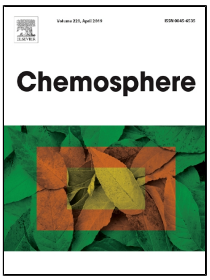
Accepted Manuscript



Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-Dichlorophenoxyacetic acid herbicide

Sonia Gaaied, Miguel Oliveira, Florane Le Bihanic, Jérôme Cachot, Mohamed Banni

PII:	S0045-6535(19)30349-2
DOI:	10.1016/j.chemosphere.2019.02.125
Reference:	CHEM 23237
To appear in:	*Chemosphere*
Received Date:	12 December 2018
Accepted Date:	20 February 2019

Please cite this article as: Sonia Gaaied, Miguel Oliveira, Florane Le Bihanic, Jérôme Cachot, Mohamed Banni, Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-Dichlorophenoxyacetic acid herbicide, *Chemosphere* (2019), doi: 10.1016/j.chemosphere.2019.02.125

Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-Dichlorophenoxyacetic acid herbicide.

Authors: Sonia GAAIED[1], Miguel OLIVEIRA[2], Florane LE BIHANIC[4], Jérôme CACHOT[4], Mohamed BANNI[1]

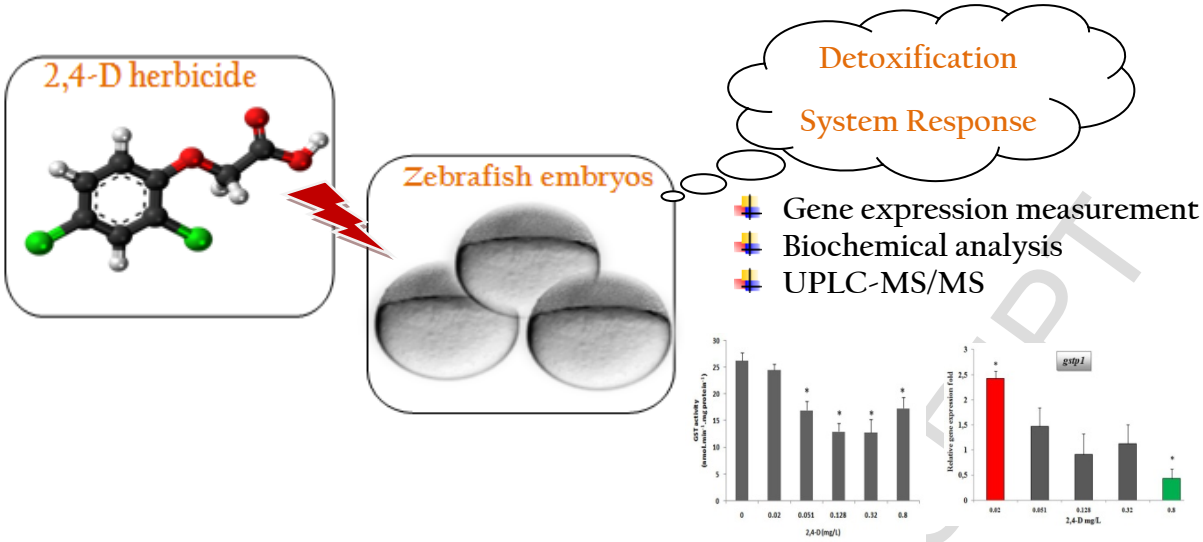
[1]Laboratory of Biochemistry and Environmental Toxicology, ISA, Chott-Mariem, Sousse, Tunisia

[2] Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[4] University Bordeaux, Laboratory of Oceanic and Continental Environments and Paleoenvironments, EPOC, UMR 5805, F-33600, Pessac, France

*Corresponding author
Laboratory of Biochemistry and Environmental
Toxicology, ISA, Chott-Mariem, Sousse, Tunisia.
Tel +216 95938898;
Email:m_banni@yahoo.fr

Running title: 2,4-D affects detoxification system in zebrafish larvae.



ABSTRACT

The present study aims to assess the effects of 2,4-D herbicide on biotransformation and oxidative stress status of zebrafish larvae. Animals were exposed to a range of sublethal concentrations (0.02 to 0.8 mg/L) and biomarkers at transcriptomic level and biochemical level were assessed. Chemical analysis with showed that the bioaccumulation of 2,4-D in 96 hpf zebrafish larvae were increased in a concentration-dependent manner. This herbicide induced significant effects at both gene expression and enzymatic activities levels after at 96 hpf. Results of mRNA expression showed a differential transcription regulation with all target genes depending on the tested concentrations. The mRNA level of *gsr* and *cyp1a* were up regulated at the highest dose of herbicide (0.8 mg/L). The gene expression of *gstp1* showed an up regulation at lower dose (0.02 mg/L) and a down regulation at the highest dose (0.8 mg/L) of 2,4-D. A significant induction of EROD activity and inhibition of GST activity were noted in groups exposed to 0.8 mg/L of 2,4-D. Considering the antioxidant defenses, the activity of CAT was increased in larvae exposed to 0.8 mg/L of herbicide and GPx activity was induced at lower doses of 2,4-D (0.02 and 0.051 mg/L). Moreover, peroxidative damage, assessed as MDA content, was markedly increased in larvae exposed to high 2,4-D concentration. Overall, the present study data indicate that bioaccumulation of 2,4-D in 96 hpf zebrafish larvae and alterations in detoxification and oxidative stress related parameters, likely associated with ROS production, which may endanger the embryo-larval stages development of fish.

Keywords: Herbicide; gene expression; biotransformation; oxidative stress; Zebrafish, early life stages.

1. Introduction

Over the last decades aquatic environmental pollution has become a worldwide problem. Currently, chemical discharge on freshwater systems displays high diversity and complexity making this ecosystem at the center of ecotoxicological interest with the aim to prevent harmful effects of toxic waste. Agricultural herbicides are among the emerging pollutants contaminating the natural habitat (Konstantinou et al., 2006; Albuquerque et al., 2016; Vieira et al., 2016). It may contaminate water surface, from point sources deriving from sewage or industrial effluents and from diffuse sources such as agricultural and domestic activities therefore endangering aquatic life (Akcha et al., 2012). 2,4-D (2,4dichlorophenoxyacetic acid) is a common herbicide widely used in agriculture and forestry since 1946 and is present in more than 1500 formulations (Garabrant, 2002; USEPA, 2005, Tayeb et al., 2011). It's a synthetic auxin, known to kill the target weed by mimicking the plant growth hormone auxin, causes uncontrolled and disorganized plant growth that leads to plant death (Tu et al., 2001; Song, 2014). Applications of 2,4-D in agriculture area include barely, wheat, oats, corn, soybeans, rice, and sugar cane (Akbulut et al., 2014). In Tunisia, the estimated quantities of the main active compounds used in the agricultural areas surrounding the Ichkeul Lake and Bizerte Lagoon was reported to be up to 2309 kg confirming its leader position in the country (Ben salem et al., 2016). Extensive application of 2,4-D may induce toxicological problems in non-target organisms at molecular and biochemical levels. Previous research report that exposure to 2,4-D enhances the production of reactive oxygen species (ROS) in cells (Atamaniuk et al., 2013; Tayeb et al., 2013). The latter's lead to the generation of oxidative stress and the resulting damage is countered by a range of cellular antioxidant defenses (Tayeb et al., 2012). Although 2,4-D toxicity on non-target organisms has been a topic of extensive research, data concerning 2,4-D sublethal effect in zebrafish larvae remains overlooked.

Therefore monitoring and assessing the toxicity of 2,4-D especially fish early life stage is crucial in protecting the aquatic environment and to larger extend human health.

The early-life stage (ELS) test is being reliable for screening the toxicity of chemical to aquatic organisms. Namely, the zebrafish embryo-larval assay has been suggested as a replacement of adult zebrafish test (OEDC., 2012, 2013). The rapid embryonic development of zebrafish enables to record the sub-lethal endpoints and to evaluate toxic potential of herbicides in the first days post fertilization (dpf) (Gellert and Heinrichsdorff, 2001; Wigh et al., 2015). Moreover, *D.rerio* genome is almost fully sequenced (Shin & Fishman, 2002). The conservation of several molecular pathways and basic cellular process, involved in the response to chemicals, between zebrafish and humans (Ahmad et al., 2012; Howe et al., 2013) allow the use of gene expression analysis for studying toxic mechanisms of pollutants.

Several pollutants are known to modulate organisms' metabolism promoting the reactive oxygen molecules (ROS) inducing oxidative alterations to aquatic biota including zebrafish embryos (Livingstone, 2003; Jin et al., 2013). For a better evaluation of 2,4-D effects on zebrafish larvae, various markers of detoxification and oxidative stress were chosen. Firstly, we studied responses at the transcriptomic level. The endpoints for gene expression related to detoxification process (cytochrome P450 (*cyp1a*), glutathione-S transferase (*gstp1*)), and oxidative stress responses (catalase (*cat*), glutathione peroxidase (*gpx1a*) and additionally glutathione reductase (*gsr*)) were analyzed.

