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PESQUISA

TOXICITY OF NAPHTHALENE IN THE NEOTROPICAL FISH *ASTYANAX LACUSTRIS* (CHARACIFORMES: CHARACIDAE) AND *GEOPHAGUS BRASILIENSIS* (PERCIFORMES: CICHLIDAE)

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Abstract

Polycyclic aromatic hydrocarbons are one of the most important organic pollutants in environmental studies. The aim of this study was to assess the naphthalene acute toxicity in two fish species, Astyanax lacustris (LLcust, 1875) and Geophagus brasiliensis (Quoy & Gaimard, 1824). The fish were exposed to naphthalene (0.005, 0.03, 0.3, and 3 mgL^{-1}) in water and after that the piscine micronucleus test in erythrocytes, comet assay in blood, liver and gill cells, glutathione S–transferase (GST) activity in the liver, and accumulation of naphthalene in the bile were performed. The susceptibility of the two species was similar and naphthalene was not genotoxic in all tested tissues. The liver GST activity may have been responsible for less damage observed in the liver while the highest DNA damage occurred in blood cells. However, low concentrations of naphthalene in water can stimulate apparent benefits, such as less DNA damage, which would be a compensatory response to an imbalance of homeostasis.

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The naphthalene is absorbed and can accumulate in the gall bladder, a greater accumulation of PAH was observed in A. lacustris, while G. brasiliensis did not differ from the control. The naphthalene concentrations are not genotoxic to the tested species, although they can potentially accumulate into the body.
Keywords: Comet assay. Ecotoxicology. Fish. Genotoxicity. Hormesis.

Toxicidade do naftaleno em peixes neotropicais astyanax lacustris (characiformes: characidae) e geophagus brasiliensis (perciformes: cichlidae)

Resumo

Hidrocarbonetos Policíclicos Aromáticos (HPAS) são um dos poluentes orgânicos mais importantes em estudos ambientais. O objetivo com este estudo foi avaliar a toxicidade aguda (96 h) do naftaleno em duas espécies de peixes, Astyanax lacustris (LLcust, 1875) e Geophagus brasiliensis (Quoy & Gaimard, 1824). Os peixes foram expostos ao naftaleno (0,005, 0,03, 0,3, e 3 mgL^{-1}) em água e, após a exposição, foram realizados os testes do micronúcleo písceo em eritrócitos, ensaio cometa em células do sangue, do fígado e brânquia, atividade da Glutationa S-transferase (GST) no fígado e acumulação de naftaleno na bile. As duas espécies foram similares na susceptibilidade à exposição ao naftaleno e este não foi genotóxico sobre todos os tecidos testados. A atividade de GST hepática pode ter sido responsável por baixos danos observados no fígado, enquanto o dano ao DNA mais elevado ocorreu em eritrócitos. No entanto, as baixas concentrações de naftaleno em água podem estimular aparentes benefícios (hormese), como menores danos ao DNA, o que seria uma resposta compensatória a um desequilíbrio da homeostase. O naftaleno é absorvido e pode acumular-se na vesícula biliar dos peixes. A maior acumulação de HPAs foi observada em A. lacustris, enquanto em G. Brasiliensis não houve diferença em relação ao controle. As concentrações de naftaleno não foram genotóxicas para as espécies testadas, embora possam potencialmente acumular-se nos animais.
Palavras-chave: Ensaio cometa. Ecotoxicologia. Peixes. Genotoxicidade. Hormese.

1 **INTRODUCTION**

Polycyclic aromatic hydrocarbons (PAHs) are the most important organic pollutants in environmental studies.[1] They might be responsible for mutagenic and carcinogenic effects, besides their environmental persistence, and high potential for toxicity.[2]

The PAHs are composed of carbon and hydrogen atoms, with a variable number of aromatic (benzene) rings, organized in the form of linear or branched molecules. Among all PAHs, sixteen of them are extensively studied, due to their industrial, environmental, and toxicological importance.[3] Naphthalene was included in this select group, it is a bicyclic aromatic hydrocarbon, naturally obtained from coal tar or crude oil.[4,5]

The increasing pollution of aromatic hydrocarbons in aquatic environment has become more relevant since the beginning of oil exploitation. Aquatic ecosystems are generally the main industrial and urban waste receptors, so the study of the effects of pollutants on these sites is of great interest. This plays a role in the human exposure and consumption of contaminated water or contaminated organisms. Naphthalene enter the environment through vehicle exhausts, asphalt roads, coal, coal tar, wildfires, agricultural burning, residential wood burning, municipal and industrial waste incineration, and hazardous waste sites.

