Toxicity assessment of Dibutyl phthalate in Grass carp: an integrated biomarker approach

Statement of novelty

Phthalates are emerging contaminants and are ubiquitous in the aquatic environment. Recently lot of attention is given to phthalate toxicity in fish. However no work is done in regard of oxidative and biochemical studies. Moreover, integrated biomarker approach is an emerging techniques that is now being used to determined an overall toxicity analysis of any toxicant. Therefore, present work was designed to study effect of DBP (low molecular weight phthalate) on Grass carp, a commercially important fish.This work will be of interest to readers in the areas of fish toxicology and biochemistry.

ABSTRACT

Phthalates are the common plasticisers used around the globe. Dibutyl phthalate (DBP) is a ubiquitous, extensively used in cosmatics and frequently present in the aquatic environment. Therefore, toxic effects of DBP were evaluated in term of oxidative stress and biochemical biomarkers. For this reason, a 21 day exposure was conducted by exposing grass carp with graded concentrations of DBP (1, 10, 100 and 1000 µg/L). After 21days, stress biomarkers:, lipid peroxidation (LPO), catalase (CAT) activity, glutathione-*S*-transferases (GST) activity and level of reduced glutathione was evaluated in liver, kidney and gills. Alkaline phosphatase (ALP), aspartate transaminase (AST), urea and creatinine were evaluated in liver and kidney homogenates respectively. Moreover, effect of DBP on all biomarkers were evaluated through integrated biomarker response (IBR). Exposure of fish to DBP resulted in oxidative stress in grass carp as evidenced by an increase in lipid peroxidation and decrease in antioxidant enzymes. DBP exposure also resulted in increased liver's ALT and AST levels. Urea and creatinine were also significantly increased in kidney after exposure to DBP. The IBR showed bad scores as the DBP concentration increased, with the highest one (1000 µg/L) presenting a score >250x the value for the control treatment. Additionally, the IBR/n showed that the most impacted organ was the kidney, followed by the liver and the gills. The obtained results show the need for deeper research into the effects of DBP on fish and their impact on different organs.

Keywords: Phthalates; Dibutyl Pthalate; Grass carp; Oxidative stress

1. INTRODUCTION

Phthalates, commonly known as phthalate esters, are the alkyl/aryl esters of phthalic acids (Bello *et al.*, 2014). They are common plasticisers, being used since 1930, and are generally added to polyvinyl chloride (PVC) to make them soft and durable (Gao and Wen, 2015). PVC may contain up to 50% phthalate plasticisers and are used in a variety of everyday products such as lubricants, adhesives, paints, waxes, medical tubing and many personal care products (Fromme *et al.*, 2002; Schettler *et al.*, 2006; Paluselli *et al.*, 2018). The production of phthalates has increased from 1.8 million tons in 1975 to 8 million tons in 2011 (Peijnenburg and Struijs 2006; Net *et al.,* 2015). Every year approximately 470 million pounds of phthalates are produced globally (Agency, 2012). Since phthalate are not chemically bound, immediate leaching to the surrounding environment occur through microbial action, photo-degradation, hydrolysis and adsorption (Zhao *et al.,* 2004; Ayranci and Bayram 2005; Jonsson *et al.,* 2006). Phthalates are generally classified on the base of their molecular weight. Dibutyl phthalate (DBT) is a low molecular weight phthalate and used in the production of caulk, varnish, cosmetics, food packing, textiles and food wrappings (Agency, 2012) and is listed on EPA as toxic chemical (Heise and Litz, 2004). Previous studies have shown that DBP induced reproductive and developmental toxicity in three-spined sticklebacks (*Gasterosteus aculetaus* - (Aoki *et al.*, 2011), fathead minnow (*Pimephales promelas*- (Crago and Klaper, 2012), murray rainbowfish (*Melanotaenia fluviatilis* - (Bhatia, 2014) neuro and immunotoxicity in zebrafish (*Danio rerio* - (Xu *et al.,* 2013a, 2015) and oxidative stress in Nile tilapia (*Oreochromis niloticus* - (Erkmen *et al.*, 2015).

Biomarkers or biological markers can be defined as a set of changes in organism's physiology, biochemistry and histology after exposure to contaminants (Peakall 1994; Quesada-García *et al.,* 2013). Biomarkers are used to evaluate the effects of sub-lethal or chronic exposure of a contaminant (van der Oost *et al.,* 2003). They provide early warning signals to exposure of a contaminant and are used extensively in toxicological studies and environmental monitoring (van der Oost *et al.,* 2003; Cravo *et al.,* 2011; Hook *et al.,* 2016). Early biological signals range from the molecular and subcellular level to organismal and population level (Beliaeff and Burgeot 2002; Marigómez *et al.,* 2013). The selection of suitable biomarkers and integration of their responses is a reliable and powerfull tool that can help in data interpretation. One group of biomarkers normally used in toxicological assays is the one related to oxidative stress. Oxidative stress is induced by

free radicals and reactive oxygen species (ROS) and is defined by the imbalance between production and elimination of these free radicals and ROS (Valavanidis *et al.,* 2006). To surmount the free radicles and ROS, the body has an antioxidant defence system that includes enzymatic (catalase; glutathione peroxidase; superoxide dismutase) and non-enzymatic antioxidants (glutathione; vitamin C - (de Zwart *et al.,* 1999; Valavanidis *et al.,* 2006). Oxidative stress results in DNA damage (Gào *et al.,*, 2019; Santos *et al.,*, 2016) and inflammation (Reuter *et al.,* 2010). Many anthropogenic chemicals induced the production of ROS in vital fish organs that leads to detrimental effects on fish health (Faheem and Lone 2017; Abd-Elkareem *et al.,* 2018; Abdel-Tawwab and Hamed 2018).

