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HILDA VANESSA POQUIOMA HERNANDEZ

# BIOCHEMICAL EFFECT OF 2,4-D IN ANIMALS: META-ANALYTIC REVIEW, AND *IN VIVO* STUDY THROUGH TROPHIC ROUTE

THESIS (MASTER'S DEGREE)

**DOIS VIZINHOS**
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Thesis presented to the Postgraduate Program in Biotechnology, Universidade Tecnológica Federal do Paraná as a partial requirement to obtain a Master's Degree in Biotechnology.
Concentration Area: Biotechnology

Advisor: Profa. Dra. Nédia de Castilhos Ghisi
Co-advisor: Prof. Dr. Ciro Alberto de Oliveira Ribeiro

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## BIOCHEMICAL EFFECT OF 2,4-D IN ANIMALS: META-ANALYTIC REVIEW, AND IN VIVO STUDY THROUGH TROPHIC ROUTE

por

**Hilda Vanessa Poquioma Hernandez**

Esta dissertação foi apresentada às quatorze horas do dia 28 de fevereiro de 2020, no Mini Auditório V - UTFPR-DV, como requisito parcial para a obtenção do título de MESTRE EM BIOTECNOLOGIA, Programa de Pós-Graduação em Biotecnologia. A candidata foi arguida pela Banca Examinadora, composta pelos professores abaixo assinados. Após deliberação, a Banca Examinadora considerou o trabalho aprovado.

Profa. Dra. Nédia De Castilhos Ghisi
(UTFPR- DV)

Profa. Dr. Ricardo Yuji Sado
(UTFPR - DV )

Profa. Dra. Flávia Yoshie Yamamoto
(UNESP - Litoral Paulista)


Prof. Dr. Andreia Anschau (UTFPR)
Coordenadora do PPGBIOTEC
UTFPR – Câmpus Dois Vizinhos

- A Folha de Aprovação assinada encontra-se na Coordenação do Programa -

Dedico este trabalho aos meus pais e irmã pelo apoio inesgotável que eles me dão, por estar sempre ao meu lado, apesar de minhas decisões me afastarem deles, mas que também me encheram de experiências maravilhosas e inesquecíveis.

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## ABSTRACT

HERNÁNDEZ, Hilda Vanessa Poquioma. **Biochemical effect of 2,4-D in animals:** meta-analytic review, and *in vivo* study through trophic route. 126 p. Thesis (Master's Degree in Biotechnology) - Universidade Tecnológica Federal do Paraná, Dois Vizinhos, 2020.

The 2,4-D is one of the most important agrochemical in Brasil, since its use increased with the development of 2,4-D resistant crops to the large monocultureover. However, its effect on non-target species is still under study. There are many research studying the effect of 2,4-D in the body, however all of them use different species of a large range of age, applying the xenobiotic by different routes and using many tissues to analyze the effect of 2,4-D on the enzymatic activity. In some of them, the agrotoxic is applied in combination with other compounds using high concentration for few hours, while in others are used more dilute concentrations of the herbicide pure, for several months. All this information makes it difficult the understanding of the real effect of 2,4-D over the oxidative stress. In order to disentangle all this information, it was don a meta analytici study and an in vivo study in fish. In the first part of this research it was studied the response of CAT, SOD, GST, GST, GR, GPx, and the level of GSH in organisms exposed to 2,4-D, in order to determine if variables such as phylogenetical group, age, exposure route, tissue of study, mixture with other compounds, dose applied and exposure time would be influencing the activity of action of those molecules, as a way to provide more conclusive information.In the second part, Rhamdia quelen was exposed to 2,4-D and there were assessed the same biochemical biomarkers from the fist part. In the meta analytical study it was found a clear difference in the response pattern from mammals and no-mammal animals. In the first group it seen a decrease in the activity of SOD and CAT, while in the second group the opposite is appreciated. The mixture with other additives is not a differential factor, while the age is a factor still unclear, studies using young animals were sparse. The hydric exposure causes increase in CAT, SOD and GST activity, while the oral one a drop in the activity of CAT, SOD, GPx and in the level of GSH. The response of the enzymatic and non-enzymatic molecules differs within the tissues however it is clear that male reproductive organs are more sensible regarding female organs. Finally, while the doses exposition is not determinant factor to increase or decrease the enzymatic activity, as longer the exposition time, lesser the activity of CAT, SOD and GPx is seen, while the GSH increases in larger exposition periods. From the in vivo study, there was found no oxidative stress in *R. quelen* exposed to doses of 20, 200 and 2000µg/kg for a period of 22 and 42 days. From these last results, corresponded with the data found in the first part of this investigation. Oxidative stress was not determined since the exposure occurred for a short period of time.



**Keywords**: Oxidative stress. Enzymatic biomarker. Herbicid.

## RESUMO

HERNÁNDEZ, Hilda Vanessa Poquioma. **Efeito bioquímico do 2,4-D em animais**: revisão meta-analítica e estudo *in vivo* por via trófica. 126 f. Dissertação (Mestrado em Biotecnologia) - Universidade Tecnológica Federal do Paraná, Dois Vizinhos, 2020.

O 2,4-D é um dos agroquímicos mais importantes do Brasil, pois seu uso aumentou com o desenvolvimento de culturas resistentes ao 2,4-D para a grande monocultura. No entanto, seu efeito sobre espécies não-alvo ainda está sendo estudado. Existem muitas pesquisas estudando o efeito do 2,4-D no organismo, no entanto, todos eles usam espécies diferentes de uma grande faixa etária, aplicando o xenobiótico por diferentes rotas e usando muitos tecidos para analisar o efeito do 2,4-D. D sobre a atividade enzimática. Em alguns deles, o agrotóxico é aplicado em combinação com outros compostos, utilizando alta concentração por algumas horas, enquanto em outros são utilizadas concentrações mais diluídas do herbicida puro, por vários meses. Toda essa informação dificulta a compreensão do efeito real do 2,4-D sobre o estresse oxidativo. Para desmembrar todas essas informações, foi realizado um estudo meta-analítico e um estudo *in vivo* em peixes. Na primeira parte desta pesquisa, estudou-se a resposta de CAT, SOD, GST, GST, GR, GPx e o nível de GSH em organismos expostos ao 2,4-D, a fim de determinar se variáveis como grupo filogenético , idade, via de exposição, tecido do estudo, mistura com outros compostos, dose aplicada e tempo de exposição influenciam a atividade de ação dessas moléculas, como forma de fornecer informações mais conclusivas. Na segunda parte, *Rhamdia quelen* foi exposto ao 2,4-D e foram avaliados os mesmos biomarcadores bioquímicos da primeira parte. No estudo meta-analítico, foi encontrada uma clara diferença no padrão de resposta de mamíferos e animais não mamíferos. No primeiro grupo, houve uma diminuição na atividade de SOD e CAT, enquanto no segundo grupo, o oposto é apreciado. A mistura com outros aditivos não é um fator diferencial, enquanto a idade ainda é um fator incerto, estudos com animais jovens foram escassos. A exposição hídrica causa aumento da atividade de CAT, SOD e GST, enquanto a exposição oral diminui a atividade de CAT, SOD, GPx e o nível de GSH. A resposta das moléculas enzimáticas e não enzimáticas difere dentro dos tecidos, porém é claro que os órgãos reprodutores masculinos são mais sensíveis em relação aos órgãos femininos. Finalmente, enquanto a exposição das doses não é fator determinante para aumentar ou diminuir a atividade enzimática, quanto maior o tempo de exposição, menor é a atividade de CAT, SOD e GPx, enquanto o GSH aumenta em períodos de exposição maiores. No estudo in vivo, não foi encontrado estresse oxidativo em R. quelen exposto a doses de 20, 200 e 2000µg / kg por um período de 22 e 42 dias. A partir de esses últimos resutaldos pode se conferir os dados encontrados na primeira parte de esta investigação. Não foi determinado estresse oxidativo já que a exposição fue por courto período de tempo.



**Palavras-chave:** Estres oxidativo.  Biomarcador enzimatico. Herbicida.

## FIGURE INDEX

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2,4-D. | 2,4-Dichlorophenoxyacetic acid |
| 2B. | Possibly carcinogenica to humans |
| AChE. | Acetylcholinestarse |
| CAT. | Catalase |
| CEUA. | Ethics Committee on Animal Use |
| CI. | Confidance intervale |
| GM. | Grand mean |
| GPX. | Glutathione peroxides |
| GR. | Glutathione reductase |
| GSH. | Reduced glutathione |
| GSSG. | Glutathione disulphide |
| GST. | Glutathione-S- transferase |
| H202. | Hydrogen Peroxide radicals |
| HOCI. | Hypochlorous acid |
| IARC. | Agency for Research of Cancer, Agency for Research of Cancer |
| LOOH. | Lipid peroxide |
| LPO. | Lipid peroxidation |
| NADPH. | Nicotinamide adenine dinucleotide phosphate |
| $NH_4^+$. | Ammonium |
| NO2−. | Nitrite |
| O2-. | Superoxide radical |
| OH*. | Hydroxyl radicales |
| PCO. | Protein carboxylation |
| PMRA. | Pest Managment Regulatory Agency of Health Canada |
| ROO. | Peroxyl Radical |
| ROS. | Reactive oxygen species |
| SOD. | Superoxide dismutase |
| T°. | Temperature |
| USEPA. | The United States Environmental Protection Agency |
| UTFPR. | Universidade Tecnológica Federal do Paraná |
| WHO. | World Healt Organization |

**INDEX**

# 1. INTRODUCTION

The 2,4-Dichlorophenoxyacetic acid (2,4-D) is an herbicide used in Brazil for being a country with large agricultural extensions and with economy predominantly agricultural. The mishandling of 2,4-D for the eradication of herbs has an effect on non-target species. Several studies aimed at evaluate the carcinogenic effect of 2,4-D, that have been reviewed by several regulatory groups, such as The United States Environmental Protection Agency (USEPA), Pest Management Regulatory Agency of Health Canada (PMRA), World Health Organization (WHO) (1996), Environment The Risk Management Authority (New Zealand), and the European Commission (2001), but the evidences in different references does not agree about the effects. Thus, in 2015, the International Agency for Research on Cancer (IARC) assessed the danger of 2,4-D in relation to cancer by placing it in the classification of "2B - possibly carcinogenic to humans" (LOOMIS *et al*., 2015).

However, there is evidence that supports the 2,4-D negative effects not only in humans, but to the environment and in non-target animals too. The literature indicates that despite its rapid excretion after long periods of exposure, it can be accumulated in the body and associated with neurotoxicity (BONGIOVANNI *et al*., 2007) and genotoxicity in humans (ADAMS *et al*., 2001), nephrotoxicity in mice (TROUDI *et al*., 2011), and with production of abnormalities in the sperm head of males treated with 2,4-D (AMER; ALY, 2001; VENKOV *et al*., 2000). Similarly, negative damage is produced in aquatic animals such as the aquatic snake exposed to 60–120 mg / L 2,4-D (KUMARI; KHARE; DANGE, 2014), in aquatic snails at 75–100 mg / L (ESTEVAM *et al*., 2006) and in different fish tissues at 10–100 mg / L (MATVIISHYN *et al*., 2014).

In addition, the toxic effect of 2,4-D was demonstrated during the chemical and biological war in World War II and continued to be used after the Vietnam War (1959-1975) where it was a component of the "Agent Orange." Several years later it was determinated the development of several disease in war veterans associated with the high level of exposition of 2,4-D they received during its application (YI *et al*., 2014).

The toxicological effect of a compound can be measured at different levels, one of them is through the assess of the main antioxidant enzymes response. This biochemical mechanism is highly conserved in vertebrates and invertebrate and can

be compared among groups. The enzymes that are part of this system are the superoxide dismutase (SOD), catalase (CAT) and glutathione-S- transferase (GST) (EL-BELTAGI; MOHAMED, 2013). The GSH tripeptide (reduced glutathione) is also part of this system, even it has not an enzymatic activity, its function is important for the detoxification process (NORRIS *et al*., 2009; OTTO; MOON, 1996). An effect of the presence of a contaminant in the body is the breakdown of proteins and lipids which can inhibit or change the shape and/or function of important enzymes like the acetylcholinesterase (AChE), so another way to measure the damage it can cause is through the study of biomarker of effect.

The *Rhamdia quelen*, called in Brazil as “jundiá”, is an organism of study of environmental pollution. Widely used in ecotoxicology studies, but with insufficient information regarding its response to 2,4-D at biochemical level. The objective of this study is to evaluate the effects of 2,4-D in jundiá, administered orally using enzymatic biomarkers. Likewise, the present investigation had a second objective: a meta-analytical study about the effect of this herbicide in different species, evaluating the variables of age, route of exposure, combination with other herbicides, dose and exposure time.

# 2. EFFECT OF 2,4-D OVER THE ANTIOXIDANT ACTIVITY IN DIFFERENT SPECIES: A META-ANALYTICAL STUDY

## 2.1 ABSTRACT

Nowadays, every organism around the world is subject to environmental pollutants. In Brazil, the herbicide 2,4-D is one of the most important for being widely used, however, its effect on non-target species is still under study. While all cells have a system that counteracts the effects of free radicals, the response of that system, which is made up of a series of enzymatic and non-enzymatic molecules, could vary depending on different variables. In order this study aimed to compile and systematize the effect of the phylogenetic group, age, exposure route, dose and exposure time variables on the response of SOD, GST, AChE, LPO, PCO and GSH in individuals exposed to 2,4-D. Within the results found, it was seen that there is a different response between mammals and aquatic animals. While CAT and SOD have great activity to counteract ROS levels, in mammals these enzymes are blocked in the presence of 2,4-D, on the contrary, in fish the activity tends to increase in order to control oxidative stress. The oral administration route showed differential response patterns regarding the others. However, this might be caused by the fact that the research that used gavage, limited their analysis to the use of mammals, who apparently have a different response patterns in presence of 2,4-D. There are necessary more studies in aquatic animals using oral route to determinate if the administration mean has an effect over the enzymatic activity. Regarding the organs, it is clear that in most of the tissue analysed the activity of each enzyme if what was expected, just in the case of kidney was seen decrease or inhibition in the activity of SOD. For the case of the age, it was shown information gaps in young animals, so there are necessary more studies in growing animals. Finally, it was seen that the doses increase cause damage in proteins and lipids the inhibition of AChE. While the exposition time reduces the activity of CAT, SOD, and AChE, and also LPO and PCO as it increases.

## 2.2 INTRODUCTION

All aerobic individuals consistently produce small amounts of reactive oxygen species (ROS) in response to internal and external stimuli. Among the main varieties of ROS are hydroxyl radicals (OH*), their peroxide radicals ($H_20_2$), and superoxide anions ($O_2^-$) (EL-BELTAGI; MOHAMED, 2013). At low levels, the presence of ROS is of great importance since it allows different cellular processes to be generated, such as intracellular communication allowing proliferation or apoptosis, generating immunity, and defense against microorganisms (YANG *et al*., 2013). On the contrary, when there is a high level of ROS in the body and / or when the removal mechanisms work improperly, there is a phenomenon called oxidative stress, which can cause metabolic malfunction and damage at the level of macromolecules (PATEL *et al*., 2018).

The presence of agrochemicals in the environment, such as herbicides, can have an action in a non-target organism such as fish, rodents, annelids, birds, etc. Among the most used in Brazil2,4-Dichlorophenoxyacetic acid is one of the most important because it is widely used, and because negative effects on health have been reported causing enzymatic deregulation that can trigger diseases (ADAPAR, 2020). This is a synthetic auxin herbicide that belongs to the phenoxy family of herbicides, with the chemical formula C8H6Cl2O3 which is usually referred to by its ISO common name 2,4-D (MUNRO *et al*., 1992).

Any chemical agent that enters in the body is normally metabolized by cells, since there is a detoxidation system that is formed by a series of non-enzymatic and enzymatic molecules, which work in a series of reactions that have four stages (0-III reduced glutathione) (GLISIC *et al*., 2015). During stage 0 the chemical compound is taken by means of membrane transporters; the stage I is enzymatic biotransformation that consists in the union of the enzyme p450 with the xenobiotic, generating a substrate-enzyme complex, a process that uses the presence of NADPH and molecular oxygen. As a result, FeIII reduction of cytochrome p450 is generated. Subsequently there will be a reduction of the complex, which still goes through another reduction process due to an $O_2$ molecule. In this way an oxidized complex is generated which can be dissociated (ANDERSSON; FÖRLIN, 1992; ARELLANO-AGUILAR; MONTOYA; GARCIA, 2009). As a result of this stage, free radicals are generated that

together with the oxidized complex are degraded due to a series of endogenous molecules that participate in stage II such as superoxide dismutase (SOD), glutathione peroxides (GPX), catalase (CAT), glutathione (GSH), in addition to a series of vitamins. Finally, all oxidized and reduced metabolites are excreted in stage III through the cell membrane (GLISIC *et al*., 2015; HODGSON, 2010).

The imbalance in the ROS level results in damage to the membrane and protein level. The prevention of lipid peroxidation (LPO) and protein carboxylation (PCO) is an essential process since the damage can reach the DNA (CADET; RICHARD WAGNER, 2013). LPO can cause DNA damage since they can react with the nucleophilic centers in the cell and generate bonds with DNA, RNA and proteins, this could generate cytotoxicity, mutagenicity and / or carcinogenicity (WHYTE *et al*., 2000). On the other hand, this imbalance can also have repercussions at the neuronal level causing locomotion problems as demonstrated in fish (DA FONSECA *et al*., 2008), mice (BERNARD *et al*., 1985a) and humans (MURUSSII *et al*., 2014), because the exposure to herbicides such as 2,4-D also generates an imbalance at the ROS level, which causes inhibition of the enzyme acetylcholinesterase (AChE). For this reason, the antioxidant action of the second stage enzymes of the detoxification process is of vital importance for the organisms.

The enzymes of stage I and II, are found mainly in the reticulum of hepatocytes. However they can also be synthesized in organs such as the intestine, kidney, lung, brain, heart, skin, and even in gonads (VAN DER OOST; BEYER; VERMEULEN, 2003).

While the enzymes that are part of this system are highly conserved within vertebrates and invertebrates, there are still discrepancies in their response when exposed to different xenobiotic of interest such as 2,4-D. In fact, certain enzymes such as catalase are broadly diversified evolutionarily within eukaryotes (ZÁMOCKÝ *et al*., 2012), after being evaluated from bacteria. Similar to catalase, GPx is an enzyme that participate of the detoxification system, and this enzyme has acquired different enzymatic properties depending on the selection pressure and adaptation of individuals to the environment (BAE *et al*., 2009). On the other hand, p450 from vertebrates would have emerged from genes type independently of genes from invertebrates, because the vertebrate type genes would have had a different origin from that of invertebrate type genes (KAWASHIMA; SATTA, 2014). This understanding is also important cause it could indicate the different responses in vertebrate and

invertebrate species in the presence of xenobiotics. However not only the phylogenetic group might determinate a response pattern, factor like age, administration route, mixture with other compounds, administration and time exposure are also variables that might influence the enzymatic activity. In fact, this could contribute to the development of drugs or herbicides that can prevent diseases to which 2,4-D may be related.

The present study aimed through a meta-analytical study to better elucidate the biochemical response of CAT, SOD, GST, AChE, LPO, PCO and GSH at organisms exposed to different concentrations of 2,4-D, in order to determine if variables such as phylogenetically group, age, exposure route, organ, dose and exposure time would be related to the levels of action of these parameters.

## 2.3 MATERIAL AND METHODS

### 2.3.1 Variables Assessed

The variables to be evaluated were the activity of CAT, SOD, GST, AChE, and on the levels of LPO, PCO and GSH, were the phylogenetic group; age (that is, if they are youth or adults); the route of administration of 2,4-D (orally, in the environment where individuals are found, or intraperitoneal); the organ used for enzyme activity analysis; the effect of herbicide application (pure or in combination with other herbicides); the concentration or dose and the exposure time.

### 2.3.2 Inclusion Criteria

In order to limit the chosen variables by the study, it was sought that factors such as technique, or type of sample do not influence the choice of the articles, so researches that used tissue cell lines or blood cells were not considered, since the interpretation of the results could generate variables, only items that have worked with animals *in vivo* and have removed the organs for enzymatic analysis were considered. There was no limit on animal species, nor age. Although not all articles analyzed all the molecules, the present study considers those that analyzed at least one of them within the meta-analysis. Likewise, articles where they have worked with 2,4-D in

combination with other herbicides were also considered, while studies using any other non-herbicidal compound were discarded. It was necessary to select numerical values of the results, and for this reason revisions, notes, book chapters, or book reviews were not included, because they did not contain the necessary values for the purpose of this analysis. Finally, only articles containing the necessary data (mean, standard deviation, standard error, sample size) were selected. There was no limitation regarding country, author or language. All the selected articles answered the main question of the objective of the investigation that was: The 2,4-D cause effect over the antioxidant enzymes?

### 2.3.3 Meta-analysis

For the data collection the databases of Web of Science (http://www.webofscience.com), Scopus and Science Direct were used. The boolean script used was "2,4-D", "OR", "2,4-Dichlorophenoxyacetic acid", "AND" and "effect". A single list of articles was generated in the Excel software and the presence of repeated articles was filtered. In the case of articles that showed only graphic data as bars, the images were loaded into a WebPlotDigitizer program which converted the graphic values to numerical values. From all numerical results an array was generated in Excel. MetaWin software was used to conduct the meta-analysis tests.

### 2.3.4 Publication Bias

Considering the criteria for paper selection in the systematic reviews, it is difficult to obtain all published data, which results in a lack of information and meta-analytic power. Additionally, as most published data commonly present positive results, it publication bias is expected considering the lack of negative published results (BORENSTEIN *et al*., 2009). In order to determine if the results for each variable had showed publication bias, the Kendall's Tau test and the Spearman Rank-Order correlation test were performed, while Rosenthal and Orwin's methods were used to determine how many studies are necessary to do not have publication bias.

## 2.4 RESULTS AND DISCUSSION

### 2.4.1 Articles found in the Databases

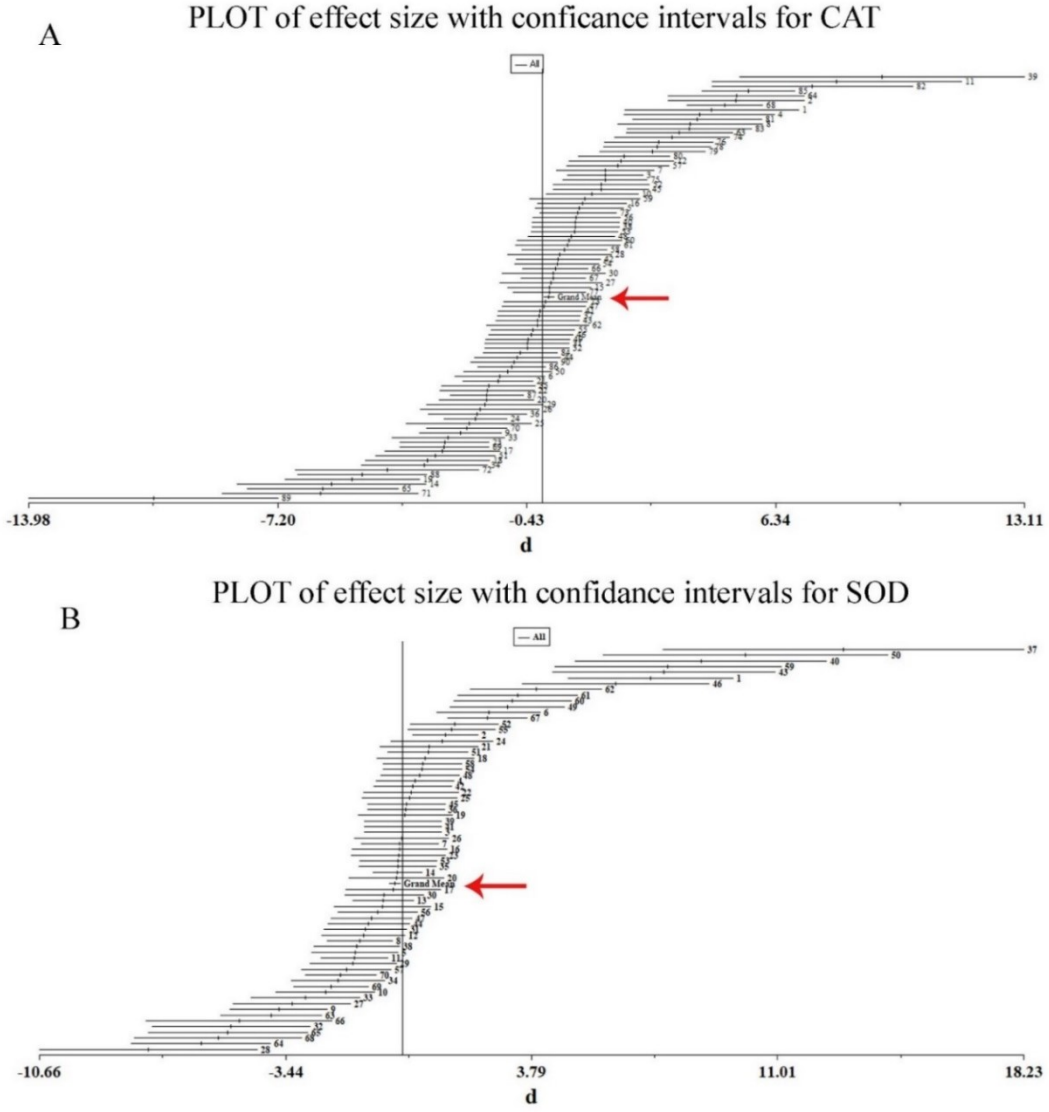
There were found 10,034 articles Scopus database, 8,762 in Web of Science, and 19,1714 in the Science Direct database. Articles without enzymatic biomarkers have been removed, so the number of articles in Scopus decreased to 200 after the first filter, the second to 180, and the third to 153. In a next filter, articles duplicated and those with incomplete data were also discarded, decreasing the number of articles to 78.

### 2.4.2. Effect Size and Confidence Intervals

To obtain the Summary effect (E), it was used random-effect model using Hedges estimator. According to the Grande Media (GM) in the analysis of CAT, there was no significant difference between the control and the treatments with 2,4-D ($p<0.05$) since the was E = -0.1586, IC 95%= -0.8955 to 0.5784.

In almost all the studies the activity of CAT increases when the animals are exposed to 2,4-D (Fig. 1A), where the Grande Mean (GM) (E+ =0.229) and the confidence interval (CI) (95% CI-0.234 to 0.693) passed positively over the 0, don't bracketing the null. On the other hand, the GM in the plot of SOD is below 0 (E+= -0.0257; 95%CI =-0.499 to 0.448), so it could be said that the SOD in most of the individuals is inhibited by the presence of the herbicide (Fig. 1B). Thus, is clear the CAT tries to counteract the action of the $H_20_2$ produced by the presence of 2,4-D (JAWED *et al*., 2000) in the different researches, however there might be some factors that could make the SOD to be inactivate. is important to note that the SOD inaction might have serious effects in lipids, proteins and DNA (CEDERBAUM, 2017). This is because of the fact that the superoxide ($O2^{-)}$ (produced by endogen and exogen sources) do not metabolized by the SOD can react with $H_20_2$ through the Haber-Wess reaction producing hydroxyl radicals (–HO) which is one of the most reactive ROS (KEHRER, 2000). On the other hand the CAT inactivation might cause an excess of $H_20_2$ what thanks to the Fenton reaction (AWADALLAH, 2013) will also have a repercussion over phospholipids, proteins and nucleic acids.

**Figure 1. Florest plot of studies evaluating the CAT activity (A), and SOD activity (B) in 2,4-D exposure, arranged by effect size.**




**Source: The author**
**Note: estimator of response ratio (effects size - d) and 95% confidence interval (CI) of each experiment included in the meta-analysis are presented. The number beside the bars represents the reference number of each experiment as in Supplementary table 1 and 2. Grand Mean (marked with a red arrow) is the overall mean effects size of all studies**

Regarding the level of GSH it drops in presence of 2,4-D as can be seen in most of the research. This can be seen in the Fig. 2A, where the GM is below 0 and the CI don't bracket the null (E+: -1.652E; 95%CI = -2.215 to-1.088) that GSH is highly consumed in this process of exposure to 2,4-D. As it is know the main function of GSH is to produce water-soluble complexes from the xenobiotic, hydrolysing it in presence or absence of GST *(LI et al*., 2007).

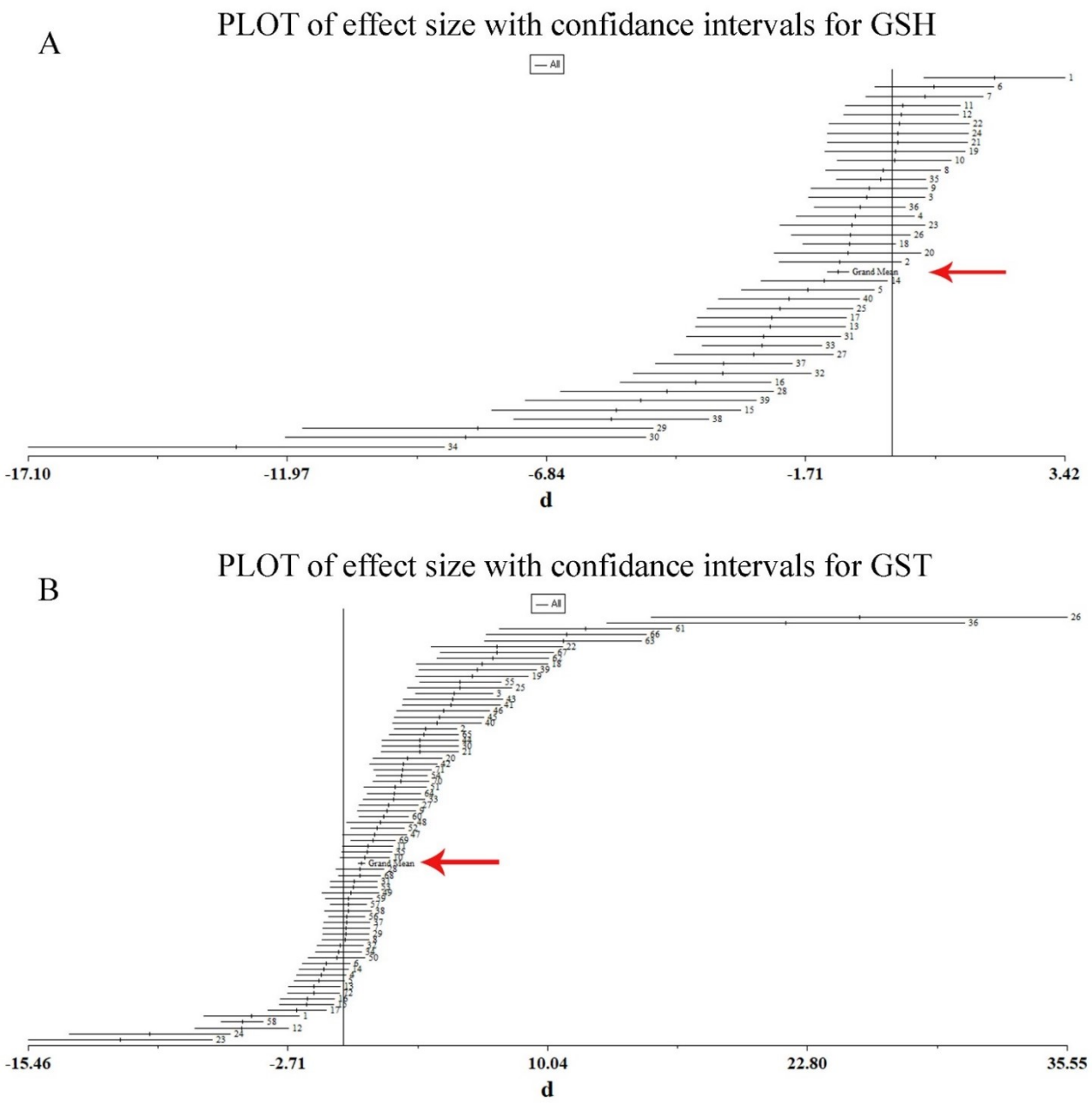
The reduction in the levels of GSH is because it is trying to control the level of 2,4-D in order to avoid oxidative stress (E+: -1.652E; 95%CI = -2.215 to-1.088) (AGAR;

MAEDEI, 1992). On the other hand the level of GST (E+: 1.865 95%CI = 1.246 to 2.485) seems to increase in most research (Fig. 2B), what might be attributed to the fact that its high level might guarantee the effectiveness of GSH to metabolizing the herbicide (AGAR; MAEDEI, 1992).

Nevertheless, is important to note that in some researches the levels of GSH seems too low. This would be dangerous because the activity of GST, SOD, CAT reaches its maximum efficiency to prevent oxidative stress, as long as GSH can control the amount of the main cause of ROS production (BINDOLI; FUKUTO; FORMAN, 2008), which in this case is 2,4-D.

The lipid peroxidation (LPO) and the protein carboxylation (PCO) are two of the main consequence of the high level of ROS. As can be seen in the Fig. 3A and 3B, it is obvious the 2,4-D has effect over the cellular lipids and proteins (E+=+1.687; 95%CI =1.291 to 2.083 to LPO and E+=1.118; 95%CI =0.298 to 1.938 to PCO). While the level of CAT might be high as was seen in the Fig. 1A, is seems that those levels are not enough to counteract the PCO and LPO.