Secondly, we studied the responses at the biochemical level: enzymatic response. The cytochrome P4501A, one of the main Phase (I) monoxygenase enzymes, involved in metabolisation of xenobiotics as well in bioactivation of pro-carcinogens. The CYP4501A synthesis is inducible by multiple organic compounds and can be quantified by ethoxyresorufin-O-deethylase (EROD) activity measurement. The enzymatic activity of glutathione S- transferase (GST), a phase II enzymes, involved in the detoxification of

numerous environmental chemicals, through the conjugation of electrophilic xenobiotic and oxidized metabolites with glutathione (GSH) (Hyne and Maher, 2003; Pan et al., 2009). Catalase (CAT), an important antioxidant enzyme defense, prevents ROS accumulation by converting hydrogen peroxide in water and oxygen (Livingstone, 2001). Glutathione redox cycle enzymes: glutathione peroxidase (GPx) participates in the reduction of hydrogen peroxide and glutathione reductase (GR) reduces the oxidized glutathion to GSH. Lactate dehydrogenase (LDH) was indicative of anaerobic metabolism (Saravanan et al., 2011). To evaluate peroxidative damage, lipid peroxidation (LPO) was used as marker of membrane phospholipid oxidation (Kamel et al., 2014).

Therefore the major concern for this work was to assess the molecular and sub-cellular responses aiming to shed the light on the molecular mechanisms by which 2,4-D may be harmful on zebrafish early life stages.

2. Materiel and Methods

2.1. Test chemicals and preparation of test solutions

Commercial formulation of 2,4-D (Désormone lourd EC; SEPCM company) consists of 600 g/L 2,4-D. Working solutions were prepared by dissolving the herbicide in dimethyl sulfoxide (DMSO).

2.2. Adult maintenance

Adults Zebrafish "*Danio rerio*" were kept in a ZebTEC (Tecniplast) recirculating system established at the Department of Biology, University of Aveiro (Portugal). A photoperiod cycle of 16:8 h (light:dark) was maintained. Culture water was purified by reverse osmosis and activated carbon filtered tap water, complemented with 0.34 mg/L salt ''Instant Ocean Synthetic Sea Salt'' (Spectrum Brands, USA) and automatically adjusted for

pH and conductivity. Water pH was 7.5 ± 0.5, conductivity 750 ± 50 lS, salinity 0.35 ppt. Temperature was 26.0 ± 1 °C and dissolved oxygen equal or above 95 % saturation. Nitrogen compounds were kept below 0.01 mg/L (nitrite and ammonia) and 0.1 mg/L (nitrate). The culture water was monitored daily and was used as dilution water for the preparation of the test solutions. The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd).

2.3. Embryo toxicity assay

The experimental was based on the Organisation for Economic Co-operation and Development (OECD) draft guideline on fish embryo toxicity (FET) test (OECD, 2012). Sexually mature male and female of the same size and age (between 16 and 18 weeks) were selected for spawning. Marbles were used in the spawning aquariums; the zebrafish embryos were collected immediately after natural mating, rinsed in water system and checked under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). Only fertilized and normal developed embryos (in the blastula stage; 3 hpf) were used in the tests. Newly Fertilized eggs previously selected, were exposed for 96 h in static conditions to five sublethal concentrations of 2,4-D (0.02, 0.051, 0.128, 0.32, 0.8 mg L^{-1}). The sublethal assay design included also a negative control (water system only) and solvent control (0.3 µL/L of DMSO, the maximal concentration of DMSO used in the 2,4-D treatments). The sublethal concentrations tested were selected based on the LC_{50} determined in a previous study (Gaaied et al., 2019). The exposure was done with the aim of collecting material for biomarkers analysis, transcriptomic analysis and chemical analysis. Eggs were exposed into small petri dishes the numbers of individuals and replicates used differed according to the test. Larvae at 96 hpf for each biomarkers and chemicals analysis were snap frozen in microtubes and kept at - 80 °C until analysis. For transcriptomic analysis larvae were frozen in RNA Later at -20 °C.

2.4. Chemicals analysis

30 larvae per experimental condition (3 biological replicates) were homogenized in 0.2 mL of 50% acetonitrile (diluted in ultrapure water). The chromatographic separation was performed on a C18 column (ACQUITY UPLC BEH), with a gradient elution using a mobile phase made of acetonitrile and 0.02 M ammonium acetate with 0.1% (v/v) formic acid (Li et al., 2017). The injection volume was 5 mL and the flow rate was 0.25 mL/min. An internal standard made of a serial dilution of a 2,4-D stock solution was used (0, 1, 5, 10 and 15 mg/mL). The extraction recovery of 2,4-D was performed using five replicates of each Quality Control samples. The recoveries (mean) of 2,4-D were found to be in the range from 100.4% to 101.8%.

2.5. Gene expression measurements

Total RNA was extracted from 20 larvae (n=4 for each exposure condition) using acidphenol–chloroform precipitation according to Chomczynski and Sacchi (1987) with TRI-Reagent (Sigma-Aldrich). RNA was further processed as described by Banni et al (2011, 2014). Relative mRNA abundances of the zebrafish genes encoding *gstp1*, *cat*, *gpx1a*, *gsr*, *Cyp1a* were evaluated with SYBR Green I chemistry (EvaGreen®dye; Bio-Rad Laboratories; Banni et al., 2007, 2014). Primer pairs (Table 1) were designed using Beacon Designer v3.0 (PremierBiosoft International, Inc.).

The PCR efficiencies used to calculate relative fold induction levels in relation to the control represent the median value obtained from at least four independent experiments, and they were 2.0, 1.96 and 1.95, 1.88 and 1.92 respectively for *gstp1*, *cat*, *gpx1a*, *gsr*, Cyp1a. Moreover, the relative expression stability of the three reference genes was calculated in our experimental conditions using geNorm. Our data showed expression stability values of 0.23, 0.37 and 0.41 respectively for *eef1a112*, *ß-actin* and *gapdh*. Relative expression data were

146 geometrically normalized to *eef1a112*, *ß-actin* and *gapdh* (Perrichon et al., 2016). qRT-PCR
147 was performed with four biological replicates and three technical replicates. Statistical
148 analyses were carried out on the group mean values using a random reallocation test (Pfaffl et
149 al., 2002).

150 **2.6. Biochemical endpoints**

151 GST, CAT, GR, GPx, LDH activities were analyzed from whole zebrafish larvae. Enzymatic
152 determinations were made spectrophotometrically (Labsystem Multiskan EX microplate
153 reader) using 96 well microplate. Samples were defrosted on ice in the day of enzymatic
154 analyses, homogenized in K-phosphate buffer (0.1 M, pH 7.4) using a sonicator (Branson S-
155 250A) and centrifuged (4 °C, 9000 g, 20 min) to isolate the post-mitochondrial supernatant
156 (PMS) later used for enzyme activity determinations. The protein concentration in the PMS
157 was determined based on the Bradford method (1976), using a wavelength of 595 nm and γ-
158 globulin as standard.

159 CAT, GST, CAT, GR, GPx, LDH activities and LPO content were determined respectively
160 according to Clairborne (1985), Habig and Jakoby (1981), Athar and Iqbal (1998), Lima et al.
161 (2007), Diamantino et al. (2001) and Livingstone et al., (1993).

162 In vivo EROD activity measurements were performed according to Le Bihanic et al. (2013)
163 with some modifications. Measurements were conducted on living larvae at 96 hpf. Larvae
164 were randomly distributed into 48 well plates and incubated in 1 µM 7-ethoxyresorufin
165 solution for 1 h at 28 ± 0.5 °C in obscurity. Then the old 7-ER was removed and replaced
166 with 600 µL fresh 1 µM 7-ER solution. The microplate was then incubated for 4 h at 28°C
167 and protected from light with aluminum foil Process fluorescence reading was conducted with
168 the microplate reader exc./em.: 560/590 nm and EROD activity was expressed as
169 pmol/well/larvae.

2.7. Statistics analysis

Statistically significant effects were assessed using SPSS 20.0 software package. Differences between controls (negative and solvent) were carried out using a Student t-test ($p < 0.05$). The normality of the distribution was tested using the Shapiro–Wilk test. Significant differences between the control and experimental groups were assessed by the parametric one-way analysis of variance (ANOVA) with Tukey's test. All values were expressed as the mean ± standard deviation (SD). Values were considered statistically significant when p was less than 0.05.

3. Results

3.1. The concentration of 2,4-D in the exposure medium

Chemical analysis results of 2,4-D in the exposure medium are presented in table 2. The exposure medium showed stable concentrations and within 95.13–110 % of the nominal concentrations in what refers the sublethal test (0.02–0.8 mg/L).