Amino transferases and phosphatases are important liver functioning enzymes and are considered potential candidates for assessing liver health (McGill, 2016).

In this study, we investigate the effects of DBP in grass carp (*Ctenopharyngodon idella*) when exposed for 21 days. Biomarkers of oxidative stress (Lipid peroxidation, reduced glutathione level, catalase and glutathione-*S*-transferases activity), nephrotoxicity (creatinine, uric acid) and hepatotoxicity (alkaline phosphatase, aspartate transaminase) were evaluated. The results were then integrated into the Integrated Biomarker Response index (IBR).

MATERIALS AND METHODS

Grass carp (*Ctenopharyngodon idella*) weighing 17.08 ± 1.01g, length of 11.8 ± 0.44cm, were placed to glass aquaria containing 60 L of tap water. A total of 6 fish were placed in each aquarium and acclimatized for a week. fish were exposed to different concentrations of dibutyl phthalate (DBP). DBP stock solution (10mg/mL) was prepared in 80% DMSO. Desired DBP concentrations were obtained by adding an appropriate volume of stock to aquaria water. Fishes in the control group were exposed to the maximum level of DMSO used for dilution (0.5 ml/L). The experiment was conducted in duplicate.

Fishes were exposed to 1, 10, 100 and 1000µg/L for 21 days in a semi static system in duplicate. Approximately ¾ water were renewed every day with a new DBP solution. Dissolve oxygen was maintained in the aquarium by air stones provided with air pumps. All experiments were performed at room temperature (28.35 ± 1.25 ºC) and 13:11h (light: dark) photoperiod. Fish

were observed for mortality and abnormal behaviour regularly during the experimental period. Fishes with abnormal swimming pattern were removed immediately.

Sample collection

After 21-days, fish were euthanized using clove oil (Latif *et al.,* 2021) according to ethic regimentation and its length and weight were recorded. Fish liver, gills and kidney were dissected and used for the biomarkers analysis. Organs were washed with chilled 0.9% saline solution to remove exogenous materials and snap-frozen in liquid nitrogen.

Organs (gills, liver and kidney) were weighed and homogenised in chilled phosphate buffer (0.1M) using a mechanical homogeniser (Scilogex D160, USA). All procedure was performed on ice. After homogenization, 1mL of the homogenate was used for the measurement of lipid peroxidation and the remaining homogenate was centrifuged for 30 min at 13,000rpm (4^{o}C) to get the post-mitochondrial supernatant (PMS) (Faheem & Lone, 2017; Latif et al., 2019).

Biochemical analysis

Lipid peroxidation was measured using the thiobarbituric acid method described by (Wright *et al.,* 1981). Tissue homogenate was mixed with an equal volume of trichloroacetic acid (TCA – 10%) and thiobarbituric acid (TBA – 0.67%). After incubation for 45min in a boiling water bath, the mixture was then centrifuged for 10 min. The supernatant was collected, and absorbance was recorded at 532nm on a Hitachi U-2000 spectrophotometer. Lipid peroxidation was measured using a molar extinction coefficient of 1.56 x 10^5/M/ cm and expressed as nmol TBARS/g tissue.

Glutathione was quantified using an adaption of the method from (Jollow *et al.,* 1974) as described earlier (Latif *et al.,* 2019) Briefly, PMS was incubated with an equal volume of 4% sulphosalicylic acid and incubated at 4^{o}C for 60 min. The mixture was centrifuged at 1200 rpm for 15 minutes (room temperature) and the supernatant was collected. To the supernatant, DNTB [5,5-dithio-bis-(2-nitrobenzoic acid)] and phosphate buffer (0.1M) were added, and absorbance read at 412nm. Reduced Glutathione content was expressed as nmol GSH/g tissue using a molar extinction coefficient of 1.36x104/M/cm.

Catalase activity (CAT) was measured using the method of (Claiborne, 1985) as explained by Faheem & Lone (2017). The reaction mixture consisted of 0.09M H_2O_2, 0.1 M phosphate buffer and PMS (10%) in a total volume of 3 ml. Change in absorbance was recorded every 30 seconds

at 240nm in a double beam spectrophotometer (Hitachi U-2000). Catalase activity was expressed in terms of nmol H_2O_2 consumed/min/mg protein.

The glutathione-*S*-transferases activity was measured kinetically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Briefly, the reaction mixture (2ml) containing 0.1M phosphate buffer, reduced gluthathione (GSH - 1 mM), 2,4-Dinitrochlorobenzene (CDNB - 1 mM) and PMS (10%). The change in absorbance was recorded at 340 nm, and the enzyme activity was expressed as nmol CDNB conjugates formed/min/mg protein (Faheem & Lone, 2017).

Protein content of the homogenate was quantified using Bradford reagent as described by (He, 2011) using bovine serum albumin as standard. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) , creatinine and uric acid were quantified using the commercial kits from Randox.