**Figure 2. Florest plot of studies evaluating the GSH amount (A), and GST activity (B) in 2,4-D exposure, arranged by effect size.**



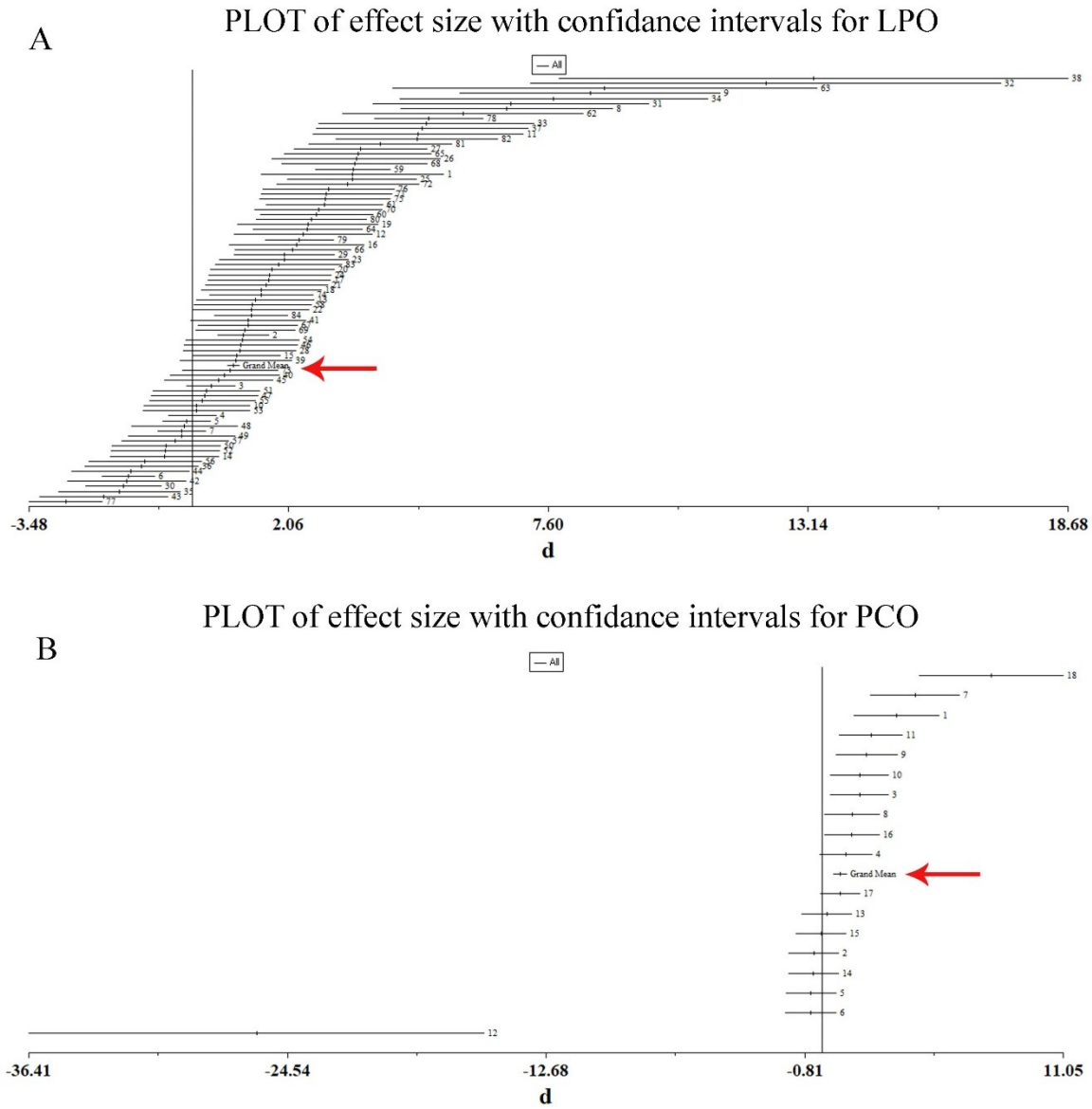

**Source: The author**
**Note: estimator of response ratio (effects size - d) and 95% confidence interval (CI) of each experiment included in the meta-analysis are presented. The number beside the bars represents the reference number of each experiment as in Supplementary table 3 and 4. Grand Mean (marked with a red arrow) is the overall mean effects size of all studies.**

Every cell has several mechanisms to repair the damage caused in the cell membrane. One example of them is the apoptosis or the repair action of the glutathione peroxidase (GPx), which in addition to its action as a cofactor to produce GSH, it can reduce the fatty acid hydroperoxides (DAVIES, 2000). This reduction might stop the

lipid peroxidation, however it seems the level of ROS produced by the presence of 2,4-D exceeds the repair capacity of the cell, causing LPO and PCO. It is interesting to see that in hardly all the research the effect over the proteins and lipids is similar, just in few research there was seen no damage over them.

Regarding to the AChE, it is clear the 2,4-D cause an inhibition, even the different variables like phylogenetic group, age, administration via, organ, dose or exposition time, the inactivation of it is evident (Fig. 4 E+=-1.226; 95%CI =-1.552 to -0.9). This is an alarming result because the effect of this might be associated with diseases such as the Alzheimer (HOLSCHNEIDER *et al*., 2011).

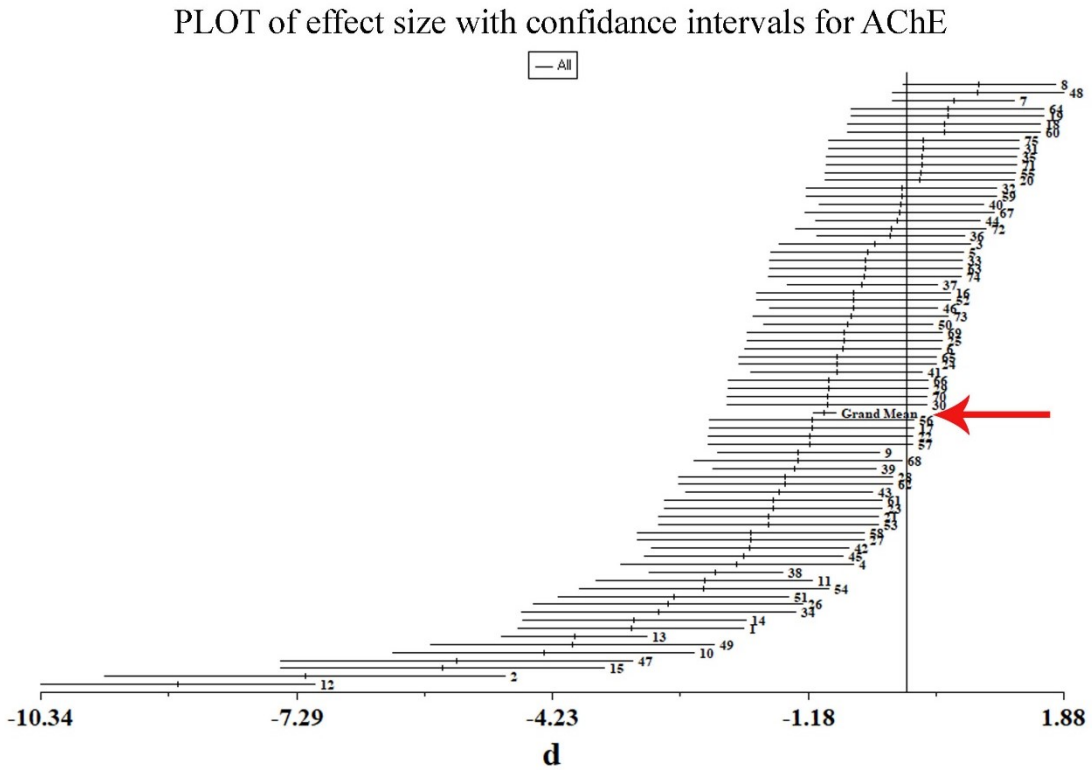
**Figure 3. Florest plot of studies evaluating the lipid peroxidation (A), and protein carboxylation (B) in 2,4-D exposure, arranged by effect size.**




**Source: The author**
**Note: estimator of response ratio (effects size - d) and 95% confidence interval (CI) of each experiment included in the meta-analysis are presented. The number beside the bars represents the reference number of each experiment as in Supplementary table 5 and 6. Grand Mean (marked with a red arrow) is the overall mean effects size of all studies**

Regarding to the AChE, it is clear the 2,4-D cause an inhibition, even the different variables like phylogenetic group, age, administration via, organ, dose or exposition time, the inactivation of it is evident (Fig. 4 E+=-1.226; 95%CI =-1.552 to -0.9). This result is alarming because the effect of this might be associated with diseases such as the Alzheimer (HOLSCHNEIDER *et al.*, 2011).

**Figure 4. Florest plot of studies evaluating the acetylcholinesterase activity in 2,4-D exposure, arranged by effect size**




**Source: The author**
**Note: estimator of response ratio (effects size - d) and 95% confidence interval (CI) of each experiment included in the meta-analysis are presented. The number beside the bars represents the reference number of each experiment as in Supplementary table 7. Grand Mean (marked with a red arrow) is the overall mean effects size of all studies**

The cognitive processes of every organism is mainly determinate by the action of the acetylcholine (Ach) which is part of the cholinergic system, and whose main action is to allow the nerve impulse (DVIR *et al.*, 2010). However, it is necessary its inactivation to produce serial nerve impulse. This inactivation consist in the formation of choline and acetate in the intersinaptic space due to the action of the acetylcholinesterase (AChE) (DOWNES; GRANATO, 2004). This allow the choline to return to neuron to form the acetylcholine again to the next nerve impulse. The excess of acetylcholine in the intersinaptic space can affect the locomotion and equilibrium of organisms, as a result of AChE inhibition (BRETAUD; TOUTANT; SAGLIO, 2000).

### 2.4.3 Effect of the Phylogenetic Group on Enzymatic Activity

Although the GM shows no difference regarding the control in the CAT activity of individuals exposed to 2,4-D (E+=0.226; 95% CI= -0.178 to 0.631), in rodents the activity had an opposite effect (Fig. 5A). When compared to the control, the enzyme activity decreases, that could be justified in those mammals as the catalase may notacting in defense of the organism or it might have been inhibited. The susceptibility of these animals compared to fish, bivalves and even annelids could be higher, what in long terms might cause the accumulation of hydrogen peroxide.

Similar results were found in SOD levels, where a differential result is also shown in rodents, the presence of 2,4-D causes a decrease in its activity (Fig. 5B). An explanation is that in these mammals, other mechanism might be counteracting the level of the agrotoxic. An example might be the vitamins activity, as was shown in cellular culture when exposed to similar herbicides(GEHIN *et al*., 2005). Among the vitamins with the highest antioxidant activity there are the vitamin C (ascorbic acid) and vitamin E. The first one is a potent reducing agent of ROS such as $O_2^-$, - OH, ROO -, and HOCl (BILLER; TAKAHASHI, 2018; ZHONG *et al*., 2017) while the last one is characterized by capturing proxy radicals, whereby damage at the membrane level can be controlled. Although they are not naturally produced, they are absorbed by the diet (DI GIULIO; MEYER, 2008), so it could be that the basal food received by rodents in experimentation might have been providing with sufficient levels of vitamins to control the possible imbalance of ROS. Nevertheless, there are necessary deeper studies, in fact this theory might be defeated by other research, where by contrary, according to their results the 2,4.-D has also the capacity to decrease the level of vitamin C (TROUDI *et al*., 2011). Whiteout the action of SOD and vitamins in mammals, the organism leaves in risk of oxidative stress

Another factor that might be reducing the SOD activity is a diet deficient in several metals or the inhibition in the absorption of them (LI *et al*., 2010). As it is known the SOD activity is reduced in animals fed diets deficient in copper and Zinc (HARVEY, 2008). This lack of absorption could be caused by the presence of herbicides, since it is reported that 2,4-D similar herbicides *i.e.*, like Glyphosate are abductor of micronutrients like zinc, cobalt and manganses. (PETERSON MYERS *et al*., 2016). In addition to that it is been seen that the oxygen species giving rise to complexes

between the ROS and the metal ions (CHAUDHARY; HASSAN; GRIMES, 2009), what is also a consequence of the presence of 2,4-D and this reduces the action of SOD.

In the case of fish, bivalves and annelids, it was seen an increase in the levels of CAT and SOD regarding the control, which would confirm the antioxidant action of those enzymes. It could be concluded that the antioxidant enzymes in aquatic animals showed a better response in the presence of 2,4-D when compared to the same enzymes in rodents.

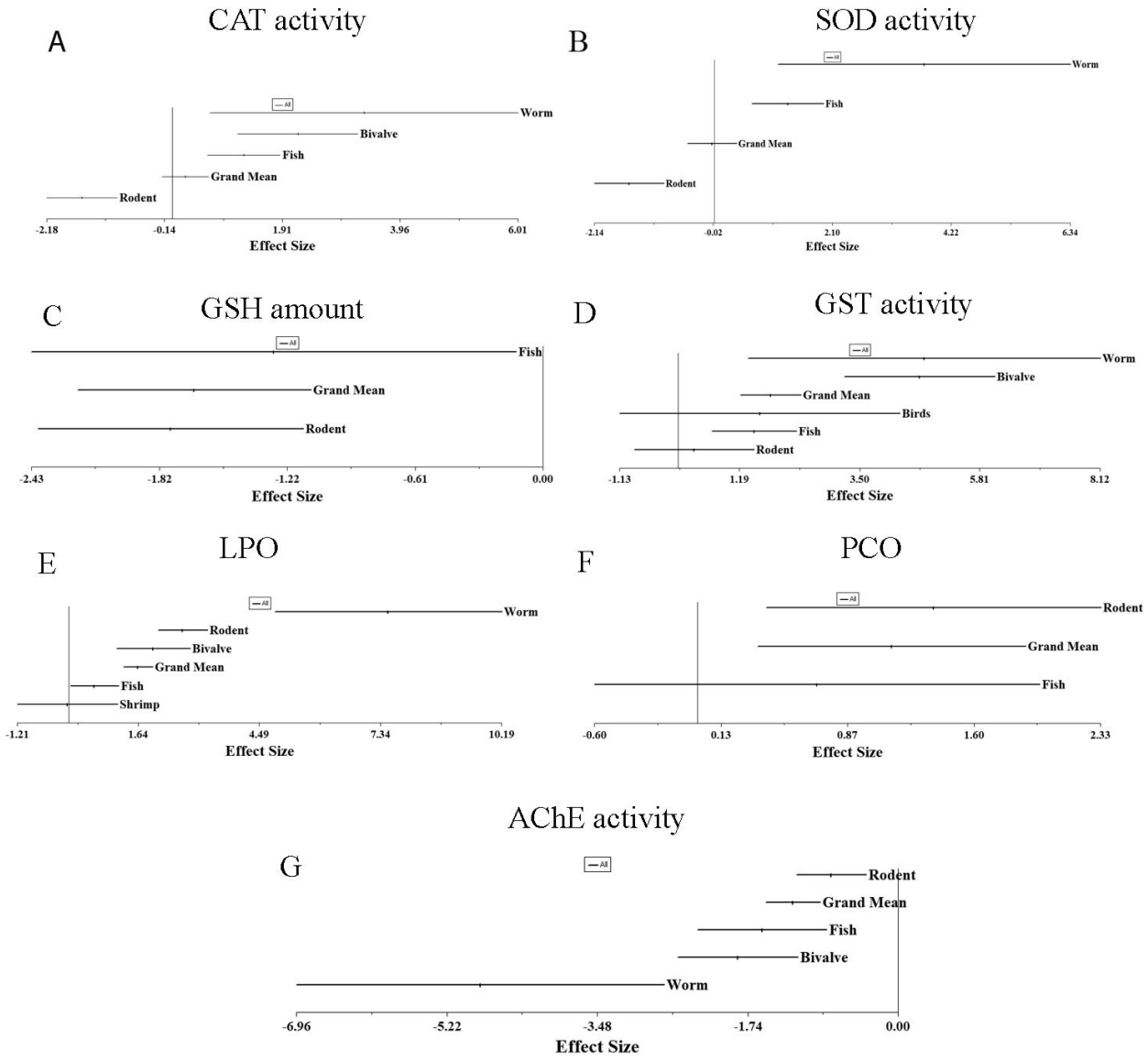
The GSH levels in all the species diminished in the presence of xenobiotic (Fig. 5C) (GM E+=-1.658; 95% CI= -2.228 to-1.088), this could be due to an inhibition in its action due to the high levels of ROS. Another possibility could be that the glutathione reserves is used to generate water-soluble complexes with the xenobiotic, since it has the ability to hydrolyse the compound directly or through the catalysing action of GST (LI *et al*., 2007). However the inhibition of important enzymes like GR is also an option, since it is responsible to catalyses the reduction of glutathione disulphide (GSSG) to GSH (FAGAN; PALFEY, 2010).

Regarding GST, it is shown difference in its level in mammals and birds, regarding GST levels in bivalves and fish, although the GM (E+=1.781; 95% CI= 1.192 to 2.37), indicates an increase in action of the enzyme in the presence of 2,4-D (Fig. 5D). The similarity in the response of birds and mammals could be due to the low number of articles found in birds, or because both show similar enzymatic behaviour. However, another explanation could be due to phylogenetic proximity. In fact there are reports that support that these two groups share orthologous genes on sex chromosomes (SMITH; VOSS, 2007). Nevertheless, more studies are necessary.

In birds and rodents, it is shown that in the presence of 2,4-D baseline levels of GSH are maintained (that is, they maintain control similar levels). This could be due to the fact that the action of vitamins and GSH is sufficient for the oxidative stress control.

On the other hand, the level of GST in aquatic animals and worms acts efficiently, trying to increase in order to be in enough amount to serve as catalyst of the GSH-mediated hydrolysis of the herbicide (RUZZA; CALDERAN, 2013).

**Figure 5. Forest plot representing the categorization by phylogenetic group of CAT (A), SOD (B), GSH (C), GST (D), LPO (E), PCO (F), AChE (G) in 2,4-D exposure.**




**Source: The author**
**Note:The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.**

Although the action of CAT and SOD try to control the levels of $H_2O_2$ and $O_2$ in order to protect from the effects of the oxidative stress in lipids and protein, it was observed that the presence of 2,4-D generates LPO ((E+=1.63; 95% CI=1.279 to 1.98), and PCO (E+=1.120; 95% CI= 0.288 to 1.951), regardless of the phylogenetic group (Fig 5 E – F). As expected ROS are produced by the presence of 2,4-D, in spite the action of the antioxidant enzyme, they can cause damage in membranes; effect that is widely reported in the literature both *in vitro* and *in vivo* (BUSSOLARO; FILIPAK NETO;

OLIVEIRA RIBEIRO, 2010; FILIPAK NETO *et al*., 2007; SCHIEBER; CHANDEL, 2014; VINAGRE *et al*., 2012; WAFA *et al*., 2012). In fish it was seen that the level of PCO was similar to the control showing that the reparation mechanism might be working efficiently at protein level.

Regarding the AChE activity (E+=-1.219; 95% CI=-1.533 to -0.905), it was seen an inhibitory action when 2,4-D is present in all groups (Fig 5G). That inhibition can generate locomotor defects such as lethargy or difficulty in swimming, as observed in fish (CATTANEO *et al*., 2008).

### 2.4.4 Effect of Age on Enzyme Activity

The levels of CAT and SOD (E+= -0.023; 95% CI= -0.501 to 0.454 for SOD, E+= 0.229; 95% CI= -0.236 to 0.696) showed no differences regarding the control even in adults and young's (Fig. 6A and B). Despite elevated CI in young's is high, it could be justified by the small amount of studies using young animals. It can be applied to all enzymes analysed here in relation to animal age, because low number of studies with small samples make the CI higher in meta-analysis (BORENSTEIN *et al*., 2009). That might be making the GM to be over the 0, so the age might not be a useful variable to determining if there is or not effect over the CAT and SOD activity. There are necessary more studies in young animals to see a real similitude or difference regarding the control, because it is widely reported the action of CAT and SOD to counteract the indirect effects of herbicides, such as ROS formation (DAVIES, 2000; HE *et al*., 2017; HSIEH; HSU, 2013; IGHODARO; AKINLOYE, 2018; MA; DENG; CHEN, 2017; WANG *et al*., 2018).

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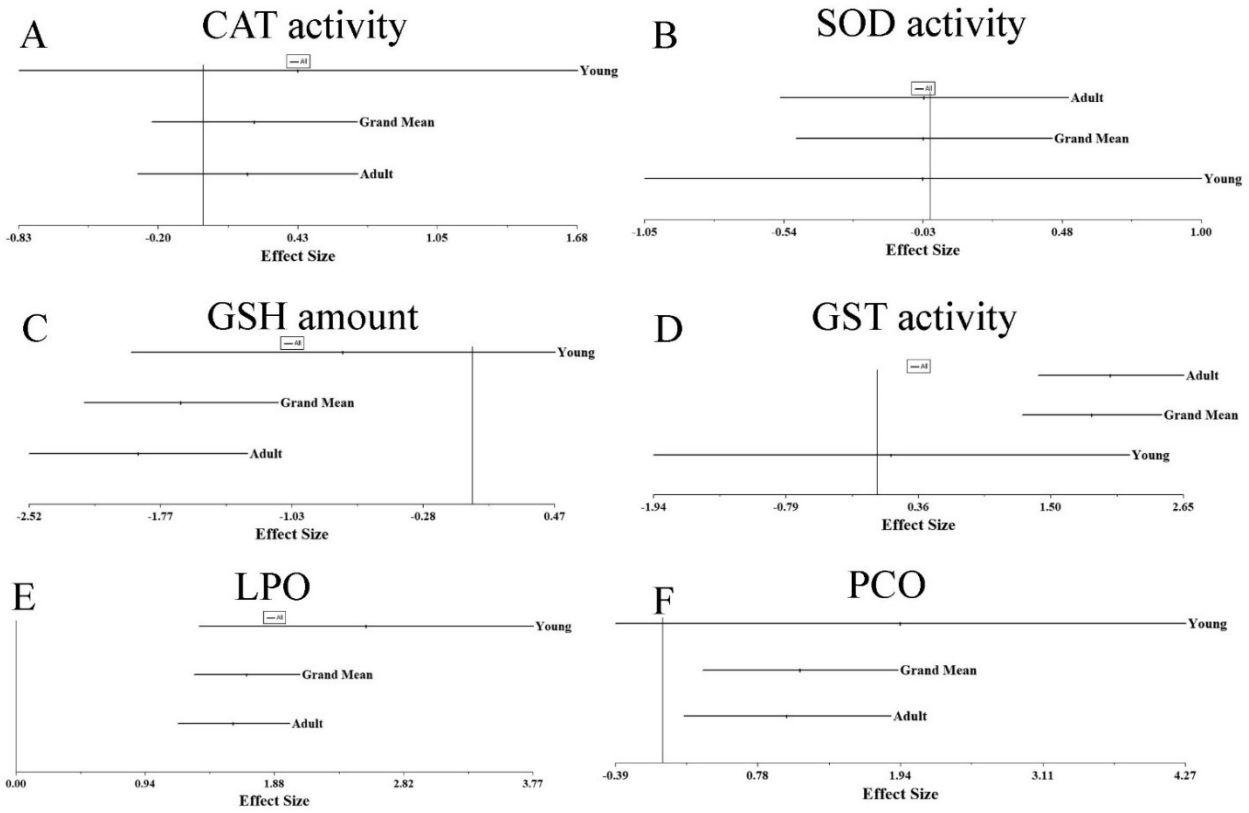
The role of GSH in young seems to be similar to the control when the individuals are exposed to the herbicide, however the amount of research using young animals was small, and push the mean values of GSH on the plot to over the 0. On the other hand, there were a huge number of research using adults, and it can be seen that the amount of GSH is reduced in presence of 2,4-D (Fig.6C). Although the low number of research using young animals, the GM is shown below 0 (E+=-1.656; 95% CI= -2.254 to -1.088). That could be because this molecule reduces the herbicide in order to make it a more degradable compound, that make it to be shown in a low level. Some research showed similar results in young but some other showed the opposite,

making the IC to be large. In this context, the function of this enzyme is the same in all the organisms and age independent, however studies in young animals are still scarce

**Figure 6. Forest plot representing the categorization by age of CAT (A), SOD (B), GSH (C), GST (D), LPO (E), PCO (F), in 2,4-D exposure**




**Source: The author**
**Note: The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.**

In the case of GST, in adults it is seen the opposite from what was seen in GSH (Fig. 6D). In them the activity of GST is increased in presence of 2,4-D. This could be because its function is to conjugate the GSH with the xenobiotic (LI *et al*., 2007), but also it has peroxidase activity over the lipid peroxide (LOOH) produced during the initial step of the LPO (DI GIULIO; MEYER, 2008), so its activity should be high (AGAR; MAEDEI, 1992). In the case of young animals, the activity of GST in the control and in the treatment is the same, however the CI is also elevated, and studies on 2,4-D effects on GST in young animals is also scarce.

As expected the 2,4-D cause damage in lipids and proteins regardless in adults (Fig. 6E), this suggests that the 2,4-D cause high levels of ROS in the organism. Just in the case of young it is not seen protein carboxylation, however the IC is large, so it

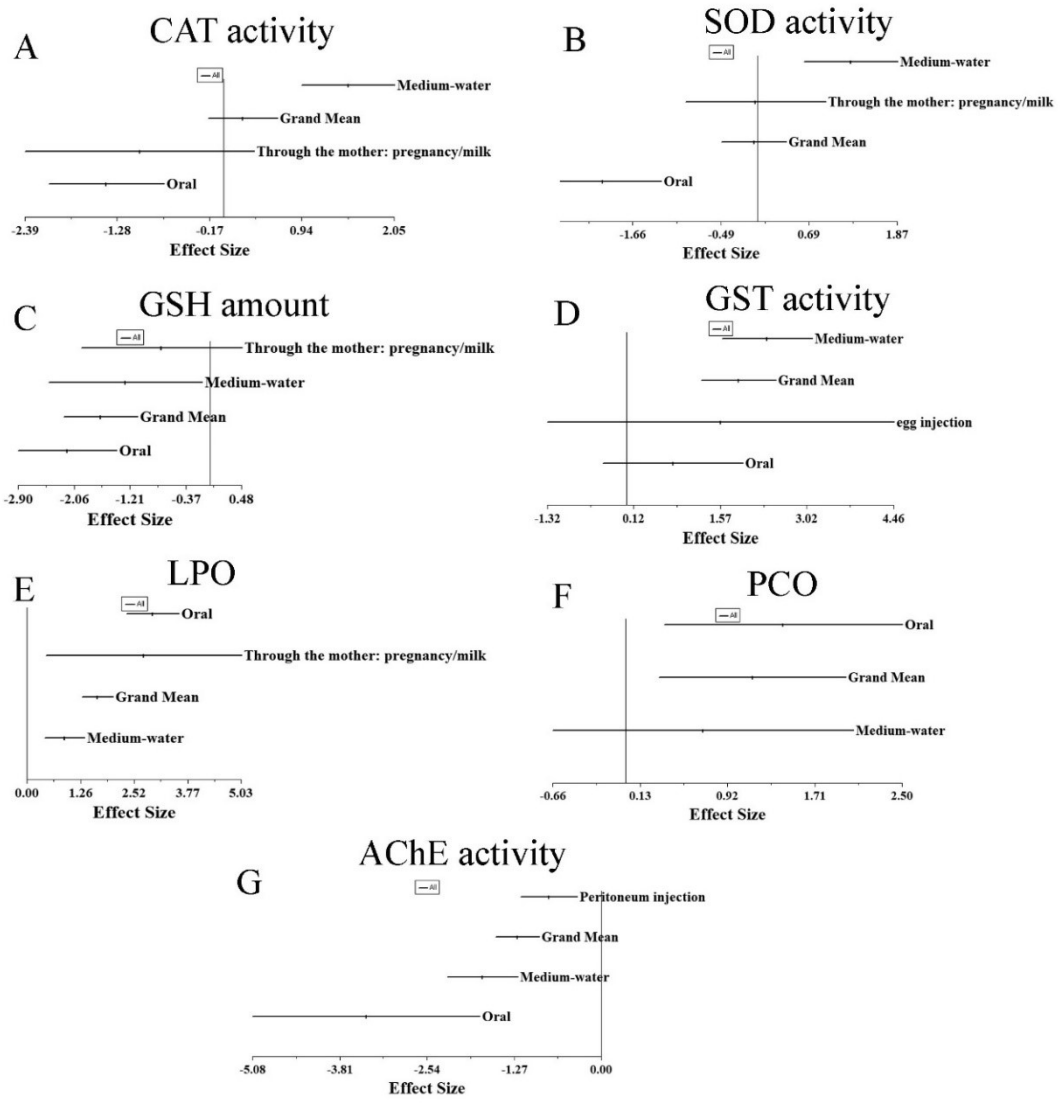
is suggested more studies. The effect of the age over the AChE activity could not be analysed because there were few records studying the effect of 2,4-D in young animals for this enzymes. The efficiency of the antioxidant enzymes reduce with the age because the ROS production increases (BAEK *et al*., 2016; RUDNEVA *et al*., 2010). White more studies reordering the changes in enzyme activity as the age rises, it is could be done a more robust analysis.

### 2.4.5 Effect of the Administration Route on Antioxidant Activity

The administration ways used in the different research were through gavage, by direct exposure in the water, through the mother, *i.e.*, in pregnant females, and by egg injection, however there are few research using these last two methods.

In studies that used the oral route, the results showed lower enzyme activity of CAT and SOD with respect to the control while in water exposure, the activity of this enzymes were elevated
(Fig. 7A and B). Similar result were found by GHISI; OLIVEIRA; PRIOLI, (2016), where the frequency of micronucleus was different in oral regarding the hydric exposure to glyphosate. This reflects that the administration route holds a pressure over the SOD and CAT activity.

**Figure 7. Forest plot representing the categorization by administration route of CAT (A), SOD (B), GSH (C), GST (D), LPO (E), PCO (F), AChE (G) in 2,4-D exposure.**




**Source: The author**
**Note: The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.**

The level of GSH is shown in decreased levels in animal exposed by oral and by hydric medium, as can be seen in the Fig. 7C where GM is below the 0. The individuals exposed to 2,4-D through the mother did not presented significate difference with the two control, however it is important to note the wide IC. Even although there were found different response regarding the administration route, the GM is below 0, what means the exposure route is not an important source of variation, especially because the number so records with different response is low.

The GM is above 0 in the case of GST of animals exposed to 2,4-D (Fig 7D), this could be because the presence of that herbicide make it increase to control the

effect of ROS. However, it can be seen that in research that used oral or egg injection administration the results show that the GST do not vary from the control, what could be because in some studies the basal level of it was enough to fulfil its function.

The administration route has no influence on the lipid peroxidation, because as can be seen in the Fig. 7E. Although there were studied different routes, the lipids are always harmed in presence of 2,4-D. While the GM of PCO is similar to that found in LPO (Fig. 7F), there were some research that report no damage over protein when used the water as a mean of administration. Those might be the case of some aquatic animals were the damage might have been repaired efficiently (PACIFICI; DAVIES, 1991). Even those are few studies, that could be the cause why it brackets the null. The administration route has no effect over the inhibition that 2,4-D has over AChE, what reaffirms the negative effect it has (Fig. 7G).

2.4.6 Effect of Organ of Study on Antioxidant Activity.

The liver is the main detoxifying organ (BAEK *et al*., 2016), however some other organs might have similar action since all cells have a detoxification system. According to the Grand Mean there is no difference between the levels of CAT and SOD when compared to the control in liver (Fig. 8A – B). This could be due to the fact that rodents could be much more susceptible to the presence of 2,4-D, while the activity in fish increases in presence of herbicide, making the GM appear over 0. The inactivation of CAT in rodents results critic due to de facts that in similar mammals like humans, it is reported that certain organs have limited capacity to remove hydroxide peroxide (HALLIWELL; CLEMENT; LONG, 2000). Most or all human cells are expose to some level of $H_2O_2$, with the mitochondria being an important source, however certain tissues may be exposed to higher $H_2O_2$ concentrations because of the limited capacity to remove it. The hydrogen peroxide can produce hydroxyl radicals (OH*) what might react and inactivates or disrupts proteins, lipids, DNA, and RNA (CEDERBAUM, 2017).

