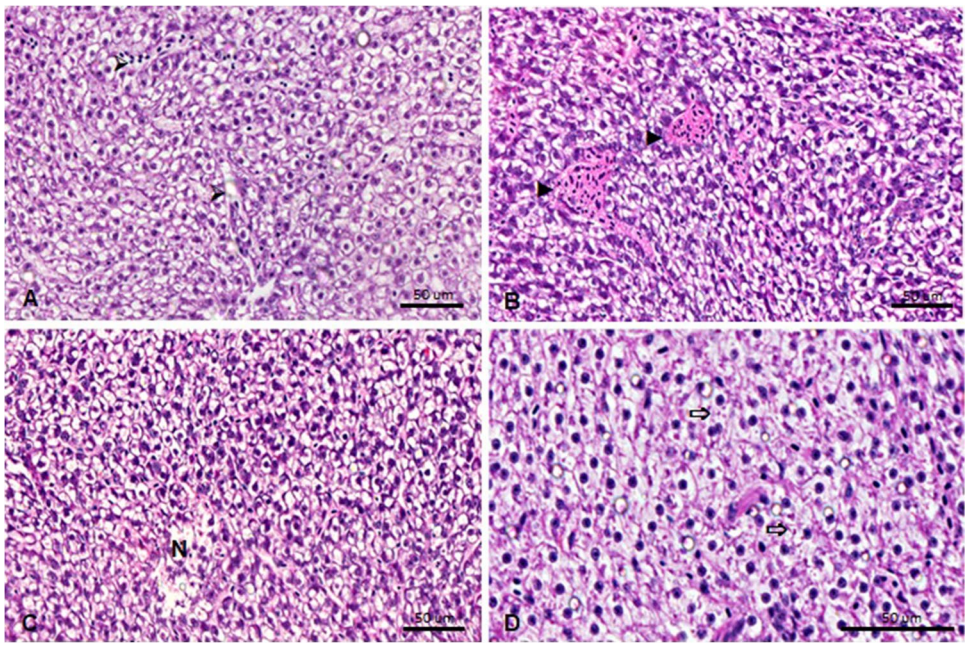

Fig. 6 Structural organization of the liver of *Danio rerio* stained with hematoxylin and eosin. **A** Liver of control individual. Sinusoids in the liver parenchyma (). **B** Liver of an individual from the group was exposed to 125 µg/L of DiPeP. Vascular congestion (►). **C** Liver from an individual from the group exposed to DiPeP 125 µg/L. Presence of necrotic area (N). **D** Liver from an individual from the group exposed to DiPeP 125 µg/L. Loss of hepatocyte cell limits (⇨). $n = 7–10$

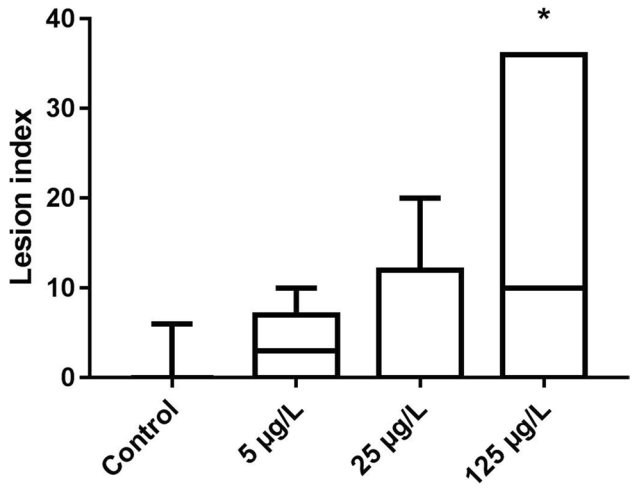

Fig. 7 Liver Injury Index of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). Results are expressed as median ± interquartile range. Kruskal–Wallis followed by Dunn's test. $n = 7–10$. Asterisk indicates a statistical difference from the control group ($p < 0.05$)

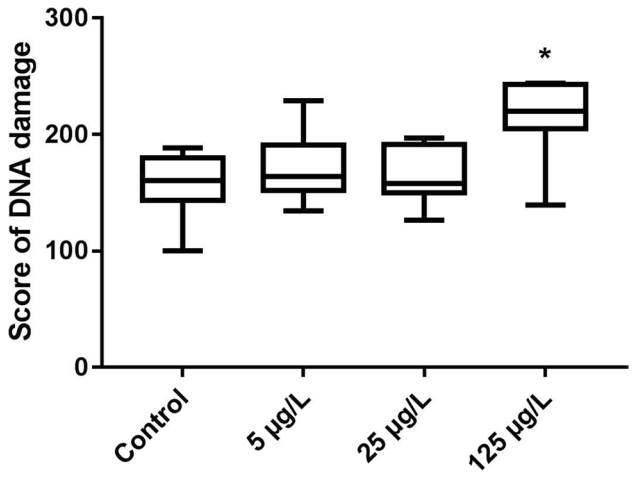

Fig. 8 DNA damage scores in gills of *Danio rerio* after 14 days of exposure to di-iso-pentyl-phthalate (DiPeP). Results are expressed as median ± interquartile range. Kruskal–Wallis followed by Dunn's test. $n = 7–10$. Asterisk indicates a statistical difference from the control group ($p < 0.05$)