Integrated biomarker response analysis

The integrated biomarker response (IBR) was calculated according to (Beliaeff and Burgeot, 2002), and can be used for field and laboratory studies (i.e. (Wang *et al.,* 2011; Morgado *et al.,* 2013; Ferreira *et al.,* 2015). Briefly, the IBR was calculated based on the score of each biomarker. The score (S) was calculated using $S = Z+|Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker at all measurements made. Since the IBR is obtained by summing up all the parameters, to allow a correct and more accurate comparison, IBR was divided by the number of biomarkers and presented as IBR/n (Broeg and Lehtonen, 2006), thus allowing an overall state of organisms for each concentration and each organ. The IBR is reported as Star Plot.

Statistical analysis

Data analyses were performed using Sigmaplot (SPSS 1999). Data was checked for normality and homoscedasticity, followed by One-way analysis of variance (ANOVA) or by ANOVA on ranks whenever these parameters were not met.A Tukey's Post Hoc was then used to determine statistical differences among the various exposure groups.

2. RESULTS

153 Grass carp responses to DBP are shown in Table 1. Oxidative damage in various organs of
154 grass carp was assessed using LPO. Lipid peroxidation increased in all organs of grass carp after
155 exposure to DBP for 21-days. The highest exposure concentrations (100 and 1000µg/L) resulted
156 in significant increase in all organs, and gills showed a significant increase for all the treatments.
157 Higher values of LPO were observed for gills, followed by liver and kidneys. Catalase showed
158 significant inhibition of its activity only in kidneys for all exposure concentrations (Table 1).
159 Higher activities of catalase were observed for liver and kidneys. Gills showed activities one
160 magnitude lower than then other organs. As for GST, a bell-shaped response was observed.
161 Significant inductions in its activity for liver and kidneys in 10 µg/L or higher concentration were
162 recorded (except for liver at 1000 µg/L when the activity was dropping and reach values near the
163 ones observed for the control). As for gills, the induction in the activity of GST was observed for
164 the two highest concentrations (100 and 1000 µg/L). As expected, the higher activities in these
165 enzymes were observed for the liver. Liver function biomarker ALP, showed a significant increase
166 in exoposures with DPB concentrations of 10 µg/L or higher (Table 1). As for the biomarker AST,
167 although a bell-shaped pattern is observed, significant differences were observed only for the DBP
168 exposure at 100 µg/L (Table 1). Creatinine also showed a bell-shaped curve with significant
169 differences to all exposures (Table 1). Moreover, uric acid showed an increasing pattern with the
170 increase of DBP concentrations but with significant differences only for the highest concentration
171 (1000 µg/L – Table 1).
172 The integration of the previous results into the IBR index allowed a better understanding of
173 the organism condition (Fig. 1 and 2). The IBR index that integrates all biomarkers and all tissues
174 (Fig. 1) showed similar values for control and the lower concentration (1 µg/L). This similar score
175 then increased up to more than 250x. A closer look also showed that some biomarkers in the lower
176 concentration (i.e. CAT in liver and gills; GSH and ALP in the liver) have better scores than the
177 control. As for the lowest score for GSH in gills, it was observed for the highest concentration
178 (1000 µg/L). It is clear that the scores increased with the increase of DBP concentrations. When
179 looking to scores of each organ (Fig. 2), the control and the lower exposure concentration (1 µg/L)
180 appear with lower scores than the other treatments.
181 In order to be able also to perform a direct comparison (due to the different number of
182 integrated biomarkers: six for kidney and liver, four for gills) the IBR/n was used. This index
183 showed similar patterns to the ones observed for IBR for what reports the increasing scores with

184 the increase of concentrations. Nonetheless, when looking to the IBR/n of liver and gills when
185 exposed to 10 and 1000 µg/L, the scores are more similar when compared to the IBR scores.
186
187 ## 3. DISCUSSION
188 Exposure biomarkers can reflect the early biological response after exposure to contaminant
189 and have been used widely in laboratory and field studies (Hook *et al.,* 2016). Integration of data
190 obtained from biomarker response of tissues, exposure chemicals and concentrations is an easier
191 way to interpret and comprehend data (Beliaeff and Burgeot, 2002). In this study, juvenile grass
192 carps were exposed to graded concentrations of di-butyl phthalate and its effect were evaluated
193 using biomarkers in vital organs (liver, gills and kidney). Although the most of concentrations of
194 DBP to which the fishes were exposed are higher than the maximum found in literature in waters
195 (3.1 µg/L - Vethaak *et al.,* 2005), the high persistence of these pollutants and resuspension from
196 sediments (30.3 µg/L - Yuan *et al.,* 2002) may lead to higher concentrations. Nonetheless it is also
197 important to highlight that to understand the impact of pollutants ecotoxicological assays use
198 higher concentrations to have a full description of their toxicity.
199 Lipid peroxidation is a biomarker of oxidative stress, commonly used in ecotoxicological
200 studies (van der Oost *et al.,* 2003; Carvalho *et al.,* 2012; Faheem and Lone 2017; Ghisi *et al.,*
201 2017). LPO results from free radicals and reactive oxygen species that react with membrane lipids
202 (Regoli and Giuliani, 2014). Whenever antioxidant defences cannot handle oxidative stress from
203 reactive oxygen species (ROS), damage can be assessed using this biomarker. In this study, it is
204 possible to observe an increase in LPO rates in all organs with the increase of DBP concentrations.
205 Gills was the most sensitive organ to respond, showing an induction in LPO rates even at 1 µg/L,
206 followed by liver (the organ were the detoxifications processes are expected to be more intensive
207 and finally the kidneys, the excretory organ. These results are not unexpected, as gills are the first
208 organ to be in contract with DBP an after the biotransformation in liver should affect in a lesser
209 level the kidneys. Nile tilapia (*Oreochromis niloticus*) showed similar levels of LPO between the
210 gills and liver when exposed to DBP (Erkmen *et al.,* 2015). LPO rates increased in most fish
211 species after exposure to different phthalates (e.g. (Kang *et al.,* 2010; Mankidy *et al.,* 2013; Xu *et*
212 *al.,* 2013b).
213 The previously observed damage (evidenced by the LPO rates) is in accordance with the GST
214 activities. GST is an important biomarker of exposure and is involved in the detoxification of