In organs such as brain and heart the activity of CAT and SOD is similar to the control. This could be because they are not the main organs involved in the detoxification process. In the case of the kidney, that also participate in the detoxification process (KIEFFER; MARTIN; ADAMS, 2016), both enzymes are inhibit

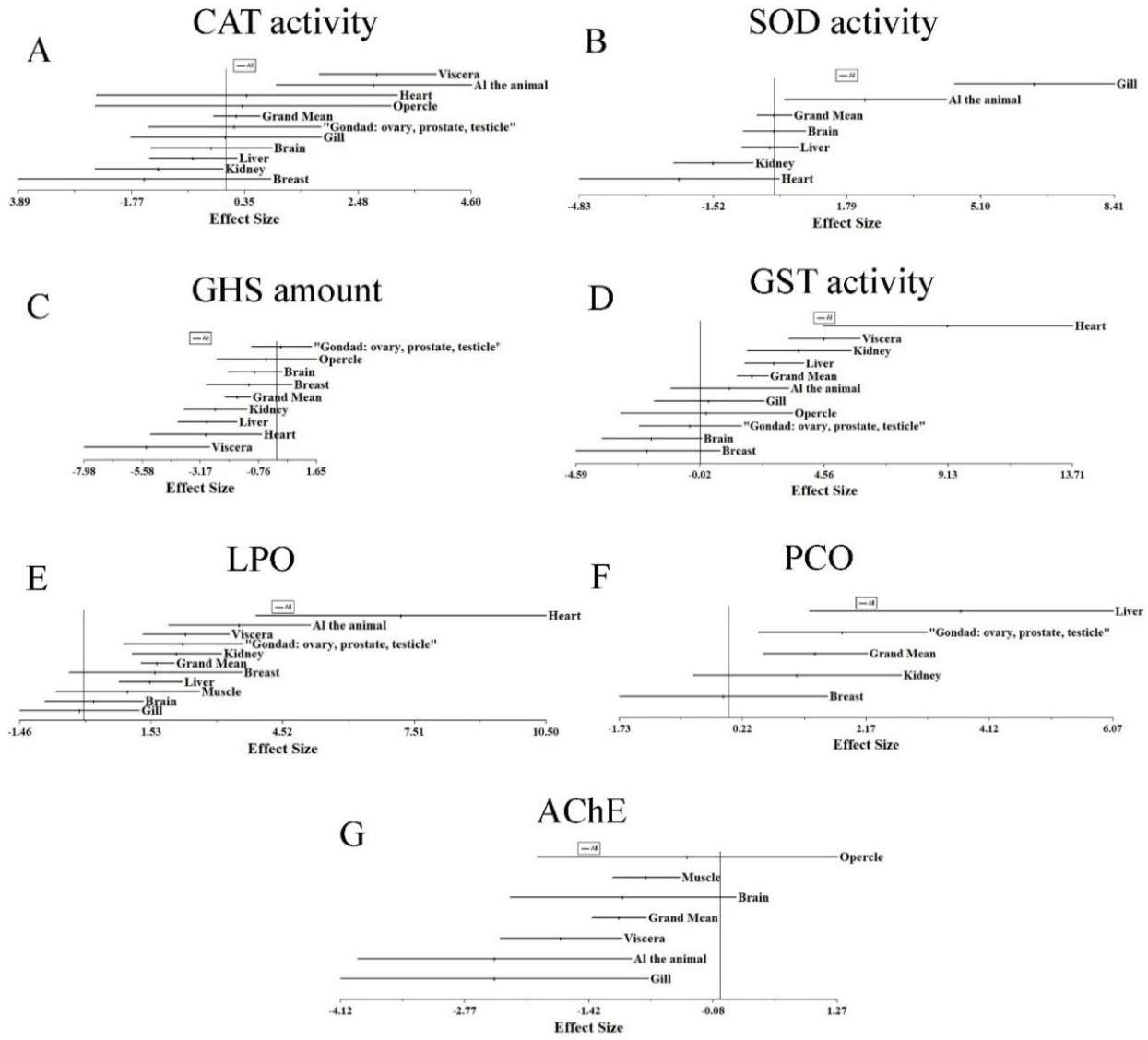
in presence of the herbicide, what might be dangerous for the survival of the animal. In the case of the gill, the SOD activity increases while the CAT activity do no change comparing to the control, however is important to note the large IC for both enzymes in gill. It could be because activities of certain biomarkers in determinate fish species are more sensitive to pesticides than in other fishes (OZCAN ORUC; SEVGILER; UNER, 2004). In the case of breast and opercula, the activity of both enzymes is inhibited, even so is clear the IC is also large, so there are necessary more studies in this organ.

The level of GSH in liver, kidney and heart in animals exposed to the xenobiotic is low, while in the same organs the level of GST is high regarding the control (Fig. 8C – D). This could be because the fact that liver and kidney are the main organs of detoxification so the consumption of GSH and activity of GST should be higher in them. The fact that the heart showed the same pattern, could compromise organism survival (MÜNZEL *et al*., 2017). In other organs like brain, gill, breast, gonad or opercle, the activity did not differ from the levels found in the control.

The Fig. 8E and F show that the 2,4-D causes LPO and PCO increase in most organs. This may be suggested from the GM value which is above 0. Just in the case of breast and kidney (to PCO) and breast, muscle, brain and gill (to LPO) there was not reported damage over protein or lipids. This could be because they are not part of the main organs of detoxification. The only one organ that showed a different response from what was expected was the kidney. In this organ the level of PCO did no differ from the control. A possible reason for that could be that the damage could have been repaired (NITA; GRZYBOWSKI, 2016; PIZZIMENTI *et al*., 2010).

In the case of AChE, in the most of the organ the enzyme is inhibited (Fig. 8G). Although in the case of the brain that is the main centre of neurotransmitters it is shown that the level of the enzymes in individuals exposed to 2,4-D does not differ from the control, that could be due to the fact that in some animals or after the exposition to certain doses, the effect over it can be controlled or repaired (NITA; GRZYBOWSKI, 2016). Similar results were found in opercula, however the IC is large, making it necessary more studies to make this result more robust.

**Figure 8. Forest plot representing the categorization by organ studied of CAT (A), SOD (B), GSH (C), GST (D), LPO (E), PCO (F), AChE (G) in 2,4-D exposure**




**Source: The author**
**Note**: **The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.**

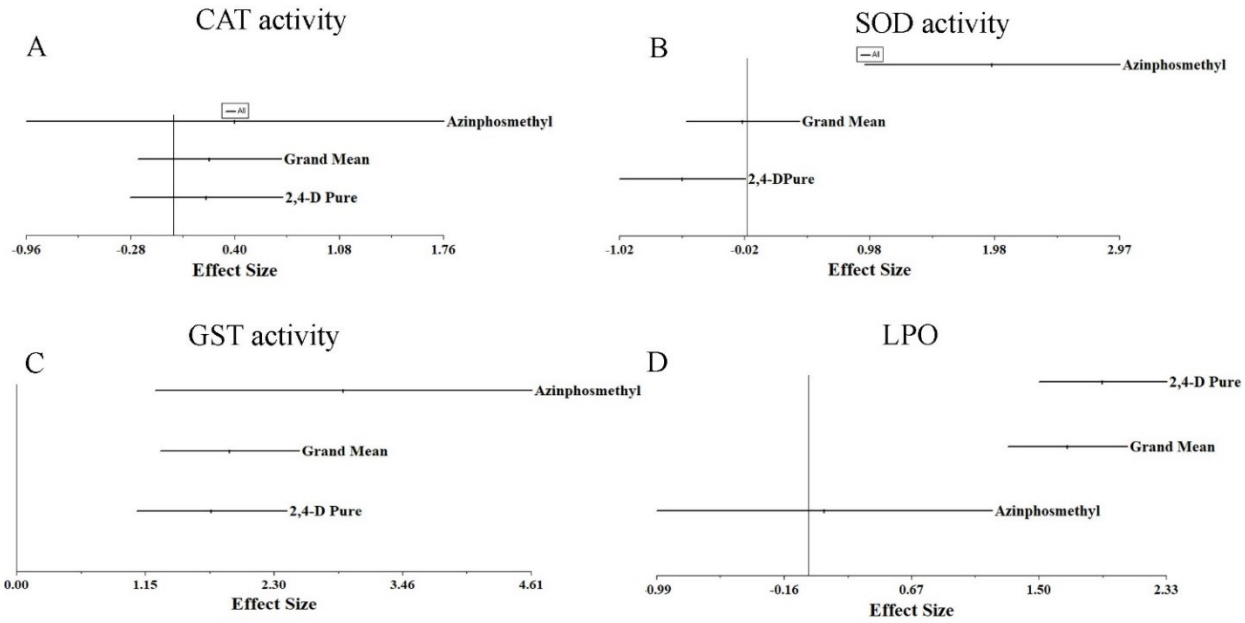
### 2.4.7 Effect of the Mixture with Azinphosmethyls

The 2,4-D is a chlorophenoxy compound (BURNS; SWAEN, 2012) while the Azinphosmethyls (Azp) is a organophosphate agent. The Azp is one of the most persistent organophosphate insecticide with a half-life of almost a month in aquatic environment (CASTRO *et al*., 2017) while the 2,4-D has a short half-time that goes from 1.5 to 16 days (DEHGHANI; NASSERI; KARAMIMANESH, 2014). They are

usually used in combination in order to attack weed and insects in the same time, however they have also effect over non-target species (COSSI *et al*., 2018), at immune (CASTRO *et al*., 2017) and enzymatic level (ORUÇ; ÜNER, 2000).

According the analysis, when the 2,4-D is applied in combination with Azinphosmethyls or alone the activity of CAT does no differ from the control (Fig. 9A), so even the presence of this other compound, the action of it remain at basal level. On the other hand, the action of SOD is inhibiting when 2,4-D is applied alone, while the presence of Azinphismethyls make it to increase (Fig. 9B). This shows that the combined treatment exerted synergistic effects (ORUÇ; ÜNER, 2000), producing a fast adaptive mechanism making the SOD activity to increases. Even so, in the plot of both enzymes the GM is over 0. It can be say that the activity of CAT does no change in presence of 2,4-D pure or in combination with AZP, however the fact that GM in SOD is over 0, could be because the opposite response between the action of 2,4-D in pure and in combined application.

**Figure 9. Forest plot representing the categorization by the mixture of 2,4-D with Azinphosmethyls or not for CAT (A), SOD (B), GST (C), LPO (D) in 2,4-D exposure.**




**Source: The author**

**Note: The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.**

The activity of GST increases in presence of 2,4-D pure or in combination with Azinphosmethyls. This could be because the cell recognizes the presence of the

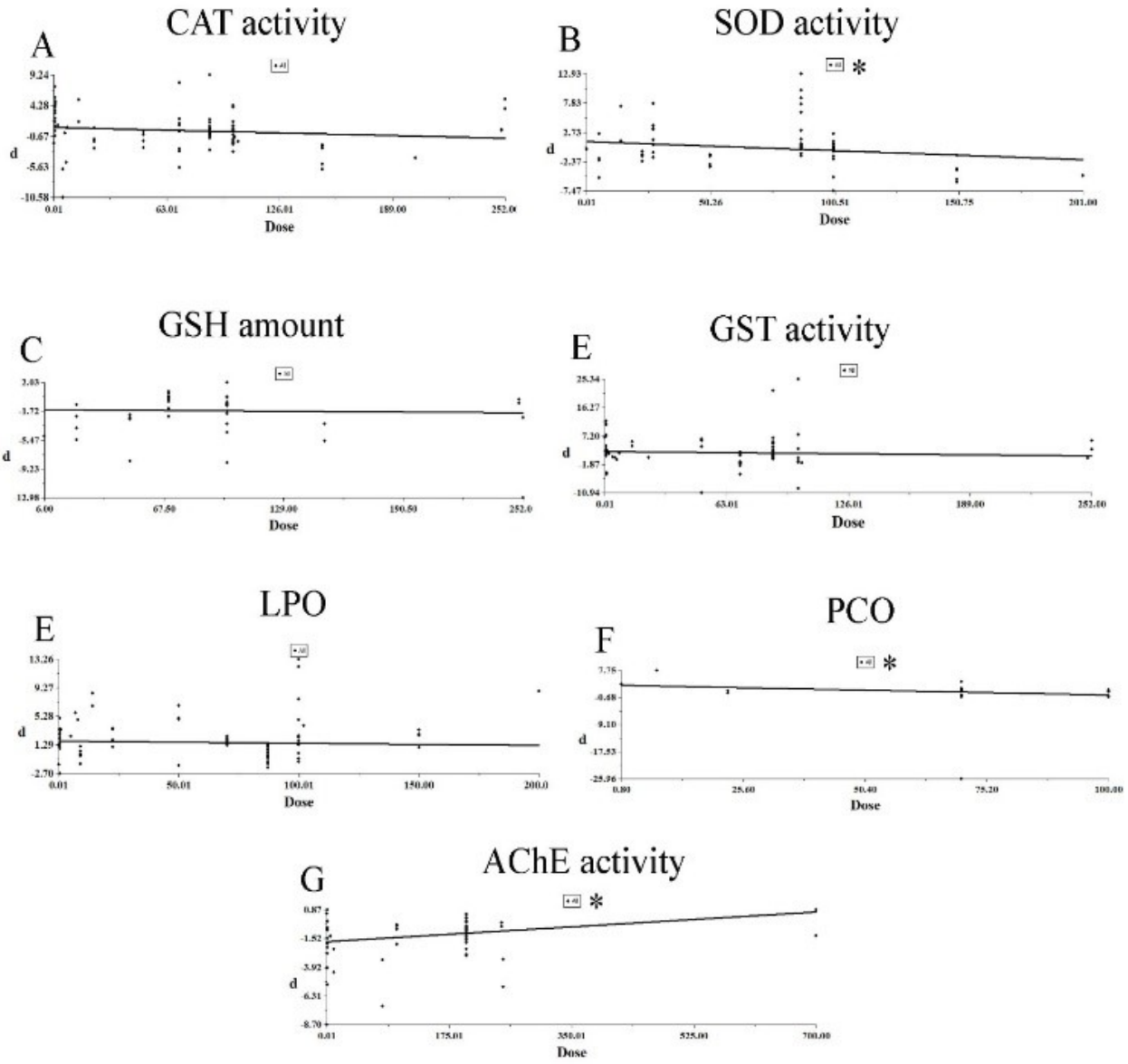
xenobiotic regardless it is pure or in combination with AZP making the GST to increase, in order to be in enough amount to be used for the GSH mediated hydrolysis (AGAR; MAEDEI, 1992). As the SOD level increases in animal exposed to 2,4-D in mixture, the level of LPO is better controlled, that's is why the level of it is lower when this mixture is used. On the other hand, the application of 2,4-D alone makes increase the level of PCO, what might confirm the effect of the herbicide over proteins. Even so, it can be seen that the CI is large in AZP in all the cases, what means there are necessary more studies were 2,4-D is applied in combination. The analysis in AChE, PCO and GSH could not be done because of the low amount of studies done over them in analysis using this mixture.

### 2.4.8 Effect of Concentration over the Antioxidant Activity

The dose used in all the studies ranged from 0.01 to 700 mg/L and from 5 to 202mg/ Kg, and from 0.5 to 200 mg. No significant change is seen in the level of CAT, GSH, GST, regarding the doses (Fig. 10A, C and D). This could reflect that despite being exposed to different doses of 2,4-D, these enzymes try to counteract the effects of the herbicide and secondary compounds. Likewise, the levels of LPO is controlled despite applying high concentrations of 2,4-D (Fig. 10E). The only enzyme that shows a differential pattern was SOD. The SOD activity is inversely proportional to 2,4-D concentration (Fig. 10B). The main function of SOD is to catalyse the reaction of reduction of the superoxide anion to water-soluble $H_2O_2$ (NORRIS *et al*., 2009). Indirectly this allows to maintain the level of GSH, that is why GSH levels may not vary with concentration. In addition, SOD has the ability to generate an early adaptive response to oxidative stress (SLANINOVÁ *et al*., 2009), that might justify why the levels of GST and CAT are maintained.

Regarding the AChE, its increase correlated directly with the 2,4-D concentration (Fig. 10G). This may be because of the adaptive capacity of the antioxidant enzymes (BAGNYUKOVA; STOREY; LUSHCHAK, 2005). Although the dose might be high, the organisms tries to counteract the action of it, increasing the level of AChE so as the organism could survive. Since the action of the AChE is important for the body locomotion, the survive of the animal might be affected since damage over it might difficult the capacity to look for the food (HOLSCHNEIDER *et al*., 2011).

**Figure 10. Regression Graphic showing the relationship between the effects size (d) and the doses used in 2,4-D exposure for CAT (A), SOD (B), GSH (C), GST (E), LPO (E), PCO (F) an Ache (G).**



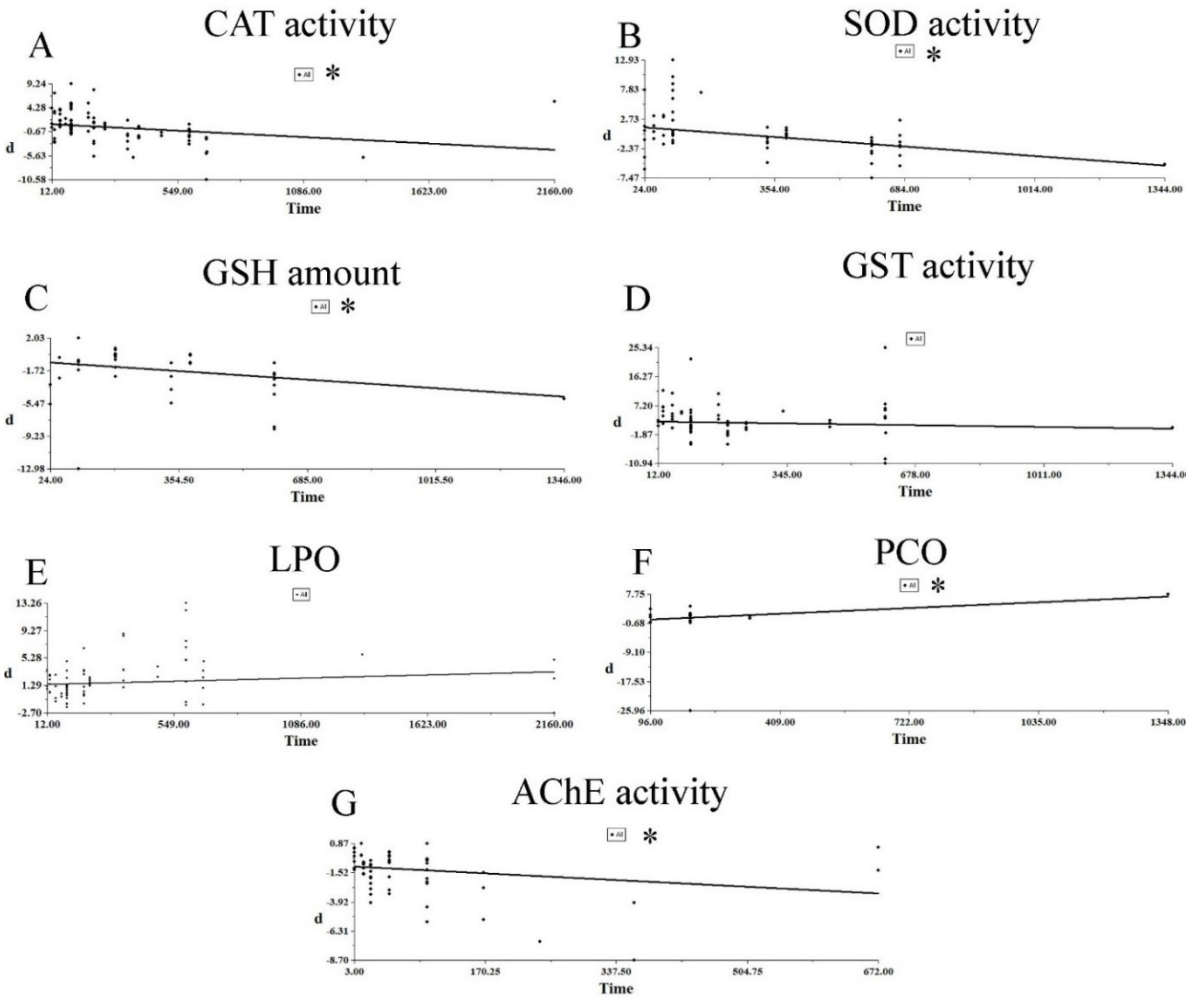

**Source: The author**
**Note: The asterisk represents the slope with p<0.05 of the effects size.**

## 2.4.9 Effect of the Exposition Time over the Antioxidant Activity

As can be seen in the Fig. 11A – C, the longer the exposure time, the higher effects are found in CAT, SOD, *i. e*., their activity decreases, likely the amount of GSH drops. The GST, and LPO was not affected by time but PCO was proportionally increased and AChE was inversely decreased. As it is known if an effects at the enzymatic level is not repaired, or in case it occurs during vulnerable periods of the

organism's development (such as reproduction), higher levels of the organism's internal organization can be affected (DAVIES, 2000; FRÄNZLE, 2003).

**Figure 11. Regression Graphic showing the relationship between the effects size (d) and the exposition time used in 2,4-D exposure for CAT (A), SOD (B), GSH (C), GST (E), LPO (E), PCO (F) and AChE (G).**




**Source: The author**
**Note: The asterisk represent slope with p<0.05 of the effects size.**

This problem in enzymes during in chronic exposure can have an impact on the survival of the individual. In addition, if this effect is seen in several individuals, higher levels of biological organization of living beings such as communities and populations may be affected (FRÄNZLE, 2003). So while the dose of 2,4-D to which any individual is exposed (up to 700mg / L, 202 mg / Kg or 200 mg, as found in this research) can be controlled in a short time, if exposure maintained for prolonged periods the antioxidant capacity may be affected.

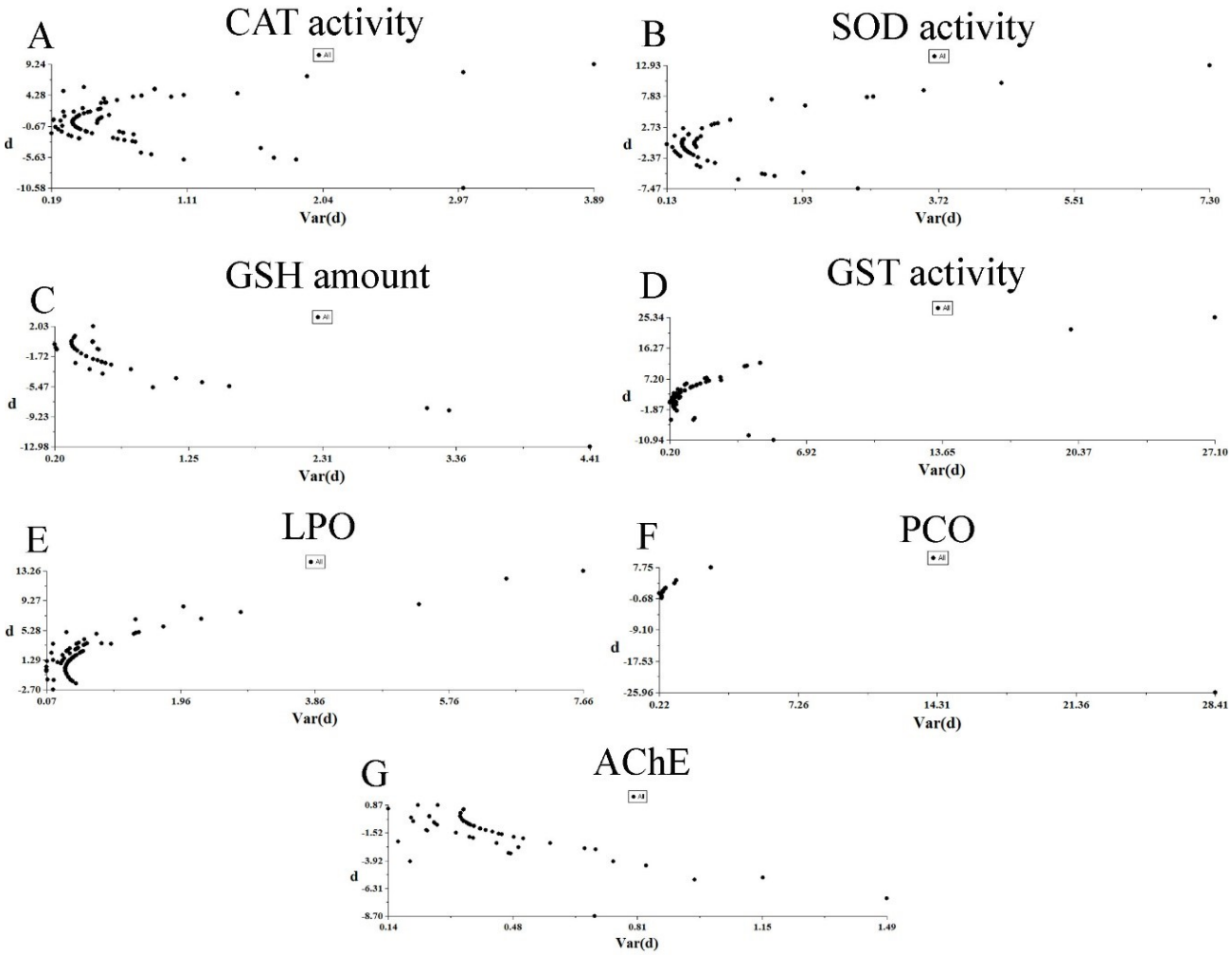
### 2.4.10 Publication bias

In a general way, there is a shape of funnil in the plot of CAT and SOD (Fig. 12A and B). These result coincide with the results given by the Spearman rank correlation coefficient (effect versus sample size) and Kendall's Tau, two statistic method to test publication bias. Both method did no revealed a significant correlation for CAT (0.191 and 0.294 respectively) and for SOD (0.204 and 0.240 respectively) (Table 1) indicating there was no publication bias.

On the other hand, there is no clear asymmetry in the funnil plot of LPO, PCO, GSH, GST and AChE (Fig. 12C - G). This correlates with the results given by the Spearman rank correlation and Kendall's Tau methods, that showed significant correlation (Table 1). That means there were publication bias. The failsafe number were 2360.7, 5927.1, 12779.9, 177.8, 7266 (by Rosenthal's Method) respectively. However according to Orwin's Methods the missing studies that would need to be added to meta-analysis to change the results from significant to no significant are 0, 249.6, 284.8, 55.8 and 0 respectively. As these numbers are larger in comparison to the number of observed studies, the observed results can be treated as a reliable estimate of the true effect.

Nevertheless, in the case of the results obtained for CAT and SOD are highly reliable since it is reports no publication bias, and Rosenthal's and Orwin's Methods determinate few or null amount of articles to be added to the meta-analysis (Table 1)

**Figure 12. Funnel plot showing the data distribution of the correlation between the effect size (d) and the variance D.**




**Source: The author.**

Note: **The asterisk represent slope with p<0.05 of the effects size.**

**Table 1. Effect size *vs.* Variance using Kendall's Tau and Spearman Rank- Order Correlation Methods in the study of CAT, SOD, GSH, GST, LPO, PCO and AChE.**

| RANK CORRELATION (Effect vs. Variance) | CAT | SOD | GSH | GST | LPO | PCO | AChE |
|---|---|---|---|---|---|---|---|
| | | | **Kendall's Tau** | | | | |
| **Tau** | 0.094 | 0.104 | -0.590 | 0.489 | 0.519 | 0.516 | -0.607 |
| **Z** | 1.307 | 1.269 | -5.359 | 6.077 | 6.992 | 2.992 | -7.706 |
| **Prob** | 0.191$^{ns}$ | 0.20$^{ns}$ | 0.000** | 0.000** | 0.000** | 0.003** | 0.000** |
| | | | **Spearman Rank-Order Correlation** | | | | |
| **Rs** | 0.112 | 0.142 | -0.763 | 0.603 | 0.662 | 0.571 | -0.700 |
| **Prob** | 0.294$^{ns}$ | 0.24$^{ns}$ | 0.000** | 0.000** | 0.000** | 0.013* | 0.000** |
| | | | **FAIL-SAFE NUMBERS** | | | | |
| **Rosenthal's Method** | 181.700 | 0.000 | 2360.700 | 5927.100 | 12779.900 | 177.800 | 7266.000 |
| **Orwin's Method** | 0.000 | 0.000 | 0.000 | 249.600 | 284.800 | 55.800 | 0.000 |
| **Amount of studies used for the analysis** | 90 | 70 | 40 | 72 | 84 | 16 | 75 |

**Note: The fail-safe number represent the amount of research needed to reduce the publication bias, for that were used the Rosenthal's and Orwin's methods. *= singificative difference <0.05, **= singificative difference <0.01, ns= no significant differenc2**

Even comprehensive searches of the published literature and all other sources of available data may not produce an unbiased sample of studies when conducting a meta-analysis. Statistically significant results are potentially more likely to be submitted, published, or more rapidly accepted than studies with null or no significant outcomes. This problem is known as publication bias (TATSIONI; IOANNIDIS, 2017). Although the bias is also a major threat for the meta-analysis, these systematic review offers a unique opportunity to examine the consistency of definitions and completeness of data reporting for specific results (TATSIONI; IOANNIDIS, 2017).

## 2.5 CONCLUSION

The effect that 2,4-D has over the animals is different in according the specie. The oxidative stress is clear in mammals where the activity of CAT and SOD is affected, while in aquatic animals those enzymes remained active, and also increase in presence of the herbicide. Even so, the effect over lipids, proteins and AChE is evident regardless the specie. The administration route is also an important factor when studding the effect of this xenobiotic over the body. There is a reduction in the activity of SOD and CAT when the trophic route is used, so this should be considered when measuring the negative effect of the 2,4-D.

When studding the oxidative damage that herbicides like 2,4-D have, the tissue used to analyse is relevant. While the activity of CAT and SOD is similar in most tissue, in kidney their response is reduced, so this tissue might be a candidate tissue its sensibility to this xenobiotic, however this sensibility must be studied, in order to know the marge of sensibility it has.

The age and administration route did not have effect over the antioxidant activity. Nevertheless, there are necessary more studies using young animals to get a more reliable results

On the other hand, both the dose and the exposure time are two important factors when planning an ecotoxicology research, as the time increases, the activity of the enzymes decreases. These results indicate that although in the short term there is no effect at the enzymatic level in spite of the doses, in case the exposure to this herbicide is prolonged there is a noticeable damage in the activity of CAT, SOD, GSH. The effect of 2,4-D over lipids and proteins increases as the time of exposition became longer

# 3. *IN VIVO* STUDY OF THE EFFECT OF 2,4-D THROUGH THROPIC ROUTE

## 3.1. ABSTRACT

All the cells have a detoxification system conformed for a series of enzymes such as SOD, GST, GST, as well as with molecules that allow this system to function, as the GSH.Any change in their activity might have repercussions on lipids (LPO), proteins (PCO), as well at neuronal level by inhibiting neurotransmitters (AChE). One of the most used herbicides is 2,4-D (2,4-dichlorophenoxyacetic acid) which would be associated with enzymatic damage. Within the species of fish considered bioindicators, *Rhamdia quelen* is one of them. Its one of the most important because it is highly sensible to different pesticides, but also due to the fact that it is an endemic brasilian specie. In most ecotoxicological research using *R. quelen*, it is used hidric exposition, however there no regist of the use of thropic administration in this specie. That is why the aim of this study was to evaluate the enzymatic activity in the fish *Rhamdia quelen* exposed orally to 20, 200 and 2000µg/kg during 22 and 42 days, as well as the effects at the level of LPO, PCO and AChE. No effect was found at enzymatc level within the treatments neither at 22 days nor at 42 days, however when compared the two exposure time it was seen a difference in the enzymatic response. This suggests that the change in the response is associated with the physiology and age of the animal more than to the 2,4-D exposure.

Keywords: herbicide, biochemical biomarkers, oral exposure, fish.

## 3.2 INTRODUCTION

During the physiological metabolism of living beings, biochemical reactions are continuously produced which generate reactive oxygen species (ROS) (FORRESTER *et al*., 2018). They are also produced during the metabolism of chemical pollutants to which most organisms are exposed as a result of anthropic activities. Generally, there is a balance between the rate of production and elimination of ROS. However an imbalance in the level of ROS, the normal metabolism of the cells can suffer alterations (MARTÍNEZ-ÁLVAREZ; MORALES; SANZ, 2005) and generate a phenomenon known as oxidative stress (DAVIES, 1995).

The generation of ROS in the body normally increases under physiological conditions, which is known as cellular “endogenous resources of reactive oxygen species” (DI GIULIO; HINTON, 2008). The main sources of ROS are the electron transport chain in the mitochondria, the endoplasmic reticulum, the function of cytochrome P450 and the performance of antioxidant enzymes during catalysis in animals (HALLIWELL; GUTTERIDGE, 1999). In fact, cytochrome P450 metabolizes a large variety of lipophilic compounds that may or may not have structural similarity.

The most common substrates of cytochrome P450 include endogenous metabolites (steroids, fatty acids, prostaglandins, etc.), and xenobiotic compounds (drugs, carcinogens, environmental contaminants, etc.) (ZIMNIAK; WAXMAN, 1993). This can generate accumulation of reduced intermediate compounds (STOLZE; NOHL, 1994), redox reactions, facilitation of the Fenton reaction, and activation of antioxidant enzymes, as well as depletion of free radical uptake (HALLIWELL; GUTTERIDGE, 1999). This can have an impact on the level of cell membranes causing lipid peroxidation, protein peroxidation, gene expression modulation, certain diseases (SLANINOVÁ *et al*., 2009).

The 2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most important phytosanitary products used in Brazil. Even it is an effective herbicide, it has also negative effects in non-target species. It was recently replaced from class 1 (Extremely Toxic) to in class 4 (Low Toxic Product – blue label), (ANVISA, 2019) although being persistent in acidic soils (MIERZEJEWSKA *et al*., 2019) and able to reach rivers and lakes. It has effects at molecular level increasing the frequency of micronuclei, what reflects its genotoxicity (RUIZ DE ARCAUTE; SOLONESKI; LARRAMENDY, 2016).