3.2. The concentration of 2,4-D in larvae after 96 h of exposure

The loads of 2,4-D in zebrafish larvae after the exposure period are reported in Table 2. 2,4-D concentrations were significantly increased in dose-dependent manner in 96 hpf larvae when compared to control. The 2,4-D accumulation reached a maximum in the group exposed to 0.8 mg/L with up to 54.44 ± 4.78 ng/mg dry weight.

3.3. Effects of 2,4-D on detoxification and oxidative stress-related gene transcription:

Expression analysis of genes was performed by real time quantitative PCR on zebrafish larvae transcripts using *eef1a1l2*, *ß-actin*, and *gapdh* mRNA as reference genes. The mRNA expression levels are expressed in relation to the average expression of the 3 reference genes.

Overall the mRNA level of various genes encoding detoxification proteins (*cyp1a* and *gstp1)* and antioxidant proteins (*cat, gpx1a* and *gsr*) were altered after 96 h of exposure to 2,4-D (Fig 1 and 2). The results demonstrated that the relative mRNA level of *cyp1a* was up-regulated in larvae exposed to a concentration equal or above 0.128 of 2,4-D.The transcription level of the gene encoding *gstp1* significantly increased at lower dose (0.02 mg/L) however significantly decreased at higher dose (0.8 mg/L). In addition, a significant up-regulation of *cat* expression was observed in larvae exposed to 0.02 and 0.051 mg/L. The relative mRNA level of *gpx1a* was significantly increased in groups exposed to 0.128 and 0.32 mg/L of 2,4-D. Moreover, The transcripts of *gsr* in zebrafish larvae was significantly up regulated after 96 h of exposure at concentration equal or above to 0.32 mg/L.

3.4. Effect of 2,4-D on detoxification and oxidative stress enzyme activities:

Enzymes involved in detoxification process are presented in figure 3. A significant increase in EROD activity was found in groups exposed to (0.02, 0.051, 0.128 and 0.8 mg/L) compared to the control. GST activity showed an inhibition for all tested concentrations (0.02 to 0.8 mg/L) compared to control and results were statistically significant at concentration equal or above 0.051 mg/L ($p < 0.05$). Enzymes involved in oxidative stress process are presented in figure 4. GR activity was not statistically significant in all tested concentrations compared to the control group. However, a significant ($p < 0.05$) increase in the activity of GPx was found in groups exposed to (0.02 and 0.051 mg/L) when compared to the control. The activity of CAT was significantly higher ($p < 0.05$) in groups exposed to the highest tested dose of 2,4-D (0.8 mg/L) compared to the control group. Regarding LDH activity no significant differences were found when compared to the control.

3.5. Effect of 2,4-D on lipid peroxidation:

Lipid oxidative damage was investigated by the evaluation of the MDA accumulation in larvae tissues. Results shows a significant increase ($p < 0.05$) in MDA levels in groups exposed to (0.02, 0.051, 0.128 and 0.8 mg/L) compared to control group (Fig. 5).

4. Discussion

Oxidative stress has become an interesting parameter in aquatic toxicology assessment and is known to occur when the rate of ROS production exceeds the activities of antioxidant systems (Livingstone, 2003; Adeyemi et al., 2015)**.** Previous studies reported that pesticides are able to enter redox cycles by accepting/donating electrons and consequently they may increase ROS level causing oxidative damage in fish (Slaninova et al., 2009; Lushchak, 2011). Thus, the effects of pro-oxidant chemicals in fish can be used to assess water pollution through monitoring the modification of the antioxidant activity or the expression of the encoding gene. From this idea, we aimed to investigate, for the first time, the impact of 2,4-D herbicide on mRNA expression of target genes associated with biotransformation and oxidative stress and their related enzyme activities in 96 hpf *D.rerio* larvae.

In this study, transcriptomic analysis provided considerate evidence that exposure to sublethal concentrations of 2,4-D modulate mRNA levels of genes encoding detoxification (*cyp1a and gstp1*) and antioxidant proteins (*cat, gsr and gpx1a*) in zebrafish embryos after 96 h. Nonetheless, there is no dose response expression in targeted genes, we can conclude that this compound contribute to oxidative stress in zebrafish larvae.

In this work, we evaluated the *cyp1a* gene expression, a subfamily of *cyp 450* known to serve as bioindicators of environmental stress in fish (Brammell and Andrew, 2010; Xing et al., 2014). Relative gene expression analyses revealed that, compared to the control, *cyp1a* was significantly up-regulated in response to 2,4-D at concentrations equal or above to 0.128 mg/L. Similar enhanced expression of *cyp1a* gene was reported in zebrafish larvae after exposure to chlorpyrifos and diurion (Jeon et al., 2016; Velki et al., 2017). Considering the mechanism associated in *cyp1a* gene expression, the up regulation of *cyp1a* gene may be dependent on the response of Pregnane X receptor (PXR), a member of nuclear receptor (NR) family. PXR plays a pivotal role in regulation of xenobiotic metabolism enzymes (Timsit and

Negishi, 2007) and any change of PXR signaling pathway may be linked with the biotransformation and detoxification of xenobiotic (Wang et al., 2019). It is well documented that the gene expression of *cyp1a* is regulated partly by PXR (Kamenickova et al., 2012) and according to Kojima et al. (2010) the 2,4-D represent an agonist to PXR. Thus, the *cyp1a* regulation in this study seems to be partly controlled by the PXR. Upon activation, the PXR displace to the nucleus and bind to DNA response elements in promoters generating the expression of several metabolizing enzymes including cytochrome P4501A (Staudinger et al., 2010). Regarding *gstp1* the gene expression pattern was up regulated at lower concentration of herbicide (0.02 mg/L) however a significant decrease of *gstp1* mRNA expression was observed in larvae exposed to the highest concentration (0.8 mg/L). It is possible that in the present work, the increase in the transcription of the detoxifying and anti-oxidative stress genes would contribute to the elimination of ROS from the cell (Banni et al., 2014). However, the reduced mRNA level demonstrated a transcriptional regulation of gene expression in larvae. The gene expression of *cat* and *gpx1a* was up regulated at lower (0.02 and 0.051 mg/L) and medium concentrations (0.128 and 0.32 mg/L) respectively. However, a clear return to baseline expression of reference genes was noted with *cat* and *gpx1a* genes in larvae exposed to the highest concentration (0.8 mg/L). Thus, the change in the expression of *cat* and *gpx1a* indicates the implication of oxidative stress in the toxicity mechanism of 2,4-D. Li et al. (2017) found a down regulation of *gpx1a* and *cat* 2,4-D in zebrafish embryos exposed to 25 mg/L of 2,4-D. The up regulation of *cat* and *gpx1*a genes observed in this study was also reported in other studies with pesticides, Jiang et al. (2015a; 2015b and 2016) also found that carbendazim, acetochlor and pretilachlore induced an up-regulation of *cat* and *gpx* genes in zebrafish embryos. Further, Jamil et al. (2015) reported that at 100 µg/L of 2,4-D there is an induction of *gpx* gene in 72 hpf zebrafish larvae, indicating its affinity for oxidative stress.

In this study, the expression of the *gsr* was up-regulated after 96 h exposure. The observed increase in the transcription of the *gsr* gene suggests its implication in antioxidative defense and its contribution to protect larvae from oxidative stress damage induced by herbicide exposure. Also, it is well known that the signaling pathways of oxidative stress enzyme may be activated by redox-sensitive proteins (Den Hertog et al., 2005) and this may explain the increase of *gsr* level. The results reported by Velki et al. (2017), assessing the effect of diuron in zebrafish embryos, are similar to our findings.

In addition to the gene expression analysis, phase I and phase II detoxification enzymes, oxidative stress enzymes as well as lipid peroxidation level were also assessed in zebrafish larvae.

Xenobiotic metabolism enzymes, including cytochrome P450 (CYP), play a central role in the biotransformation of foreign compounds (Gueguen et al., 2006). In our work the EROD activity was increased and this effect may be considerate as a toxicity mechanism in exposed larvae. Being a xenobiotic, 2,4-D is metabolized by organisms and 2,4-D or its metabolites may affect normal detoxification processes (Atamaniuk et al., 2013). In this study, the *cyp1a* mRNA abundance and the EROD enzyme showed a disparity and the induction was markedly observed at the transcriptomic level than at the biochemical level.

Glutathione S-transferases (GST) are among the primary antioxidant enzymes and the first-line indicators of the antioxidant state (Yang and Lee, 2015). In fact, in phase II of biotransformation, the GST plays a central role through the detoxification of xenobiotic such as herbicides (Higgins and Hayes, 2011) and endogenic compounds that conjugate with glutathione (Domingues et al., 2010). In this study the oxidative stress enzyme GST were inhibited at concentrations equal and higher than 0.128 mg/L. This inhibition could be due to an impairment of the GST pathway in response to a substantial stress increase as previously reported by Oliveira et al. (2013). Similar results were found in *D.rerio* embryos after 96 h

exposure to rotenone and potassium dichromate (Melo et al., 2015; Domingues et al., 2010). Further, it was observed that the mRNA level of *gstp1* correlate well with the GST enzyme activity suggesting that this enzyme was controlled at least by transcription regulation rather than at the protein level (Jiang et al., 2016).