xenobiotics (Regoli and Giuliani, 2014). In the liver and kidney, the enzymes show a bell-shaped pattern. The pattern is typical for enzymatic activity curves, where at high concentrations, the enzyme is inhibited and may even reach values below the control. These patterns are observed for phthalates (Latif *et al.,* 2019) but also for other xenobiotics (e.g.(Ferreira *et al.,* 2015).

Reduced glutathione is involved in vital aspects of cellular homeostasis (Pompella *et al.,* 2003) and is essential in detoxification processes. A decrease in reduced glutathione content was recorded in gills of Nile tilapia (*Oreochromis niloticus*) exposed to DBP for 96 hours (Erkmen *et al.,* 2015). On the contrary, an increase in GSH levels was recorded in Nile tilapia (*Oreochromis niloticus*) exposed to 590 and 1180 µg/L DBP for eight weeks (Abu Zeid and Khalil, 2015). Still, the observed differences may result from the extensive exposure period that could result in the inactivity of enzymes that use GSH as a substrate. In the present study, apart from small exceptions, GSH levels can be directly connected with GST activity. For example, in the kidney, GSH showed a decreasing pattern with a significant difference from concentrations of 10 µg/L onward, that can be a result of its consumption for GST detoxification processes. These patterns of increase GST activity and decrease GSH can be seen for all the three sampled organs.

Catalase, along with other antioxidants, protects the cellular components from damage (Costa-Silva *et al.,* 2015). When reporting to CAT activity in kidneys, significant decreases can be observed in all concentrations. Still, in all other tissues, no significant differences are observed, although a decreasing pattern can be noticed for the liver.

To determine liver damage, the biomarkers ALP and AST were assessed. For ALP, results showed a significant increase for all DBP concentrations except 1 µg/L. As for AST, a bell-shaped pattern is again observed. Still, only the concentration 100 µg/L showed a significant increase when compared to the control. Similarly, the kidney damage biomarkers creatinine and uric acid also show an impact on their levels. Creatinine shows a bell-shaped curve with a significant increase in its levels for all the exposure concentrations except 1000 µg/L. As for uric acid, the levels show an increasing pattern with the increase of DBP concentrations, although significant differences can only be observed for the highest concentration. An increase in AST and ALP were also observed in various fish species exposed to DBP and other phthalates (Mehta *et al.,* 2003; Kang *et al.,* 2010; Latif *et al.,* 2019).

The IBR index helped to explain and understand the results described previously. It is noticeable that when all the biomarkers measured in all organs are integrated even within realistic

environmental concentrations (1 and 10 µg/L), where up to >110x increase in the score was observed. Similarly, even the comparison between the control and 1 µg/L, showed a 4.7x increase in the score. The integration of the data into the IBR and IBR/n index also showed another interesting result. It is routinely expected that liver is most effected organ after the exposure to toxicant but interestingly, IBR/n result showed that kidneys are most impacted after exposure to DBP.

Conclusion

The impact of phthalates is an important topic that needs to be addressed urgently, and that still needs more information. This study highlights that need by showing the effects of DBP in a freshwater fish species (*Ctenopharyngodon idella*) and most importantly how the general idea that liver, the detoxification organ or gills would be the most impacted organs do not seem to be true for DBP when biomarkers data is integrated into the IBR/n index. As so, this opens the doors for studies that should focus for example on the mechanistic pathways and genes variation on these organs or even the cellular aspect of their specific cellular structure.

4. Acknowledgements

This research work was supported by ORIC, GCU. Nuno Ferreira was funded through a Marie Sklodowska Curie Fellowship (H2020-CoFund ,SIRCIW>MINT) supported by European Union, Welsh Government and Cardiff University.

Author's Contribution

ZZ performed all experimental work under supervision of MF. NGCF performed all data analysis.

REFERENCES

Abd-Elkareem M, Abou Khalil NS and Sayed AH, 2018. Hepatotoxic responses of 4-nonylphenol on African catfish (Clarias gariepinus): antixoidant and histochemical biomarkers. Fish Physiology and Biochemistry 44:969–981.

Abdel-Tawwab M and Hamed HS, 2018. Effect of bisphenol A toxicity on growth performance, biochemical variables, and oxidative stress biomarkers of Nile tilapia, Oreochromis niloticus (L.). Journal of Applied Ichthyology 34:1117–1125.