Naphthalene can directly interact with aquatic organisms causing a variety of responses both at the cellular and systemic levels. [6,7] The contaminated animals can be a source of indirect exposure to humans and other animals as well, and it can accumulate in the food chain or sediment.[8]

Fish can metabolize the pollutants in water and accumulate them. They are good bioindicators of the mutagenic and carcinogenic potential of contaminants[9] as well as biochemical parameters.[10] Fish are a large and diverse group of vertebrates present in different trophic levels, they require large amount of water to breathe therefore intensifying its exposure to contaminants in the aquatic environment.[11]

The aim of this study was to assess the acute toxicity of naphthalene in two specie of neotropical fish: *Astyanax lacustris* (LLcust, 1875) and *Geophagus brasiliensis* (Quoy & Gaimard, 1824), through genetic and biochemical endpoints.

2 **MATERIALS AND METHODS**

Juvenile fish of *A. lacustris* (weight 4.62±2.16 g and size 7.46±0.94 cm) and *G. brasiliensis* (weight 3.45±0.95 g and size 6.68±0.64 cm) were obtained from commercial pisciculture and acclimated for 30 days in tanks (2,000 L) with dechlorinated water and equipped with water filters and air pumps, at a temperature of 25±2 °C, constant aeration, and for 12:12 light/dark cycle. For each naphthalene treatment, a stock solution was prepared previously in ethanol. The fifteen specimens in each treatment were individually exposed to environmentally relevant naphthalene concentrations (CAS No 91-20-3) of 0.005,[12] 0.03, 0.3, and 3 mgL^{-1}. A negative control (NC) with water, and one with ethanol (SC) used as naphthalene's solvent was carried out. The exposure time was 96 h in a semi-static system with daily replacement of two-thirds of the water and the chemical compound. The experimental procedures performed in this study were in accordance with the ethical principles for animal testing, and approved by the Ethics Committee on Animal Use (CEUA) of the Federal Paraná University (UFPR).

The piscine micronucleus test (PMNT) in erythrocyte was applied for mutagenicity and genotoxicity analysis. The test was based on Heddle[13] and Schmid[14] methodology, with staining process proposed by Ueda[15] and the analysis of nuclear morphological abnormalities (NMA) was performed according to Carrasco.[16] DNA damage analysis was performed using comet assay in erythrocyte, liver, and gills cells. The comet assay in gills cells was performed just for *G. brasiliensis*. It was based on Singh[17] with modifications for the erythrocytes by Ferraro[18] and for the tissues Ramsdorf and coleagues.[19]

The enzymatic activity of gluthatione S-transferase (GST) in the liver was performed according to Keen[20] and the quantification of PAHs in bile was analyzed based on Hanson[21] with modifications. Gall bladder pools were performed with animals of the same group for this analysis.

For statistical analysis, first, the normality test Kolmogorov-Smirnov was applied. For data with no normal distribution, we used the non-parametric test Kruskal-Wallis, comparing treatments by Student-Newman-Keuls test. ANOVA test was used for enzymatic assays. All tests with a significance level of $p<0.05$.

3 **RESULTS**

The weight and size of the fish presented normal distribution and there were no fish deaths during the experiment. Therefore, it was observed some alterations like decrease of mobility and exploration of the aquarium, especially when animals were treated to the two highest naphthalene concentrations (0.3 and 3 mgL^{-1}).

For *A. lacustris* the PMNT and NMA in erythrocytes showed no difference in the exposed groups compared to the control in any of the tested concentrations. Both NC and SC present no difference between each other. Regarding the naphthalene genotoxicity, obtained by the comet assay in erythrocytes there was a difference only between 0.03 mgL^{-1} of naphthalene and the solvent control. The score of the group contaminated with naphthalene was slightly lower than the respective control (Figure 1).