According to studies in *Clarias batrachus* evaluating the DNA damage by comet assays, this pesticide was associated with increase in DNA bankruptcy (ATEEQ; FARAH; AHMAD, 2005). Similar effect has been shown in human cells *in vitro* (SANDAL; YILMAZ, 2011) and in *Cricetulus griseus* ovary cells (GONZÁLEZ *et al*., 2005).

At neurological level, it causes a decrease in the activity of acetylcholinesterase (AChE) after 96 hours of exposure in the fish *Cnesterodon decemmaculatus*, an impaired locomotion of individuals (ES RUIZ DE ARCAUTE *et al*., 2019), feeding, escaping, and reproductive disability (BRETAUD; TOUTANT; SAGLIO, 2000).

Additionally, it has been reported that the *Pimphales promelas* larval survival is affected by the presence of 2,4-D exposed to 0.50 ppm (DEHNERT *et al*., 2018), as well as developmental and neuronal circuits disturbance in *Danio rerio* larvae exposed to 8 ppm (DEHNERT; KARASOV; WOLMAN, 2019).

The study of the toxic effects of 2,4-D is of great importance, not only because of the direct exposure can cause damage, as seen in farmers occupationally exposed to pesticides (MARCELINO; WACHTEL; GHISI, 2019), but also the indirect one. According to a study conducted in Brazilian ublic health agents exposed only occasionally to pesticides, DNA damage was evidenced (CRAVEIRO FRANCO *et al*., 2016). For this reason, the study of the 2,4-D effects on non-target species is of main interest.

Fish are non-target organisms to herbicides (in this case 2,4-D). However they are widely used as a bioindicator of water pollution (CHOVANEC; HOFER; SCHIEMER, 2003; NAIGAGA *et al*., 2011) since they are enough sensible to the presence of chemical agents (ARAÚJO *et al*., 2017). In addition, it is an important link between the environment and human populations because it is an important food resource, the study of its behaviour and modifications allow the analysis and make inferences in gene functions in mammals involved in toxicity mechanisms. This is explained by the duplication-degeneration-complementation model (DDC), which predicts that some genes in fish would be associated with several sub-functions in mammals such as humans, that is, those would be orthologous genes that allow us to understand the behaviour of species that belong to another kingdom but that share orthologous or even paralogs genes (CARVAN, 2007; POSTLETHWAIT *et al*., 2004).

Among the fish species considered bioindicators, *Rhamdia quelen* is of great importance in Brazil with high sensitivity (GODEFROID; CASSIO, 2008). In addition it is widely used in ecotoxicological studies in southern Brazil (AZEVEDO-LINHARES *et al*., 2018; BALDISSERA *et al*., 2018; COSTA *et al*., 2010). It is an endemic species of the genus, among 11 validated species in which, 8 are found in Brazilian territory, and it is the only species that is distributed in almost all the hydrographic areas of the country (MARTINEZ *et al*., 2011).

When fish is exposed to a stress situation caused by biotic or abiotic agents, triggered three response stages: alarm, resistance and compensation or exhaustion (death), which are necessary for the fish to overcome severe challenges and if it is possible to restore homeostasis (SCHRECK; CONTRERAS-SANCHEZ; FITZPATRICK, 2001). The alarm phase consists of the positive regulation of the systems involved fighting and coping the damage. During the resistance stage, the fish can completely overcome the stressor, which allows the restoration of homeostatic norms, or it can overcome the stressor enough to allow it to recover (compensation), or in last instance it can start a pathway that leads to death (SCHRECK; TORT, 2016). According to Holden 2000, very low levels of stress are really adaptive, while higher levels of stress (distress) have maladaptive or adaptive elements (HOLDEN, 2000).

One efficient way to measure how environmental chemical compounds activate these response mechanisms in non-target species is through the use of biomarkers. It is important to understand that a biomarker allows verifying the effects of a stressor at the sub individual level or at most at individual level. By contrast the individual would be considered a bioindicator when seeking to evaluate alterations at higher levels (population level, or at the ecosystem level) (HANSSON, 2008). The study of certain biomarkers allows us to see the response in a short period of time, and predict early warning signs.

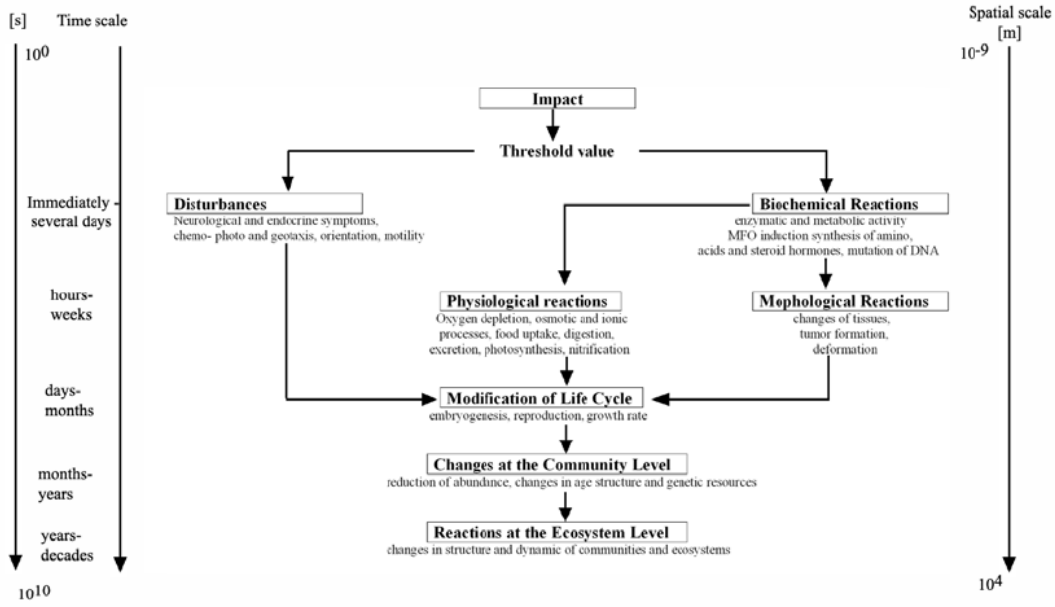
The effects of stress are manifested at low levels of the biological organization, long before reaching the level of population, community or ecosystem. These effects are observable at molecular level, with the induction of biochemical mechanisms of cellular defense, which can generate an adaptive response after exposure (PARENTE; HAUSER-DAVIS, 2014). If this first stage of defense fails, or is oversaturated, damage to higher levels can be generated, causing deterioration at histological, or physiological level, which cannot be reversible (depending on the damaged organ or system) (SCHLENK, 1999). If these processes are permanently affected or altered during

vulnerable periods of the organism's development (example: during reproduction) the survival will be affected, disturbing higher levels (example: population, community) (SCHLENK, 1999). That is, damage to the highest levels of the hierarchy of organizational levels is always preceded by changes in biological processes, so that there are signs of effects on early warning biomarkers before reaching subsequent response levels such as shown in Fig. 13.

In aerobic organisms, the first stage of defense is performed by molecules as vitamin C and E, uric acid, and glutathione (GSH), as well as antioxidant enzymes that prevent the cascade of oxidative reactions, intercepting and inactivating reactive oxygen intermediates. Together, they form the "primary antioxidants". The objective of the vitamins and GSH is to control the toxicity of ROS through its uptake (MODESTO; MARTINEZ, 2010), and generate more water-soluble compounds from xenobiotic due to the action of GST (glutathione transferase). On the other hand, the enzyme molecule system, in addition to having ROS detoxifying activity, also fulfills the adaptation function, and it is formed by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and GST (EL-BELTAGI; MOHAMED, 2013).

SOD protects against oxidative damage by the superoxide anion reduction reaction catalysis to $H_2O_2$ which is then degraded by CAT to $H_2O$. GPx reduces both hydrogen peroxide and hydroperoxides. GR maintains the GSG / GSSG ratios, it is the main regulatory enzyme that regenerates GSH from GSSG. GST plays a fundamental role in detoxification as it catalyses the conjugation of the GSH tripeptide with endogenous toxic metabolites and environmental pollutants, making them more water soluble (NORRIS *et al*., 2009; OTTO; MOON, 1996).

**Figure 13. Stress response time of biotic systems related to the magnitude of exposure and complexity**




**Source: modified from FRÄNZLE (2003) in KORTE & BAHADIR (1992)**

The activities of these antioxidant enzymes are used as biomarkers, being specifically "biomarkers of oxidative stress". However, the oxidative stress caused by the imbalance of ROS can cause damage which can be measured studying "biomarkers of damage by ROS" Determining the effects at the membrane level by the assessment of lipid peroxidation (LPO) and protein carboxylation (PCO) is an example of the second type of biomarkers (SLANINOVÁ *et al*., 2009). The damage can also be measured at the level of neurotransmitters, such as acetylcholinesterase (AChE), which is of great importance for the higher levels of biological organizations. Disturbance at AChE level can cause instability due to problems in its locomotion and balance (BRETAUD; TOUTANT; SAGLIO, 2000), this in turn hinders their ability to capture or search for food, putting organism survival at risk.

According the literature these enzymes are used as biomarkers of contamination, since the use of biomarkers increases the possibility of identifying the causes behind the toxic effects and providing information on the bioavailability of a contaminant and its potential ecological damage (ALBERTSSON *et al*., 2007). Indeed, in studies of fish exposed to environmental contaminants such as heavy metals (SEVCIKOVA *et al*., 2011), organochlorine pesticides (SULFATH; SANKAR; NANDAN, 2013), organophosphates (ABHIJITH; RAMESH; POOPAL, 2016;

MATVIISHYN *et al*., 2014; THOMAZ *et al*., 2009), and carbamates (ATAMANIUK *et al*., 2014), there are changes in the patterns of these enzymes making them good biomarkers of contamination.

Although the abiotic factors can generate ROS, there are biotic factors that can also cause an imbalance, which will have an effect on the antioxidant activity of the fish, and oxidative stress can be achieved if the system fails to control this flow of ROS to avoid oxidative stress (DAVIES, 1995). So the presence of a xenobiotic does not necessarily ensure harmful effects (VAN DER OOST; BEYER; VERMEULEN, 2003). In fact, it is shown that factors such as phylogenetic position, age (that is, it is in the stage of eggs, larvae, juvenile or adult) (RUDNEVA *et al*., 2010; ZENGIN *et al*., 2015), eating behavior, nutritional factors (MARTÍNEZ-ÁLVAREZ; MORALES; SANZ, 2005) and intracellular sources of ROS as a result of metabolism would cause fluctuations in the activity of the antioxidant enzymes.

Knowing the importance of the study of *Rhamdia quelen*, and how the biotic and abiotic factors to which it may be exposed generate might cause an imbalance in the protective enzymes this study had as objective the study oxidative stress in jundia exposed to 2,4-D. To resolve this objective, it was assessed the level of the main antioxidant enzymes (SOD, CAT, GST, AChE) and the effect on the level of non-enzymatic molecules, that also participate in the detoxification process as reduced glutathione (GSH), carboxylic proteins (PCO) and lipoperoxidation (LPO) in Neotropical fish *Rhamdia quelen* exposed to 2,4-D by trophic route.

## 3.3 METHODS AND MATERIALS

### 3.3.1 Bioassay

The bioassay was done at Laboratory of Fish Nutrition and Health of the Universidad Technological Federal do Paraná (UTFPR). The enzymatic evaluation was done in the Cellular Biology Laboratory of the Federal University of Paraná - Curitiba. This research was approved by the Ethics Committee on Animal Use (CEUA) of UTFPR (protocol 2016-18 – ANEXO 1)

The individuals of *Rhamdia quelen* (mean ± SD length, weight) were acquired in a commercial aquaculture farmer (Universidade Estadual do Paraná Pisciculture,

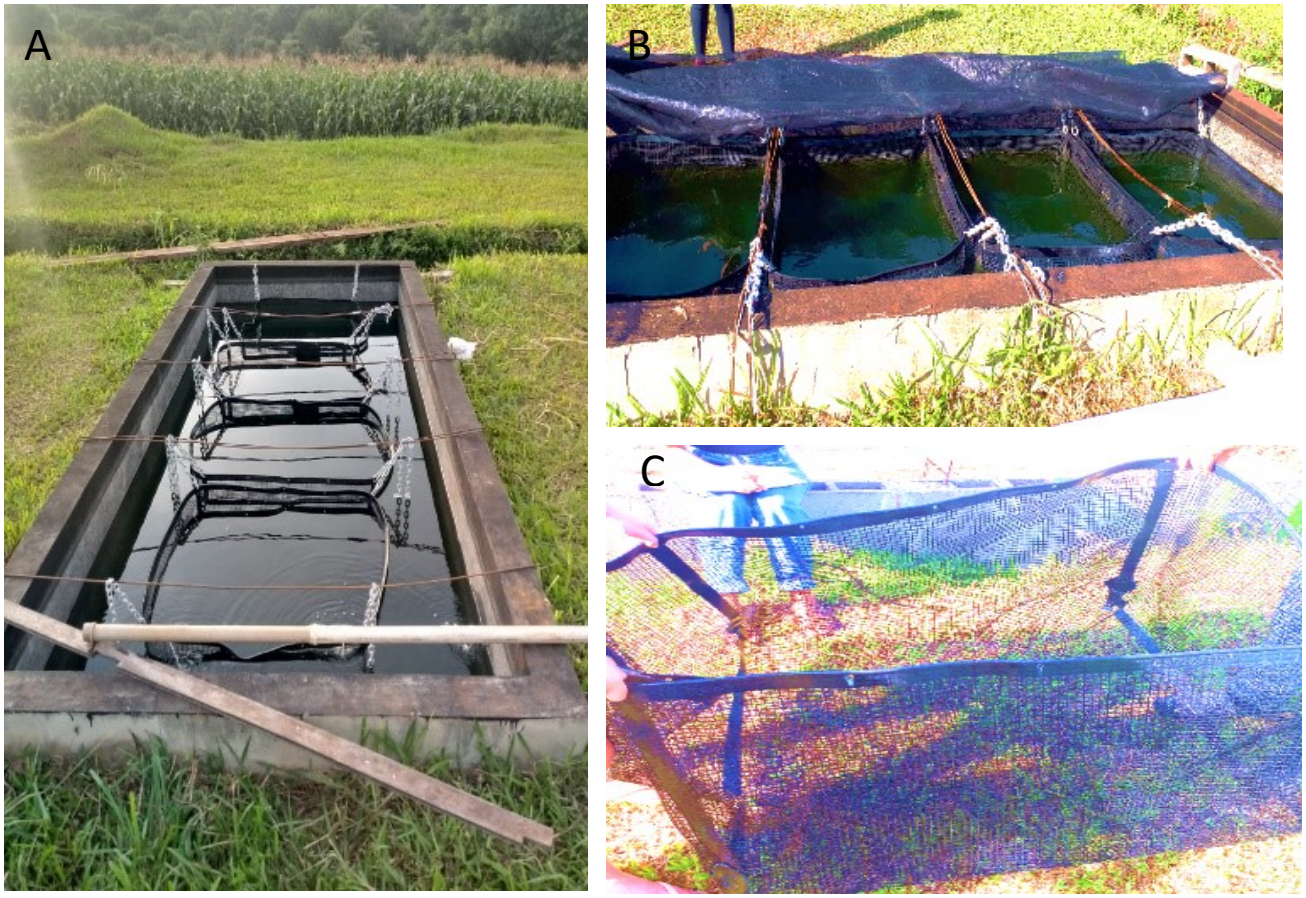
União da Vitória) and transported in aired bags to the UTFPR. As soon as the animals arrived, they were placed in circular polyethylene tanks, and exposed to a bath of iodized sodium chloride in order to control the possible stress caused by the transport (Fig. 14). The animals were all young our young adults (size range between 13 and 18 cm.

**Figure 14. The bucket with sodium chloride, where the fishes were placed to counteract the transporting stress**

**Source: The author, 2019.**

To install the system, 16 net tank flexible bags were used (Sansuy®) (one for each treatment), made of PVC of 1 x 1 x 0.6m (length, width, depth, respectively) (Fig. 15A-C). The rectangular net tanks were previously washed and installed, using wire harnesses and metal wires to separate each treatment (Fig. 15). Mesh cover were used to protect the net tank bags from the light during the day.

**Figure 15. Picture of the net tank used to separate each treatment (C) and system installed (A**

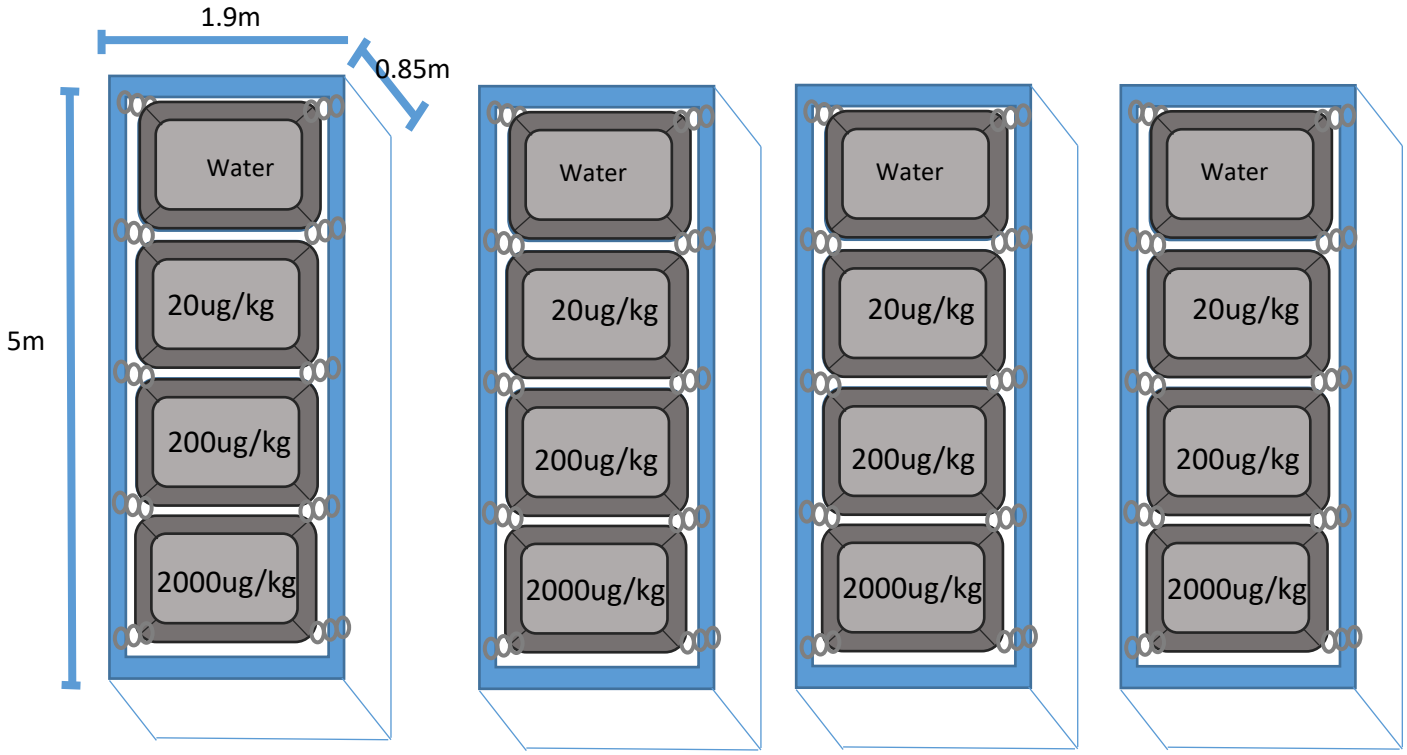



**Source: The author**

### 3.3.2 Experimental design

After the arrival and sodium chloride bathing, the individuals were distributed in the net tank bags (in order to separate each treatment) in four outdoor tanks (5.0 x 1.9 x 0.85 m) with a capacity of 8,075 L. The experiment was done in quadruplicate and 4 treatments: Control treatment (Water), treatment with lower dose (20µg/Kg), treatment with medium dose (200µg/Kg) and treatment with high dose (2000µg/kg), as it can be seen in the fig 16.

**Figure 16. Scheme of the 4 treatments (Control, 20ug/kg, 200ug/kg, 200ug/kg) and the 4 replicates used in the study.**




**Source: The author.**

Each net tank had 23 individuals which were kept under observation and acclimatization for 14 days before starting the experiment. The animals were fed with commercial diet (32% crude protein) twice a day until apparent satiation (during the morning between 7 and 9 a.m., and during the afternoon between 5 and 7pm), leaving a time lapse of 45 minutes for them to eat. After that, the rest of the food was removed. After that, the rest of the food was removed. After the first feed of the day 5kg of sodium chloride per tank were placed, while the measurement of the parameters of temperature (T°), pH, oxygen(O2), nitrite (NO2−) and ammonium ($NH_4^+$) was done twice a week. The tanks were kept covered with meshes in order to generate a dark environment, since the Rhamdia quelen ideal habitat is in dark environments after feeding and after measuring the parameters.

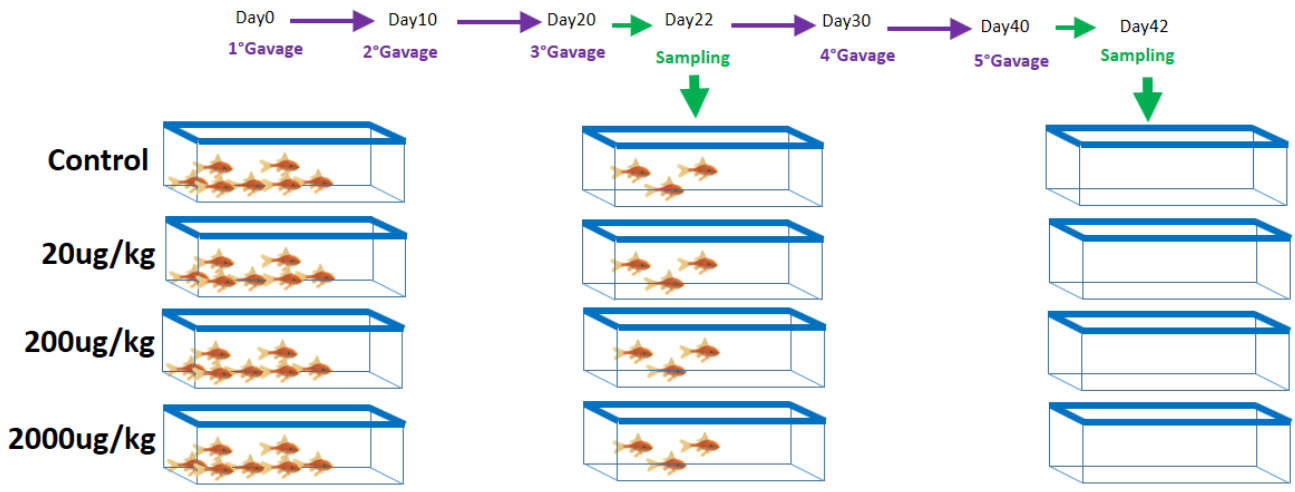
The doses were determined according to the Brazilian norm and the physiology of fish. It was calculating the volume of urine excreted by Rhamdia quelen individuals with around 50g, since that was the average weight that the animals had when arrived. According to Bolner and Baldisserotto, (2007), an individual of 1kg

excretes around 60mL/kg/day; so an individual of 50g excretes 3ml/day. Considering that the values allowed by the Brazilian legislation for waterbodies for standard class III allow 30µg/L of 2,4-D, that in 3mL corresponds to 0.09 µg. We rounded this value to 0.1 µg of 2,4-D by day to fish with 50g or a dose 2,4-D of 2 µg/day/kg. As the gavage were performed each 10 days (Fig. 17), we administrated the doses of 20, 200 and 2,000 µg/kg.

The 2,4 – D analytical standard– Sigma Aldrich® was used in this experiment. The control treatment was treated only with distilled water (Fig. 18). After preliminary tests with food dye in the R. quelen it was concluded that the maximum total volume possible to be orally administered was 100µL of liquid to avoid the regurgitation of the doses, so each dose was prepared in a final volume of 100µL in insulin syringe.

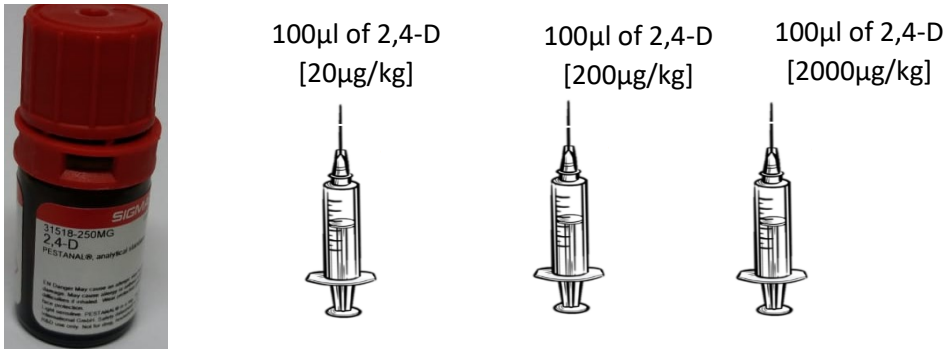
For the gavage, the animals were captured using a fishing net and placed in a plastic bucket previously prepared with a mix of water and sodium chloride to reduce the fish's stress (TAVARES-DIAS; MONTAGNER, 2015). After that, each fish was placed in a plastic bag of approximately 20×20cm, that had a cut in one of its the corners through which the animal was crossed just until the gills, and suggested with the rest of the bag to facilitate handling.

**Figure 17. Scheme of the chronogram of gavage and sampling data of liver, muscle and brain, that were used for the enzymatic analysis.**




**Source: The author**

**Figure 18. The 2,4 – D analytical standard– Sigma Aldrich® used to prepare the different doses. And a scheme of the three doses used in the study. In the control treatment it was applied 100 ul of distillate water.**




**Source: The author.**

Having secure the animal, it was used a 1mL syringe with a round-tipped needle at the end, so as to do not harming the animal (Oral Gavage Needles) (Fig. 19). The process can be seen in the next link:

https://photos.google.com/share/AF1QipP79pT2gwm4qERvxnKvMRVsFEKezEAYPEEpWu0XrB4bgO9X0O6h46R8OKD7_UCi2w?key=Ul9ORUVPQkFYYzhYMHdTU0ctV0w1QURISjdjblRn

**Figure 19. Picture of the way it was done the gavage using a plastic bag with a cut in one of the four corner. The herbicide was applied using an oral gavage needle**

**Source: The author.**

### 3.3.3 Tissue Collection

According to the activities chronogram, two euthanasia and tissues collection were performed. During each one, five animals were collected from each treatment and placed in an 8L plastic box with 100mg/L Benzocaine (DALCIN *et al*., 2018). After about 3 to 5 minutes, the animals lost stability, after that, the animal was placed in another plastic bucket with 250 mg/L Benzocaine to euthanasia, before the biometric measurement be recorded. Then, the necropsy was made so as to take the data of weight (g) of the liver, muscle, and brain. All the organs were stored in cryotubes in liquid nitrogen until the enzymatic protocols.

### 3.3.4 Enzymatic Assay

The tissues were homogenized in a Tissue Lyser, using a metal bead per sample. After that, the amount of protein was measured following the Bradford protocol (BRADFORD, 1976). The average protein of each sample was determined by comparison with a calibration curve of albumin from Bovine serum albumin (BSA) at 594 nm. The catalase (CAT) was measured regarding the decrease in the absorbance at 240 nm due to the degradation of the hydrogen peroxide in water (AEBI, 1984).

To measure the level of superoxide dismutase (SOD), it was used the capacity of the enzyme to inhibit a reduction of Nitroblue tetrazolium in blue formazan, due to the presence of $O_2^-$ produced by hydroxylamine in an alkaline solution. The absorbance was determined at 560nm (CROUCH *et al*., 1981). The level of Glutathione S-transferase (GST) was measured using the capacity of the enzyme to catalyse the reaction of 1 chlorine2-4 dinitrobenzene with GSH producing a thioether, which can be seen by the increase in the absorbance at 340nm (KEEN; HABIG; JAKOBY, 1976).

The amount of carbonylated proteins (PCO) react with 2,4-dinitrofenil-hidrazin producing dinitrophenyl hydrazine and could be determined at 358-370nm (LEVINE *et al*., 1994). To recognize if there were lipid peroxidation (LPO), it was used a method based in the fast oxidation of $Fe^{+2}$ in presence of an acid media, and the formation of a complex $Fe^{+3}$ – (Xylenol orange), which in presence of Butilhidroxitolueno it can be measured at 550-570nm. Using the protein capacity to precipitate and the later

reaction of non- protein Thiols with 5,5'-dithiobis-[2-nitrobenzoic acid] giving a product that absorbs at 414 nm, it was measured the amount of non-protein Thiols (GSH)(SEDLAK; LINDSAY, 1968). To measure the activity of acetylcholinesterase (AChE), it was used a method based in detect 2-nitrobenzoato-5-mercaptotiocolina and 5-tio-2-nitrobenzoato which are produced by the action between the thicholine (product of the hydrolysis of acetylcholine thanks to the AChE activity) and the acid 5,5'-ditiobis-2-nitrobenzóico at 405 nm (ELLMAN *et al*., 1961).

### 3.3.5 Statistical analysis

The statistical analyses of the antioxidant enzyme activities were performed by using Statistica 8 (STATSOFT, 2007). A value of $p < 0.05$ was considered a statistically significant difference. It was done the Levene's test for Homogenity of Variances and ANOVA for parametric dates, and Kruskal-Walls ANOVA for nonparametric dates.

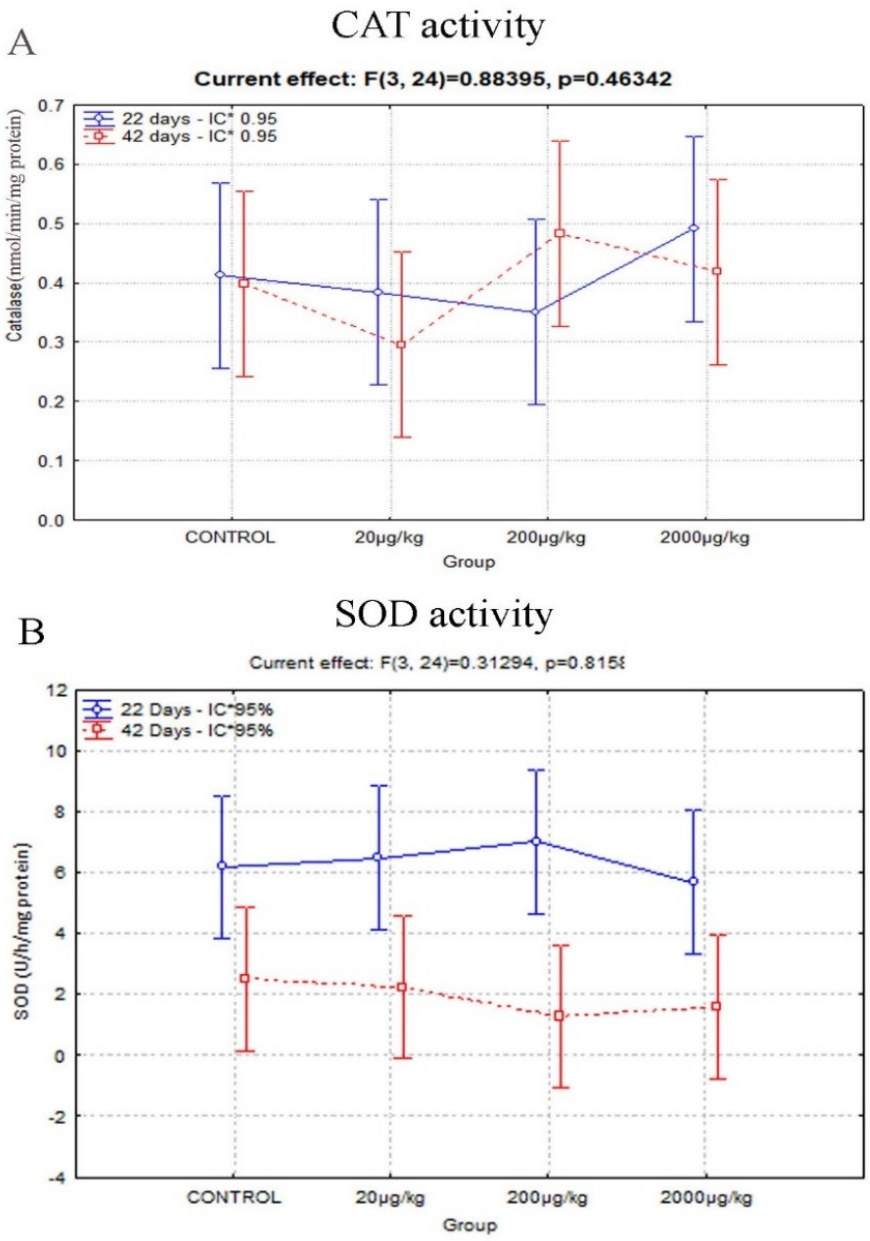
## 3.4 RESULTS AND DISCUSSION

In the present study, no significant difference was found between the control and the groups exposed to 2,4-D, either after 22 days, or after 42 days (Fig. 20A) for the SOD activity. However, it is important to emphasize that the exposure was not done continuously; on the contrary, there was an interval of 10 days between exposures. So the different doses really remained in the body for at least a day, since an individual of 50mg (which is the approximate weight that the individuals had at the beginning of the experiment) has the ability to excrete 3ml / day (BOLNER; BALDISSEROTTO, 2007), and in the present study, 100 µL of each dose (2µg/kg, 20µg/kg, 200µg/kg of 2,4-D per animal weight) were placed. The SOD is a first response enzymes, to reduce the $O_2^-$ that is generated during the first stage of the biodegradation process. Its performance is immediate, trying to catch and unfold the superoxide free radicals it finds, to generate $H_20_2$.

