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

3 Statement of novelty

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

12 ABSTRACT

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

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

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1. INTRODUCTION

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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 aculeatus* - (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).

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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

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

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

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

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80 MATERIALS AND METHODS

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

89 Fishes were exposed to 1, 10, 100 and 1000 μ g/L for 21 days in a semi static system in
90 duplicate. Approximately $\frac{3}{4}$ water were renewed every day with a new DBP solution. Dissolve
91 oxygen was maintained in the aquarium by air stones provided with air pumps. All experiments
92 were performed at room temperature (28.35 ± 1.25 °C) and 13:11h (light: dark) photoperiod. Fish

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

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96 **Sample collection**

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

101 Organs (gills, liver and kidney) were weighed and homogenised in chilled phosphate buffer
102 (0.1M) using a mechanical homogeniser (Scilogex D160, USA). All procedure was performed on
103 ice. After homogenization, 1mL of the homogenate was used for the measurement of lipid
104 peroxidation and the remaining homogenate was centrifuged for 30 min at 13,000rpm (4°C) to get
105 the post-mitochondrial supernatant (PMS) (Faheem & Lone, 2017; Latif *et al.*, 2019).

106 **Biochemical analysis**

107 Lipid peroxidation was measured using the thiobarbituric acid method described by (Wright
108 *et al.*, 1981). Tissue homogenate was mixed with an equal volume of trichloroacetic acid (TCA –
109 10%) and thiobarbituric acid (TBA – 0.67%). After incubation for 45min in a boiling water bath,
110 the mixture was then centrifuged for 10 min. The supernatant was collected, and absorbance was
111 recorded at 532nm on a Hitachi U-2000 spectrophotometer. Lipid peroxidation was measured
112 using a molar extinction coefficient of 1.56×10^5 /M/ cm and expressed as nmol TBARS/g tissue.

113 Glutathione was quantified using an adaption of the method from (Jollow *et al.*, 1974) as
114 described earlier (Latif *et al.*, 2019) Briefly, PMS was incubated with an equal volume of 4%
115 sulphosalicylic acid and incubated at 4°C for 60 min. The mixture was centrifuged at 1200 rpm for
116 15 minutes (room temperature) and the supernatant was collected. To the supernatant, DNTB [5,5-
117 dithio-bis-(2-nitrobenzoic acid)] and phosphate buffer (0.1M) were added, and absorbance read at
118 412nm. Reduced Glutathione content was expressed as nmol GSH/g tissue using a molar
119 extinction coefficient of 1.36×10^4 /M/cm.

120 Catalase activity (CAT) was measured using the method of (Claiborne, 1985) as explained by
121 Faheem & Lone (2017). The reaction mixture consisted of 0.09M H₂O₂, 0.1 M phosphate buffer
122 and PMS (10%) in a total volume of 3 ml. Change in absorbance was recorded every 30 seconds

123 at 240nm in a double beam spectrophotometer (Hitachi U-2000). Catalase activity was expressed
124 in terms of nmol H₂O₂ consumed/min/mg protein.

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

130 Protein content of the homogenate was quantified using Bradford reagent as described by (He,
131 2011) using bovine serum albumin as standard. Alanine aminotransferase (ALT) and Aspartate
132 aminotransferase (AST) , creatinine and uric acid were quantified using the commercial kits from
133 Randox.

134 **Integrated biomarker response analysis**

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

144 **Statistical analysis**

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

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2. RESULTS

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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 1000 μ g/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 μ g/L or higher concentration were
162 recorded (except for liver at 1000 μ g/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 μ g/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 μ g/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 μ g/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 μ g/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 μ g/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 μ g/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 μ g/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 where the detoxification 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 contact with DBP and 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

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

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

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

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

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

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

252 **Conclusion**

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

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264 **Author's Contribution**

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

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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*