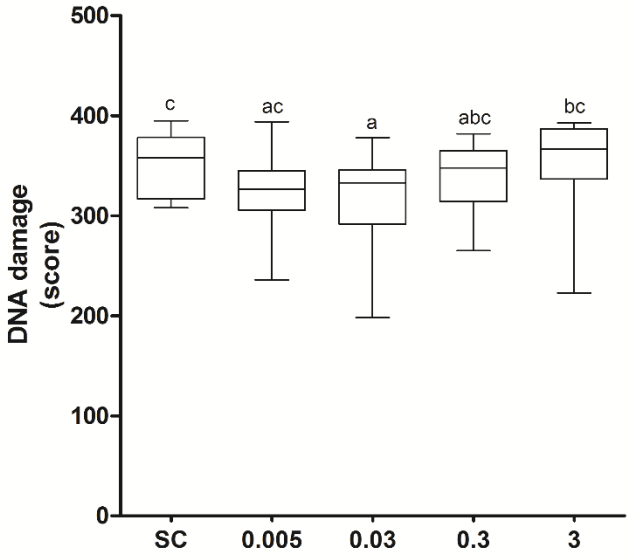

Figure 1 – Comet assay scores from erythrocytes of *Astyanax lacustris.* Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as median and quartiles

The scores obtained at comet assay by *A. lacustris*' liver cells analysis showed no difference among any of the groups tested, neither among groups exposed to naphthalene nor between these groups and the control. The data for comet assay in gill cells of *A. lacustris* is not presented.

Two benzene rings compounds accumulated in the bile of *A. lacustris*, either naphthalene or metabolites, indicate that the three largest concentrations of naphthalene (0.03, 0.3, and 3 mgL^{-1}) presented significant differences comparing with solvent control (Figure 2).

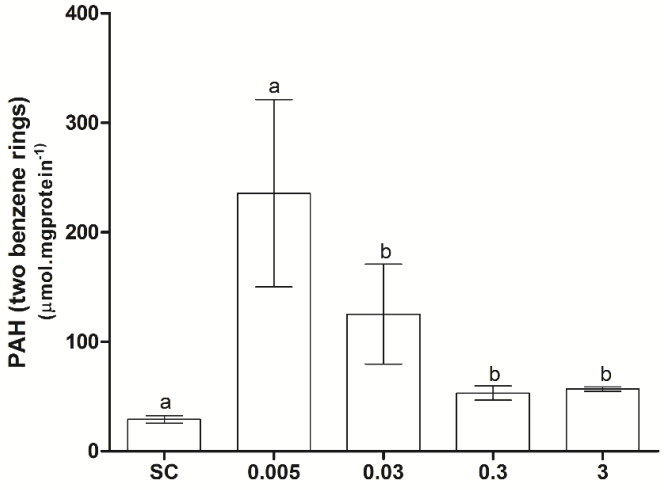

Figure 2 – Accumulation of two benzene rings compounds in the bile of *Astyanax lacustris*. Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as the mean and standard deviation

The GST activity in *A. lacustris* liver tissue measured by biochemical analysis was increased in the treatment of 3 mgL^{-1} when compared to solvent control, indicating that the naphthalene, especially in higher concentration, has the ability to increase the activation of GST enzyme activity (Figure 3).

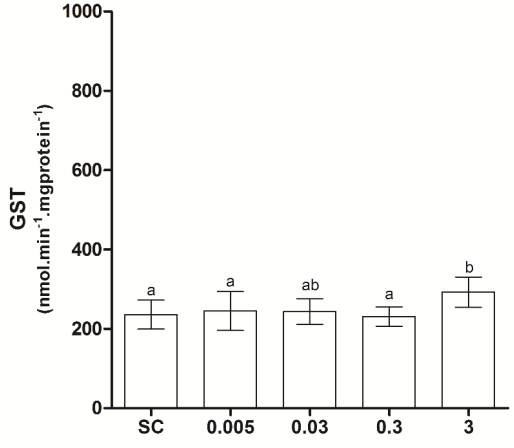

Figure 3 – Enzyme glutationa S-transferase (GST) activity from *Astyanax lacustris* liver. Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as the mean and standard deviation

When PMNT and NMAs test were performed in *G. brasiliensis*, there was no statistical difference between the exposed groups compared to the control in any of the tested concentrations, neither between controls. The results of the genotoxicity of naphthalene by the comet assay of *G. brasiliensis* erythrocytes showed a significant different between the negative control and solvent control, ethanol by itself increases the DNA damage. All treatments with naphthalene were different to the solvent control, which presented a very high score. A comparison among the groups treated with naphthalene shows a higher DNA damage at the 0.03 mgL^{-1} concentration (Figure 4).