The lack of difference between the control and groups does not mean that there would have been no activity of them. In fact, it could be that within the first hours the level of SOD could have increased, however later it would have stabilized reaching

levels similar to the control after the post-gavage recovery process, as demonstrated in gill cells of *Carassius auratus*, which were exposed for 96 hours at doses of high toxicity in the water (100 mg/L 2,4-D), and subsequently 96 h without exposure to toxic to evaluate the recovery process (ATAMANIUK *et al*., 2013). This could have been due to the fact that SOD has the ability to generate an early adaptive response to oxidative stress (SLANINOVÁ *et al*., 2009). That would justify why after the two first gavages, and after the 5 gavages there was no significant difference. The same has been verified when *Carassius auratus* was exposed for a longer period of time at doses of 1mg/L 2,4-D also in the same species, where could been control-similar-level post-exposure, as evidenced of the adaptive response of SOD (ZHANG *et al*., 2004). Results with the same pattern were found in *Cherax destructor* exposed to atrazine and also with a recovery stage (STARA; KOUBA; VELISEK, 2018).

**Figure 20. The SOD and CAT activity in liver of *Rhmadia quelen* exposed to 2,4-D during 22 and 42 days.**




**Source: The author**
**Note: Significant difference p<0.05 and confidence interval (IC) of 95%.**

SOD catalyses the transformation of superoxide radicals into water and oxygen and it is the first enzyme to act against oxygen radicals (KAPPUS, 1985). Introduction in SOD levels occurs with the increase in ROS levels (LENÁRTOVÁ *et al*., 1997) clearly demonstrated by the study of Oruc and Uner (2002). On the other hand, the superoxide radicals by itself or after its conversion to $H_2O_2$ cause oxidation of cysteine in the enzyme which decreases the activity of SOD (ST. DIMITROVA; TISHINOVA; VELCHEVA, 1994). This increase serves as a protection response to the

presence of free radicals (CHEUNG *et al*., 2001), while the decrease after the exposure is associated with the consumption of SOD and return of its baseline values.

Regarding to CAT activity, non-significant difference was found among treatments, when levels were compared separately after 22 days, and after 42 days (Figure 20B). Similar results were evidenced after exposure with the herbicide tebuthiuron in *Oreochromis niloticus* after a continuous exposure of 62.5 mg/L and 125mg/L in water for a period of 72 hours, which is a longer exposure time compared to the conditions of the present experiment (FRANCO-BERNARDES, 2014).

However, it cannot be assured that there was no activity of this enzyme immediately after administration of 2,4-D doses. As mentioned earlier, catalase along with SOD are two of the enzymes that act in the first stage of defence, and the CAT function lies in generating $H_20$ from $H_2O_2$, which catalysis was previously done by SOD in a previous stage (ABELE; VÁZQUEZ-MEDINA; ZENTENO-SAVÍN, 2011). According to studies that record the CAT activity in the first hours during and after exposure, there is still a dilemma regarding to the type of agent, time and dose. According to studies that contemplate post-exposure recovery treatment in crustaceans and fish that were exposed to 1.21mg/L of atrazine (STARA; KOUBA; VELISEK, 2018) and 100mg/L of 2,4-D (ATAMANIUK *et al*., 2013; KUBRAK *et al*., 2013c) for a period of 14 days and 96 hours respectively and in a continuous way, the CAT activity during the exposure time increased above the control levels, but after the recovery period (without exposure), its level decreased until control-similar-levels.

These results would correspond to studies with exposure time ranging from 24 to 96 hours and concentrations ranging from 27 mg/L to 252mg/L. In them it is clear that the increase in CAT activity is directly proportional to the time, indistinct the concentrations in both liver and kidney. However, it is important to note that in those studies there is no information about the CAT activity after a recovery stage. So, that decrease could be due to the adaptive capacity that catalase also has by itself (SLANINOVÁ *et al*., 2009).

On the other hand, it is also reported that the CAT activity has no significant difference regarding the control in goldfish exposed for 96h to different doses ranging from permissible to toxic concentrations of 2,4-D. Indeed, even although they went through a recovery stage, the CAT activity did not changed neither before nor after it (MATVIISHYN *et al*., 2014). The CAT has a short term response so it would be necessary an analysis about is response in the first exposure hours.

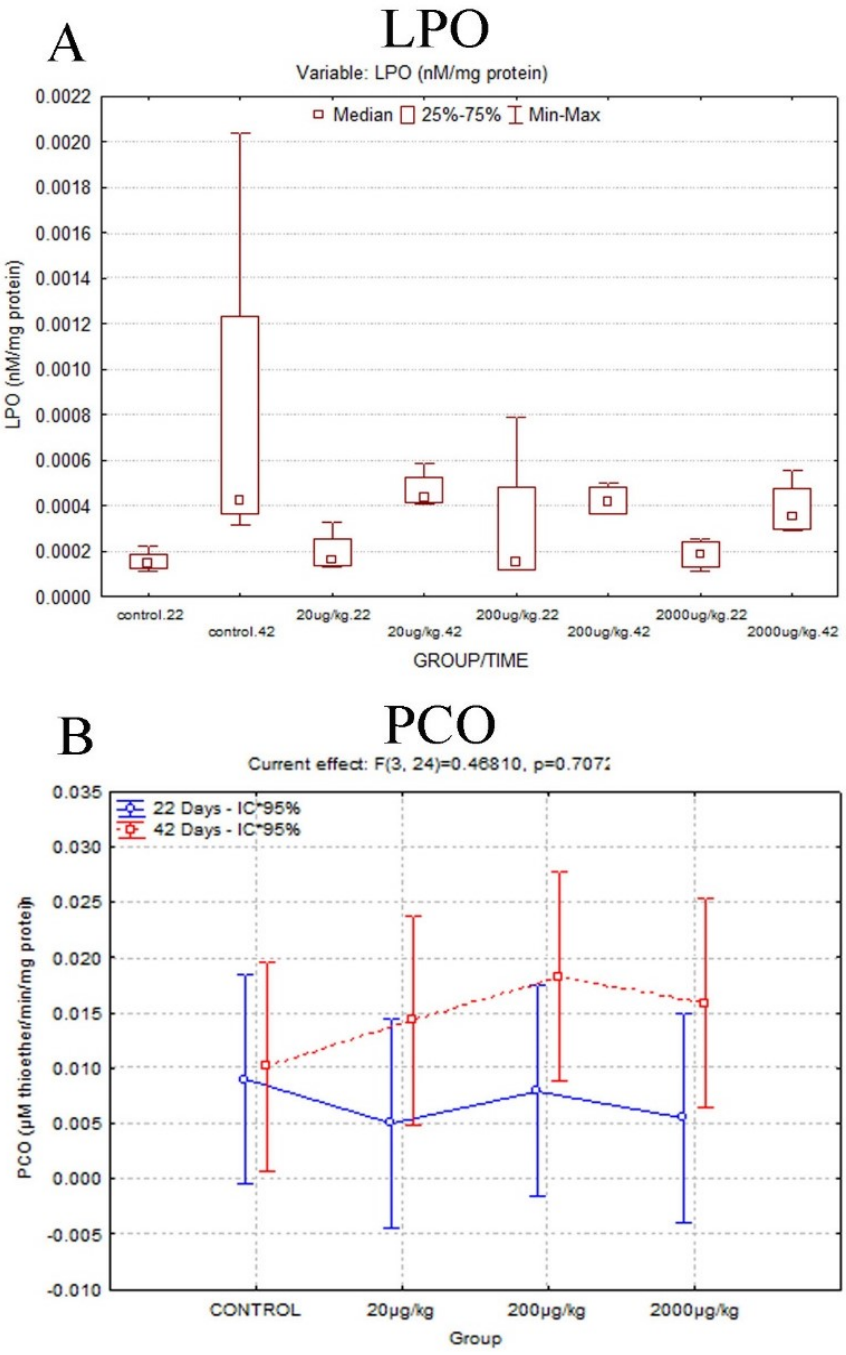
The decrease in the levels of $O2^{-}$ and $H_2O_2$ due to the SOD and CAT activity are of great importance since they prevent the production of hydroxyl radicals (OH*) through the Fenton/ Harber-Weiss reactions (BOELSTERLI, 2007). The hydroxyl radicals, as well as the alkoxy radicals (RO*) and peroxyl (ROO*), are generated by the attack of organic chemicals. They can abstract allyl hydrogens forming unstable carbon-carbon bonds in polyunsaturated fatty acids (PUFAs) that are mainly associated with cell membranes, which are in most of organelles such as lysosomes and endoplasmic reticulum (TEJERO *et al*., 2007). This abstraction is the initial step of a lipid peroxidation, which generates lipid radicals (R*), that can react with $O_2$ generating lipid peroxyl radical (ROO *), what in turn can react with another PUFA by removing a hydrogen and generating a lipid peroxide (LOOH), and generating R*, also will continue with the propagation phase of lipid peroxidation, and cascade reactions in several PUFAs (CASTELL *et al*., 1997). Lipid peroxidation can finish when lipid radicals produce non-radical products due to the action of an α-tocopherol that donates a hydrogen to form ROOH, and due to the action of another antioxidant enzyme, the GPx that reduces ROOH, and thus prevents the lipid peroxidation in others membranes (DI GIULIO; MEYER, 2008). So a low or no LPO levels indicate the protective effect of antioxidant enzymes (OZCAN ORUC; SEVGILER; UNER, 2004).

In the present study, no significant difference was found between the LPO levels in the control and the treatments with 2,4-D (Fig. 21A). It could be due to the fact that if there was any damage immediately after exposure, it could have been repaired due to lysophospholipids that could have been free in the membrane, or due the antioxidant enzymes was efficient in protect the organism (DAVIES, 2000), or in turns that there was no negative effects at membrane levels. Similar results were seen in fish *Oreochromis niloticus* exposed during 72h to doses of 125, 250 and 500 µg/L 2,4-D in water, although the levels of SOD and CAT also showed no increase, however this lack of protection by antioxidant enzymes suggests that there was no alteration in ROS levels (FRANCO-BERNARDES, 2014), which is consistent with the lack of lipid peroxidation. This could also be justified by the fact that perhaps the baseline levels of antioxidant enzymes are sufficient to cope with any changes resulting from ROS production (ORUÇ; ÜNER, 2002).

It has also been shown that when there is a recovery process, the LPO levels return to similar amounts to those of the control, which would indicate that the damage could have been repaired (MATVIISHYN *et al*., 2014). On the other hand, when the

control of radicals generated as a function of concentration exceeds the capacity of antioxidant enzymes at baseline, their levels may increase without causing effect on the LPO levels, which would indicate that their protective activity is acting efficiently even their in basal level (ORUÇ; ÜNER, 2000). But if LPO is evidenced, that would indicate that although antioxidant enzymes tried to control ROS levels, oxidative stress was such that it generated a membrane level effect (GAAIED *et al*., 2019). Similar pattern to the latter is seen when the time is longer despite the lower doses (example 14mg/L) in the earthworm after exposure to 2,4-D (HATTAB *et al*., 2015).

**Figure 21. The LPO and PCO level in liver of *Rhmadia quelen* exposed to 2,4-D during 22 and 42 days.**




**Source: The author**
**Note: Significant difference p<0.05 and confidence interval (IC) of 95%.**

Similar as the affinity ROS have for PUFAs, causing peroxidation, reactive oxygen species can also generate deleterious consequences on proteins, mainly protein carboxylation (PCO), causing enzyme inactivation, receptor disruption and other proteins involved in translation and disturb homeostasis (ZHANG; XIAO; AHN,

2013). There are few references that determine the effect of 2,4-D at the protein level, most of them only study the effects at the membrane level.

The damage that can be generated in situations of stress, tends to be recovered reaching similar levels to the control after the contact with the xenophobic is eliminated, as it was demonstrated in goldfish where after an exposure for 96 h at a dose of 100,000µg/L of 2,4-D. Both in blood and gills, in the first stage the increase in PCO levels is evident, but apparently in the recovery stage, there was a repair of these proteins, which generated PCO levels similar to those of control in the recovery stage (ATAMANIUK *et al*., 2013; KUBRAK *et al*., 2013a). From our results, it could be seen there was no significat difference in the level of PCO within the treatments, that can reflect the absence of stress in presence of 2,4-D, or that the damage could had been repaired in a early stage (Figure 21B).

GSH also has protective and adaptive function, which is widely established in aquatic animals (OLIVEIRA; PACHECO; SANTOS, 2008; SAERA-VILA *et al*., 2009). The environmental contaminants that come into contact with the cells are removed by conjugation with GSH directly or by the catalytic action of GST. Thus, it can cause a decrease in GSH and that is why GST is considered a detoxification enzyme (ELIA *et al*., 2003; LI *et al*., 2007).

In the present study, no significant difference was found between the levels of GST and GSH, both in individuals collected after 22 days, or within those collected after 42 days (Fig 22 A – B). There is increase in GST levels after exposures with 2,4-D for 48 and 96 hours at concentrations higher than 200,000µg/L (OZCAN ORUC; SEVGILER; UNER, 2004). Despite GSH levels were similar to those of the control, this could be explained by the fact that GST would be acting to counteract the effects of xenobiotic. The two main function of GST is to serve as a GSH catalyst, and as a remover of LOOH because its peroxidase activity (DI GIULIO; MEYER, 2008), so its presence in high level is important.

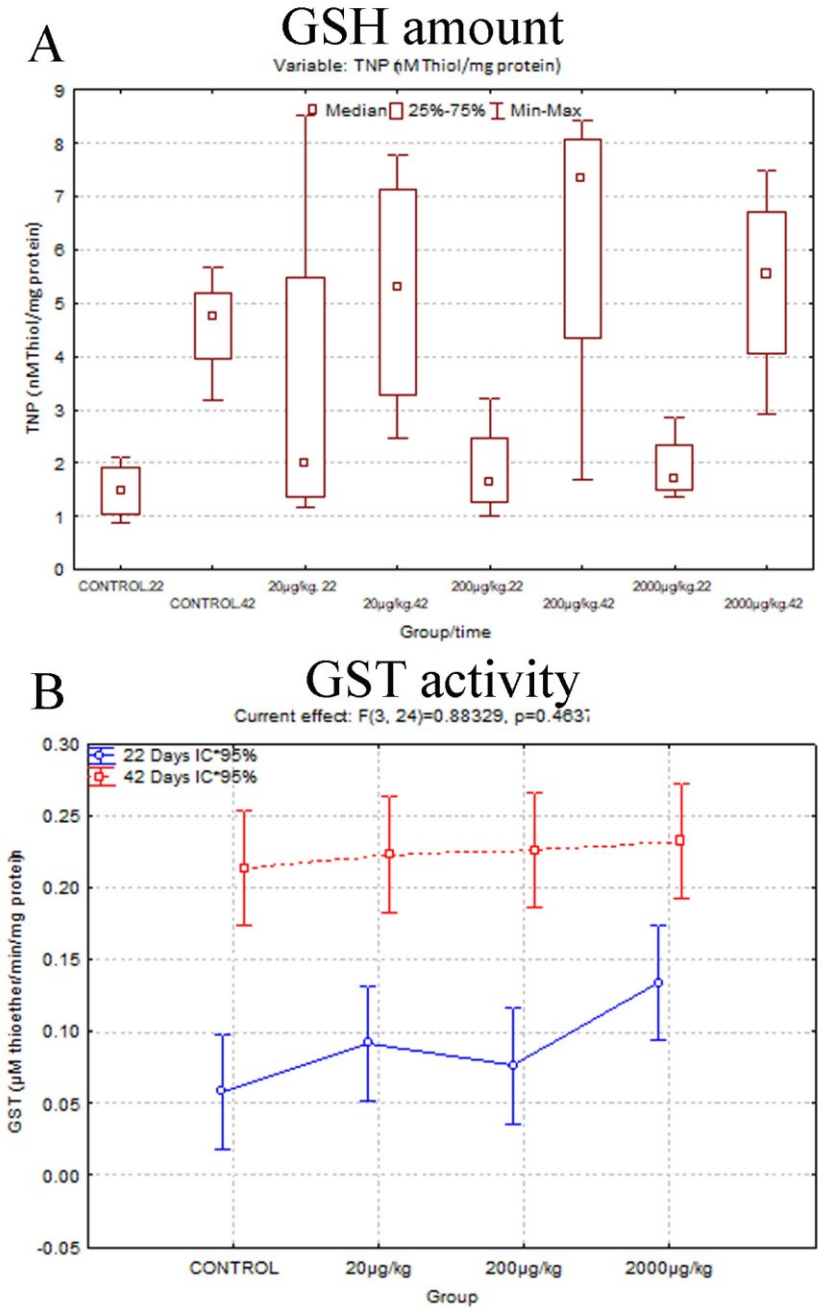
Furthermore, after a recovery stage the GST levels match those of the control, which suggests a compensatory action after the stress stage. Similar results have also been reported with exposure to other electrophilic compounds such as clomazone and endosulfan in fairly low concentrations (0.00045 and 0.005 µg/L respectively) (DE MENEZES *et al*., 2011b; PANDEY *et al*., 2001). On the other hand, it has also been reported that the values for GST would not increase or decrease significantly after exposures ranging from 0.001 to 500µg/L 2,4-D for periods of 48, 72 and 96h

(FRANCO-BERNARDES, 2014; FRANCO-BERNARDES *et al*., 2015; KUBRAK *et al*., 2013b; MATVIISHYN *et al*., 2014).

In some cases accompanied by total GSH values also similar to those of the control which reflects that the basal levels could be controlling the effects of the herbicide (ATAMANIUK *et al*., 2013; STARA; KOUBA; VELISEK, 2018). In another case accompanied by low GSH values which would confirm their ability to act on the xenobiotic without the need for the GST cofactor (KUBRAK *et al*., 2013b). However, in all cases it can be seen that the levels return to values similar to those of the control after a stage without exposure to 2,4-D and other xenobiotic (DANION *et al*., 2014; DE MENEZES *et al*., 2011a, 2011b; KUBRAK *et al*., 2013b). It indicates that at this stage it was possible cope with the effects of ROS generated by the environmental pollutant.

There are a few records showing the decreased total GSH values after the recovery stage, however that reflects possible GR action to repair any damage generated (ATAMANIUK *et al*., 2013). In case there was oxidative stress (in GSH and GST) within the first hour of exposure, their activity recovered, in case there was not reduction in their activity, the 2,4-D had no effect over GST and GSH at 20, 200 or 2000µg/kg.

**Figure 22. The GSH level and GST activity in liver of *Rhmadia quelen* exposed to 2,4-D during 22 and 42 days.**




**Source: The author**
**Note: Significant difference p<0.05 and confidence interval (IC) of 95%**

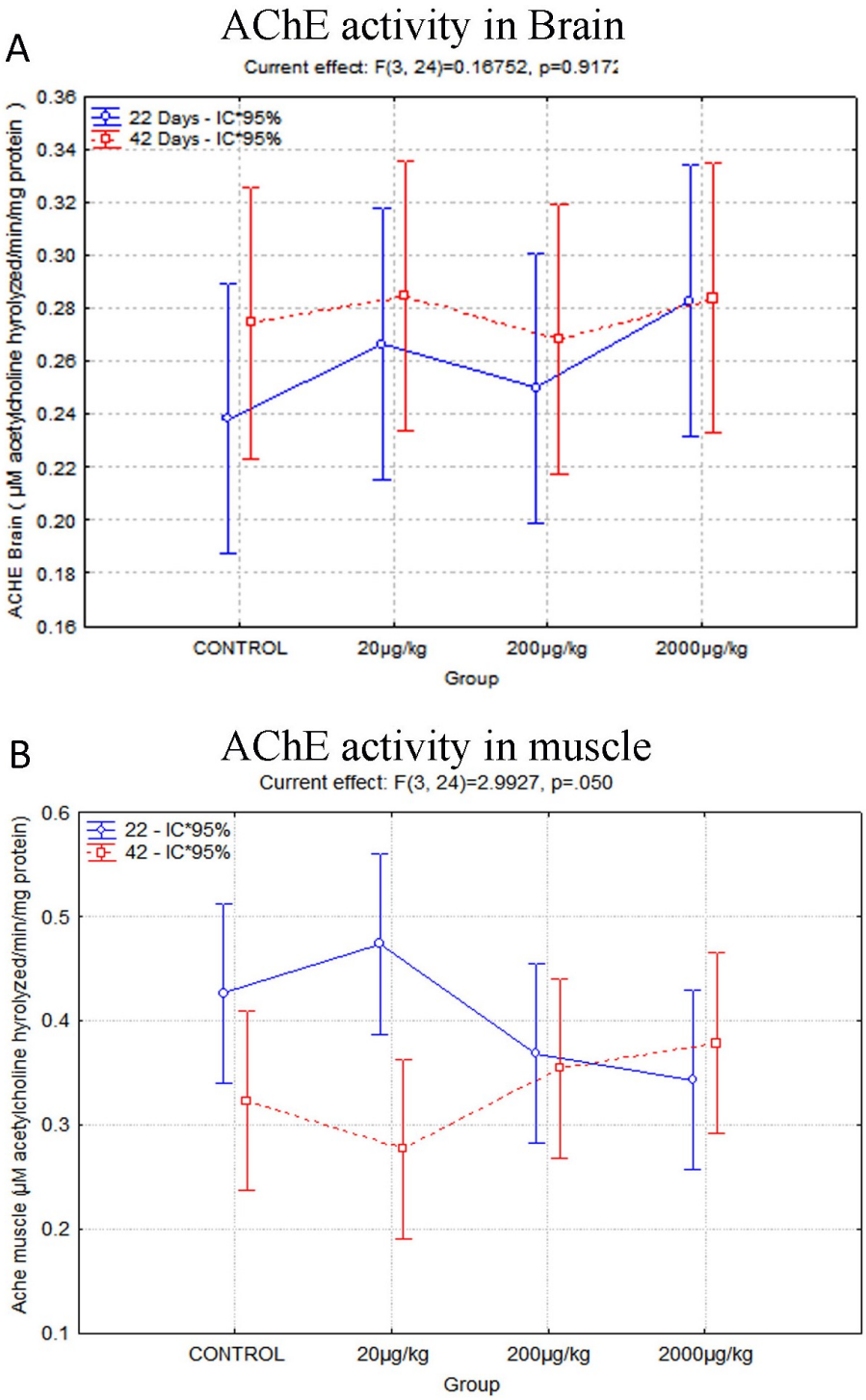
The AChE is an enzyme responsible for splitting acetylcholine (ACh) in choline and acetic acid. ACh is found in the central and peripheral nervous system, during the muscle synapse and in the red blood cells (LUNESTAD; SAMUELSEN, 2008). Inhibition in its action generates an increase in acetylcholine in the synaptic space

which can overstimulate the synaptic nerve, this can cause behavioural changes, tremors, loss of balance and even the death of the animal (ÜNER *et al*., 2006).

In the present study, no significant differences were found regarding the control, between the treatments after day 22, nor between the treatments collected after day 42 in both muscle and brain (Fig. 23 A – B). That could have been due to the fact that antioxidant enzymes were able to control the effects generated by the presence of 2,4-D in the organism, and the damages were repaired, considering that the 2,4-D target organ is the nervous system and the cardiovascular system (BENLI *et al*., 2007), or that in the experimental doses evaluated, 2,4-D had no effect over AChE on any of the organs studied.

There is a disagreement in the literature about the effect at AChE regarding the species. In goldfish exposed for 96 hours to 1000µg/L, 10000µg/L and 100000µg/L, AChE levels show no difference, even after the recovery stage, both in brain and muscle (ATAMANIUK *et al*., 2013; KUBRAK *et al*., 2013b; MATVIISHYN *et al*., 2014). On the other hand, in *Leporinus obtusidens* exposed to the same concentrations and time, had AChE inhibition in muscle, while in brain still from moderately toxic concentrations, although there were no behavioural changes (DA FONSECA *et al*., 2008).

**Figure 23. The GSH level and GST activity in liver of *Rhmadia quelen* exposed to 2,4-D during 22 and 42 days.**




**Source: The author**
**Note: Significant difference p<0.05 and confidence interval (IC) of 95%**

In a previous study, effects at AChE level in *Rhamdia quelen* have not been reported at concentrations below 100,000µg/L, however when exposed to values greater than 400,000µg/L, enzyme inhibition in muscle was observed. Despite this, in

brain an over activity was seen, and also changes in behaviour such as lethargy, and difference in the swimming pattern. This could be due to the effects of the xenobiotic and secondary compound generated, and the oxidative stress generated by them as was seen also in *R. quelen* exposed to LC50 levels (from 1 to 780 mg/L) (CATTANEO *et al*., 2008). There are no reports on the time of restoration of AChE activity in *Rhamdia sp.* after exposure to 2,4-D. However, according to a study in which 2,4-D is exposed at environmentally permissible concentrations, after 96 hours of recovery, the levels of AChE in the brain recover, but the same does not happen in the muscle. This would indicate that the muscle in *R. quelen* would be more sensitive to the effects of xenobiotic when compared with the brain (CRESTANI *et al*., 2007).

Although there was no difference among treatments of individuals collected after day 22, and among the groups collected after 42 days of exposure, when the it was compared the activity of SOD, GST, CAT, AChE and the amount of GSH within the two exposition times, it could be seen a significant difference. This could only be due to age factors in fish rather than the effects of 2,4-D. As it is known, physiological factors can cause changes in enzyme activity levels, and age is an example of that since the production of free radicals increases with age (ASHOK; ALI, 1999), so the level of enzymatic activity would also be increased (MARTÍNEZ-ÁLVAREZ; MORALES; SANZ, 2005; RUDNEVA, 1999).

There are several reports in the literature related to changes in the activity of antioxidant enzymes with respect to age and aging, but most of them are made in humans (GIERGIEL *et al*., 2012; RIZVI; MAURYA, 2007) rats (ÖZTÜRK; GÜMÜŞLÜ, 2004; YANG *et al*., 2015) and even camelids (GÓRECKA; SITARSKA; KLUCIŃSKI, 2002; MOUSA *et al*., 2006). However, in fish the number of researches is low, even more in species like *Rhamdia quelen*. According to a recent study in teleost's species, the *Scorpaena porcus* (which is the most *R.quele*n similar specie because of its benthic behaviour) the antioxidant enzymes activity changes as the age increases (GOMES *et al*., 2006; GOMIERO; SOUZA; BRAGA, 2007; MALABARBA, 2006). On the one hand, the activity of SOD is higher at 2 years old, while during the following years the levels tend to decrease, similar to the case of the CAT where the levels in the juveniles is higher when compared to the adults. For the case of GR, the levels remain similar in individuals of different ages, while GST levels decrease with age (RUDNEVA *et al*., 2010). Partially similar results were found in trout, which is a much more similar

species, where SOD levels also show decrease according to age increase, while CAT does not show significant dependence with age (PAROLINI *et al*., 2019).

This result leads us to understand that the imbalance between ROS levels and the antioxidant defence generates consequences at the physiological level, which would be the main cause of senescence, that is, the activity of enzymes shows a different pattern according to age. However, it might also depend on the species.

An important variable to consider is the type of administration, in this study it was discussed the results of studies in which the route of exposure was by immersion in water containing the pesticide. According Santana 2018 there is no differential effect with regarding the route of administration, once no relationship is shown between the route and the enzymatic activity (SANTANA *et al*., 2018). The meta-analysis performed in the first part of this dissertation it was shown that the administration route is also a differential factor. Studies using gavage in *Rhamdia quelen* (Fig. 7) is scarce. This is the first report where the doses are administered orally in *Rhamdia quelen*, however further studies are necessary to determine if the routes of 2,4-D administration do not influence the enzymatic response in fish, especially in shorter time period.

## 3.5. CONCLUSION

According to our results the there was no oxidative stress in R. quelen exposed to 2,4-D at doses of 20, 200 and 2000ug/kg. In case there were an imbalance within the first hours of exposure the antioxidant enzyme generated an adaptation to the presence of xenobiotic, because although they received several exposures no effect was found

On the other hand, differences were found in the levels of enzymes with respect to the lifetime of individuals. In this sense more studies are needed to measure the levels of these enzymes in juvenile and adult R. quelen. In addition, there are needed more researches where the oral route is used as a means of administration, in order to understand if the route of administration is an important variant to consider when ecotoxicology studies are done.

## 4. GENERAL CONCLUSION

The bioassay allowed us to understand that after the application of the different doses for 22 and 42 days of exposure every 10 days did not register any effect on the activity of the antioxidant enzymes. Furthermore, this exposition did no registered any effect at the lipid level, proteins or at the level of AChE. However, studies that record response in a shorter period of time are necessary since these enzymes are short-response.

The meta-analysis allowed us a better understanding of this, since it allowed us to understand that although the susceptibility to the different enzymes to 2,4-D depends on the species, it is also suggested that the time of exposure is an important variant, since the fact that the longer the exposure time the worst the damages caused at enzymatic level.

## REFERENCE

ABELE, D.; VÁZQUEZ-MEDINA, J. P.; ZENTENO-SAVÍN, T. Oxidative Stress in Aquatic Ecosystems. Oxidative Stress in Aquatic Ecosystems, p. 1–524, 2011.

ABHIJITH, B. D.; RAMESH, M.; POOPAL, R. K. Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of Catla catla during methyl parathion exposure. The Journal of Basic & Applied Zoology, v. 77, p. 31–40, out. 2016.

ADAMS, S. M. *et al*. The use of biomarkers in ecological risk assessment: recommendations from the Christchurch conference on Biomarkers in Ecotoxicology. Biomarkers, v. 6, n. 1, p. 1–6, 29 jan. 2001.

ADAPAR. Agência de Defesa Agropecuária do Paraná - ADAPAR. Disponível em: <http://www.adapar.pr.gov.br/>. Acesso em: 7 fev. 2020.

AEBI, H. [13] Catalase in Vitro. Methods in Enzymology, v. 105, n. C, p. 121–126, 1 jan. 1984.

AGAR, N.; MAEDEI, Y. THE RELATIONSHIP BETWEEN REDUCED GLUTATHIONE LEVEL AND GLUTATHIONE S-TRANSFERASE ACTIVITY IN SHEEP ERYTHROCYTESHokkaidoUniversity JPn,J· Vet.Res. [s.l: s.n.].

AL-BAROUDI, D. A. A.; ARAFAT, R. A.; EL-KHOLY, T. A. Hepatoprotective effect of chamomile capitula extract against 2, 4-dichlorophenoxyacetic acid-induced hepatotoxicity in rats. Life Science Journal, v. 11, n. 8, p. 34–40, 2014.

ALBERTSSON, E. *et al*. Proteomic analyses indicate induction of hepatic carbonyl reductase/20β-hydroxysteroid dehydrogenase B in rainbow trout exposed to sewage effluent. Ecotoxicology and Environmental Safety, v. 68, n. 1, p. 33–39, set. 2007.

AMEL, N. *et al*. Extra virgin olive oil modulates brain docosahexaenoic acid level and oxidative damage caused by 2,4-Dichlorophenoxyacetic acid in rats. Journal of Food Science and Technology, v. 53, n. 3, p. 1454–1464, 1 mar. 2016.

AMER, S. M.; ALY, F. A. Genotoxic effect of 2,4-dichlorophenoxy acetic acid and its metabolite 2,4-dichlorophenol in mouse. Mutation research, v. 494, n. 1–2, p. 1–12, 25 jul. 2001.

ANDERSSON, T.; FÖRLIN, L. Regulation of the cytochrome P450 enzyme system in fish. Aquatic Toxicology, v. 24, n. 1–2, p. 1–19, nov. 1992.

ARAÚJO, F. G. *et al*. Biomarkers and bioindicators of the environmental condition using a fish species (Pimelodus maculatus Lacepède, 1803) in a tropical reservoir in Southeastern Brazil. Brazilian Journal of Biology, v. 78, n. 2, p. 351–359, 2017.

ARELLANO-AGUILAR, O.; MONTOYA, R. M.; GARCIA, C. M. Endogenous Functions and Expression of Cytochrome P450 Enzymes in Teleost Fish: A Review. Reviews in Fisheries Science, v. 17, n. 4, p. 541–556, 2 out. 2009.

ASHOK, B. T.; ALI, R. The aging paradox: free radical theory of aging. Experimental gerontology, v. 34, n. 3, p. 293–303, jun. 1999.

ATAMANIUK, T. M. *et al*. Oxidative stress as a mechanism for toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D): Studies with goldfish gills. Ecotoxicology, v. 22, n. 10, p. 1498–1508, 2013.

ATAMANIUK, T. M. *et al*. The mancozeb-containing carbamate fungicide tattoo induces mild oxidative stress in goldfish brain, liver, and kidney. Environmental Toxicology, v. 29, n. 11, p. 1227–1235, 1 nov. 2014.