Abu Zeid EH and Khalil ASA, 2015. Toxicological Consequences of Di-N-Butyl-Phthalate (DBP)

277 on Health of Nile Tilapia Fingerlings. American Journal of Animal and Veterinary
278 Sciences 9:269–276.
279 Agency USEP, 2012. U.S. Environmental Protection Agency - Phthalates Action Plan, 2012,
280 www.epa.gov, acessado em 03/06/2017. 1–16.
281 Aoki KAA, Harris CA, Katsiadaki I, *et al.*, 2011. Evidence suggesting that di-n-butyl phthalate
282 has antiandrogenic effects in fish. Environmental Toxicology and Chemistry 30:1338–
283 1345.
284 Ayranci E and Bayram E, 2005. Adsorption of phthalic acid and its esters onto high-area activated
285 carbon-cloth studied by in situ UV-spectroscopy. Journal of Hazardous Materials 122:147–
286 153.
287 Beliaeff B and Burgeot T, 2002. Integrated biomarker response: A useful tool for ecological risk
288 assessment. Environmental Toxicology and Chemistry 21:1316–1322.
289 Bello UM, Madekurozwa MC, Groenewald HB, *et al.*, 2014. The effects on steroidogenesis and
290 histopathology of adult male Japanese quails (Coturnix coturnix japonica) testis following
291 pre-pubertal exposure to di(n-butyl) phthalate (DBP). Comparative Biochemistry and
292 Physiology Part - C: Toxicology and Pharmacology 166:24–33.
293 Bhatia H, 2014. Effects of exposures to the plasticiser , di-n-butyl phthalate and the pharmaceutical
294 , flutamide on the biomarkers of reproduction in Australian freshwater fish species , Murray
295 rainbowfish (Melanotaenia fluviatilis) Submitted for the degree of Doctor .
296 Broeg K and Lehtonen KK, 2006. Indices for the assessment of environmental pollution of the
297 Baltic Sea coasts: Integrated assessment of a multi-biomarker approach. Marine Pollution
298 Bulletin 53:508–522.
299 Carvalho C dos S, Bernusso VA, Araújo HSS de, *et al.*, 2012. Biomarker responses as indication
300 of contaminant effects in Oreochromis niloticus. Chemosphere 89:60–69.
301 Claiborne A, 1985. Catalase activityIn: Greenwald RA, editor. CRC handbook of methods in
302 oxygen radical research. CRC Press: Boca Raton.
303 Costa-Silva DG, Nunes MEM, Wallau GL, *et al.*, 2015. Oxidative stress markers in fish (Astyanax
304 sp. and Danio rerio) exposed to urban and agricultural effluents in the Brazilian Pampa
305 biome. Environmental Science and Pollution Research 22:15526–15535.
306 Crago J and Klaper R, 2012. A mixture of an environmentally realistic concentration of a phthalate
307 and herbicide reduces testosterone in male fathead minnow (Pimephales promelas) through

a novel mechanism of action. Aquatic Toxicology 110–111:74–83.

Cravo A, Pereira C, Medeiros A, *et al.*, 2011. A multibiomarker approach in the clam Ruditapes decussatus to assess the impact of pollution in the Ria Formosa lagoon, South Coast of Portugal. Marine Environmental Research 75:23–34.

Erkmen B, Yıldırım Z, Mert R, *et al.*, 2015. Impact of sublethal di-n-butyl phthalate on the aquaculture fish species Nile tilapia (Oreochromis niloticus): histopathology and oxidative stress assessment . Aquaculture Research 48:675–685.

Faheem M and Lone KP, 2017. Oxidative stress and histopathologic biomarkers of exposure to bisphenol-A in the freshwater fish , Ctenopharyngodon idella. 53:1–9.

Ferreira NGC, Cardoso DN, Morgado R, *et al.*, 2015. Long-term exposure of the isopod Porcellionides pruinosus to nickel: Costs in the energy budget and detoxification enzymes. Chemosphere 135:354–362.

Fromme H, Küchler T, Otto T, *et al.*, 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Research 36:1429–1438.

Gao D-W and Wen Z-D, 2015. Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. Science of the Total Environment 541:986–1001.

Gào X, Zhang Y, Burwinkel B, *et al.*, 2019. The associations of DNA methylation alterations in oxidative stress-related genes with cancer incidence and mortality outcomes: a population-based cohort study. Clinical Epigenetics 11:14.

Ghisi NC, Oliveira EC, Guiloski IC, *et al.*, 2017. Multivariate and integrative approach to analyze multiple biomarkers in ecotoxicology: A field study in Neotropical region. Science of The Total Environment 609:1208–1218.

Habig WH, Pabst MJ and Jakoby WB, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. The Journal of Biological Chemistry 249:7130–7139.

He F, 2011. Bradford Protein Assay. BIO-PROTOCOL 1.

Heise S and Litz N, 2004. Deskstudy Phthlates. German Federal Environmental 14:9–11.

Hook SE, Gallagher EP and Batley GE, 2016. HHS Public Access. 10:327–341.

Jollow DJ, Mitchell JR, Zampaglione N, *et al.*, 1974. Bromobenzene-Induced Liver Necrosis. Protective Role of Glutathione and Evidence for 3,4-Bromobenzene Oxide as the Hepatotoxic Metabolite. Pharmacology 11:151–169.