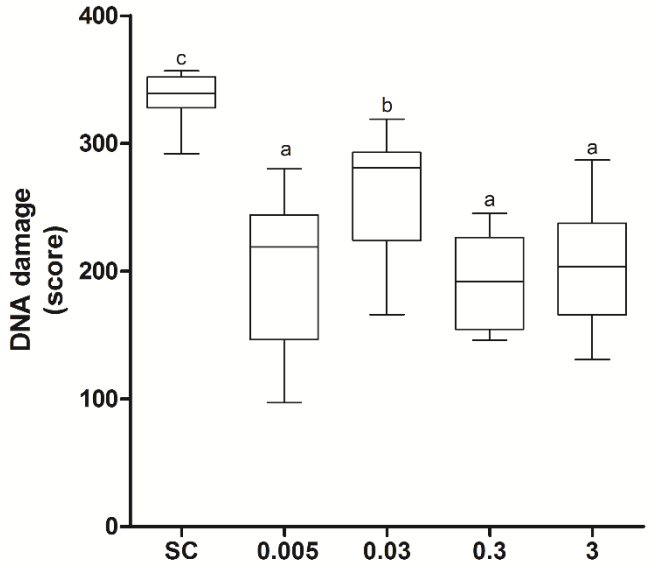

Figure 4 – Comet assay scores from erythrocytes of *Geophagus brasiliensis*. Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as the median and quartiles

The comet assay scores obtained with *G. brasiliensis* liver cell analysis showed no difference between any of the tested concentrations.

The *G. brasiliensis* gill cells were also analyzed by the comet assay. Information related to genotoxicity shows a lower DNA damage for all groups exposed to naphthalene in relation to solvent control. Among the naphthalene exposed groups treated with different concentrations there were no statistical difference (Figure 5).

It was investigated the presence of compounds with two benzene rings in the bile of *G. brasiliensis* and the data suggest the lack of difference between the treatments compared to control. The GST activity in *G. brasiliensis* increases at the groups of 0.005 and 0.3 mgL^{-1} compared to the control (Figure 6). For both fish species when the enzymatic parameter was evaluated, just the ethanol alone (solvent control) in the water was able to increase the activity of the GST.

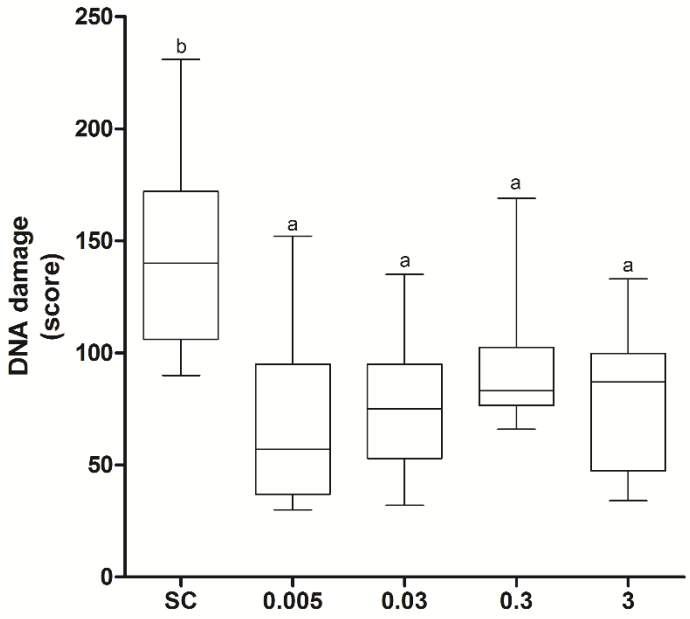

Figure 5 – Comet assay scores from gill cells of *Geophagus brasiliensis.* Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as the median and quartiles