ATEEQ, B.; FARAH, M. A.; AHMAD, W. Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlorophenoxyacetic-acid- and butachlor-exposed erythrocytes of Clarias batrachus. Ecotoxicology and Environmental Safety, v. 62, n. 3, p. 348–354, 2005.

AWADALLAH, S. Protein Antioxidants in Thalassemia. In: Advances in Clinical Chemistry. [s.l.] Academic Press Inc., 2013. v. 60p. 85–128.

AZEVEDO-LINHARES, M. *et al*. Microcystin and pyriproxyfen are toxic to early stages of development in Rhamdia quelen: An experimental and modelling study. Ecotoxicology and Environmental Safety, v. 166, p. 311–319, 30 dez. 2018.

BAE, Y.-A. *et al*. Modular evolution of glutathione peroxidase genes in association with different biochemical properties of their encoded proteins in invertebrate animals. 2009.

BAEK, M. K. *et al*. Age-related changes in antioxidative enzyme capacity in tongue of fischer 344 rats. Clinical and Experimental Otorhinolaryngology, v. 9, n. 4, p. 352–357, 2016.

BAGNYUKOVA, T. V.; STOREY, K. B.; LUSHCHAK, V. I. Adaptive response of antioxidant enzymes to catalase inhibition by aminotriazole in goldfish liver and kidney. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, v. 142, n. 3, p. 335–341, nov. 2005.

BALDISSERA, M. D. *et al*. Gill bioenergetics dysfunction and oxidative damage induced by thiamethoxam exposure as relevant toxicological mechanisms in freshwater silver catfish Rhamdia quelen. Science of The Total Environment, v. 636, p. 420–426, 15 set. 2018.

BANAOUI, A. *et al*. Assessment of multimarker responses in Perna perna, Mytilus galloprovincialis and Donax trunculus bivalves exposed to malathion and 2,4-dichlorophenoxyacetic acid pesticides. Journal of Materials and Environmental Science, v. 6, n. 6, p. 1678–1683, 2015.

BENLI, A. Ç. K. *et al*. Investigation of acute toxicity of (2,4-dichlorophenoxy)acetic acid (2,4-D) herbicide on crayfish (Astacus leptodactylus Esch. 1823). Pesticide Biochemistry and Physiology, v. 88, n. 3, p. 296–299, jul. 2007.

BENLI, A. Ç. K. *et al*. The sublethal effects of (2,4-Dichlorophenoxy) acetic acid (2,4-D) on narrow-clawed crayfish (Astacus leptodactylus Eschscholtz, 1823). Arhiv za Higijenu Rada i Toksikologiju, v. 67, n. 4, p. 289–296, 1 dez. 2016.

BERNARD, P. A. *et al*. 2,4-Dichlorophenoxyacetic acid (2,4-D) reduces acetylcholinesterase activity in rat muscle. Experimental Neurology, v. 87, n. 3, p. 544–556, 1985a.

BERNARD, P. A. *et al*. 2,4-Dichlorophenoxyacetic acid (2,4-D) reduces acetylcholinesterase activity in rat muscle. Experimental Neurology, v. 87, n. 3, p. 544–556, mar. 1985b.

BILLER, J. D.; TAKAHASHI, L. S. Oxidative stress and fish immune system: Phagocytosis and leukocyte respiratory burst activity. Anais da Academia Brasileira de Ciencias, v. 90, n. 4, p. 3403–3414, 1 out. 2018.

BINDOLI, A.; FUKUTO, J. M.; FORMAN, H. J. Forum Review Thiol Chemistry in Peroxidase Catalysis and Redox Signaling. ANTIOXIDANTS & REDOX SIGNALING, v. 10, n. 9, 2008.

BOELSTERLI, U. A. Mechanistic toxicology : the molecular basis of how chemicals disrupt biological targets /. 2nd ed. ed. Boca Raton, FL : CRC Press, 2007.

BOLNER, K.; BALDISSEROTTO, B. Water pH and urinary excretion in silver catfish Rhamdia quelen. Journal of Fish Biology, v. 70, n. 1, p. 50–64, 2007.

BONGIOVANNI, B. *et al*. Melatonin decreases the oxidative stress produced by 2,4-dichlorophenoxyacetic acid in rat cerebellar granule cells. Neurotoxicity research, v. 11, n. 2, p. 93–9, fev. 2007.

BORENSTEIN, M. *et al*. Introduction to Meta-Analysis. John Wiley ed. Chichester: [s.n.].

BRADFORD, M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry, v. 72, n. 1–2, p. 248–254, 7 maio 1976.

BRETAUD, S.; TOUTANT, J.-P.; SAGLIO, P. Effects of Carbofuran, Diuron, and Nicosulfuron on Acetylcholinesterase Activity in Goldfish (Carassius auratus). Ecotoxicology and Environmental Safety, v. 47, n. 2, p. 117–124, out. 2000.

BURNS, C. J.; SWAEN, G. M. H. Review of 2,4-dichlorophenoxyacetic acid (2,4-D) biomonitoring and epidemiologyCritical Reviews in ToxicologyTaylor & Francis, , out. 2012.

BUSSOLARO, D.; FILIPAK NETO, F.; OLIVEIRA RIBEIRO, C. A. Responses of hepatocytes to DDT and methyl mercury exposure. Toxicology in Vitro, v. 24, n. 6, p. 1491–1497, set. 2010.

CADET, J.; RICHARD WAGNER, J. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harbor Perspectives in Biology, v. 5, n. 2, fev. 2013.

CARVAN, M. J. Fish Models in Toxicology. Zebrafish, v. 4, n. 1, p. 9–20, 2007.

CASTELL, J. V. *et al*. In vitro Investigation of the Molecular Mechanisms of Hepatotoxicity. In: Archives of toxicology. Supplement. = Archiv fur Toxikologie. Supplement. [s.l: s.n.]. v. 19p. 313–321.

CASTRO, J. M. *et al*. Modulation of immune and antioxidant responses by azinphos-methyl in the freshwater mussel Diplodon chilensis challenged with Escherichia coli. Environmental Toxicology and Chemistry, v. 36, n. 7, p. 1785–1794, 1 jul. 2017.

CATTANEO, R. *et al*. Metabolic and histological parameters of silver catfish (Rhamdia quelen) exposed to commercial formulation of 2,4-dichlorophenoxiacetic acid (2,4-D) herbicide. Pesticide Biochemistry and Physiology, v. 92, n. 3, p. 133–137, nov. 2008.

CEDERBAUM, A. I. Cytochrome P450 and Oxidative Stress in the Liver. In: Liver Pathophysiology: Therapies and Antioxidants. [s.l.] Elsevier, 2017. p. 401–419.

CELIK, I.; TULUCE, Y. Determination of toxicity of subacute treatment of some plant growth regulators on rats. Environmental Toxicology, v. 22, n. 6, p. 613–619, dez. 2007.

CELIK, I.; TULUCE, Y.; ISIK, I. Influence of subacute treatment of some plant growth regulators on serum marker enzymes and erythrocyte and tissue antioxidant defense and lipid peroxidation in rats. Journal of Biochemical and Molecular Toxicology, v. 20, n. 4, p. 174–182, 2006.

CHAUDHARY, A. J.; HASSAN, M. UL; GRIMES, S. M. Simultaneous recovery of metals and degradation of organic species: Copper and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Journal of Hazardous Materials, v. 165, n. 1–3, p. 825–831, 15 jun. 2009.

CHEUNG, C. C. C. *et al*. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, Perna viridis. Aquatic Toxicology, v. 52, n. 3–4, p. 189–203, 1 maio 2001.

CHOVANEC, A.; HOFER, R.; SCHIEMER, F. Chapter 18 Fish as bioindicators. Trace Metals and other Contaminants in the Environment, v. 6, n. C, p. 639–676, 2003.

COSSI, P. F. *et al*. Environmental concentrations of azinphos-methyl cause different toxic effects without affecting the main target (cholinesterases) in the freshwater gastropod Biomphalaria straminea. Ecotoxicology and Environmental Safety, v. 162, p. 287–295, 30 out. 2018.

COSTA, D. D. M. *et al*. Vitellogenesis and other physiological responses induced by 17-β-estradiol in males of freshwater fish Rhamdia quelen. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, v. 151, n. 2, p. 248–257, 2010.

CRAVEIRO FRANCO, F. *et al*. Evaluating genotoxic risks in Brazilian public health agents occupationally exposed to pesticides: a multi-biomarker approach. Environmental Science and Pollution Research, v. 23, n. 19, p. 19723–19734, 2016.

CRESTANI, M. *et al*. Effect of clomazone herbicide on biochemical and histological aspects of silver catfish (Rhamdia quelen) and recovery pattern. Chemosphere, v. 67, n. 11, p. 2305–2311, 2007.

CROUCH, R. K. *et al*. The inhibition of islet superoxide dismutase by diabetogenic drugs. Diabetes, v. 30, n. 3, p. 235–241, 1981.

DA FONSECA, M. B. *et al*. The 2,4-D herbicide effects on acetylcholinesterase activity and metabolic parameters of piava freshwater fish (Leporinus obtusidens). Ecotoxicology and Environmental Safety, v. 69, n. 3, p. 416–420, 2008.

DALCIN, M. O. *et al*. Concentrado proteico de arroz na alimentação do jundiá (Rhamdia quelen). Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v. 70, n. 1, p. 306–314, 2018.

DANION, M. *et al*. Effects of in vivo chronic exposure to pendimethalin on EROD activity and antioxidant defenses in rainbow trout (Oncorhynchus mykiss). Ecotoxicology and Environmental Safety, v. 99, p. 21–27, 2014.

DAVIES, K. J. Oxidative stress: the paradox of aerobic life.Biochemical Society symposium, 1995.

DAVIES, K. J. A. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life, v. 50, n. 4–5, p. 279–289, 2000.

DE MENEZES, C. C. *et al*. Roundup effects on oxidative stress parameters and recovery pattern of Rhamdia quelen. Archives of Environmental Contamination and Toxicology, v. 60, n. 4, p. 665–671, 2011a.

DE MENEZES, C. C. *et al*. Oxidative parameters of Rhamdia quelen in response to commercial herbicide containing clomazone and recovery pattern. Pesticide Biochemistry and Physiology, v. 100, n. 2, p. 145–150, jun. 2011b.

DEHGHANI, M.; NASSERI, S.; KARAMIMANESH, M. Removal of 2,4-Dichlorophenolyxacetic acid (2,4-D) herbicide in the aqueous phase using modified granular activated carbon. Journal of Environmental Health Science and Engineering, v. 12, n. 1, p. 28, 2014.

DEHNERT, G. K. *et al*. Effects of low, subchronic exposure of 2,4-Dichlorophenoxyacetic acid (2,4-D) and commercial 2,4-D formulations on early life stages of fathead minnows ( Pimephales promelas ). Environmental Toxicology and Chemistry, v. 37, n. 10, p. 2550–2559, out. 2018.

DEHNERT, G. K.; KARASOV, W. H.; WOLMAN, M. A. 2,4-Dichlorophenoxyacetic acid containing herbicide impairs essential visually guided behaviors of larval fish. Aquatic Toxicology, v. 209, p. 1–12, 1 abr. 2019.

DI GIULIO, R. T.; HINTON, D. E. The toxicology of fishes. In: The Toxicology of Fishes. Boca Raton: [s.n.]. p. 1096.

DI GIULIO, R. T.; MEYER, J. N. Reactive oxygen species and oxidative stress. The Toxicology of Fishes, p. 273–324, 2008.

DOWNES, G. B.; GRANATO, M. Acetylcholinesterase function is dispensable for sensory neurite growth but is critical for neuromuscular synapse stability. Developmental Biology, v. 270, n. 1, p. 232–245, 1 jun. 2004.

DVIR, H. *et al*. Acetylcholinesterase: From 3D Structure to Function. Chem Biol Interact, v. 187, p. 10–22, 2010.

EL-BELTAGI, H. S.; MOHAMED, H. I. Reactive oxygen species, lipid peroxidation and antioxidative defense mechanism. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, v. 41, n. 1, p. 44–57, 2013.

ELIA, A. C. *et al*. Antioxidant responses and bioaccumulation in Ictalurus melas under mercury exposure. Ecotoxicology and environmental safety, v. 55, n. 2, p. 162–7, jun. 2003.

ELLMAN, G. L. *et al*. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, v. 7, n. 2, p. 88–95, 1 jul. 1961.

ES RUIZ DE ARCAUTE, C. *et al*. Auxinic herbicides induce oxidative stress on Cnesterodon decemmaculatus (Pisces: Poeciliidae). Environmental Science and Pollution Research, v. 26, n. 20, p. 20485–20498, 2019.

ESTEVAM, E. C. *et al*. Dominant lethal effects of 2,4-D in Biomphalaria glabrata. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, v. 611, n. 1–2, p. 83–88, dez. 2006.

FAGAN, R. L.; PALFEY, B. A. Flavin-dependent enzymes. In: Comprehensive Natural Products II: Chemistry and Biology. [s.l.] Elsevier Ltd, 2010. v. 7p. 37–113.

FERRI, A.; DUFFARD, R.; DE DUFFARD, A. M. E. Selective oxidative stress in brain areas of neonate rats exposed to 2,4-dichlorophenoxyacetic acid through mother's milk. Drug and Chemical Toxicology, v. 30, n. 1, p. 17–30, 24 jan. 2007.

FILIPAK NETO, F. *et al*. Use of hepatocytes from Hoplias malabaricus to characterize the toxicity of a complex mixture of lipophilic halogenated compounds. Toxicology in Vitro, v. 21, n. 4, p. 706–715, jun. 2007.

FORRESTER, S. J. *et al*. Reactive oxygen species in metabolic and inflammatory signalingCirculation ResearchLippincott Williams and Wilkins, , 1 mar. 2018.

FRANCO-BERNARDES, M. F. Biochemical and genotoxic effects of a commercial formulation of the herbicide tebuthiuron in Oreochromis niloticus of different sizes. Ecotoxicology and Environmental Contamination, v. 9, n. 1, p. 59–67, 2014.

FRANCO-BERNARDES, M. F. *et al*. The use of biomarkers to study the effects of the mixture of diuron and hexazinone on small and large O. niloticus. Ecotoxicology and Environmental Contamination, v. 10, n. 1, p. 83–92, 2015.

FRÄNZLE, O. Chapter 2 Bioindicators and environmental stress assessment. Trace Metals and other Contaminants in the Environment, v. 6, n. C, p. 41–84, 2003.

GAAIED, S. *et al*. 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, v. 224, p. 289–297, 2019.

GALLAGHER, E. P.; DI GIULIO, R. T. Effects of 2,4-dichlorophenoxyacetic acid and picloram on biotransformation, peroxisomal and serum enzyme activities in channel catfish (Ictalurus punctatus). Toxicology letters, v. 57, n. 1, p. 65–72, jun. 1991.

GEHIN, A. *et al*. Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: A biochemometric approach. International Journal of Pharmaceutics, v. 288, n. 2, p. 219–226, 20 jan. 2005.

GHISI, N. DE C.; OLIVEIRA, E. C. DE; PRIOLI, A. J. Does exposure to glyphosate lead to an increase in the micronuclei frequency? A systematic and meta-analytic review. Chemosphere, v. 145, p. 42–54, 2016.

GIERGIEL, M. *et al*. The influence of age and gender on antioxidant enzyme activities in humans and laboratory animalsAging Clinical and Experimental Research, dez. 2012.

GLISIC, B. *et al*. Characterization of glutathione-S-transferases in zebrafish (Danio rerio). Aquatic Toxicology, v. 158, p. 50–62, 2015.

GODEFROID, R. S.; CASSIO, R. UTILIZAÇÃO DOS PEIXES DO RIO BACACHERI COMO INDICADORES DA QUALIDADE AMBIENTAL BACACHERI RIVER FISH USED AS INDICATOR OF ENVIRONMENTAL QUALITY UTILIZACIÓN DE LOS PECES DEL RÍO BACACHERI COMO INDICADORES DE LA CALIDAD AMBIENTAL sem tratamento adequado tem . n. 2000, 2008.

GOMES, L. DE C. *et al*. Biologia do jundiá Rhamdia quelen (Teleostei, Pimelodidae). Ciência Rural, v. 30, n. 1, p. 179–185, 2006.

GOMIERO, L. M.; SOUZA, U. P.; BRAGA, F. M. DE S. Reprodução e alimentação de Rhamdia quelen (Quoy & Gaimard, 1824) em rios do Núcleo Santa Virgínia, Parque Estadual da Serra do Mar, São Paulo, SP. Biota Neotropica, v. 7, n. 3, p. 127–133, 2007.

GONZÁLEZ, M. *et al*. Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic and a commercial formulation, 2,4-dichlorophenoxyacetic acid dimethylamine salt. I. Evaluation of DNA damage and cytogenetic endpoints in Chinese Hamster ovary (CHO) cells. Toxicology in Vitro, v. 19, n. 2, p. 289–297, mar. 2005.

GÓRECKA, R.; SITARSKA, E.; KLUCIŃSKI, W. Antioxidant parameters of horses according to age, sex, breed and environment. Polish journal of veterinary sciences, v. 5, n. 4, p. 209–16, 2002.

GRECO, L. *et al*. Physiological effects of temperature and a herbicide mixture on the soft-shell clam Mya arenaria (Mollusca, Bivalvia). Environmental Toxicology and Chemistry, v. 30, n. 1, p. 132–141, jan. 2011.

HALLIWELL, B.; CLEMENT, M. V.; LONG, L. H. Hydrogen peroxide in the human bodyFEBS Letters, 1 dez. 2000.

HALLIWELL, B.; GUTTERIDGE, J. M. C. Free radicals in biology and medicine. [s.l.] Clarendon Press, 1999.

HANSSON, N. Does fish health matter? : the utility of biomarkers in fish for environmental assessment. [s.l.] Department of plant and environmental sciences, University of Gothenburg, 2008.

HARVEY, J. W. The Erythrocyte: Physiology, Metabolism, and Biochemical Disorders. In: Clinical Biochemistry of Domestic Animals. [s.l.] Elsevier Inc., 2008. p. 173–240.

HATTAB, S. *et al*. Transcriptional expression levels and biochemical markers of oxidative stress in the earthworm Eisenia andrei after exposure to 2,4-dichlorophenoxyacetic acid (2,4-D). Ecotoxicology and Environmental Safety, v. 122, p. 76–82, 2015.

HE, L. *et al*. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen SpeciesCellular Physiology and BiochemistryS. Karger AG, , 1 dez. 2017.

HODGSON, E. Introduction to Biotransformation (Metabolism). In: Hayes' Handbook of Pesticide Toxicology. [s.l.] Elsevier, 2010. p. 865–875.

HOLDEN, C. LABORATORY ANIMALS: Researchers Pained by Effort to Define Distress Precisely. Science, v. 290, n. 5496, p. 1474–1475, 24 nov. 2000.

HOLSCHNEIDER, D. P. *et al*. Acetylcholinesterase inhibition and locomotor function after motor-sensory cortex impact injury. Journal of Neurotrauma, v. 28, n. 9, p. 1909–1919, 1 set. 2011.

HSIEH, Y. S.; HSU, C. Y. Oxidative stress and anti-oxidant enzyme activities in the trophocytes and fat cells of queen honeybees (Apis mellifera). Rejuvenation Research, v. 16, n. 4, p. 295–303, 1 ago. 2013.

IGHODARO, O. M.; AKINLOYE, O. A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine, v. 54, n. 4, p. 287–293, 1 dez. 2018.

JAWED, S. *et al*. Hypoxia, oxidative stress and exercise in rheumatoid arthritis. In: Handbook of Oxidants and Antioxidants in Exercise. [s.l.] Elsevier, 2000. p. 1147–1188.

KAPPUS, H. Lipid Peroxidation: Mechanisms, Analysis, Enzymology and Biological Relevance. In: Oxidative Stress. [s.l.] Elsevier, 1985. p. 273–310.

KAWASHIMA, A.; SATTA, Y. Substrate-Dependent Evolution of Cytochrome P450: Rapid Turnover of the Detoxification-Type and Conservation of the Biosynthesis-Type. PLoS ONE, v. 9, n. 6, p. 100059, 2014.

KEEN, J. H.; HABIG, W. H.; JAKOBY, W. B. Mechanism for the several activities of the glutathione S transferases. Journal of Biological Chemistry, v. 251, n. 20, p. 6183–6188, 1976.

KEHRER, J. P. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology, v. 149, n. 1, p. 43–50, 14 ago. 2000.

KIEFFER, D. A.; MARTIN, R. J.; ADAMS, S. H. Impact of Dietary Fibers on Nutrient Management and Detoxification Organs: Gut, Liver, and Kidneys. Advances in Nutrition: An International Review Journal, v. 7, n. 6, p. 1111–1121, nov. 2016.

KORTE, F.; BAHADIR, M. Lehrbuch der Ökologischen Chemie : Grundlagen und Konzepte für die ökologische Beurteilung von Chemikalien. [s.l.] G. Thieme, 1992.

KUBRAK, O. I. *et al*. Goldfish can recover after short-term exposure to 2,4-dichlorophenoxyacetate: Use of blood parameters as vital biomarkers. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, v. 157, n. 3, p. 259–265, 2013a.

KUBRAK, O. I. *et al*. Transient effects of 2,4-dichlorophenoxyacetic acid (2,4-D) exposure on some metabolic and free radical processes in goldfish white muscle. Food and Chemical Toxicology, v. 59, p. 356–361, 2013b.

KUBRAK, O. I. *et al*. Transient effects of 2,4-dichlorophenoxyacetic acid (2,4-D) exposure on some metabolic and free radical processes in goldfish white muscle. Food and Chemical Toxicology, v. 59, p. 356–361, set. 2013c.

KUMARI, K.; KHARE, A.; DANGE, S.  The Applicability of Oxidative Stress Biomarkers in Assessing Chromium Induced Toxicity in the Fish Labeo rohita . BioMed Research International, v. 2014, p. 1–11, 2014.

LAKSHMANA, M. K.; RAJU, T. R. 2, 4-dichloro phenoxy acetic acid alters monoamine levels, acetylcholinesterase activity & operant learning in rats. The Indian journal of medical research, v. 104, p. 234–9, set. 1996.

LENÁRTOVÁ, V. *et al*. Antioxidant and detoxifying fish enzymes as biomarkers of river pollution. Biomarkers, v. 2, n. 4, p. 247–252, 1997.

LEVINE, R. L. *et al*. Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology, v. 233, n. C, p. 346–357, 1 jan. 1994.

LI, F. *et al*. Hydroxyl radical generation and oxidative stress in Carassius auratus liver as affected by 2,4,6-trichlorophenol. Chemosphere, v. 67, n. 1, p. 13–19, fev. 2007.

LI, H.-T. *et al*. Roles of zinc and copper in modulating the oxidative refolding of bovine copper, zinc superoxide dismutase. Acta Biochim Biophys Sin, p. : 183–194, 2010.

LOOMIS, D. *et al*. Carcinogenicity of lindane, DDT, and 2,4-dichlorophenoxyacetic acid. The Lancet Oncology, v. 16, n. 8, p. 891–892, ago. 2015.

LUNESTAD, B. T.; SAMUELSEN, O. Veterinary drug use in aquaculture. In: Improving Farmed Fish Quality and Safety. [s.l.] Elsevier, 2008. p. 97–127.

MA, X.; DENG, D.; CHEN, W. Inhibitors and Activators of SOD, GSH-Px, and CAT Provisional chapter Inhibitors and Activators of SOD, GSH-Px, and CAT. 2017.

MALABARBA, L. R. Peixes de Riachos da Mata Atlântica nas Unidades de Conservação do Vale do Rio Ribeira de Iguape no Estado de São Paulo. Neotropical Ichthyology, v. 4, n. 3, p. 377–377, set. 2006.

MARCELINO, A. F.; WACHTEL, C. C.; GHISI, N. DE C. Are our farm workers in danger? Genetic damage in farmers exposed to pesticides. International Journal of Environmental Research and Public Health, v. 16, n. 3, 2019.

MARTÍNEZ-ÁLVAREZ, R. M.; MORALES, A. E.; SANZ, A. Antioxidant defenses in fish: Biotic and abiotic factors. Reviews in Fish Biology and Fisheries, v. 15, n. 1–2, p. 75–88, fev. 2005.

MARTINEZ, J. F. *et al*. Comparative cytogenetics of three populations from the Rhamdia quelen species complex (Siluriformes, Heptapteridae) in two Brazilian hydrographic basins. Caryologia, v. 64, n. 1, p. 121–128, 2011.

MATVIISHYN, T. M. T. M. *et al*. Tissue-specific induction of oxidative stress in goldfish by 2,4-dichlorophenoxyacetic acid: Mild in brain and moderate in liver and kidney. Environmental Toxicology and Pharmacology, v. 37, n. 2, p. 861–869, mar. 2014.

MENEZES, C. *et al*. Commercial formulation containing 2,4-D affects biochemical parameters and morphological indices of silver catfish exposed for 90 days. Fish Physiology and Biochemistry, v. 41, n. 2, p. 323–330, abr. 2015.

MIERZEJEWSKA, E. *et al*. Removal and ecotoxicity of 2,4-D and MCPA in microbial cultures enriched with structurally-similar plant secondary metabolites. Water (Switzerland), v. 11, n. 7, p. 1–16, 2019.

MODESTO, K. A.; MARTINEZ, C. B. R. Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish Prochilodus lineatus. Chemosphere, v. 78, n. 3, p. 294–299, jan. 2010.

MOUSA, H. M. *et al*. Antioxidant levels in tissues of young and adult camels (Camelus dromedarius). Journal of physiology and biochemistry, v. 62, n. 3, p. 213–8, set. 2006.

MUNRO, I. C. *et al*. A Comprehensive, Integrated Review and Evaluation of the Scientific Evidence Relating to the Safety of the Herbicide 2,4-D. Journal of the American College of Toxicology, v. 11, n. 5, p. 559–664, 4 set. 1992.

MÜNZEL, T. *et al*. Impact of Oxidative Stress on the Heart and Vasculature: Part 2 of a 3-Part SeriesJournal of the American College of CardiologyElsevier USA, , 11 jul. 2017. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/28683969>. Acesso em: 14 fev. 2020

MURUSSII, C. *et al*. Alterações em marcadores oxidativos, antioxidantes endógenos e na atividade da enzima acetilcolinesterase em trabalhadores rurais expostos a pesticidas agrícolas - Um estudo piloto. Ciencia Rural, v. 44, n. 7, p. 1186–1193, 2014.

NAIGAGA, I. *et al*. Fish as bioindicators in aquatic environmental pollution assessment: A case study in Lake Victoria wetlands, Uganda. Physics and Chemistry of the Earth, v. 36, n. 14–15, p. 918–928, 2011.

NAKBI, A. *et al*. Effects of olive oil and its fractions on oxidative stress and the liver's fatty acid composition in 2,4-Dichlorophenoxyacetic acid-treated rats. Nutrition and Metabolism, v. 7, p. 80, 29 out. 2010.

NAKBI, A. *et al*. Olive oil protects against 2,4-dichlorophenoxyacetic acid-induced oxidative renal dysfunction in adult rats. European Journal of Lipid Science and Technology, v. 114, n. 4, p. 469–478, 1 abr. 2012.

NITA, M.; GRZYBOWSKI, A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in AdultsOxidative Medicine and Cellular LongevityHindawi Limited, , 2016.

NORRIS, L. E. *et al*. Comparison of dietary conjugated linoleic acid with safflower oil on body composition in obese postmenopausal women with type 2 diabetes mellitus. AMERICAN JOURNAL OF CLINICAL NUTRITION, v. 90, n. 3, p. 468–476, 2009.

OLIVEIRA, M.; PACHECO, M.; SANTOS, M. A. Organ specific antioxidant responses in golden grey mullet (Liza aurata) following a short-term exposure to phenanthrene. Science of the Total Environment, v. 396, n. 1, p. 70–78, 15 jun. 2008.

ORUÇ, E. Ö.; ÜNER, N. Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of Oreochromis niloticus. Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology, v. 127, n. 3, p. 291–296, 2000.

ORUÇ, E. Ö.; ÜNER, N. Marker enzyme assesment in the liver of Cyprinus carpio (L.) exposed to 2,4-D and azinphosmethyl. Journal of Biochemical and Molecular Toxicology, v. 16, n. 4, p. 182–188, 2002.

OTTO, D. M. E.; MOON, T. W. Endogenous antioxidant systems of two teleost fish, the rainbow trout and the black bullhead, and the effect of age. Fish Physiology and Biochemistry, v. 15, n. 4, p. 349–358, 1996.

OZCAN ORUC, E.; SEVGILER, Y.; UNER, N. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, v. 137, n. 1, p. 43–51, 2004.

ÖZTÜRK, O.; GÜMÜŞLÜ, S. Age-related changes of antioxidant enzyme activities, glutathione status and lipid peroxidation in rat erythrocytes after heat stress. Life Sciences, v. 75, n. 13, p. 1551–1565, 13 ago. 2004.

PACIFICI, R. E.; DAVIES, K. J. A. Protein, lipid and DNA repair systems in oxidative stress: The free-radical theory of aging revisitedGerontology, 1991. Disponível em: <https://www.karger.com/Article/FullText/213257>. Acesso em: 18 fev. 2020

PANDEY, S. *et al*. Effect of endosulfan on antioxidants of freshwater fish Channa punctatus bloch: 1. Protection against lipid peroxidation in liver by copper preexposure. Archives of Environmental Contamination and Toxicology, v. 41, n. 3, p. 345–352, 2001.

PARENTE, T. E. M.; HAUSER-DAVIS, R. A. The Use of Fish Biomarkers in the Evaluation of Watter Pollution. Pollution and Fish Health in Tropical Ecosystems, n. December, p. 164–181, 2014.

PARK, K. *et al*. Biological and molecular responses of Chironomus riparius (Diptera, Chironomidae) to herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, v. 151, n. 4, p. 439–446, maio 2010.

PAROLINI, M. *et al*. Age- and sex-dependent variation in the activity of antioxidant enzymes in the brown trout (Salmo trutta). Fish Physiology and Biochemistry, v. 45, n. 1, p. 145–154, 15 fev. 2019.

PATEL, R. *et al*. Reactive Oxygen Species: The Good and the Bad. In: Reactive Oxygen Species (ROS) in Living Cells. [s.l.] InTech, 2018.

PETERSON MYERS, J. *et al*. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. 2016.

PIZZIMENTI, S. *et al*. The “Two-Faced” effects of reactive oxygen species and the lipid peroxidation product 4-Hydroxynonenal in the hallmarks of cancerCancersMultidisciplinary Digital Publishing Institute (MDPI), , jun. 2010.

POCHETTINO, A. A. *et al*. Oxidative stress in ventral prostate, ovary, and breast by 2,4-dichlorophenoxyacetic acid in pre- and postnatal exposed rats. Environmental Toxicology, v. 28, n. 1, p. 1–10, 1 jan. 2013.

POSTLETHWAIT, J. *et al*. Subfunction partitioning, the teleost radiation and the annotation of the human genome. Trends in Genetics, v. 20, n. 10, p. 481–490, 2004.

RAFTOPOULOU, E. K. *et al*. Introduction of cAMP and establishment of neutral lipids alterations as pollution biomarkers using the mussel Mytilus galloprovincialis. Correlation with a battery of biomarkers. Science of the Total Environment, v. 368, n. 2–3, p. 597–614, 15 set. 2006.

RIZVI, S. I.; MAURYA, P. K. Alterations in antioxidant enzymes during aging in humans. Molecular Biotechnology. Anais...set. 2007

RUDNEVA, I. I. Antioxidant system of Black Sea animals in early development. Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology, v. 122, n. 2, p. 265–271, fev. 1999.

RUDNEVA, I. I. *et al*. Age composition and antioxidant enzyme activities in blood of Black Sea teleosts. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, v. 151, n. 2, p. 229–239, mar. 2010.

RUIZ DE ARCAUTE, C.; SOLONESKI, S.; LARRAMENDY, M. L. Toxic and genotoxic effects of the 2,4-dichlorophenoxyacetic acid (2,4-D)-based herbicide on the Neotropical fish Cnesterodon decemmaculatus. Ecotoxicology and Environmental Safety, v. 128, p. 222–229, 2016.

RUZZA, P.; CALDERAN, A. Glutathione transferase (GST)-activated prodrugsPharmaceuticsMultidisciplinary Digital Publishing Institute (MDPI), , 2 abr. 2013.

SAERA-VILA, A. *et al*. Assessment of the health and antioxidant trade-off in gilthead sea bream (Sparus aurata L.) fed alternative diets with low levels of contaminants. Aquaculture, v. 296, n. 1–2, p. 87–95, 1 nov. 2009.

SANDAL, S.; YILMAZ, B. Genotoxic effects of chlorpyrifos, cypermethrin, endosulfan and 2,4-D on human peripheral lymphocytes cultured from smokers and nonsmokers. Environmental Toxicology, v. 26, n. 5, p. 433–442, out. 2011.

SANTANA, M. S. *et al*. Biomarker responses in fish exposed to polycyclic aromatic hydrocarbons (PAHs): Systematic review and meta-analysis. Environmental Pollution, v. 242, p. 449–461, 1 nov. 2018.

SCHIEBER, M.; CHANDEL, N. S. ROS function in redox signaling and oxidative stressCurrent BiologyCell Press, , 19 maio 2014.