339 Jonsson S, Vavilin VA and Svensson BH, 2006. Phthalate hydrolysis under landfill conditions.
340 Water Science and Technology : A Journal of the International Association on Water
341 Pollution Research 53:119–127.
342 Kang J-C, Jee J-H, Koo J-G, *et al.*, 2010. Anti-oxidative status and hepatic enzymes following
343 acute administration of diethyl phthalate in olive flounder Paralichthys olivaceus, a marine
344 culture fish. Ecotoxicology and Environmental Safety 73:1449–1455.
345 Latif M, Faheem M and Asmatullah, 2019. Study of oxidative stress and histo-biochemical
346 biomarkers of diethyl phthalate induced toxicity in a cultureable fish, *Labeo rohita*. Pak
347 Vet J. http://dx.doi.org/10.29261/pakvetj/2019.108
348 Latif M, Faheem M, Asmatullah, *et al.*, 2021. Protective efficacy of Nigella sativa seeds against
349 diethyl phthalate induced growth retardation, oxidative stress and histo-biochemical
350 damages in *Labeo rohita.* Aquaculture 533:736065
351 Mankidy R, Wiseman S, Ma H, *et al.*, 2013. Biological impact of phthalates. Toxicology Letters
352 217:50–58.
353 Marigómez I, Garmendia L, Soto M, *et al.*, 2013. Marine ecosystem health status assessment
354 through integrative biomarker indices: a comparative study after the Prestige oil spill
355 "Mussel Watch." Ecotoxicology 22:486–505.
356 Mehta V, Rao CV, Khare M, *et al.*, 2003. Toxicity Study of Diethyl Phthalate on Freshwater Fish
357 Cirrhina mrigala. Ecotoxicology and Environmental Safety 53:255–258.
358 Morgado R, Ferreira NGC, Tourinho P, *et al.*, 2013. Environmental- and growth stage-related
359 differences in the susceptibility of terrestrial isopods to UV radiation. Journal of
360 Photochemistry and Photobiology B: Biology 126:60–71.
361 Net S, Sempéré R, Delmont A, *et al.*, 2015. Occurrence, Fate, Behavior and Ecotoxicological State
362 of Phthalates in Different Environmental Matrices. Environmental Science & Technology
363 49:4019–4035.
364 van der Oost R, Beyer J and Vermeulen NP., 2003. Fish bioaccumulation and biomarkers in
365 environmental risk assessment: a review. Environmental Toxicology and Pharmacology
366 13:57–149.
367 Paluselli A, Aminot Y, Galgani F, *et al.*, 2018. Occurrence of phthalate acid esters (PAEs) in the
368 northwestern Mediterranean Sea and the Rhone River. Progress in Oceanography 163:221–
369 231.

370 Peakall DB, 1994. The role of biomarkers in environmental assessment (1). Introduction.
371 Ecotoxicology 3:157–160.
372 Peijnenburg WJGM and Struijs J, 2006. Occurrence of phthalate esters in the environment of the
373 Netherlands. Ecotoxicology and Environmental Safety 63:204–215.
374 Pompella A, Visvikis A, Paolicchi A, *et al.*, 2003. The changing faces of glutathione, a cellular
375 protagonist. Biochemical Pharmacology 66:1499–1503.
376 Quesada-García A, Valdehita A, Torrent F, *et al.*, 2013. Use of fish farms to assess river
377 contamination: Combining biomarker responses, active biomonitoring, and chemical
378 analysis. Aquatic Toxicology 439–448.
379 Regoli F and Giuliani ME, 2014. Oxidative pathways of chemical toxicity and oxidative stress
380 biomarkers in marine organisms. Marine Environmental Research 93:106–117.
381 Reuter S, Gupta SC, Chaturvedi MM, *et al.*, 2010. Oxidative stress, inflammation, and cancer:
382 how are they linked? Free Radical Biology & Medicine 49:1603–1616.
383 Santos TN dos, Duarte FB, Maia Filho PA, *et al.*, 2016. Association of oxidative stress and DNA
384 damage with grafting time in patients with multiple myeloma and lymphoma submitted to
385 autologous hematopoietic stem cell transplantation. Revista Da Associação Médica
386 Brasileira 62:39–43.
387 Schettler T, Skakkebæk NE, De Kretser D, *et al.*, 2006. Human exposure to phthalates via
388 consumer products. International Journal of Andrology 29:134–139.
389 Valavanidis A, Vlahogianni T, Dassenakis M, *et al.*, 2006. Molecular biomarkers of oxidative
390 stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology
391 and Environmental Safety 64:178–189.
392 Vethaak AD, Lahr J, Schrap SM, *et al.*, 2005. An integrated assessment of estrogenic
393 contamination and biological effects in the aquatic environment of The Netherlands.
394 Chemosphere 59:511–524.
395 Wang C, Lu G, Peifang W, *et al.*, 2011. Assessment of environmental pollution of Taihu Lake by
396 combining active biomonitoring and integrated biomarker response. Environmental
397 Science and Technology 45:3746–3752.
398 Wright JR, Colby HD and Miles PR, 1981. Cytosolic factors which affect microsomal lipid
399 peroxidation in lung and liver. Archives of Biochemistry and Biophysics 206:296–304.
400 Xu H, Yang M, Qiu W, *et al.*, 2013a. The impact of endocrine-disrupting chemicals on oxidative