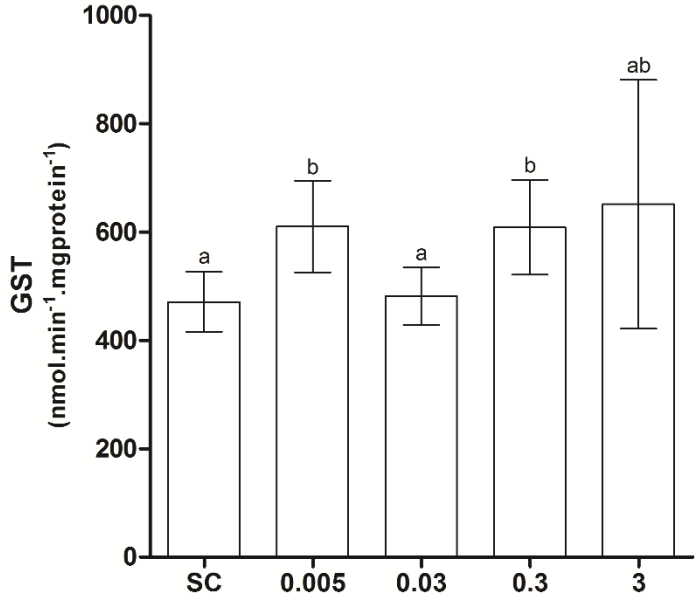

Figure 6 – Enzyme glutationa S-transferase (GST) activity from *Geophagus brasiliensis* liver. Solvent control (SC) and treated groups to different concentration of naphthalene at mgL^{-1}. Different letters mean statistical difference ($p<0.05$), data as the mean and standard

4 **DISCUSSION**

Naphthalene is the main PAH found in crude oil, petroleum, and its derivatives, associated with extensive capacity of dispersion that makes it one of the most important chemicals in environmental impact studies. In this work, we chose to test low naphthalene concentrations, even lower than

those present in environments with historical or present contamination. Some studies report that, in general, fish populations can be exposed to PAH concentration ranging from 50 to 410 mgL^{-1}.[22,23]

In this study, no difference was observed in micronucleus test as well as NMAs between the treated and control group. Because only cells in division can potentially present micronuclei might be an explanation for these results.[24] Fish in general have low hematopoietic index and their erythrocytes may remain in circulation for times ranging from 60 to 160 days depending on the specie.[25] Due to a 96 h exposure time, few erythrocytes were produced during the experiment, explaining the absence of abnormalities and the few micronucleus.

This suggestion is supported by several studies in which the fish exposure was acute and the researchers found no significant increase in the frequency of micronuclei in the groups exposed to xenobiotics. As an example, experiments by Disner[26] with *A. lacustris* exposed to Roundup®, Ghisi[27] testing *Corydoras paleatus* also exposed to Roundup®, Winter's[28] study where *Pimephales promelas* were exposed to cyclophosphamide and Bücker's[29] work evaluating benzene on *Eingenmannia virescens*, also observed no induction of micronucleus.

The results of comet assay suggest the absence of genotoxic effects of all naphthalene concentrations on the genetic material of the two fish species used in this study. The statistical analysis of genotoxic damage experiments shows that groups exposed to varying concentrations of naphthalene presented no difference from control or the difference representing less DNA damage at the treated groups. Similar information may be found in the literature regarding the naphthalene toxic response. As suggested by Scheiner,[30] DNA fragmentation and chromosome breakage are consistent with indirect mechanisms related to the contaminant response and this may take longer to be displayed. In this study, the absence of pronounced effects after xenobiotic exposure is probably due to the low concentrations. However, the naphthalene concentrations tested were realistic and close to environmental conditions than the concentrations used in acute toxicity tests. Degraeve[31] observed the acute naphthalene toxicity in two fish species, with LC50 values of 1.6 mgL^{-1} for *Oncorhynchus mykisse* and 7.9 mgL^{-1} for *Pimephales minnows*.

Despite the heterogeneity of the data found on naphthalene toxicity, it seems to have no specific affinity to directly bind the DNA. Studies indicate that only the metabolites are capable of binding to DNA.[32]

Brusick[1] conducted a critical review of dozens of studies in genetic toxicology of naphthalene and they found that 80% of the studies have no evidence of genotoxicity. Still according to this research, studies that found no evidence of genotoxicity were: the point mutation test in bacteria and cell cultures, the Ames test, chromosome breaks test, DNA repair tests, and cell morphological transformation test. Thus, naphthalene responses in most assays indicate this component is non-reactive with DNA and therefore is not properly genotoxic.