The ROS detoxification mechanisms are associated with a combination of multiple enzymes such as CAT, and GPx. Hence, CAT and GPx can be considered as the first line of defense against the deleterious effect provoked by excessive production of ROS in response to chemical exposure (Shukla et al., 2017). Regarding, CAT activity our result shows a significant induction at the highest level of 2,4-D (0.8 mg/L). The induction of CAT enzyme can be explained by its catalytic activity to convert hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Pi et al., 2010). Our results are similar to the findings of Jiang et al. (2016) in which pretilachlor exposure lead to a significant CAT activity in *D.rerio* larvae. Others studies with adult fish showed that 2,4-D exposure increased CAT activity in the kidney of *Cyprinus carpio* and in the gills of goldfish, *Carassius auratus* (Oruc et al., 2004; Atamaniuk et al., 2013). We have noticed that the gene expression level of *cat* showed opposite trend to the activity of corresponding enzyme at 0.8 mg/L. This can be explained by the high CAT activity which may cause a decrease of *cat* mRNA level by the negative feedback mechanism (Xia et al., 2017). Conversely, to CAT enzyme activity, a bell shaped curve response of GPx enzyme was observed in this study. This finding could be an indication that the rate of production of H_2O_2 in exposed larvae surpassed the scavenging ability of GPx, and consequently resulting in oxidative stress. In terms of comparison between Gr activity and *gsr* mRNA expression, the up regulation of this gene was not translated into higher activity of GR enzyme. We can suggest that the transcriptional up-regulation of *gsr* genes may be initiated to compensate the low levels of enzymes (Gagnaire et al., 2015).

Lipid peroxidation (LPO) is an oxidative stress marker, indicating that ROS production exceeded antioxidant defenses (Ahmad et al., 2008; Oliveira et al., 2009). MDA is the end product of LPO and considered as an indicator of oxidative stress, which results from the free radical damage to membrane components of cells (Kuder and Philip, 2017). In the present study, the MDA content in zebrafish larvae was increased following exposure to 2,4-D which indicated that the zebrafish larvae suffered from oxidative damage. Previous investigations have also reported that 25 mg/L of 2,4-D induce malondialdehyde (MDA) accumulation in 72 hpf zebrafish embryos (Li et al., 2017).

5. Conclusions

In summary, it can be concluded that sublethal exposure to 2,4-D herbicide contribute to oxidative stress in 96 hpf zebrafish larvae. The changes in detoxifying and oxidative stress-genes as well as related enzyme activities could be an adaptive and protective response of larvae from 2,4-D toxicity. However, the mRNA induction patterns were not in conformity with the changes in levels of antioxidant enzymes in most cases, suggesting that certain antioxidant enzymes are controlled by enzyme activity and not by transcriptional regulation. The discrepancy between antioxidant enzymes level and the gene expression level must be taken into consideration when evaluating the oxidative stress in organisms. Thus, a combined stress biomarker pattern at different biological levels provides a reliable prediction of adverse effects of herbicide and emerging pollutants to aquatic organisms.

Acknowledgements

This work was supported by funds from the «Ministère de l'Enseignement Supérieur et de la Recherche Scientifique; UR13A6R08, Biochimie et Toxicologie Environnementale» and the Department of Biology, University of Aveiro Portugal.

References

Adeyemi, J.A., Martins-Junior, A.C., Barbosa, F.Jr., 2015. Teratogenicity, genotoxicity and oxidative stress in zebrafish embryos (*Danio rerio*) co-exposed to arsenic and atrazine. Comp. Biochem. Physiol. C. 172, 7–12.

Ahmad, F., Noldus, L.P.J.J., Tegelenbosch, R., Richardson, M., 2012. Zebrafish embryos and larvae in behavioural assays. Behaviour 149, 1241–1281.

Ahmad, I., Maria, V.L., Oliveira, M., Serafim, A., Bebianno, M.J., Pacheco, M., Santos, M.A., 2008. DNA damage and lipid peroxidation vs protection responses in the gill of *Dicentrarchus labrax L*. from a contaminated coastal lagoon (Ria de Aveiro, Portugal). Sci. Total. Environ. 406, 298–307.

Akbulut, C., 2014. The histopathological effects of 2,4- dichlorophenoxyacetic acid on intestine tissue of zebrafish (*Danio rerio*). Elixir Pollut. 74, 27021–27024.

Akcha, F., C. Spagnol and J. Rouxel., 2012. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. Aquat. Toxicol. 106-107: 104–113.

Albuquerque, A.F., Ribeiro, J.S., Kummrow, F., Nogueira, A.J.A., Montagner, C.C., & Umbuzeiro, G.A. 2016. Pesticides in Brazilian freshwaters: a critical review. Environ. Sci. Process. Impact 18, 779-787.

Atamaniuk, T.M., Kubrak, O.I., Storey, K.B., Lushchak, V.I., 2013. Oxidative stress as a mechanism for toxicity of 2, 4-dichlorophenoxyacetic acid (2, 4-D): studies with goldfish gills. Ecotoxicology 22, 1498–1508.

Athar, M., Iqbal, M., 1998. Ferric nitrilotriacetate promotes Ndiethylnitrosamine- induced renal tumorigenesis in the rat: implications for the involvement of oxidative stress. Carcinogenesis 19, 1133-1139.

Banni, M., Dondero, F., Jebali, J., Guerbej, H., Boussetta, H., Viarengo, A., 2007. Assessment of heavy metal contamination using real-time PCR analysis of mussel metallothionein mt10 and mt20 expression: a validation along the Tunisian coast. Biomarkers 12, 369–383. http://dx.doi.org/10.1080/13547500701217061.

Banni, M., Negri, A., Mignone, F., Boussetta, H., Viarengo, A., Dondero, F., 2011. Gene expression rhythms in the mussel Mytilus galloprovincialis (Lam.) across an annual cycle. PLoS One 6, e18904. http://dx.doi.org/10.1371/journal.pone.0018904.

Banni, M., Hajer, A., Sforzini, S., Oliveri, C., Boussetta, H., Viarengo, A., 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus*

galloprovincialis exposed to nickel and heat stress. Comp. Biochem. Phys C. 160, 23–29. http://dx.doi.org/10.1016/j.cbpc.2013.11.005.

Ben Salem, F., Ben Said, O., Aissa, P., Mahmoudi, E., Monperrus, M., Grunberger, O., Duran, R., 2016. Pesticides in Ichkeul Lake–Bizerta LagoonWatershed in Tunisia: use, occurrence, and effects on bacteria and free-living marine nematodes. Environ. Sci. Pollut. Res. 23 , 36–48. http://dx.doi.org/10.1007/s11356-015-4991-8.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brammel, B.F., Wigginton, A.J., 2010. Differential gene expression in zebrafish (*Danio rerio*) following exposure to gaseous diffusion plant effluent and effluent receiving stream water. Am. J. Environ. Sci. 6, 286–294.

Claiborne, A. 1985. Catalase activity. CRC handbook of methods for oxygen radical research 1, 283-284.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159. doi:10.1016/0003-2697(87)90021-2.

Den Hertog, J., Groen, A.,Van Der Wijk, T., 2005. Redox regulation of protein-tyrosine phosphatases. Arch. Biochem. Biophy S. 434, 11–15.

Diamantino, T.C., Almeida, E., Soares, A.M., Guilhermino, L., 2001. Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. Chemosphere 45, 553–560.

Domingues, I., Oliveira, R., Lourenc¸ o, J., Grisolia, C.K., Mendo, S., Soares, M.V.M., 2010. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comp. Biochem. Phys C. 152, 338–345, http://dx.doi.org/10.1016/j.cbpc.2010.05.010.