401 stress and innate immune response in zebrafish embryos. Environmental Toxicology and
402 Chemistry 32:1793–1799.
403 Xu H, Yang M, Qiu W, *et al.*, 2013b. The impact of endocrine-disrupting chemicals on oxidative
404 stress and innate immune response in zebrafish embryos. Environmental Toxicology and
405 Chemistry 32:1793–1799.
406 Xu H, Dong X, Zhang Z, *et al.*, 2015. Assessment of immunotoxicity of dibutyl phthalate using
407 live zebrafish embryos. Fish and Shellfish Immunology 45:286–292.
408 Yuan SY, Liu C, Liao CS, *et al.*, 2002. Occurrence and microbial degradation of phthalate esters
409 in Taiwan river sediments. Chemosphere 49:1295–1299.
410 Zhao X-K, Yang G-P, Wang Y-J, *et al.*, 2004. Photochemical degradation of dimethyl phthalate
411 by Fenton reagent. Journal of Photochemistry and Photobiology A: Chemistry 161:215–
412 220.
413 de Zwart LL, Meerman JH., Commandeur JN., *et al.*, 1999. Biomarkers of free radical damage:
414 Applications in experimental animals and in humans. Free Radical Biology and Medicine
415 26:202–226.
416

Table 1. Average values of lipid peroxidation, antioxidant enzymes, ALT, AST, Creatinine and uric acid in different organs of grass carp exposed to DBP for 21 days. Results of ANOVA and Tukey's post hoc test. *= $p < 0.05$						
Biomarker	**Organ/tissue**	**Control**	**1µg/l DBP**	**10µg/l DBP**	**100µg/l DBP**	**1000µg/l DBP**
Lipid peroxidation (nmol TBARS /g tissue)	**liver**	30.84±5.21	56.51±5.68	59.90±8.65	**73.03±6.88***	**101.1±15.45***
	Gills	21.57± 6.24	**84.01±23.56***	**114.7±11.46***	**90.73±12.08***	**106.8±23.00***
	kidney	34.72±7.15	47.93±8.86	44.25±10.51	**66.79±4.01***	**88.17±5.19***
Catalase	**liver**	0.3433±0.05	0.4947±0.12	0.3402±0.09	0.1142±0.07	0.04637±0.01
	Gills	0.06564±0.03	0.06649±0.02	0.03743±0.01	0.06352±0.02	0.02308±0.009
	Kidney	0.3151±0.04	**0.1518±0.01***	**0.1110±0.03***	**0.1536±0.02***	**0.06902±0.02***
Reduced Glutathione	**Liver**	5.760±1.37	5.201±0.98	**1.434±0.42***	3.109±0.87	**1.122±0.24***
	Gills	2.998±0.35	4.113±0.57	1.734±0.09	**1.158±0.32***	**1.040±0.13***
	Kidney	17.26±3.68	10.81±4.0	**5.021±1.67***	**1.845±0.33***	**1.621±0.33***
Glutathione-*S*-transferases	**Liver**	75.18±9.42	123.4±23.09	**163.4±15.60***	**184.5±18.97***	88.20±10.74
	Gills	50.50±7.13	43.12±3.15	69.86±6.65	**74.74±7.06***	**72.28±6.37***
	Kidney	69.12±15.73	26.58±4.13	**34.67±2.05***	**61.68±5.20***	**23.70±2.95***
ALP (U/L)	**Liver**	44.79± 6.553	40.48±6.557	**317.7±64.78***	**186.0±40.17***	**363.8±24.05***
AST (U/L)		16.66±4.87	30.46±10.08	55.75±14.42	**141.3± 6.98***	61.88±24.30
Creatinine (mg/dl)	**kidney**	0.5202±0.04	**1.104±0.166***	**1.337±0.07***	**1.387±0.14***	**0.9619±0.14***
Uric acid (mg/dl)		6.680± 0.24	6.880±0.75	9.880±0.43	9.040±0.86	**13.12±0.43***