environmental stress, aiming to reduce cell damage in the *Danio rerio* brain. Also, in the gills, there was an increase in SOD activity after 14 days of exposure to DiPeP at concentrations of 25 and 125 µg/L, which demonstrates a defense mechanism of the gills in trying to remove the free radicals formed. Different from gills and brain, there was no change in intestinal and hepatic SOD in the groups exposed to DiPeP. Other studies have reported changes in hepatic SOD in *Danio rerio* (Lu et al. 2016), *Dicentrarchus labrax* (Espinosa et al. 2019), and *Clarias gariepinus* (Iheanacho and Odo 2020) fish exposed to polyvinyl chloride (PVC) microparticles and microplastics.

Regarding the neurotoxicity evaluated through acetylcholinesterase in the brain, there were no significant changes compared to the control group, but it is not excluded that there is an effect of DiPeP in the brain of *Danio rerio*, as there was a change in antioxidant enzymes. Although in our study with DiPeP there were no changes in AChE activity, an increase of this enzyme has already been observed in *Pseudobagrus fulvidraco* chronically exposed to 100, 500, and 1000 mg/kg of dibutyl phthalate (DBP) for 8 weeks (Jee

Table 2 Frequency of erythrocytic nuclear alterations (ENAs) in *Danio rerio* separated into binuclei, blebbed, notched, vacuolated, and the total amount of erythrocytic nuclear alterations

Group	Binuclei	Blebbed	Notched	Vacuolated	Total ENAs
Control	0 (0/1)	11 (8.75/12.25)	4 (1.75/7.25)	1 (0.5/2.5)	15 (11.5/20.5)
5 µg/L	0 (0/0)	14.5 (9.75/20.75)	2.5 (0.75/4.75)	2 (1/6.5)	19 (11.5/23)
25 µg/L	0 (0/1)	17.5 (12.75/19.75)*	4 (1.75/7.25)	10 (1.5/11)*	33 (16.5/43)*
125 µg/L	1 (0/2)	11.5 (8/17.25)	0 (0/1)	1.5 (1/2.75)	20 (15.5/23.75)

Data expressed as median and quartiles (1st *Q*/3rd *Q*). Kruskal–Wallis followed by Dunn's test ($p < 0.05$). $n = 7–10$

et al. 2009). As an enzyme that degrades the neurotransmitter acetylcholine (ACh), AChE is important because when ACh is released by presynaptic neurons, it regulates the concentration of this neurotransmitter (Soreqh and Seidman 2001).

In the histopathological evaluation of the intestine, the group exposed to 125 µg/L of DiPeP showed the presence of macrophages in the lumen. These cells moved from the connective tissue to the epithelium and thus reached the lumen. The intestine is one of the major organ systems of fish that interact with the environment and plays a critical role in survival under stressful environments. Although we did not observe morphological changes in the intestinal epithelium after 14 days, the contact of the pollutant with this tissue probably triggered an inflammatory process, being the first line of defense to act in this organ, before morphological lesions are established.

Marinsek et al. (2018) also observed a strong inflammatory response in the intestine, reinforcing the influence of polluted environments on the gastrointestinal tract of fish. According to Brierley and Linden (2014), the environmental contaminants may be responsible for the recruitment of cells from the intestinal immune system, causing an increase in the inflammatory process.

In the hepatic tissue, severe alterations were observed such as loss of cell limit that can precede necrosis, blood congestion, and necrotic areas. All these findings were significant at exposure to 125 µg/L of DiPeP. Blood congestion is liver dysfunction caused by venous congestion, usually as a result of heart dysfunction, also known as congestive heart failure (Barja-Fernández et al. 2013). The fish liver is especially susceptible to environmental pollutants thanks to slow blood flow relative to cardiac output (Leão-Buchir et al. 2021).

Confirming the hepatotoxic effects induced by phthalates, *Clarias gariepinus* exposed to different concentrations (30 µg/L, 40 µg/L, 60 µg/L, and 80 µg/L) of DEP showed congestion, necrosis, and degeneration of hepatocytes (Obiezue et al. 2014). In *Channa striatus* fish exposed to 0.4 mg/L, 4 mg/L, and 40 mg/L of DEP for 7, 14, and 21 days, hepatic necrosis was also observed in addition to cytoplasmic vacuolization (George et al. 2017). These data demonstrate the toxicological potential of phthalates for the liver tissue of fish.