Gaaied, S., Oliveira, M., Inês Domingues, I., Mohamed Banni, M., 2019. 2,4-Dichlorophenoxyacetic acid herbicide effects on zebrafish larvae: Development, neurotransmission and behavior as sensitive endpoints. Environ. Sci. Pollut. R. DOI: 10.1007/s11356-019-04488-5

Gagnaire, B., Cavalié, I., Pereira, S., Floriani, M., Dubourg, N., Camilleri, V., Adam, G.C., 2015. External gamma irradiation-induced effects in early-life stages of zebrafish, *Danio rerio*. Aquat. Toxicol. 169, 69–78. http://dx.doi.org/10.1016/j. aquatox.2015.10.005

406 Garabrant, D., Philbert, M., 2002. Review of 2, 4-Dichlorophenoxyacetic acid (2,4-D)
407 epidemiology and toxicology, Critical. Review. Toxicol. 32, 233-257.
408 Gellert, G., Heinrichsdorff, J., 2001. Effect of age on the susceptibility of zebrafish eggs to
409 industrial waste water. Water Res. 35, 3754-3757.
410 Gueguen, Y., Mouzat, K., Ferrari, L., Tissandie, E., Lobaccaro, J.M., Batt, A.M., Paquet, F.,
411 Voisin, P., Aigueperse, J., Gourmelon, P., and Souidi, M., 2006. Cytochromes P450:
412 xenobiotic metabolism, regulation and clinical importance. Ann. Biol. Clin. (Paris) 64,
413 535-548.
414 Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases.
415 Methods Enzymol. 77, 398–405.
416 Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., et al., 2013.
417 The zebrafish reference genome sequence and its relationship to the human genome.
418 Nature; 496, 498–503.
419 Higgins, L.G., Hayes, J.D., 2011. Mechanisms of induction of cytosolic and microsomal
420 glutathione transferase (GST) genes by xenobiotics and proinflammatory agents. Drug
421 Metab. Rev. 43, 92–137, http://dx.doi.org/10.3109/ 03602532.2011.567391
422 Hyne, R.V., Maher, W.A., 2003. Invertebrate biomarkers: links to toxicosis that predict
423 population decline. Ecotoxicol. Environ. Saf. 54, 366–374.
424 Jamil, A., Saeed, A., Hashmi, I., Habibi, H. R., Yu, L., Achari, G. 2015. Photocatalytic
425 Degradation of 2, 4-D and Transition of Endocrine Disruptive Activity Using
426 Transcriptome Based Bioassay in Zebrafish Embryos [A]. Int. J. Environ. Sci. Dev. Vol.6
427 No.2[C].
428 Jeon, H.J., Lee, Y.H., Kim, M.J., Choi, S.D., Park, B.J., Lee, S.E., 2016. Integrated
429 biomarkers induced by chlorpyrifos in two different life stages of zebrafish (*Danio rerio*)
430 for environmental risk assessment. Environ. Toxicol. Phar. 43, 166–174.
431 Jiang, J., Chen, Y., Yu, R., Zhao, X., Wang, Q., Cai, L., 2016. Pretilachlor has the potential to
432 induce endocrine disruption, oxidative stress, apoptosis and immunotoxicity during
433 zebrafish embryo development. Environ. Toxicol. Phar. 42, 125–134.
434 Jiang, J., Wu, S., Liu, X., Wang, Y., An, X., Cai, L., Zhao, X., 2015b. Effect of acetochlor on
435 transcription of genes associated with oxidative stress, apoptosis, immuno-toxicity and
436 endocrine disruption in the early life stage of zebrafish. Environ. Toxicol. Phar. 40, 516–
437 523.
438 Jiang, J., Wu, S., Wang, Y., An, X., Cai, L., Zhao, X., Wu, C., 2015a. Carbendazim has the
439 potential to induce oxidative stress, apoptosis, immunotoxicity and endocrine disruption

during zebrafish larvae development. Toxicol. In Vitro 29, 1473–1481, http://dx.doi.org/10.1016/j.tiv.2015.06.003.

Jin, Y., Pan, X., Cao, L., Ma, B., Fu, Z., 2013. Embryonic exposure to cis-bifenthrin enantioselectively induces the transcription of genes related to oxidative stress, apoptosis and immunotoxicity in zebrafish (*Danio rerio*). Fish Shell fish Immunol. 34, 717–723.

Kamel, N., Burgeot, T., Banni, M., Chalghaf, M., Devin, S., Minier, C., Boussetta, H., 2014. Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from Bizerte lagoon. Environ. Sci. Pollut. Res. 21, 6108–6123.

Kamenickova , A., Vrzal, R., Dvorak, Z., 2012. Effects of ready to drink teas on AhR- and PXR-mediated expression of cytochromes P450 CYP1A1 and CYP3A4 in human cancer cell lines and primary human hepatocytes. Food Chem. 131, 1201–1206.

Kojima, H., Takeuchi, S., Nagaui, T., 2010. Endocrine disrupting potential of pesticides via nuclear receptors and aryl hydrocarbon receptor. J. Health. Sci. 56, 374–86.

Konstantinou, I.K., Hela, D.G., & Albanis, T.A., 2006. The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. Environ. Pollut, 141, 555-570.

Kuder, R.S & Gundala Harold Philip, G.P., 2017. Antioxidant enzymatic activities and lipid peroxidation in liver and ovary of zebrafish (*Danio rerio*) exposed to deltamethrin, Chem. and Ecol. 33, 739-749. DOI: 10.1080/02757540.2017.1359263

Le Bihanic, F., Couillard, C.M., Rigaud, C., Legare, B., 2013. A simple and reliable in vivo EROD activity measurement in single *Fundulus heteroclitus* embryo and larva. Mar. Environ. Res. 84, 17-23.

Li, K., Wu, J.Q., Jiang, L.L., Shen, L.Z., Li, J.Y., He, Z.H., Wei, P., Lv, Z. & He, M.F., 2017. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. Chemosphere 171, 40-48. http://dx.doi.org/10.1016/j. chemosphere.2016.12.032

Lima, I., Moreira, S.M., Osten, J.R.V., Soares, A.M.V.M and L. Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the Northwestern coast of Portugal. Chemosphere 66, 1230-1242.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Tech. Biotechnol. 57, 195-211.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Livingstone, D.R.., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue Med. Vet. 154, 427–430.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Melo, K.M., Oliveira, R., Grisolia, C.K., Domingues, I., Pieczarka, J.C., Filho, J.D.S., Nagamachi, C.Y., 2015. Short-term exposure to low doses of rotenone induces developmental, biochemical, behavioral, and histological changes in fish. Environ. Sci. Pollut. Res. 22, 13926–13938.

OECD (2012) Fish Embryo Toxicity (FET) test, draft proposal for a new guideline. OECD Guideline for the Testing of Chemicals. Organization for Economic Cooperation and Development. http:// www.oecd.org/env/chemicalsafetyandbiosafety/testingofchemicals/ 2012-07-09_Draft_FET_TG_v8_FINAL.pdf. Accessed July 2012.

OECD Organization for Economic Co-Operation and Development (2013) Guidelines for the testing of chemicals, section 2: effects on biotic systems. Test No. 236: Fish Embryo Toxicity Test. DOI: 10.1787/9789264203709-en.

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M., Santos, M.A., 2009. Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata*: an integrated biomarker approach. Environ. Pollut. 157, 959–967.

Oliveira, R., McDonough, S., Ladewig, J.C.L., Soares, A.M.V.M., Nogueira, A.J.A., Domingues, I., 2013. Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 36, 903–912.

Oruc¸ E.O., Sevgiler, Y., Uner, N., 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp. Biochem. Physiol. C 137, 43–51.

Pan, L., Ren, J., Zheng, D., 2009. Effects of benzo(a)pyrene exposure on the antioxidant enzyme activity of scallop *Chlamys farreri*. Chin. J Oceanol. Limnol. 27, 43–53.

Perrichon, P., Menach, K.L., Akcha, F., Cachot, J., Budzinski, H., Bustamante, P., 2016. Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (*Danio rerio*) embryo-larval bioassay with a multilevel approach. Sci. Total. Environ. 568, 952–966.

505 Pfaffl, M.W., Horgan, G., Dempfle, L., 2002. Relative expression software tool (rest(C)) for
506 group-wise comparison and statistical analysis of relative expression results in real time
507 PCR. Nucleic Acids Res. 30. http://dx.doi.org/10.1093/nar/30.9.e36.
508 Pi, J., Zhang, Q., Fu, J.G., Woods, C.G., Hou, Y., Corkey, B.E., Collins, S., Andersen, M.E.,
509 2010. ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function. Toxicol.
510 Appl. Pharmacol. 244, 77–83.
511 Saravanan, M., Karthika, S., Malarvizhi, A., Ramesh, M., 2011. Ecotoxicological impacts of
512 clofibricacid and diclofenac in common carp (*Cyprinus carpio*) finger lings:
513 Hematological, biochemical, ionoregulatory and enzymological responses. J. Hazard.
514 Mater.195, 188–194.
515 Shin, J.T. & Fishman, M.C., 2002. From zebrafish to human: modular medical models. Annu.
516 Rev. Genom. Hum. Genet. 3, 311-340.
517 Shukla, S., Jhamtani, R.C., Dahiya, M.S., Agarwal, R., 2017. Oxidative injury caused by
518 individual and combined exposure of neonicotinoid, organophosphate and herbicide in
519 zebrafish. Toxicol. Rep. 4, 240-244. http://dx.doi.org/10.1016/j.toxrep.2017.05.002.
520 Slaninova, A., Smutna, M., Modra, H., Svobodova, Z., 2009. A review: oxidative stress in
521 fish induced by pesticides. Neuroendocrinol. Lett. 30, 2–12.
522 Song, Y., 2014. Insight into the mode of action of 2, 4-dichlorophenoxyacetic acid (2, 4-D) as
523 an herbicide. J. Integr. Plant. Biol. 56, 106-113.
524 Staudinger, J. L., Chenshu, X., Cui, Y.J., Klaasen, C.D., 2010. Nuclear receptor 521 mediated
525 regulation of carboxylesterase expression and activity. Expert Opin. Drug Metab. 522
526 Toxicol. 6, 261-271.
527 Tayeb, W., Nakbi, A., Chargui, I., Cheraief, I., Miled, A., Hammami, M., 2011. Subacute
528 effects of 2,4-dichlorophenoxyacetic herbicide on antioxidant defense system and lipid
529 peroxidation in rat erythrocytes. Pestic. Biochem. Phys. 99, 256–64.
530 Tayeb, W., Nakbi, A., Chaieb, I., Hammami, M., 2012. Oxidative stress induced by the 2,4-
531 dichlorophenoxyacetic herbicide. In: Lushchak, V. (Ed.), Oxidative Stress –
532 Environmental Induction and Dietary Antioxidants. In Tech, Croatia, pp. 115–130, ISBN:
533 978-953-51-0553-4.
534 Tayeb, W., Nakbi, A., Cheraief, I., Miled, A., Hammami, M., 2013. Alteration of lipid status
535 and lipid metabolism, induction of oxidative stress and lipid peroxidation by 2,4-
536 dichlorophenoxyacetic herbicide in rat liver. Toxicol. Mech. Methods, 23, 449–458.
537 Tu, M., Hurd, C., Randall, J.M 2001. Weed Control Methods Handbook, The Nature
538 Conservancy. Retrieved December, 27, 2007.