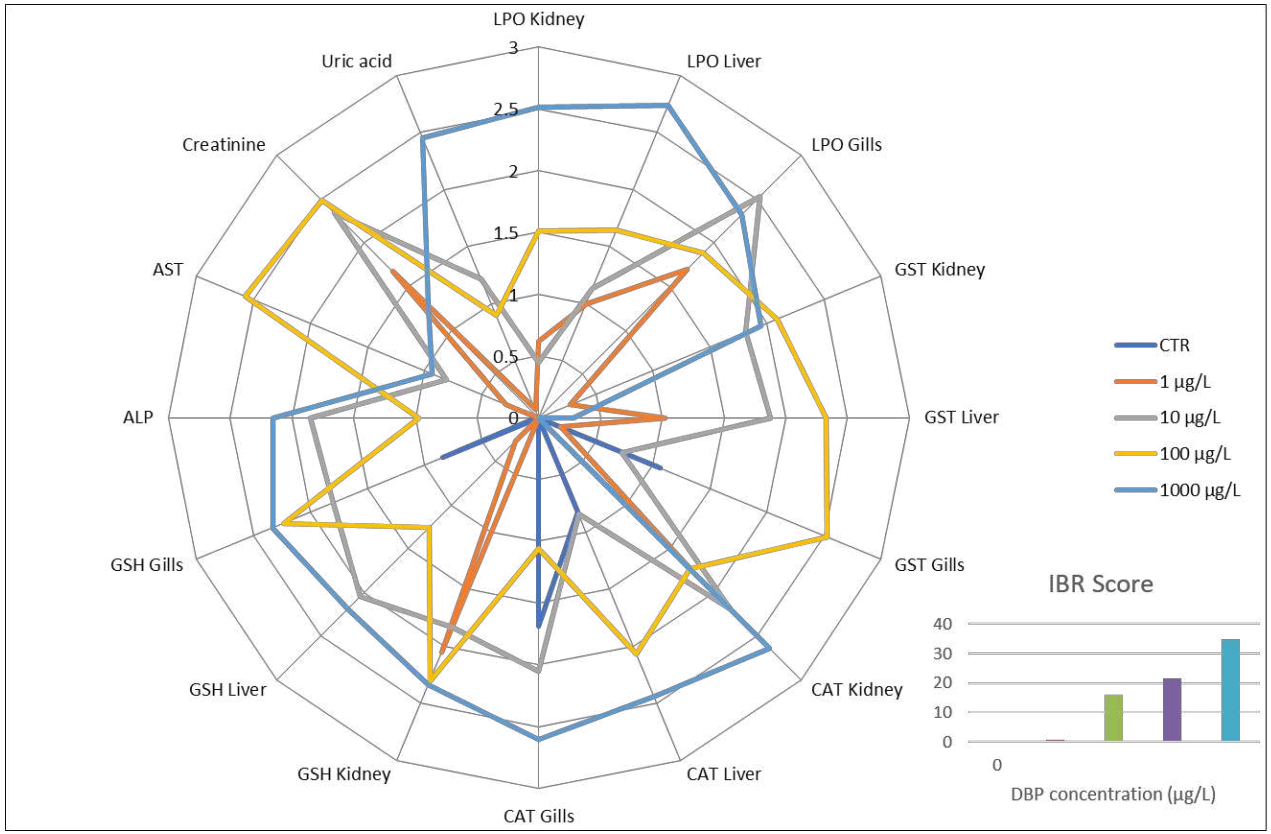

Figure 1- Integrated biomarker response (IBR) represented by star plot and histogram of grass carp *(Ctenopharyngodon idella)* exposed to different concentrations of DBP. LPO- Lipid peroxidation; GST- glutathione-*S*-transferases; CAT- catalase; GSH- reduced glutathione; ALP- Alanine aminotransferase; AST- Aspartate aminotransferase.

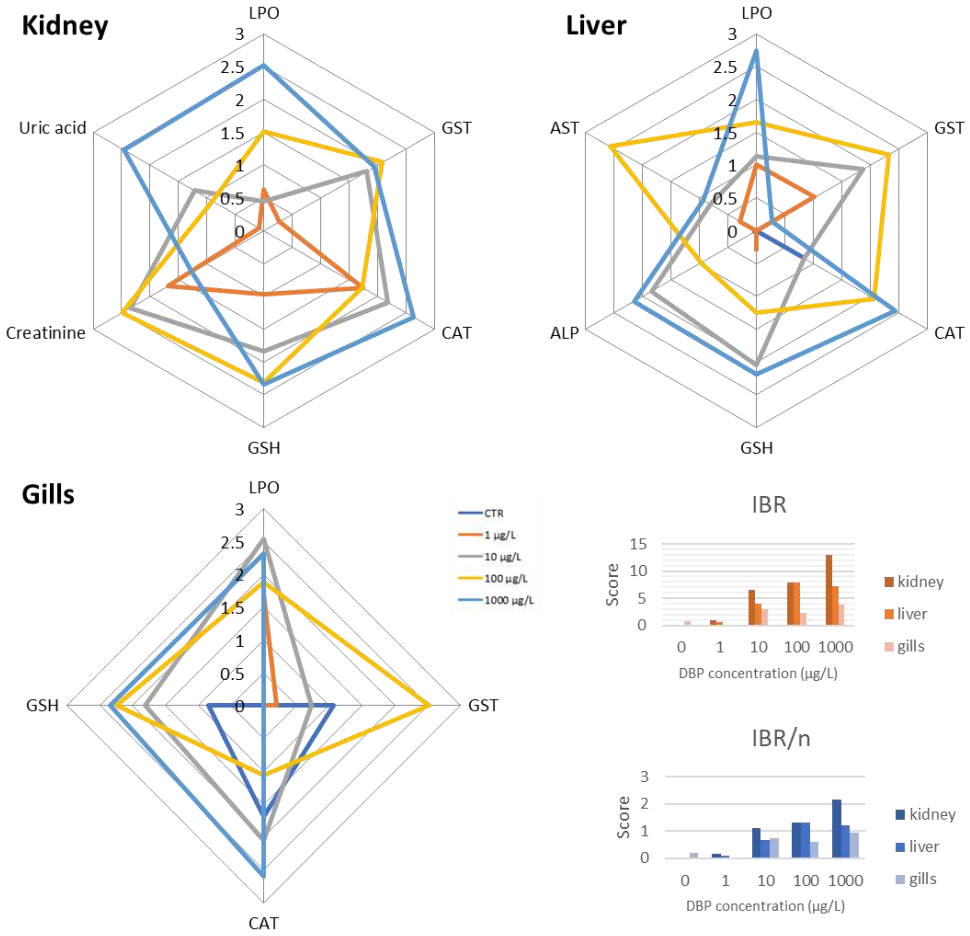

Figure 2- Integrated biomarker response (IBR) and Integrated biomarker response per biomarker (IBR/n) of the different organs (kidney, liver and gills) represented by star plot and histograms of grass carp *(Ctenopharyngodon idella)* exposed to different concentrations of DBP. LPO- Lipid peroxidation; GST- glutathione-*S*-transferases; CAT- catalase; GSH- reduced glutathione; ALP- Alanine aminotransferase; AST- Aspartate aminotransferase.