The gills are an organ of great importance for responding effectively to environmental variations in aquatic organisms, ensuring that the animal's physiological functions remain preserved (Virgens et al. 2015). As they are the first organs to have contact with contaminants, they may change due to defense responses and injuries caused by contaminants (Gomes et al. 2012).

Regarding the analysis of genotoxicity in *Danio rerio* gills, there was a significant increase in DNA breaks at the concentration of 125 µg/L of DiPeP. According to our knowledge, no study has evaluated the genotoxic effects of DiPeP in *Danio rerio*; however, genotoxicity by other phthalates has been observed. *Oreochromis niloticus* exposed to 0.59 and 1.18 mg/L of DBP had an increase in DNA damage in gills (Zeid et al. 2014). Sublethal concentrations of DBP (1/2 LC_{50} 96 h and 1/3 LC_{50} 96 h) were also genotoxic to fish gills (Khalil et al. 2016). In these two studies, the authors evaluated the effect of high concentrations of DBP. Our study showed DNA damage even when *Danio rerio* were exposed to concentrations a thousand times lower.

In the blood, there was an increase in blebbed (evagination), vacuolated (vacuole in the middle of the nucleus), and total erythrocytic nuclear alterations in the group exposed to 25 µg/L of DiPeP, demonstrating a mutagenic potential of DiPeP. Environmental contaminants can induce damage to DNA and proteins involved in the cell division process through several mechanisms that cause the loss of genome integrity and the formation of micronuclei and nuclear morphological changes (Iheanacho et al. 2021). Possible damages will depend on whether the contaminant is clastogenic (micronuclei originate from acentric chromosomal fragments) and/or aneugenic (whole chromosomes that do not complete the anaphasic migration of cell division), according to the stage of the cell cycle affected (Canedo et al. 2021). Mutagenic effects of phthalates have already been demonstrated in the exposure of Nile tilapia (*Oreochromis niloticus*) to the sublethal concentration of 10 mg/L of DBP, obtaining a greater difference in the Notched-type abnormality (invagination) after 96-h exposure (Benli et al. 2016). The type of abnormality observed may vary depending on the exposure time and via and the contaminant class.

In the gills, carbonic anhydrase activity did not show any significant alteration at the concentrations tested. Carbonic

anhydrase regulates acid–base respiration and ion uptake in fish such as *Danio rerio*, revealing itself to be sensitive to the presence of contaminants of different classes (Lionetto et al. 2012) but it was not affected by DiPeP. Unlike what happened in our study, other contaminants such as drugs and metals can alter the activity of this enzyme (Saravanan et al. 2011; Mela et al. 2013b; Perussolo et al. 2019).

Conclusion

In the present study, phthalate deposition occurred in muscle tissue of *Danio rerio* at all concentrations tested. Therefore, our data suggest that in the long term DiPeP can cause risks to humans via bioaccumulation processes. The antioxidant system was altered in all evaluated organs (liver, intestine, gills, and brain), demonstrating to be sensitive even to exposure to low concentrations of this phthalate. DiPeP caused major tissue damage in the liver, including necrosis. In the intestine, it was possible to observe cells of the immune system in the lumen of the organ as a response to exposure to phthalate. The genotoxic potential of DiPeP was observed in the gills after exposure to the highest concentration, and, in the blood, there were nuclear morphological changes in erythrocytes in fish exposed to 25 µg/L of DiPeP.

DiPeP did not change the neurotoxicity for zebrafish or caused osmoregulatory change (inferred from carbonic anhydrase activity) at the concentrations tested. However, the occurrence of neurotoxicity should not be excluded, since the antioxidant system was altered in the zebrafish brain, proving it to be sensitive even to exposure to low concentrations of this phthalate.

Future studies evaluating the effects of chronic exposure, exposure in developmental periods, behavior, and analysis of the gonads and/or hormones, would help to clarify the toxicological potential of this phthalate.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-24071-9.

Acknowledgements We would like to thank the Instituto de Pesquisa Pelé Pequeno Príncipe for the equipment and materials used for the development of the experiment.

Author contribution Sheila Gabriel dos Santos: methodology, data curation, and writing — original draft. Marília Cristina Oliveira de Souza: investigation and writing — reviewing. Fernando Barbosa Junior: investigation and writing — reviewing. Maritana Mela Prodocimo: investigation and writing — reviewing. Fellip Rodrigues Marcondes: investigation and writing — reviewing. William de Almeida: investigation and writing — reviewing. Marta Margarete Cestari: investigation and writing — reviewing. Luciana Rodrigues de Souza-Bastos: investigation and writing — reviewing. Anderson Joel Martino Andrade: writing — reviewing. Izonete Cristina Guiloski: conceptualization, supervision, and writing — reviewing and editing. All authors read and approved the final manuscript.

Funding This study was financed in part by Instituto de Pesquisa Pelé Pequeno Príncipe (IPPPP, Curitiba, Brazil). The funders were not involved in the design of the study; data collection, analysis, and interpretation of the data; and the writing of the manuscript.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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