539 Timsit, Y.E., Negishi, M., 2007. CAR and PXR: the xenobiotic-sensing receptors.
540 Steroids, 72 (2007), pp. 231-246.
541 US Environmental Protection Agency., 2005. Reregistration Eligibility Decision for 2, 4-D.
542 [cited 5 November 2009]. Available from: http://
543 nepis.epa.gov/Exe/ZyPDF.cgi/2000E8DY.PDF?Dockey¼2000E8DY.PDF
544 Velki, M., Meyer-Alert, H., Seiler, T.B., Hollert, H., 2017. Enzymatic activity and gene
545 expression changes in zebrafish embryos and larvae exposed to pesticides diazinon and
546 diuron. Aquat. Toxicol. 193, 187–200.
547 Vieira, D.C., Noldin, J.A., Deschamps, F.C., & Resgalla Jr,C. 2016. Ecological risk analysis
548 of pesticides used on irrigated rice crops in southern Brazil. Chemosphere, 162, 48-54.
549 Wang, C., Ku, P., Nie, X., Bao, S., Wang, Z., Li, K., 2019. Effects of simvastatin on the PXR
550 signaling pathway and the liver histology in *Mugilogobius abei*. Sci. Total. Environ. 651,
551 399-409. doi: 10.1016/j.scitotenv.2018.09.133.
552 Wigh, A., Devaux, A., Brosselin, V., Gonzalez-Ospina, A., Domenjoud, B., Aït-Aïssa, S.,
553 Creusot, N., Gosset, A., Bazin, C., Bony, S., 2015. Proposal to optimize ecotoxicological
554 evaluation of wastewater treated by conventional biological and ozonation
555 processes. Environ. Sci. Pollut. R., 1-10.
556 Xia, Q., Luo, J., Mei, X., Wang, Y., Huang, W., Wang, J., Yang, R., Ma, Z., Lin, R. 2017. A
557 developmental toxicity assay of Carpesii Fructus on zebrafish embryos/larvae. Toxicol
558 Res, 6, 460-467.
559 Xing, H., Zhang, Z., Yao, H., Liu, T., Wang, L., Xu, S., et al., 2014. Effects of atrazine and
560 chlorpyrifos on cytochrome P450 in common carp liver. Chemosphere; 104, 244–50.
561 Yang, H.Y., Lee, T.H., 2015. Antioxidant enzymes as redox-based biomarkers: a brief review.
562 BMB Rep.48, 200–208.
563

Figures captions

Fig.1. Relative mRNA expressions of phase I and phase II detoxification enzymes: *cyp1a* (A) and *gstp1* (B) measured in zebrafish larvae exposed to sublethal concentrations of 2,4-D for 96 h. The results of mRNA expression levels are expressed in relation to normalized gene expression average which is set to 1. Values are means ± standard deviation of 4 replicates. Asterisks indicate statistically significant difference from the control ($P < 0.05$), threshold cycle random reallocation test according to Pfaffl et al. (2002). Red colors indicate up-regulated gene and green color indicate down- regulated gene.

Fig.2. Relative mRNA expressions of oxidative stress enzymes *cat* (A), *gpx1a* (*B*), *gsr* (C) measured in zebrafish larvae exposed to sublethal concentrations of 2,4-D for 96 h. The results of mRNA expression levels are expressed in relation to normalized gene expression which is set to 1. Values are means ± standard deviation of 4 replicates. Asterisks indicate statistically significant difference from the control ($P < 0.05$), threshold cycle random reallocation test according to Pfaffl et al. (2002). Red colors indicate up-regulated gene

Fig.3. Detoxification enzymes (mean values ± standard deviation, n = 5) measured in zebrafish embryos after 96 h of exposure to 2,4-D. EROD activity (A) was expressed on pmol/well/larvae. Data, expressed as nmol/min/mg protein for GST (B). Asterisks mean significantly different from the respective control ($p < 0.05$) after one-way ANOVA using Tukey's test.

Fig.4. Biomarkers of oxidative stress (mean values ± standard deviation, n = 5) measured on zebrafish embryos after 96 h of exposure to 2,4-D. Data, expressed as nmol/min/mg protein for CAT (A), GR (B), GPx (C) and LDH (D). Asterisks mean significantly different from the respective control ($p < 0.05$) after one-way ANOVA using Tukey's test.

Fig.5. Lipid peroxidation levels (LPO) expressed on nmol/mg protein. Mean values ± standard deviation, n=5 and asterisks mean significantly different from the respective control ($p < 0.05$) after one-way ANOVA using Tukey's test.

Figure 1

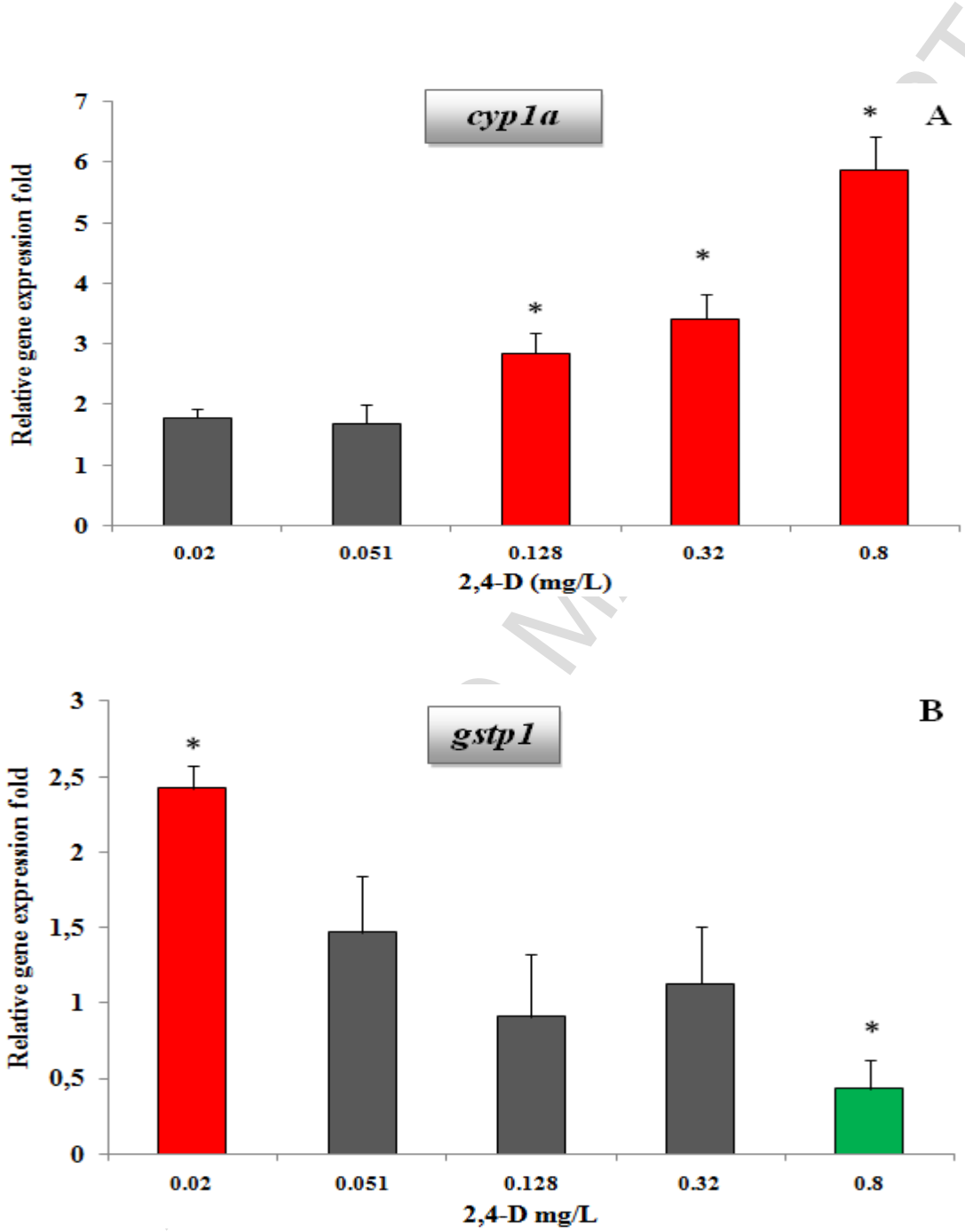

cyp1a
A
Relative gene expression fold
7
6
5
4
3
2
1
0
*
*
*
*
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
gstp1
B
Relative gene expression fold
3
2,5
2
1,5
1
0,5
0
*
*
0.02
0.051
0.128
0.32
0.8
2,4-D mg/L