SCHLENK, D. Necessity of defining biomarkers for use in ecological risk assessments. Marine Pollution Bulletin. Anais...jan. 1999

SCHRECK, C. B.; CONTRERAS-SANCHEZ, W.; FITZPATRICK, M. S. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture, v. 197, n. 1–4, p. 3–24, 1 jun. 2001.

SCHRECK, C. B.; TORT, L. The Concept of Stress in Fish. In: Fish Physiology. [s.l.] Elsevier Inc., 2016. v. 35p. 1–34.

SEDLAK, J.; LINDSAY, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical Biochemistry, v. 25, n. C, p. 192–205, 1968.

SEVCIKOVA, M. *et al*. Metals as a cause of oxidative stress in fish: a reviewVeterinarni Medicina. [s.l: s.n.].

SHAFEEQ, S.; MAHBOOB, T. Magnesium supplementation ameliorates toxic effects of 2,4-dichlorophenoxyacetic acid in rat model. Human and Experimental Toxicology, v. 39, n. 1, p. 47–58, 1 jan. 2020.

SINGH, V.; SINGH, K. Effect of sublethal exposure of herbicides on the biochemical parameters of earthworm Eutyphoeus Waltoni Michaelsen [oligochaeta: Octochaetidae]. IIOAB Journal, v. 7, n. 10, p. 55–62, 2016.

SLANINOVÁ, A. *et al*. A review: Oxidative stress in fish induced by pesticides. Neuroendocrinology Letters, v. 30, n. SUPPL.1, p. 2–12, 2009.

SMITH, J. J.; VOSS, S. R. Bird and Mammal Sex-Chromosome Orthologs Map to the Same Autosomal Region in a Salamander (Ambystoma). Genetics Society of America, 2007.

ST. DIMITROVA, M.; TISHINOVA, V.; VELCHEVA, V. Combined effect of zinc and lead on the hepatic superoxide dismutase-catalase system in carp (Cyprinus carpio). Comparative Biochemistry and Physiology. Part C: Pharmacology, v. 108, n. 1, p. 43–46, 1994.

STARA, A.; KOUBA, A.; VELISEK, J. Biochemical and histological effects of sub-chronic exposure to atrazine in crayfish Cherax destructor. Chemico-Biological Interactions, v. 291, n. January, p. 95–102, 1 ago. 2018.

STATSOFT, I. STATISTICA (data analysis software system), version 8.0, 2007.

STOLZE, K.; NOHL, H. Effect of xenobiotics on the respiratory activity of rat heart mitochondria and the concomitant formation of superoxide radicals. Environmental Toxicology and Chemistry, v. 13, n. 3, p. 499–502, mar. 1994.

SULFATH, P. P.; SANKAR, H.; NANDAN, B. EFFECT OF ORGANOCHLORINE PESTICIDE, LINDANE ON THE ANTIOXIDANT ACTIVITY OF OREOCHROMIS MOSSAMBICUS (PETERS). Journal of Aquatic Biology & Fisheries, v. 1, p. 97–105, 2013.

TATSIONI, A.; IOANNIDIS, J. P. A. Meta-analysis. In: International Encyclopedia of Public Health, Second Edition. Second Edi ed. [s.l.] Elsevier, 2017. v. 5p. 117–124.

TAVARES-DIAS, M.; MONTAGNER, D. Empresa Brasileira de Pesquisa Agropecuária Embrapa Amapá Ministério da Agricultura, Pecuária e Abastecimento Uso e Principais Aplicações do Sal Comum na Piscicultura de Água Doce. 2015.

TAYEB, W. *et al*. Biochemical and histological evaluation of kidney damage after sub-acute exposure to 2,4-dichlorophenoxyacetic herbicide in rats: Involvement of oxidative stress. Toxicology Mechanisms and Methods, v. 22, n. 9, p. 696–704, nov. 2012.

TAYEB, W. *et al*. Alteration of lipid status and lipid metabolism, induction of oxidative stress and lipid peroxidation by 2,4-dichlorophenoxyacetic herbicide in rat liver. Toxicology Mechanisms and Methods, v. 23, n. 6, p. 449–458, jul. 2013.

TEJERO, I. *et al*. Theoretical modeling of hydroxyl-radical-induced lipid peroxidation reactions. Journal of Physical Chemistry B, v. 111, n. 20, p. 5684–5693, 24 maio 2007.

THOMAZ, J. M. *et al*. Cardio-respiratory function and oxidative stress biomarkers in Nile tilapia exposed to the organophosphate insecticide trichlorfon (NEGUVON®). Ecotoxicology and Environmental Safety, v. 72, n. 5, p. 1413–1424, jul. 2009.

TICHATI, L.; TREA, F.; OUALI, K. Potential Role of Selenium Against Hepatotoxicity Induced by 2,4-Dichlorophenoxyacetic Acid in Albino Wistar Rats. Biological Trace Element Research, v. 194, n. 1, p. 228–236, 1 mar. 2020.

TROUDI, A. *et al*. 2,4-Dichlorophenoxyacetic acid effects on nephrotoxicity in rats during late pregnancy and early postnatal periods. Ecotoxicology and Environmental Safety, v. 74, n. 8, p. 2316–2323, 2011.

TROUDI, A. *et al*. Oxidative stress induced by 2,4-phenoxyacetic acid in liver of female rats and their progeny: Biochemical and histopathological studies. Environmental Toxicology, v. 27, n. 3, p. 137–145, mar. 2012.

ÜNER, N. *et al*. Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of Oreochromis niloticus. Environmental Toxicology and Pharmacology, v. 21, n. 3, p. 241–245, maio 2006.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environmental Toxicology and Pharmacology, v. 13, n. 2, p. 57–149, 2003.

VENKOV, P. *et al*. Genotoxic effect of substituted phenoxyacetic acids. Archives of Toxicology, v. 74, n. 9, p. 560–566, 20 nov. 2000.

VINAGRE, C. *et al*. Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, Dicentrarchus labrax. Ecological Indicators, v. 23, p. 274–279, dez. 2012.

WAFA, T. *et al*. Oxidative Stress Induced by the 2,4-Dichlorophenoxyacetic Herbicide. In: Oxidative Stress - Environmental Induction and Dietary Antioxidants. [s.l.] InTech, 2012.

WANG, Y. *et al*. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol, v. 217, n. 6, 2018.

WHYTE, J. J. *et al*. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposureCritical Reviews in ToxicologyInforma Healthcare, , 2000.

YANG, W. *et al*. Age-dependent changes of the antioxidant system in rat livers are accompanied by altered mapk activation and a decline in motor signaling. EXCLI Journal, v. 14, p. 1273–1290, 22 dez. 2015.

YANG, Y. *et al*. Reactive oxygen species in the immune system. International Reviews of Immunology, v. 32, n. 3, p. 249–270, jun. 2013.

YI, S. W. *et al*. Agent Orange exposure and disease prevalence in Korean Vietnam veterans: The Korean veterans health study. Environmental Research, v. 133, p. 56–65, 2014.

ZÁMOCKÝ, M. *et al*. Molecular evolution of hydrogen peroxide degrading enzymesArchives of Biochemistry and Biophysics, 15 set. 2012.

ZENGIN, H. *et al*. Antioxidant Enzymatic Defences during Embryogenesis of Rainbow Trout Oncorhynchus mykiss (Walbaum 1792). Turkish Journal of Fisheries and Aquatic Sciences, v. 15, p. 437–446, 2015.

ZHANG, D. *et al*. Exposure to 2,4-dichlorophenoxyacetic acid induces oxidative stress and apoptosis in mouse testis. Pesticide Biochemistry and Physiology, v. 141, p. 18–22, 1 set. 2017.

ZHANG, J. *et al*. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish Carassius auratus. Chemosphere, v. 55, n. 2, p. 167–174, 2004.

ZHANG, W.; XIAO, S.; AHN, D. U. Protein Oxidation: Basic Principles and Implications for Meat QualityCritical Reviews in Food Science and Nutrition, jan. 2013.

ZHONG, X. *et al*. An evaluation of the protective role of vitamin C in reactive oxygen species-induced hepatotoxicity due to hexavalent chromium in vitro and in vivo. Journal of Occupational Medicine and Toxicology, v. ) 12, n. 15, 2017.

ZIMNIAK, P.; WAXMAN, D. J. Liver Cytochrome P450 Metabolism of Endogenous Steroid Hormones, Bile Acids, and Fatty Acids. In: [s.l: s.n.]. p. 123–144.

**APPENDIX A -** Approval of the project by the ethics committee of the UTFPR

Ministério da Educação
UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ
Câmpus Dois Vizinhos
**Comissão de Ética no Uso de Animais - CEUA**

**PROJETO DE PESQUISA / AULA PRÁTICA**

| | |
|---|---|
| **Título:** | Avaliação múltipla dos efeitos tóxicos do herbicida 2,4-D (ácido diclorofenoxiacético) sobre a espécie nativa *Rhamdia quelen* (Pisces: Siluriformes) |
| **Área Temática:** | Ciências Ambientais |
| **Pesquisador / Professor:** | Profa. Dra. Nédia de Castilhos Ghisi |
| **Instituição:** | UTFPR/ Dois Vizinhos |
| **Financiamento:** | Não há |
| **Versão:** | 02 |

| **PARECER CONSUBSTANCIADO DA CEUA** | **Protocolo nº 2016-18** |
|---|---|

**Apresentação do Projeto:**

O projeto em apreciação visa avaliar os possíveis efeitos do herbicida 2,4-D (ácido diclorofenoxiacético) em *Rhamdia quelen* (Pisces: Siluriformes).

**Objetivo:**

A presente proposta tem como objetivo avaliar os efeitos tóxicos da exposição aguda e subcrônica pelas vias hídrica e trófica ao herbicida 2,4-D (ácido diclorofenoxiacético) sobre a espécie nativa *Rhamdia quelen*, através da avaliação de diferentes biomarcadores. Os objetivos específicos dessa proposta são:
1- Avaliar a exposição por via hídrica do potencial tóxico do herbicida 2,4-D, por tempos de 24, 48, 96 e 192 horas, nas concentrações de 15, 30 e 60 µL, através de biomarcadores de genotoxicidade, mutagenicidade, citotoxicidade, análises morfológicas, análises bioquímicas e análise de desregulação endócrina;
2- Avaliar a exposição por via trófica do potencial tóxico do herbicida 2,4-D, por tempos de 24, 48, 96 e 192 horas, nas concentrações posteriormente definidas, através de biomarcadores de genotoxicidade, mutagenicidade, citotoxicidade, análises morfológicas, análises bioquímicas e análise de desregulação endócrina;

3- Oferecer oportunidade e estrutura para realização de trabalhos de conclusão de curso, iniciação científica e dissertações de mestrado no campus Dois Vizinhos da UTFPR e parceiras, contribuindo para formação de recursos humanos capacitados para realizar várias técnicas de análise e com senso crítico quanto aos problemas ambientais.

**Avaliação dos Riscos e Benefícios:**

Os organismos-alvo desta proposta, os peixes *Rhamdia quelen*, serão submetidos a exposições hídrica e trófica do herbicida 2,4-D em concentrações que próximas àquelas que encontrariam em seu ambiente natural contaminado pelo mesmo. Embora esta exposição intencional vise observar os prováveis malefícios causados pelo produto nestes animais, a proposta central do trabalho muito relevante, uma vez que o 2,4-D é um produto com história curta e, consequentemente, seus efeitos de longo prazo no ambiente e também nas comunidades humanas são desconhecidos. Assim, os efeitos tóxicos que sejam eventualmente detectados nestes peixes deverão servir de parâmetro para medidas de melhor controle no uso do herbicida.

**Comentários e Considerações sobre a Pesquisa:**

A metodologia a ser aplicada é compatível com os objetivos da proposta, inclusive é semelhante ao que tem sido feito na área de pesquisa, demonstrado através de citações de publicações atualizadas (ano de 2016).

**Considerações sobre os Termos de apresentação obrigatória:**

Foram anexados à presente solicitação os seguintes termos e documentos: 1) o requerimento preenchido e assinado pelo pesquisador responsável pelo projeto; 2) o formulário unificado de encaminhamento ao CEUA-UTFPR, com os ajustes solicitados no parecer anterior (versão 01); 3) projeto de pesquisa completo no modelo da PROPPG-CEUA; 4) a declaração de não início do projeto (com assinatura e data); 5) Declaração de médico veterinário, datado e assinado, como responsável técnico pelo bem estar dos animais utilizados durante a execução do projeto; 6) o registro de projeto junto à DIRPPG-DV.

**Conclusões ou Pendências e Lista de Inadequações:**

Todos os documentos exigidos foram apresentados e em ordem e, como já comentado, os riscos inerentes aos peixes do experimentos são compensados pelos benefícios que os futuros resultados deverão trazer.

No parecer anterior (versão 1) deste mesmo protocolo foram exigidos ajustes ou justificativas para os seguintes três itens da proposta:

1) Correção nas tabelas do item 10.11 que indicam somente tratamentos com concentrações de 15 µg/L, enquanto o declarado no restante do documento seriam 15, 30 e 45 µg/L;
2) Ainda no item 10.11, solicitou-se maiores explicações que fundamentassem e justificasse o uso de tantos animais extras como controle para cada tratamento;
3) Para a segunda fase do experimento, que seria da exposição em via trófica, definir as concentrações do herbicida a serem experimentadas (eu não serem decididas *a posteriori*), ou apresentar justificativa plausível para tal indefinição.

A proponente fez todos os ajustes e explicações solicitadas. Particularmente quanto à questão do uso de triplicatas dos grupos controle, demonstrou, através de publicações atualizadas em periódicos científicos renomados, que esta é uma prática corrente em termos de delineamento amostral para o tipo de experimentação científica da área. Quanto à definição das concentrações do herbicida no experimento via trófica, a proponente seguiu as recomendações da instrução OECD 420 (da *Organisation for Economic Co-operation and Development*) que indica parâmetros de experimentação quanto a testes de toxicidade aguda em animais não-humanos. O fato de apoiar-se em parâmetros respeitados internacionalmente indicaram a esta CEUA a concordância com o procedimento.

**Situação do Parecer:**

APROVADO

**Considerações Finais a Critério da CEUA:**

Todos os procedimentos devem seguir a lei n° 11.794 de 8 de outubro de 2008.

Todos os membros presentes na reunião de 13 de setembro de 2016 acompanharam a decisão do parecerista.

Ministério da Educação
**UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ**
**Câmpus Dois Vizinhos**
**Comissão de Ética no Uso de Animais - CEUA**

UTFPR
UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ

## CERTIFICADO

Certificamos que o projeto intitulado "Avaliação múltipla dos efeitos tóxicos do herbicida 2,4-D (ácido diclorofenoxiacético) sobre a espécie nativa *Rhamdia quelen* (Pisces: Siluriformes)", protocolo nº 2016/018, sob a responsabilidade de Nédia de Castilhos Ghisi - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-UTFPR) da UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ, em reunião de 13/09/2016.

| | |
|---|---|
| **Vigência do projeto:** | De 16/10/2016 a 10/01/2019 |
| **Finalidade** | (  ) Ensino ( X ) Pesquisa Científica |
| **Espécie/linhagem:** | *Rhamdia quelen* (Pisces: Siluriformes) |
| **Número de animais:** | 576 |
| **Peso/Idade:** | 50 g |
| **Sexo:** | Masculino |
| **Origem:** | Piscicultura comercial |

Dois Vizinhos, 14 de setembro de 2016.

Assinado por:
Gustavo Sene Silva
Vice-Presidente da Comissão de Ética no Uso de Animais da Universidade Tecnológica Federal do Paraná

**APPENDIX B -** Suplementary Table 1

Suplementary Table 1: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of CAT.

| Ref | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(GAAIED *et al*., 2019)** | Fish | Youn. | 0.8 | Pure | Med-water | 96 | All animal | CAT | 5 | 660.58 | 40.14 | 5 | 839.41 | 29.20 | 4.60 | 1.46 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Brain | CAT | 6 | 9.99 | 4.89 | 6 | 35.50 | 6.09 | 4.26 | 1.09 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Liver | CAT | 6 | 84.99 | 12.24 | 6 | 95.49 | 6.14 | 1.00 | 0.38 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Kidney | CAT | 6 | 52.49 | 8.59 | 6 | 42.49 | 7.34 | -1.16 | 0.39 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Gill | CAT | 6 | 7.63 | 1.24 | 6 | 10.81 | 2.10 | 1.70 | 0.45 |
| **(KUBRAK *et al*., 2013b)** | Fish | Adult | 100 | Pure | Med-water | 96 | Muscle | CAT | 6 | 1.81 | 0.46 | 6 | 5.62 | 1.15 | 4.02 | 1.01 |
| **(GRECO *et al*., 2011)** | Bivalve | Adult | 0.01 | Pure | Med-water | 672 | All animal | CAT | 15 | 19.30 | 11.50 | 15 | 3.60 | 2.60 | -1.83 | 0.19 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | CAT | 6 | 5.90 | 0.53 | 6 | 9.78 | 0.14 | 9.24 | 3.89 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | CAT | 6 | 1.55 | 0.46 | 6 | 2.17 | 0.78 | 0.89 | 0.37 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | CAT | 6 | 4.23 | 2.79 | 6 | 4.08 | 0.53 | -0.07 | 0.33 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | CAT | 6 | 135.33 | 35.80 | 6 | 154.67 | 46.70 | 0.43 | 0.34 |
| **(OZCAN ORUC;** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | CAT | 6 | 2.30 | 0.93 | 6 | 2.17 | 0.78 | -0.14 | 0.33 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SEVGILER; UNER, 2004)** | | | | | | | | | | | | | | | |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | CAT | 6 | 7.00 | 0.22 | 6 | 6.32 | 1.27 | -0.69 | 0.35 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Kidney | CAT | 6 | 5.90 | 0.53 | 6 | 8.60 | 2.15 | 1.59 | 0.44 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Brain | CAT | 6 | 1.55 | 0.46 | 6 | 1.41 | 0.36 | -0.31 | 0.34 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Gill | CAT | 6 | 4.23 | 2.79 | 6 | 4.33 | 1.95 | 0.04 | 0.33 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Kidney | CAT | 6 | 135.33 | 35.80 | 6 | 169.00 | 42.49 | 0.79 | 0.36 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Brain | CAT | 6 | 2.30 | 0.93 | 6 | 1.98 | 0.48 | -0.40 | 0.34 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Gill | CAT | 6 | 7.00 | 0.22 | 6 | 6.46 | 0.71 | -0.95 | 0.37 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 24 | Liver | CAT | 6 | 107.39 | 7.35 | 6 | 87.67 | 4.90 | -2.91 | 0.69 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 48 | Liver | CAT | 6 | 84.38 | 29.39 | 6 | 129.31 | 22.05 | 1.60 | 0.44 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 72 | Liver | CAT | 6 | 72.87 | 7.35 | 6 | 83.83 | 14.70 | 0.87 | 0.36 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 96 | Liver | CAT | 6 | 90.95 | 9.80 | 6 | 94.79 | 8.08 | 0.39 | 0.34 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Azinpho. | Med-water | 24 | Liver | CAT | 6 | 107.39 | 7.34 | 6 | 104.10 | 14.70 | -0.26 | 0.34 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Azinpho. | Med-water | 48 | Liver | CAT | 6 | 84.38 | 29.39 | 6 | 106.30 | 9.80 | 0.92 | 0.37 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Azinpho. | Med-water | 72 | Liver | CAT | 6 | 72.87 | 7.34 | 6 | 98.63 | 14.70 | 2.05 | 0.51 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Azinpho. | Med-water | 96 | Liver | CAT | 6 | 90.95 | 9.79 | 6 | 97.53 | 10.73 | 0.59 | 0.35 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 2.25 | Pure | Med-water | 240 | Liver | CAT | 4 | 23.80 | 2.80 | 4 | 26.90 | 1.80 | 1.15 | 0.58 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 7.5 | Pure | Med-water | 240 | Liver | CAT | 4 | 23.80 | 2.80 | 4 | 28.50 | 7.40 | 0.73 | 0.53 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 22.5 | Pure | Med-water | 240 | Liver | CAT | 4 | 23.80 | 2.80 | 4 | 27.60 | 6.20 | 0.69 | 0.53 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 6 | Picloram | Med-water | 240 | Liver | CAT | 4 | 23.80 | 2.80 | 4 | 23.40 | 2.00 | -0.14 | 0.50 |
| **(ES RUIZ DE ARCAUTE *et al.*, 2019)** | Fish | Adult | 252 | Pure | Med-water | 48 | Viscera | CAT | 10 | 12.86 | 2.34 | 10 | 68.91 | 20.27 | 3.72 | 0.55 |
| **(ES RUIZ DE ARCAUTE *et al.*, 2019)** | Fish | Adult | 252 | Pure | Med-water | 96 | Viscera | CAT | 10 | 13.52 | 2.96 | 10 | 41.55 | 6.57 | 5.27 | 0.89 |
| **(ES RUIZ DE ARCAUTE *et al.*, 2019)** | Fish | Adult | 250 | Pure | Med-water | 48 | Opercle | CAT | 10 | 12.86 | 7.39 | 10 | 68.91 | 217.91 | 0.35 | 0.20 |
| **(ES RUIZ DE ARCAUTE *et al.*, 2019)** | Fish | Adult | 250 | Pure | Med-water | 96 | Opercle | CAT | 10 | 13.52 | 9.36 | 10 | 41.55 | 131.39 | 0.29 | 0.20 |
| **(GAAIED *et al.*, 2019)** | Fish | Youn. | 0.8 | Pure | Med-water | 96 | All animal | CAT | 30 | 665.02 | 39.40 | 30 | 839.90 | 29.55 | 4.96 | 0.27 |
| **(BANAOUI *et al.*, 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | CAT | 8 | 3.64 | 0.98 | 8 | 4.76 | 1.21 | 0.96 | 0.28 |
| **(BANAOUI *et al.*, 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | CAT | 8 | 3.45 | 0.84 | 8 | 6.67 | 0.89 | 3.52 | 0.64 |
| **(BANAOUI *et al.*, 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | CAT | 8 | 3.68 | 1.17 | 8 | 5.74 | 1.12 | 1.70 | 0.34 |
| **(BANAOUI *et al.*, 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | CAT | 8 | 2.52 | 0.75 | 8 | 4.34 | 0.19 | 3.16 | 0.56 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | CAT | 8 | 3.52 | 0.56 | 8 | 3.66 | 0.80 | 0.19 | 0.25 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | CAT | 8 | 3.47 | 0.52 | 8 | 5.25 | 0.56 | 3.12 | 0.55 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | CAT | 8 | 3.14 | 0.47 | 8 | 5.39 | 0.89 | 2.99 | 0.53 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | CAT | 8 | 1.87 | 0.42 | 8 | 3.23 | 0.70 | 2.22 | 0.40 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | CAT | 8 | 11.66 | 0.62 | 8 | 16.48 | 1.40 | 4.21 | 0.80 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | CAT | 8 | 12.28 | 0.78 | 8 | 17.25 | 0.47 | 7.33 | 1.93 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | CAT | 8 | 11.97 | 0.78 | 8 | 19.74 | 2.49 | 3.99 | 0.75 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | CAT | 8 | 14.77 | 0.78 | 8 | 13.52 | 2.64 | -0.60 | 0.26 |
| **(MENEZES *et al*., 2015)** | Fish | Youn. | 0.5 | Pure | Med-water | 2160 | Liver | CAT | 24 | 2.99 | 0.31 | 24 | 6.29 | 0.76 | 5.60 | 0.41 |

**APPENDIX C** - Suplementary Table 2

Suplementary Table 2: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of SOD.

| Ref. | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(HATTAB *et al*., 2015)** | Worm | Adul. | 14 | Pure | Oral | 168 | Al the animal | SOD | 10 | 31.180 | 2.120 | 10 | 56.690 | 4.250 | 7.275 | 1.523 |
| **HATTAB *et al*., 2015)** | Worm | Adul. | 14 | Pure | Oral | 336 | Al the animal | SOD | 10 | 29.050 | 2.830 | 10 | 36.140 | 7.080 | 1.260 | 0.240 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adul. | 100 | Pure | Medium-water | 96 | Brain | SOD | 6 | 62.500 | 20.400 | 6 | 62.500 | 12.730 | 0.000 | 0.333 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adul. | 100 | Pure | Medium-water | 96 | Liver | SOD | 6 | 243.750 | 46.540 | 6 | 260.410 | 38.280 | 0.361 | 0.339 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adul. | 100 | Pure | Medium-water | 96 | Kidney | SOD | 6 | 127.080 | 30.610 | 6 | 87.500 | 20.400 | -1.405 | 0.416 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adul. | 100 | Pure | Medium-water | 96 | Gill | SOD | 6 | 34.950 | 6.170 | 6 | 47.920 | 2.640 | 2.523 | 0.599 |
| **(KUBRAK *et al*., 2013b)** | Fish | Adul. | 100 | Pure | Medium-water | 96 | Muscle | SOD | 6 | 12.170 | 4.330 | 6 | 11.860 | 2.010 | -0.085 | 0.334 |
| **(TAYEB *et al*., 2013)** | Rodent | Adul. | 150 | Pure | Oral | 672 | Liver | SOD | 10 | 8.420 | 1.040 | 10 | 5.130 | 3.380 | -1.260 | 0.240 |
| **(TAYEB *et al*., 2012)** | Rodent | Adul. | 150 | Pure | Oral | 672 | Kidney | SOD | 10 | 5.310 | 0.580 | 10 | 2.740 | 0.760 | -3.641 | 0.531 |
| **(TROUDI *et al*., 2011)** | Rodent | Adul. | 22.5 | Pure | Oral | 336 | Liver | SOD | 6 | 24.440 | 5.683 | 6 | 12.780 | 3.552 | -2.272 | 0.548 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(TROUDI *et al.*, 2011)** | Rodent | Young | 22.5 | Pure | Mother | 336 | Liver | SOD | 10 | 24.290 | 9.424 | 10 | 13.750 | 3.384 | -1.426 | 0.251 |
| **(TROUDI *et al.*, 2011)** | Rodent | Adul. | 22.5 | Pure | Oral | 336 | Kidney | SOD | 6 | 21.880 | 8.034 | 6 | 12.830 | 6.369 | -1.152 | 0.389 |
| **(TROUDI *et al.*, 2011)** | Rodent | Young | 22.5 | Pure | Mother | 336 | Kidney | SOD | 10 | 19.310 | 9.392 | 10 | 13.240 | 10.973 | -0.569 | 0.208 |
| **(GRECO *et al.*, 2011)** | Bivalve | Adul. | 0.01 | Pure | Medium-water | 672 | Al the animal | SOD | 15 | 600.000 | 265.000 | 15 | 567.000 | 140.000 | -0.152 | 0.134 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 240.000 | 120.000 | 4 | 150.000 | 140.000 | -0.600 | 0.523 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 300.000 | 180.000 | 4 | 280.000 | 200.000 | -0.091 | 0.501 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 335.000 | 230.000 | 4 | 280.000 | 90.000 | -0.274 | 0.505 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 290.000 | 140.000 | 4 | 390.000 | 120.000 | 0.667 | 0.528 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 270.000 | 260.000 | 4 | 290.000 | 220.000 | 0.072 | 0.500 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 400.000 | 240.000 | 4 | 350.000 | 220.000 | -0.189 | 0.502 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2000.000 | 360.000 | 4 | 2450.000 | 610.000 | 0.781 | 0.538 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2430.000 | 350.000 | 4 | 2540.000 | 440.000 | 0.241 | 0.504 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2690.000 | 610.000 | 4 | 2610.000 | 480.000 | -0.127 | 0.501 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2290.000 | 360.000 | 4 | 2980.000 | 640.000 | 1.156 | 0.584 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2100.000 | 160.000 | 4 | 2140.000 | 180.000 | 0.204 | 0.503 |
| **(FERRI; DUFFARD; DE DUFFARD, 2007)** | Rodent | Young | 100 | Pure | Mother | 384 | Brain | SOD | 4 | 2410.000 | 300.000 | 4 | 2400.000 | 240.000 | -0.032 | 0.500 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 50 | Pure | Oral | 600 | Liver | SOD | 6 | 1986.400 | 191.900 | 6 | 835.500 | 420.800 | -3.249 | 0.773 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 100 | Pure | Oral | 600 | Liver | SOD | 6 | 1986.400 | 191.900 | 6 | 817.600 | 70.300 | -7.466 | 2.656 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 50 | Pure | Oral | 600 | Kidney | SOD | 6 | 323.100 | 70.600 | 6 | 224.500 | 53.500 | -1.453 | 0.421 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 100 | Pure | Oral | 600 | Kidney | SOD | 6 | 323.100 | 70.600 | 6 | 238.100 | 192.300 | -0.542 | 0.346 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 50 | Pure | Oral | 600 | Brain | SOD | 6 | 790.200 | 152.100 | 6 | 659.400 | 31.700 | -1.099 | 0.384 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 100 | Pure | Oral | 600 | Brain | SOD | 6 | 790.200 | 152.100 | 6 | 163.400 | 56.900 | -5.039 | 1.391 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 50 | Pure | Oral | 600 | Heart | SOD | 6 | 984.400 | 219.400 | 6 | 447.800 | 109.700 | -2.856 | 0.673 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adul. | 100 | Pure | Oral | 600 | Heart | SOD | 6 | 984.400 | 219.400 | 6 | 640.600 | 88.600 | -1.897 | 0.483 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Kidney | SOD | 6 | 0.160 | 0.004 | 6 | 0.145 | 0.090 | -0.145 | 0.334 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Brain | SOD | 6 | 0.160 | 0.020 | 6 | 0.170 | 0.140 | 0.092 | 0.334 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Gill | SOD | 6 | 0.038 | 0.022 | 6 | 0.860 | 0.075 | 12.933 | 7.303 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Kidney | SOD | 6 | 2.530 | 0.410 | 6 | 2.090 | 0.120 | -1.345 | 0.409 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Brain | SOD | 6 | 0.240 | 0.120 | 6 | 0.240 | 0.030 | 0.000 | 0.333 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Gill | SOD | 6 | 0.030 | 0.003 | 6 | 0.098 | 0.007 | 8.753 | 3.525 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Kidney | SOD | 6 | 0.160 | 0.004 | 6 | 0.155 | 0.014 | 0.000 | 0.333 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Brain | SOD | 6 | 0.160 | 0.020 | 6 | 0.170 | 0.040 | 0.292 | 0.337 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Gill | SOD | 6 | 0.038 | 0.022 | 6 | 4.030 | 0.680 | 7.660 | 2.778 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Kidney | SOD | 6 | 2.530 | 0.410 | 6 | 2.190 | 0.170 | -1.000 | 0.375 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Brain | SOD | 6 | 0.240 | 0.120 | 6 | 0.250 | 0.007 | 0.108 | 0.334 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Gill | SOD | 6 | 0.030 | 0.003 | 6 | 0.081 | 0.009 | 6.252 | 1.962 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Pure | Medium-water | 24 | Liver | SOD | 6 | 5.220 | 2.841 | 6 | 2.990 | 1.445 | -0.913 | 0.368 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Pure | Medium-water | 48 | Liver | SOD | 6 | 4.350 | 0.710 | 6 | 4.740 | 0.710 | 0.507 | 0.344 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Pure | Medium-water | 72 | Liver | SOD | 6 | 4.930 | 1.421 | 6 | 10.160 | 1.715 | 3.071 | 0.726 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Pure | Medium-water | 96 | Liver | SOD | 6 | 5.320 | 0.465 | 6 | 13.540 | 0.955 | 10.060 | 4.550 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Azin pho. | Med-water | 24 | Liver | SOD | 6 | 5.220 | 2.840 | 6 | 7.160 | 1.886 | 0.742 | 0.356 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Azin pho. | Med-water | 48 | Liver | SOD | 6 | 4.350 | 0.710 | 6 | 5.220 | 0.245 | 1.515 | 0.429 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Azin pho. | Med-water | 72 | Liver | SOD | 6 | 4.930 | 1.420 | 6 | 4.740 | 1.176 | -0.134 | 0.334 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adul. | 87 | Azin pho. | Med-water | 96 | Liver | SOD | 6 | 5.320 | 0.460 | 6 | 5.700 | 0.735 | 0.575 | 0.347 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Pure | Medium-water | 24 | Liver | SOD | 6 | 9.700 | 1.200 | 6 | 11.770 | 1.450 | 1.436 | 0.419 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Pure | Medium-water | 48 | Liver | SOD | 6 | 10.070 | 1.820 | 6 | 8.130 | 2.910 | -0.738 | 0.356 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Pure | Medium-water | 72 | Liver | SOD | 6 | 9.700 | 0.610 | 6 | 8.610 | 0.610 | -1.649 | 0.447 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Pure | Medium-water | 96 | Liver | SOD | 6 | 9.100 | 2.060 | 6 | 10.190 | 1.330 | 0.580 | 0.347 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Azin pho. | Med-water | 24 | Liver | SOD | 6 | 9.700 | 1.200 | 6 | 19.170 | 1.040 | 7.785 | 2.859 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Azin pho. | Med-water | 48 | Liver | SOD | 6 | 10.070 | 1.820 | 6 | 15.650 | 1.340 | 3.223 | 0.766 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Azin pho. | Med-water | 72 | Liver | SOD | 6 | 9.700 | 0.610 | 6 | 15.890 | 2.320 | 3.369 | 0.806 |
| **(ORUÇ; ÜNER, 2000)** | Fish | Adul. | 27 | Azin pho. | Med-water | 96 | Liver | SOD | 6 | 9.100 | 2.060 | 6 | 16.260 | 1.210 | 3.912 | 0.971 |
| **(SHAFEEQ; MAHBOOB, 2020)** | Rodent | Adul. | 150 | Pure | Oral | 24 | Liver | SOD | 10 | 1.140 | 0.160 | 10 | 0.630 | 0.080 | -3.862 | 0.573 |
| **(SHAFEEQ; MAHBOOB, 2020)** | Rodent | Adul. | 150 | Pure | Oral | 24 | Kidney | SOD | 10 | 1.220 | 0.110 | 10 | 0.570 | 0.100 | -5.922 | 1.077 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(TICHATI; TREA; OUALI, 2020)** | Rodent | Adul. | 5 | Pure | Oral | 1344 | Liver | SOD | 6 | 222.920 | 51.480 | 6 | 7.640 | 18.710 | -5.131 | 1.430 |
| **(ZHANG *et al*., 2017)** | Rodent | Adul. | 201 | Pure | Oral | 336 | Gonad | SOD | 4 | 89.180 | 3.710 | 4 | 67.170 | 4.240 | -4.804 | 1.943 |
| **(AMEL *et al*., 2016)** | Rodent | Adul. | 5 | Pure | Oral | 672 | Brain | SOD | 10 | 3.960 | 1.040 | 10 | 6.516 | 0.928 | 2.485 | 0.354 |
| **(AL-BAROUDI; ARAFAT; EL-KHOLY, 2014)** | Rodent | Adul. | 150 | Pure | Oral | 672 | Liver | SOD | 6 | 94.150 | 0.850 | 6 | 89.830 | 0.600 | -5.420 | 1.558 |
| **(NAKBI *et al*., 2012)** | Rodent | Adul. | 5 | Pure | Oral | 672 | kidney | SOD | 10 | 5.280 | 0.790 | 10 | 3.800 | 0.530 | -2.107 | 0.311 |
| **(NAKBI *et al*., 2010)** | Rodent | Adul. | 5 | Pure | Oral | 672 | Liver | SOD | 10 | 9.490 | 1.340 | 10 | 6.710 | 1.580 | -1.818 | 0.283 |