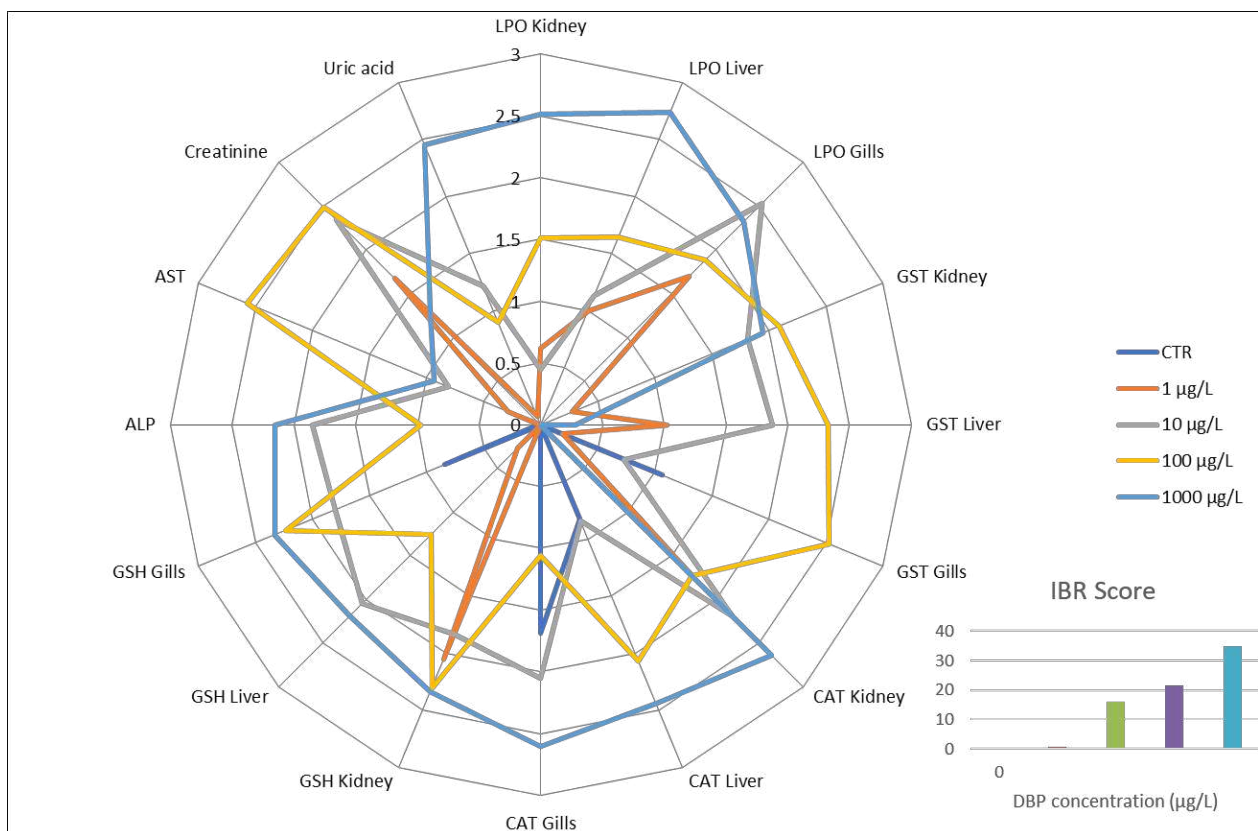


Figure 1- Integrated biomarker response (IBR) represented by star plot and histogram of grass carp (*Ctenopharyngodon idella*) exposed to different concentrations of DBP. LPO- Lipid peroxidation; GST- glutathione-S-transferases; CAT- catalase; GSH- reduced glutathione; ALP- Alanine aminotransferase;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

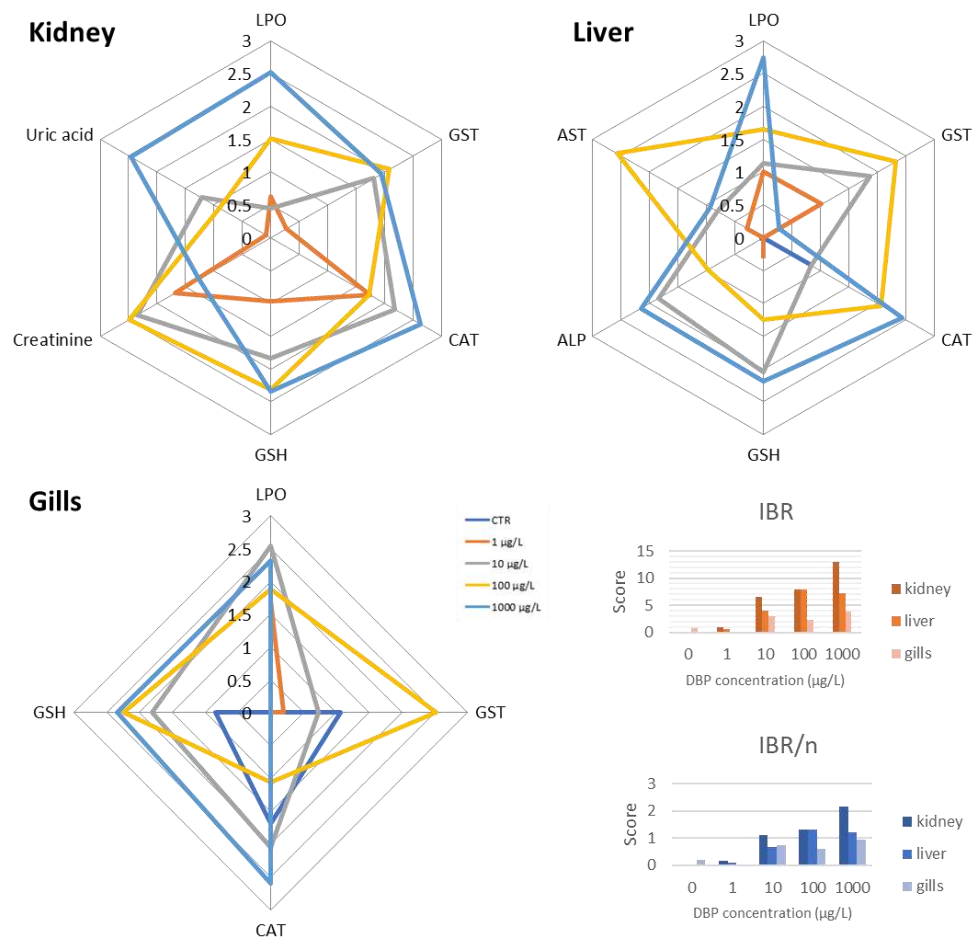


Figure 2- Integrated biomarker response (IBR) and Integrated biomarker response per biomarker (IBR/n) of the different organs (kidney, liver and gills) represented by star plot and histograms of grass carp (*Ctenopharyngodon idella*) exposed to different concentrations of DBP. LPO- Lipid peroxidation;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