Figure 2

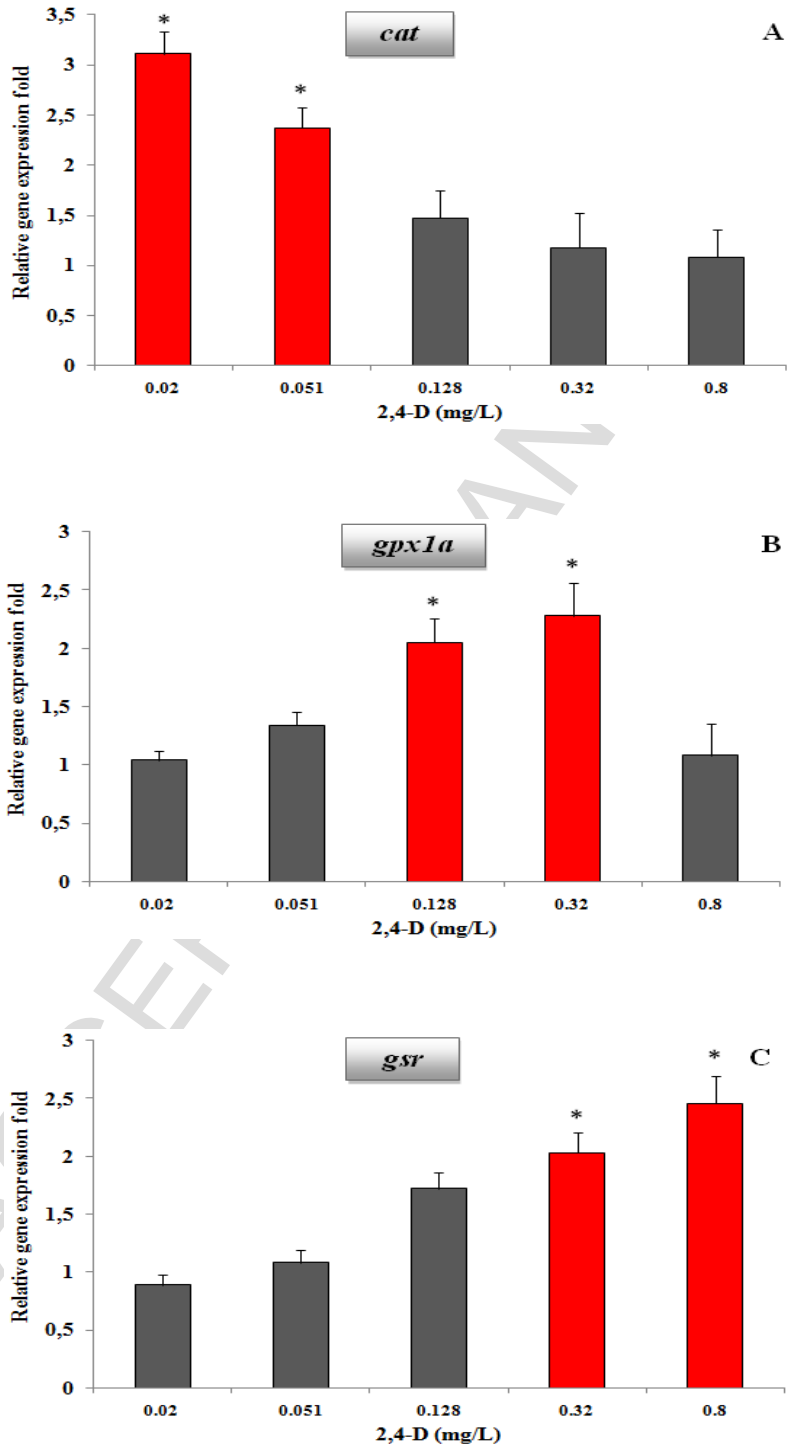

cat
A
Relative gene expression fold
3,5
3
2,5
2
1,5
1
0,5
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
gpx1a
B
Relative gene expression fold
3
2,5
2
1,5
1
0,5
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
gsr
C
Relative gene expression fold
3
2,5
2
1,5
1
0,5
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)

Figure 3

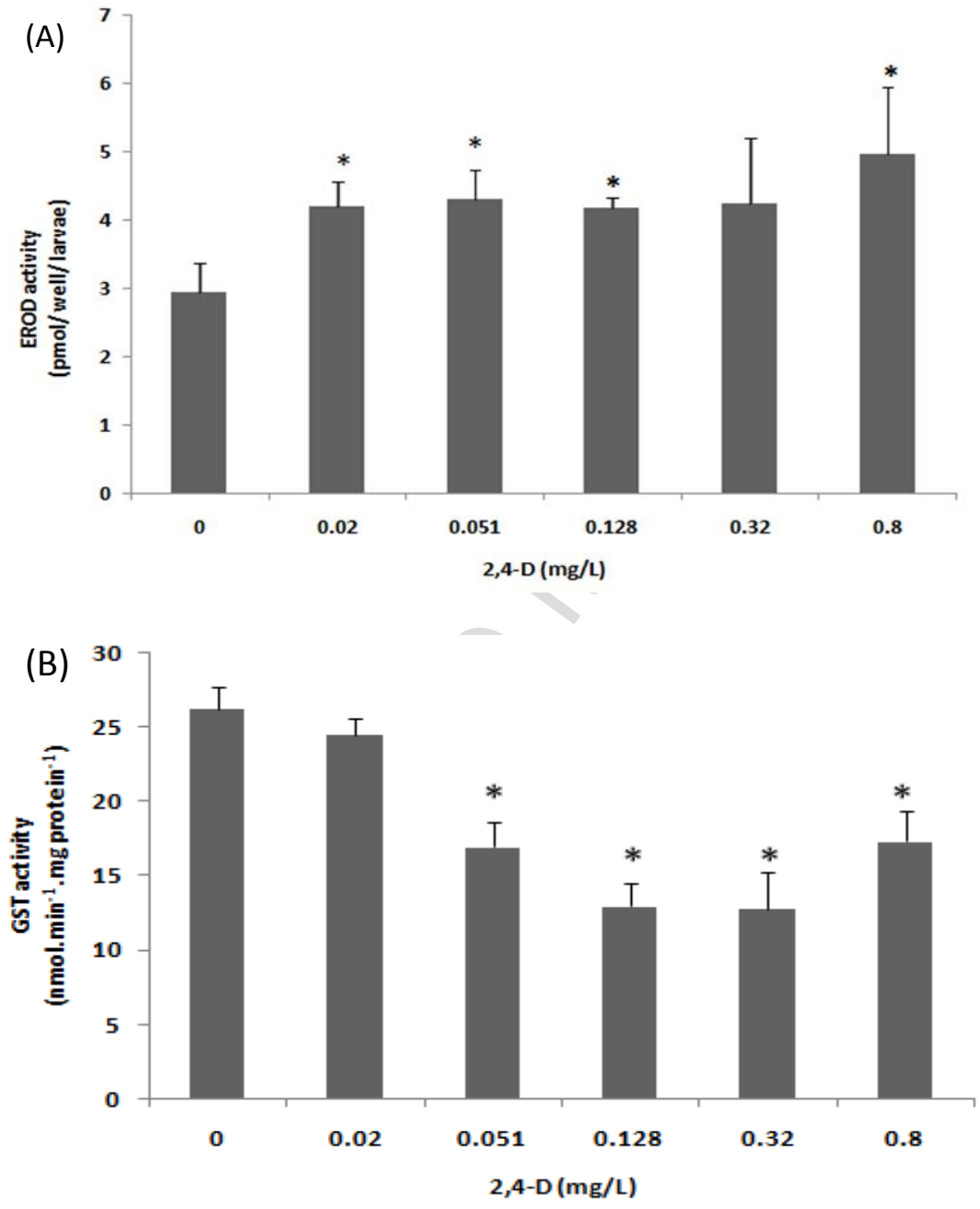

(A)
EROD activity
(pmol/well/larvae)
0
1
2
3
4
5
6
7
*
*
*
*
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
(B)
GST activity
(nmol.min-1.mg protein-1)
0
5
10
15
20
25
30
*
*
*
*
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)