**APPENDIX D -** Suplementary Table 3

Suplementary Table 3: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of GSH.

| Ref. | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Medium-water | 96 | Brain | GSH | 6 | 78.0 | 6.0 | 6 | 106.00 | 16.97 | 6 | 78.0 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Medium-water | 96 | Liver | GSH | 6 | 220.0 | 19.0 | 6 | 200.00 | 16.97 | 6 | 220.0 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Medium-water | 96 | Kidney | GSH | 6 | 177.0 | 14.7 | 6 | 169.00 | 14.69 | 6 | 177.0 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Medium-water | 96 | Gill | GSH | 6 | 65.8 | 4.4 | 6 | 60.88 | 7.64 | 6 | 65.8 |
| **(KUBRAK *et al*., 2013b)** | Fish | Adult | 100 | Pure | Medium-water | 96 | Muscle | GSH | 6 | 3.8 | 1.4 | 6 | 1.38 | 1.29 | 6 | 3.8 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 491.0 | 29.4 | 6 | 520.00 | 34.29 | 6 | 491.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 642.0 | 210.7 | 6 | 748.00 | 34.29 | 6 | 642.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 341.0 | 58.8 | 6 | 333.00 | 19.60 | 6 | 341.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 1562.0 | 396.8 | 6 | 1360.00 | 431.11 | 6 | 1562.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 672.0 | 58.8 | 6 | 676.00 | 95.53 | 6 | 672.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | GSH | 6 | 519.0 | 93.1 | 6 | 537.00 | 53.89 | 6 | 519.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | GSH | 6 | 942.0 | 12.2 | 6 | 951.00 | 61.24 | 6 | 942.0 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | GSH | 6 | 1072.0 | 188.6 | 6 | 667.00 | 112.68 | 6 | 1072.0 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | GSH | 6 | 3551.0 | 1854.3 | 6 | 1560.00 | 553.58 | 6 | 3551.0 |
| **(TROUDI *et al*., 2012)** | Rodent | Adult | 22.5 | Pure | Oral | 336 | Liver | GSH | 6 | 729.0 | 30.3 | 6 | 504.68 | 44.24 | 6 | 729.0 |
| **(TROUDI *et al*., 2011)** | Rodent | Adult | 22.5 | Pure | Oral | 336 | Kidney | GSH | 6 | 538.2 | 56.2 | 6 | 393.52 | 55.92 | 6 | 538.2 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 50 | Pure | Oral | 600 | Liver | GSH | 6 | 31.5 | 3.3 | 6 | 25.60 | 1.10 | 6 | 31.5 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 100 | Pure | Oral | 600 | Liver | GSH | 6 | 31.5 | 3.3 | 6 | 28.20 | 4.10 | 6 | 31.5 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 50 | Pure | Oral | 600 | Kidney | GSH | 6 | 43.4 | 3.2 | 6 | 26.90 | 7.20 | 6 | 43.4 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 100 | Pure | Oral | 600 | Kidney | GSH | 6 | 43.4 | 3.2 | 6 | 19.30 | 6.30 | 6 | 43.4 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 50 | Pure | Oral | 600 | Brain | GSH | 6 | 44.9 | 4.6 | 6 | 12.90 | 2.20 | 6 | 44.9 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 100 | Pure | Oral | 600 | Brain | GSH | 6 | 44.9 | 4.6 | 6 | 12.70 | 1.90 | 6 | 44.9 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 50 | Pure | Oral | 600 | Heart | GSH | 6 | 65.0 | 1.9 | 6 | 41.50 | 11.90 | 6 | 65.0 |
| **(CELIK; TULUCE; ISIK, 2006)** | Rodent | Adult | 100 | Pure | Oral | 600 | Heart | GSH | 6 | 65.0 | 1.9 | 6 | 37.80 | 10.40 | 6 | 65.0 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Medium-water | 48 | Viscera | GSH | 10 | 3.2 | 0.6 | 10 | 2.08 | 0.13 | 10 | 3.2 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Medium-water | 96 | Viscera | GSH | 10 | 2.6 | 0.2 | 10 | 1.04 | 0.03 | 10 | 2.6 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Medium-water | 48 | Opercle | GSH | 10 | 3.2 | 1.8 | 10 | 2.08 | 6.58 | 10 | 3.2 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Medium-water | 96 | Opercle | GSH | 10 | 2.6 | 0.5 | 10 | 1.04 | 3.29 | 10 | 2.6 |
| **(SHAFEEQ; MAHBOOB, 2020)** | Rodent | Adult | 150 | Pure | Oral | 24 | Liver | GSH | 10 | 91.3 | 9.9 | 10 | 52.15 | 12.41 | 10 | 91.3 |
| **(SHAFEEQ; MAHBOOB, 2020)** | Rodent | Adult | 150 | Pure | Oral | 24 | Kidney | GSH | 10 | 77.0 | 4.7 | 10 | 38.24 | 8.17 | 10 | 77.0 |
| **(TICHATI; TREA; OUALI, 2020)** | Rodent | Adult | 6 | Pure | Oral | 1346 | Liver | GSH | 6 | 1.7 | 0.1 | 6 | 1.15 | 0.05 | 6 | 1.7 |
| **(CELIK; TULUCE, 2007)** | Rodent | Adult | 100 | Pure | Oral | 600 | Spleen | GSH | 6 | 131.2 | 12.3 | 6 | 111.99 | 0.84 | 6 | 131.2 |

**APPENDIX E -** Suplementary table 4

Suplementary Table 4: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of GST.

| Ref | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(GAAIED *et al*., 2019)** | Fish | Young | 0.8 | Pure | Med-water | 96 | Al the animal | GST | 5 | 26.06 | 1.46 | 5 | 17.18 | 2.05 | 5 | 26.06 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Brain | GST | 6 | 0.66 | 0.04 | 6 | 0.60 | 0.06 | 6 | 0.66 |
| **MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Liver | GST | 6 | 1.68 | 0.22 | 6 | 1.41 | 0.20 | 6 | 1.68 |
| **MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Kidney | GST | 6 | 0.88 | 0.06 | 6 | 0.80 | 0.11 | 6 | 0.88 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Gill | GST | 6 | 521.00 | 30.20 | 6 | 528.70 | 60.42 | 6 | 521.00 |
| **(KUBRAK *et al*., 2013b)** | Fish | Adult | 100 | Pure | Med-water | 96 | Muscle | GST | 6 | 90.50 | 15.43 | 6 | 91.90 | 5.38 | 6 | 90.50 |
| **(PARK *et al*., 2010)** | Insect | Young | 0.01 | Pure | Med-water | 24 | Al the animal | GST | 5 | 0.17 | 0.04 | 5 | 0.39 | 0.01 | 5 | 0.17 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | GST | 6 | 86.82 | 23.93 | 6 | 203.83 | 64.20 | 6 | 86.82 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | GST | 6 | 168.56 | 45.82 | 6 | 199.40 | 19.66 | 6 | 168.56 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | GST | 6 | 73.68 | 28.68 | 6 | 76.91 | 8.72 | 6 | 73.68 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | GST | 6 | 98.90 | 4.80 | 6 | 201.80 | 35.27 | 6 | 98.90 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | GST | 6 | 71.70 | 21.80 | 6 | 81.35 | 10.30 | 6 | 71.70 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | GST | 6 | 61.50 | 3.47 | 6 | 60.90 | 4.40 | 6 | 61.50 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 24 | Liver | GST | 6 | 219.58 | 39.19 | 6 | 470.10 | 30.37 | 6 | 219.58 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 48 | Liver | GST | 6 | 225.77 | 31.84 | 6 | 380.41 | 30.13 | 6 | 225.77 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 72 | Liver | GST | 6 | 191.75 | 31.84 | 6 | 420.60 | 46.54 | 6 | 191.75 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 96 | Liver | GST | 6 | 204.12 | 44.09 | 6 | 361.85 | 53.89 | 6 | 204.12 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 2.25 | Pure | Med-water | 240 | Liver | GST | 4 | 1.73 | 0.26 | 4 | 2.11 | 0.16 | 4 | 1.73 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 7.5 | Pure | Med-water | 240 | Liver | GST | 4 | 1.73 | 0.26 | 4 | 2.14 | 0.10 | 4 | 1.73 |
| **(GALLAGHER; DI GIULIO, 1991)** | Fish | Adult | 22.5 | Pure | Med-water | 240 | Liver | GST | 4 | 1.73 | 0.26 | 4 | 1.85 | 0.32 | 4 | 1.73 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Med-water | 48 | Viscera | GST | 10 | 0.04 | 0.01 | 10 | 0.07 | 0.01 | 10 | 0.04 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Med-water | 96 | Viscera | GST | 10 | 0.04 | 0.01 | 10 | 0.10 | 0.01 | 10 | 0.04 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Med-water | 48 | Opercle | GST | 10 | 0.04 | 0.03 | 10 | 0.07 | 0.22 | 10 | 0.04 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Med-water | 96 | Opercle | GST | 10 | 0.04 | 0.03 | 10 | 0.10 | 0.32 | 10 | 0.04 |
| **(GAAIED *et al*., 2019)** | Fish | Young | 0.8 | Pure | Med-water | 96 | Al the animal | GST | 30 | 26.19 | 1.41 | 30 | 17.23 | 2.09 | 30 | 26.19 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | GST | 8 | 20.00 | 4.62 | 8 | 27.69 | 2.31 | 8 | 20.00 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | GST | 8 | 23.85 | 3.08 | 8 | 67.69 | 3.85 | 8 | 23.85 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | GST | 8 | 21.54 | 4.62 | 8 | 93.85 | 12.31 | 8 | 21.54 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | GST | 8 | 17.69 | 5.38 | 8 | 88.46 | 6.92 | 8 | 17.69 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | GST | 8 | 18.40 | 9.60 | 8 | 38.40 | 4.80 | 8 | 18.40 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | GST | 8 | 25.60 | 10.30 | 8 | 59.20 | 4.80 | 8 | 25.60 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | GST | 8 | 25.60 | 4.79 | 8 | 72.80 | 3.20 | 8 | 25.60 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | GST | 8 | 23.90 | 4.90 | 8 | 52.80 | 1.50 | 8 | 23.90 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | GST | 8 | 76.38 | 9.65 | 8 | 86.03 | 12.86 | 8 | 76.38 |

**APPENDIX F** - Suplementary Table 5

Suplementary Table 5: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of LPO.

| Ref. | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(GAAIED *et al*., 2019)** | Fish | Young | 0.8 | Pure | Med-water | 96 | Al the animal | LPO | 5 | 9.38 | 1.39 | 5 | 14.85 | 1.50 | 3.42 | 0.98 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Liver | LPO | 30 | 5.45 | 2.42 | 30 | 18.58 | 16.74 | 1.08 | 0.08 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Liver | LPO | 30 | 2.62 | 1.62 | 30 | 3.23 | 1.41 | 0.40 | 0.07 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Muscle | LPO | 30 | 1.61 | 0.81 | 30 | 1.61 | 1.62 | 0.00 | 0.07 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Liver | LPO | 30 | 607.36 | 165.64 | 30 | 570.55 | 404.91 | -0.12 | 0.07 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Gill | LPO | 30 | 542.94 | 55.21 | 30 | 460.12 | 64.41 | -1.36 | 0.08 |
| **(BENLI *et al*., 2016)** | Shrimp | Adult | 9 | Pure | Med-water | 168 | Muscle | LPO | 30 | 524.53 | 101.23 | 30 | 506.13 | 55.21 | -0.22 | 0.07 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Brain | LPO | 6 | 134.51 | 39.06 | 6 | 138.05 | 29.87 | 0.09 | 0.33 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Liver | LPO | 6 | 456.63 | 31.91 | 6 | 594.69 | 19.54 | 4.82 | 1.30 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Kidney | LPO | 6 | 77.87 | 15.96 | 6 | 113.27 | 11.28 | 2.36 | 0.57 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Gill | LPO | 6 | 57.14 | 5.24 | 6 | 90.00 | 31.47 | 1.34 | 0.41 |
| **(KUBRAK *et al*., 2013b)** | Fish | Adult | 100 | Pure | Med-water | 96 | Muscle | LPO | 6 | 1162.00 | 225.35 | 6 | 1003.00 | 266.94 | -0.59 | 0.35 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(GRECO *et al*., 2011)** | Bivalve | Adult | 0.01 | Pure | Med-water | 672 | Al the animal | LPO | 15 | 2.84 | 1.31 | 15 | 1.37 | 0.44 | -1.46 | 0.17 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | LPO | 6 | 2.42 | 1.49 | 6 | 3.51 | 0.36 | 0.93 | 0.37 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | LPO | 6 | 2.61 | 0.66 | 6 | 3.45 | 1.44 | 0.69 | 0.35 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | LPO | 6 | 5.33 | 1.32 | 6 | 6.54 | 0.17 | 1.19 | 0.39 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Kidney | LPO | 6 | 3.98 | 0.34 | 6 | 3.05 | 0.80 | -1.40 | 0.41 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Brain | LPO | 6 | 2.91 | 0.56 | 6 | 2.08 | 0.12 | -1.89 | 0.48 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | Pure | Med-water | 96 | Gill | LPO | 6 | 4.89 | 0.63 | 6 | 3.88 | 0.78 | -1.32 | 0.41 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Kidney | LPO | 6 | 2.42 | 1.49 | 6 | 3.18 | 0.93 | 0.56 | 0.35 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Brain | LPO | 6 | 2.61 | 0.66 | 6 | 3.64 | 1.12 | 1.03 | 0.38 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Gill | LPO | 6 | 5.33 | 1.32 | 6 | 5.90 | 2.52 | 0.26 | 0.34 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Kidney | LPO | 6 | 3.98 | 0.34 | 6 | 3.81 | 1.29 | -0.17 | 0.33 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Brain | LPO | 6 | 2.91 | 0.56 | 6 | 2.53 | 2.10 | -0.23 | 0.34 |
| **(OZCAN ORUC; SEVGILER; UNER, 2004)** | Fish | Adult | 87 | AZP | Med-water | 96 | Gill | LPO | 6 | 4.89 | 0.63 | 6 | 4.24 | 1.39 | -0.56 | 0.35 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 24 | Liver | LPO | 6 | 6.20 | 3.48 | 6 | 7.00 | 0.69 | 0.29 | 0.34 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 48 | Liver | LPO | 6 | 7.56 | 2.91 | 6 | 5.92 | 2.35 | -0.57 | 0.35 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 72 | Liver | LPO | 6 | 7.11 | 3.11 | 6 | 7.33 | 0.98 | 0.09 | 0.33 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | Pure | Med-water | 96 | Liver | LPO | 6 | 6.20 | 1.96 | 6 | 10.16 | 4.41 | 1.07 | 0.38 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | AZP | Med-water | 24 | Liver | LPO | 6 | 6.20 | 3.47 | 6 | 7.11 | 4.21 | 0.22 | 0.34 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | AZP | Med-water | 48 | Liver | LPO | 6 | 7.56 | 2.91 | 6 | 5.30 | 0.34 | -1.01 | 0.38 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | AZP | Med-water | 72 | Liver | LPO | 6 | 7.11 | 3.11 | 6 | 6.04 | 2.20 | -0.37 | 0.34 |
| **(ORUÇ; ÜNER, 2002)** | Fish | Adult | 87 | AZP | Med-water | 96 | Liver | LPO | 6 | 6.20 | 1.95 | 6 | 9.99 | 3.31 | 1.29 | 0.40 |
| **(GAAIED *et al*., 2019)** | Fish | Young | 0.8 | Pure | Med-water | 96 | Al the animal | LPO | 30 | 9.46 | 1.38 | 30 | 14.45 | 1.49 | 3.43 | 0.16 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | LPO | 8 | 1.66 | 0.38 | 8 | 2.86 | 0.25 | 3.53 | 0.64 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | LPO | 8 | 1.41 | 0.22 | 8 | 1.88 | 0.19 | 2.14 | 0.39 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | LPO | 8 | 1.26 | 0.31 | 8 | 1.63 | 0.28 | 1.19 | 0.29 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | LPO | 8 | 0.91 | 0.13 | 8 | 1.38 | 0.13 | 3.46 | 0.62 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | LPO | 8 | 6.42 | 1.17 | 8 | 8.25 | 1.83 | 1.13 | 0.29 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | LPO | 8 | 4.50 | 0.75 | 8 | 8.33 | 1.75 | 2.69 | 0.48 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | LPO | 8 | 5.42 | 1.50 | 8 | 9.83 | 1.42 | 2.86 | 0.51 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | LPO | 8 | 3.92 | 0.83 | 8 | 6.33 | 0.50 | 3.32 | 0.59 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | LPO | 8 | 76.38 | 9.65 | 8 | 86.03 | 12.86 | 0.80 | 0.27 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | LPO | 8 | 80.40 | 11.26 | 8 | 100.50 | 14.47 | 1.47 | 0.32 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | LPO | 8 | 86.03 | 10.45 | 8 | 119.80 | 12.06 | 2.83 | 0.50 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | LPO | 8 | 90.05 | 10.45 | 8 | 130.25 | 15.28 | 2.90 | 0.51 |
| **(MENEZES *et al*., 2015)** | Fish | Young | 0.5 | Pure | Med-water | 2160 | Liver | LPO | 24 | 6.52 | 0.81 | 24 | 4.62 | 0.54 | -2.70 | 0.16 |
| **(MENEZES *et al*., 2015)** | Fish | Young | 0.5 | Pure | Med-water | 2160 | Muscle | LPO | 24 | 5.20 | 0.81 | 24 | 8.51 | 0.41 | 5.05 | 0.35 |
| **(MENEZES *et al*., 2015)** | Fish | Young | 0.5 | Pure | Med-water | 2160 | Brain | LPO | 24 | 6.38 | 0.27 | 24 | 6.88 | 0.14 | 2.28 | 0.14 |

**APPENDIX G -** Suplementary table 6

Suplementary Table 6: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of PCO.

| Ref. | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(GAAIED *et al*., 2019)** | Fish | Young | 0.8 | Pure | Med-water | 96 | Al the animal | PCO | 5 | 5.60 | 1.61 | 5 | 11.66 | 1.60 | 3.41 | 0.98 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Brain | PCO | 6 | 1.55 | 0.29 | 6 | 1.42 | 0.31 | -0.40 | 0.34 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Liver | PCO | 6 | 2.18 | 0.61 | 6 | 3.35 | 0.66 | 1.70 | 0.45 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Kidney | PCO | 6 | 2.26 | 0.41 | 6 | 3.65 | 1.64 | 1.07 | 0.38 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Gill | PCO | 6 | 9.54 | 2.62 | 6 | 7.10 | 5.60 | -0.52 | 0.34 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Muscle | PCO | 6 | 1.37 | 0.34 | 6 | 1.19 | 0.29 | -0.53 | 0.34 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 3.54 | 0.29 | 6 | 4.84 | 0.27 | 4.25 | 1.09 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 10.66 | 2.62 | 6 | 15.01 | 3.23 | 1.37 | 0.41 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 7.02 | 2.16 | 6 | 12.42 | 2.72 | 2.03 | 0.51 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 14.77 | 6.74 | 6 | 23.71 | 1.15 | 1.71 | 0.45 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 5.74 | 0.32 | 6 | 8.80 | 1.76 | 2.23 | 0.54 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Gonad | PCO | 6 | 622.00 | 2.30 | 6 | 564.00 | 1.79 | -25.96 | 28.41 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | PCO | 6 | 19.25 | 2.01 | 6 | 21.34 | 13.40 | 0.20 | 0.34 |
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | PCO | 6 | 28.57 | 9.46 | 6 | 23.31 | 13.45 | -0.42 | 0.34 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(POCHETTINO *et al*., 2013)** | Rodent | Adult | 70 | Pure | Oral | 192 | Breast | PCO | 6 | 59.38 | 26.19 | 6 | 57.37 | 36.47 | -0.06 | 0.33 |
| **(TROUDI *et al*., 2011)** | Rodent | Adult | 22.5 | Pure | Oral | 336 | Kidney | PCO | 6 | 0.53 | 0.17 | 6 | 0.88 | 0.29 | 1.36 | 0.41 |
| **(TROUDI *et al*., 2011)** | Rodent | Young | 22.5 | Pure | Mother | 336 | Kidney | PCO | 10 | 0.51 | 0.32 | 10 | 0.92 | 0.60 | 0.82 | 0.22 |
| **(SHAFEEQ; MAHBOOB, 2020)** | Rodent | Adult | 8 | Pure | Oral | 1348 | Liver | PCO | 6 | 26.90 | 1.50 | 6 | 41.60 | 1.97 | 7.75 | 2.84 |

**APPENDIX H -** Suplementary table 7

Suplementary Table 7: References, studied variables, Sample Size of controls (NC), and experiment (NE), Mean of control (MC), and experiment (ME), Standard desviation of contro (SDC) and experiment (SDE), Effect size (d) and variance of d, for the analysis of AChE.

| Ref | Animal | Stage | Dose (mg/L; mg/Kg) | Mix | Route | Time (H) | Tissue | Enzyme | NC | MC | SDC | NE | ME | SDE | d | Var (d) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(SINGH; SINGH, 2016)** | Worm | Adult | 80 | Pure | Oral | 24 | Al the animal | AchE | 10 | 0.08 | 0.01 | 10 | 0.04 | 0.02 | -3.29 | 0.47 |
| **(SINGH; SINGH, 2016)** | Worm | Adult | 80 | Pure | Oral | 240 | Al the animal | AchE | 10 | 0.08 | 0.01 | 10 | 0.02 | 0.01 | -7.18 | 1.49 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Brain | AchE | 6 | 105.46 | 2.30 | 6 | 104.53 | 2.27 | -0.38 | 0.34 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Liver | AchE | 6 | 22.49 | 4.60 | 6 | 13.59 | 3.42 | -2.03 | 0.50 |
| **(MATVIISHYN *et al*., 2014)** | Fish | Adult | 100 | Pure | Med-water | 96 | Kidney | AchE | 6 | 45.93 | 16.06 | 6 | 37.49 | 17.24 | -0.47 | 0.34 |
| **(ATAMANIUK *et al*., 2013)** | Fish | Adult | 100 | Pure | Med-water | 96 | Gill | AchE | 6 | 11.50 | 0.73 | 6 | 10.80 | 0.97 | -0.75 | 0.36 |
| **(GRECO *et al*., 2011)** | Bivalve | Adult | 0.01 | Pure | Med-water | 672 | Al the animal | AchE | 15 | 0.55 | 0.34 | 15 | 0.72 | 0.24 | 0.56 | 0.14 |
| **(CATTANEO *et al*., 2008)** | Fish | Adult | 700 | Pure | Med-water | 96 | Brain | AchE | 10 | 0.02 | 0.00 | 10 | 0.03 | 0.02 | 0.87 | 0.22 |
| **(CATTANEO *et al*., 2008)** | Fish | Adult | 700 | Pure | Med-water | 96 | Muscle | AchE | 10 | 0.01 | 0.01 | 10 | 0.00 | 0.00 | -1.29 | 0.24 |
| **(DA FONSECA *et al*., 2008)** | Fish | Adult | 10 | Pure | Med-water | 96 | Brain | AchE | 8 | 15.07 | 1.41 | 8 | 10.10 | 0.60 | -4.34 | 0.84 |
| **(DA FONSECA *et al*., 2008)** | Fish | Adult | 10 | Pure | Med-water | 96 | Muscle | AchE | 8 | 5.67 | 0.87 | 8 | 3.78 | 0.58 | -2.42 | 0.43 |
| **(RAFTOPOULOU *et al*., 2006)** | Bivalve | Adult | 0.03 | Pure | Med-water | 360 | Dig.gland | AchE | 30 | 34.96 | 2.74 | 30 | 16.50 | 1.12 | -8.71 | 0.70 |
| **(RAFTOPOULOU *et al*., 2006)** | Bivalve | Adult | 0.03 | Pure | Med-water | 360 | Gill | AchE | 30 | 47.11 | 1.21 | 30 | 34.63 | 4.22 | -3.97 | 0.20 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Med-water | 48 | Viscera | AchE | 10 | 384.71 | 17.33 | 10 | 267.11 | 45.79 | -3.25 | 0.46 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 252 | Pure | Med-water | 96 | Viscera | AchE | 10 | 372.21 | 37.25 | 10 | 164.24 | 34.54 | -5.55 | 0.97 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 219.00 | 59.00 | 6 | 186.00 | 34.00 | -0.63 | 0.35 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 334.00 | 99.00 | 6 | 228.00 | 72.00 | -1.13 | 0.39 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 258.00 | 74.00 | 6 | 292.00 | 66.00 | 0.45 | 0.34 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 373.00 | 49.00 | 6 | 407.00 | 76.00 | 0.49 | 0.34 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 239.00 | 62.00 | 6 | 250.00 | 65.00 | 0.16 | 0.33 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 203.00 | 22.00 | 6 | 160.00 | 26.00 | -1.65 | 0.45 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 252.00 | 40.00 | 6 | 195.00 | 51.00 | -1.15 | 0.39 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 273.00 | 41.00 | 6 | 214.00 | 26.00 | -1.59 | 0.44 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 326.00 | 49.00 | 6 | 286.00 | 40.00 | -0.83 | 0.36 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 196.00 | 26.00 | 6 | 180.00 | 11.00 | -0.74 | 0.36 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 243.00 | 9.00 | 6 | 183.00 | 26.00 | -2.85 | 0.67 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 246.00 | 37.00 | 6 | 177.00 | 31.00 | -1.87 | 0.48 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 263.00 | 60.00 | 6 | 185.00 | 37.00 | -1.44 | 0.42 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 336.00 | 58.00 | 6 | 284.00 | 44.00 | -0.93 | 0.37 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 204.00 | 15.00 | 6 | 177.00 | 34.00 | -0.95 | 0.37 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 231.00 | 18.00 | 6 | 235.00 | 18.00 | 0.21 | 0.34 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 244.00 | 31.00 | 6 | 242.00 | 31.00 | -0.06 | 0.33 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 250.00 | 39.00 | 6 | 234.00 | 18.00 | -0.49 | 0.34 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 308.00 | 14.00 | 6 | 216.00 | 38.00 | -2.97 | 0.70 |
| **(BERNARD *et al*., 1985b)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 216.00 | 38.00 | 6 | 224.00 | 44.00 | 0.18 | 0.33 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Med-water | 48 | Opercle | AChE | 10 | 384.71 | 0.41 | 10 | 267.11 | 844.68 | -0.19 | 0.20 |
| **(ES RUIZ DE ARCAUTE *et al*., 2019)** | Fish | Adult | 250 | Pure | Med-water | 96 | Opercle | AChE | 10 | 372.21 | 117.79 | 10 | 164.24 | 519.37 | -0.53 | 0.21 |
| **(GAAIED *et al*., 2019)g** | Fish | Young | 0.8 | Pure | Med-water | 96 | Heart | AChE | 20 | 230.30 | 115.75 | 20 | 13.94 | 62.34 | -2.28 | 0.17 |
| **(AMEL *et al*., 2016)a** | Rodent | Adult | 5 | Pure | Oral | 672 | Brain | ACHE | 10 | 507.41 | 29.68 | 10 | 465.87 | 29.67 | -1.34 | 0.24 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | ACHE | 8 | 5.65 | 1.09 | 8 | 5.58 | 0.71 | -0.07 | 0.25 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | ACHE | 8 | 4.81 | 0.64 | 8 | 4.30 | 0.51 | -0.84 | 0.27 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | ACHE | 8 | 5.20 | 0.71 | 8 | 3.98 | 0.51 | -1.87 | 0.36 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | ACHE | 8 | 3.66 | 0.83 | 8 | 2.70 | 0.13 | -1.52 | 0.32 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | ACHE | 8 | 4.51 | 1.04 | 8 | 4.39 | 1.10 | -0.11 | 0.25 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | ACHE | 8 | 5.06 | 0.49 | 8 | 3.23 | 1.16 | -1.95 | 0.37 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 48 | Viscera | ACHE | 8 | 4.14 | 0.67 | 8 | 3.53 | 1.10 | -0.63 | 0.26 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 168 | Viscera | ACHE | 8 | 3.84 | 0.24 | 8 | 2.07 | 0.37 | -5.38 | 1.15 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | ACHE | 8 | 35.08 | 2.62 | 8 | 37.70 | 3.14 | 0.86 | 0.27 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 24 | Viscera | ACHE | 8 | 33.77 | 2.62 | 8 | 20.94 | 3.40 | -3.99 | 0.75 |
| **(BANAOUI *et al*., 2015)** | Bivalve | Adult | 0.5 | Pure | Med-water | 12 | Viscera | ACHE | 8 | 35.08 | 2.62 | 8 | 37.70 | 3.14 | 0.86 | 0.27 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 219.00 | 59.00 | 6 | 186.00 | 34.00 | -0.63 | 0.35 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 203.00 | 22.00 | 6 | 160.00 | 26.00 | -1.65 | 0.45 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 234.00 | 9.00 | 6 | 183.00 | 26.00 | -2.42 | 0.58 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 231.00 | 18.00 | 6 | 235.00 | 26.00 | 0.17 | 0.33 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 334.00 | 99.00 | 6 | 228.00 | 72.00 | -1.13 | 0.39 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 252.00 | 40.00 | 6 | 195.00 | 51.00 | -1.15 | 0.39 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 246.00 | 37.00 | 6 | 177.00 | 31.00 | -1.87 | 0.48 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 244.00 | 31.00 | 6 | 242.00 | 31.00 | -0.06 | 0.33 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 258.00 | 74.00 | 6 | 292.00 | 66.00 | 0.45 | 0.34 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 273.00 | 41.00 | 6 | 214.00 | 26.00 | -1.59 | 0.44 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 263.00 | 60.00 | 6 | 185.00 | 37.00 | -1.44 | 0.42 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 250.00 | 39.00 | 6 | 234.00 | 18.00 | -0.49 | 0.34 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 373.00 | 49.00 | 6 | 407.00 | 76.00 | 0.49 | 0.34 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 326.00 | 49.00 | 6 | 286.00 | 40.00 | -0.83 | 0.36 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 336.00 | 58.00 | 6 | 284.00 | 44.00 | -0.93 | 0.37 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 311.00 | 45.00 | 6 | 308.00 | 14.00 | -0.08 | 0.33 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 339.00 | 62.00 | 6 | 250.00 | 65.00 | -1.29 | 0.40 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 196.00 | 26.00 | 6 | 180.00 | 11.00 | -0.74 | 0.36 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 204.00 | 15.00 | 6 | 177.00 | 34.00 | -0.95 | 0.37 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 216.00 | 38.00 | 6 | 224.00 | 44.00 | 0.18 | 0.33 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 3 | Muscle | AchE | 6 | 920.00 | 150.00 | 6 | 890.00 | 150.00 | -0.18 | 0.33 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 15 | Muscle | AchE | 6 | 1130.00 | 180.00 | 6 | 1020.00 | 120.00 | -0.66 | 0.35 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 24 | Muscle | AchE | 6 | 1090.00 | 170.00 | 6 | 1000.00 | 160.00 | -0.50 | 0.34 |
| **(LAKSHMANA; RAJU, 1996)** | Rodent | Adult | 200 | Pure | Peritoneum injection | 48 | Muscle | AchE | 6 | 1090.00 | 260.00 | 6 | 1140.00 | 180.00 | 0.21 | 0.34 |