Figure 4

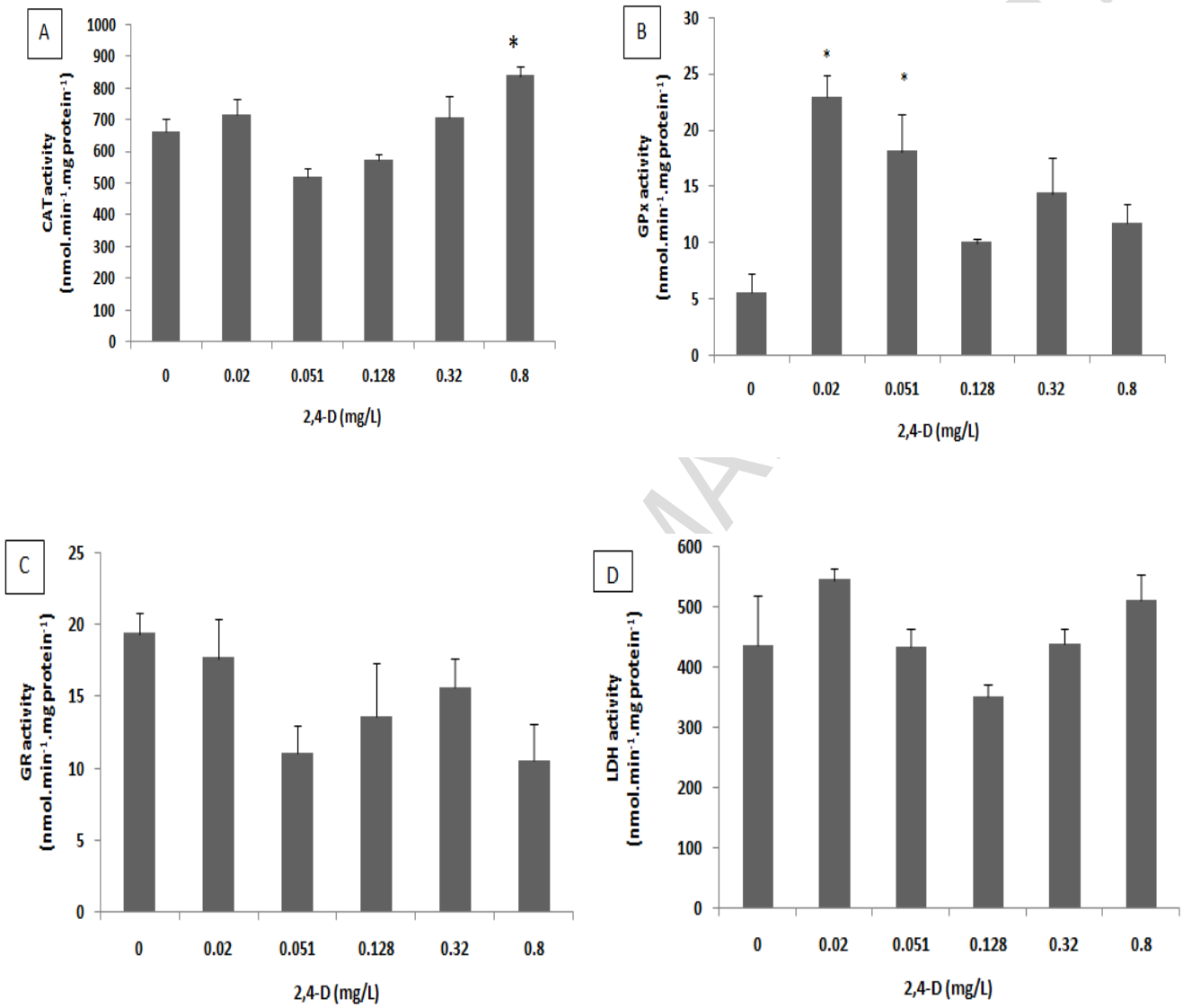
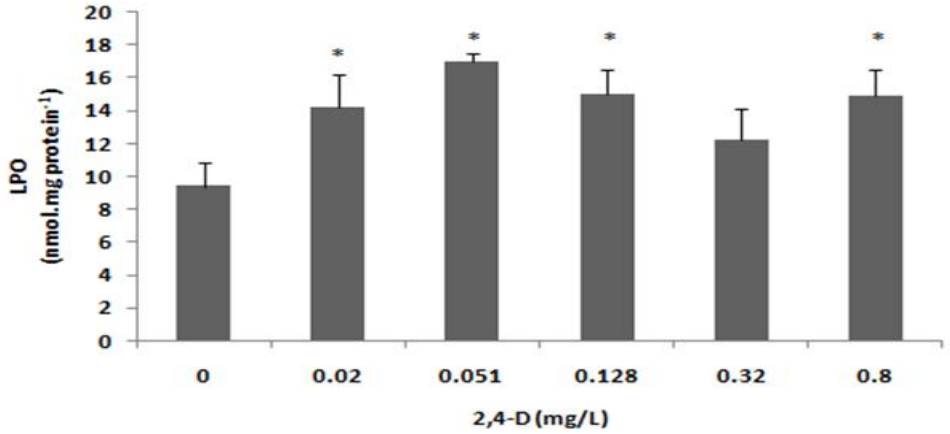

A
CAT activity (nmol.min-1.mg protein-1)
1000
900
800
700
600
500
400
300
200
100
0
*
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
B
GPx activity (nmol.min-1.mg protein-1)
30
25
20
15
10
5
0
*
*
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
C
GR activity (nmol.min-1.mg protein-1)
25
20
15
10
5
0
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)
D
LDH activity (nmol.min-1.mg protein-1)
600
500
400
300
200
100
0
0
0.02
0.051
0.128
0.32
0.8
2,4-D (mg/L)

Figure 5



Highlights

- Sublethal exposure to 2,4-D herbicide contribute to oxidative stress in zebrafish larvae.
- 2,4-D alters gene expression of detoxification and oxidative stress enzyme in zebrafish larvae.
- 2,4-D modulate detoxification and oxidative stress enzyme activities in zebrafish larvae.
- 2,4-D enhances MDA level in 96 hpf zebrafish larvae.

Table1. Primer sequences, accession numbers, amplicon sizes, and annealing temperatures used in qPCR reaction.

Target genes	Accession numbers	Primer sequence (5'- 3')	Amplicon sizes
gstp1	NM_131734.3	**F:** TGGTGCTGTTTCAGTCCAAG **R:** AGCCTCACTGTCGTTTTTGC	80
cat	NM 130912.1	**F:** TTTCTCCTACCCAGACACACA **R:** CACCTGGGTCACGTTGTCAT	169
gpx1a	NM_001007281.2	**F**: GAGGCACAACAGTCAGGGATT **R**: CTTCATTCTTGCAGTTCTCCTGGT	126
gsr	NM_001020554.1	**F:** CGGCCTCAACCTCAGTCAAA **R:** TGCTTCATCAGGTGTCAGAAG	142
cyp1a	NM_131879.2	**F :** AAAGACACCTGCGTGTTTGTAA **R :** GAGGGATCCTTCCACAGTTCT	68
eef1a1l2	NM_001039985.1	**F:** GTGGTAATGTGGCTGGAGAC **R:** TGTGAGCAGTGTGGCAATC	138
ß-actin	AF025305	**F:** CGAGCTGTCTTCCCATCCA **R:**TCACCAACCTAGCTGTCTTTCTG	100
gapdh	BC083506	**F:** GTGGAGTCTACTGGTGTCTTC **R:** GTGCAGGAGGCATTGCTTACA	173

Target genes abbreviations: *gstp1* (glutathione S-transferase pi 1); *cat* (catalase); *gpx* (glutathione peroxidase); *gsr* (glutathione reductase); *cyp1a* (cytochrome P450, family 1, subfamily A).

References genes abbreviations: *eef1a1l2* (eukaryotic translation elongation); *gapdh* (Glyceraldehyde-3-phosphate dehydrogenase); *ß-actin* (Beta-actin).

Table2. Data of chemical analysis of 2,4-D in working solutions and in 96 hpf zebrafish larvae. Data of 2,4-D content in larvae are expressed as ng/mg dry weight (n=3); asterisks mean significantly different from the respective control ($p<0.05$) after one-way ANOVA using Tukey's test.

Nominal concentration of 2,4-D in water (mg/L)	Measured Concentration of 2,4-D in water (mg/L)	% Recovery	2,4-D accumulation in larvae (ng/mg dry weight)
0	0	0	0
0.02	0.022	110	1.42 ± 0.12*
0.051	0.055	107.84	3.58 ± 0.42*
0.128	0.125	97.66	8.47 ±0.91*
0.32	0.311	97.19	20.38 ± 2.34*
0.8	0.761	95.13	54.44 ± 4.78*