Regarding the assessment of genotoxicity, there is no linear response. It was not consistently observed the concentration-response effect. The data suggest the beginning of a U-shaped curve, which can be explained by hormesis phenomenon. Hormesis is defined as a dose-response event,

characterized by stimulation at lower doses and inhibition at high doses. It is highly general, and it has been often observed irrespective of chemical or physical agent, biological model, measured biomarker, chemical class, and inter-individual variability.[33] Calabrese & Baldwin[34] suggest that hormesis bring a "dose-response revolution", where there is no linear response, but biphasic-U-shaped curve. The observed response may be due to the direct stimulation of hormesis (as an active phenomenon) or the result of a compensatory biological process following an initial disruption of homeostasis.[35] In short, hormesis is a stress response.[36]

According to Chapman,[36] studies with hormesis curves are not commonly published because the lack of consensus on data interpretation, and papers discussing the importance and relevance of this phenomenon are not common in ecotoxicology literature. Also on this subject there is a lack of consensus and consistency of information for a complete understanding. What should be taken into consideration to avoid misinterpretation is that the beneficial effects caused by low doses during the response process is not permanent and is not due to the contaminant itself, but rather the intrinsic system of protecting the body. Perhaps, the most correct is not to say that low doses in any exposure may cause beneficial effect. For example, the stimulation caused by low doses of radiation, occurs only as a result of reparative compensation,[35] and not as a benefit conferred upon exposure. This kind of results can usually lead to confusion in the concept.

Another evidence seems to support the idea that naphthalene is quickly eliminated from the organism, thus, the resulting damage is usually reduced due the short period of contact with the contaminant. Eisele[37] conducted a study where chickens were exposed to naphthalene by oral gavage for 31 days, after 48 h stopping exposure the animals had already eliminated between 75-80% of the compound, showing a low permanence of this agent in the animals. Varanasi[38] similarly reported great loss of the contaminant in a short time. These researchers exposed rainbow trout (*Oncorhynchus mykiss*) to naphthalene for 96 h and found that after the exposure a great amount of the compound had been eliminated and the epidermal mucus was suggested as a major route of excretion. Similarly, Melancon & Lech[12] reported a half-life of naphthalene and methyl-naphthalene in rainbow trout tissues to be less than 24 h, except for fats. Using ^{14}C-naphthalene elimination data these researchers could distinguish disposal after acute or chronic exposure, and due to the metabolism of the compound which generally occurs in chronic exposure it is expected that the loss occurs more slowly.

Even knowing the naphthalene is unstable in water solution, the exposure was tested at very low concentrations because it represents environmentally realistic conditions in natural water systems. This issue was also relevant to Melancon & Lech[12] who studied the accumulation and elimination of ^{14}C in rainbow trout tissues after exposure to ^{14}C-naphthalene and ^{14}C-2-methyl-naphthalene. After 8 h of exposure to 0.005 or 0.023 mgL^{-1} ^{14}C-naphthalene, the studied tissues contained ^{14}C at a range of 20 to 100 times higher than water levels, while fat and bile presented an increase higher than a hundred times compared to water content. This is basically due to the lipophilic nature of PAHs. Research has shown that naphthalene was rapidly absorbed after exposure, therefore this study supports the

exposure method used (hydric) and leaves no doubt that the naphthalene is readily available before suffering degradation.

The distribution and kinetics of naphthalene, labeled with C^{14}, was also studied by Domingos,[39] the results after 24 h exposure shows that all fish accumulated radiolabeled compounds. The highest rates of absorption were found in the gall bladder, liver, and intestine, but in a week, there was a great decrease due to the process of elimination.

In addition to the biological evidence on disposal of naphthalene, it seems that volatilization plays an important role in the chemical transformation. This can be explained from its small molecular mass, valued at 128.18 $gmol^{-1}$.[40] The vapor pressure, that is, how easily the naphthalene has lost depends directly on its molecular weight. Thus, by having low molecular weight, naphthalene can be easily volatilized from the water in natural systems, as indicated by the volatilization half-lives of 0.4-3.2 h.[41] Furthermore, the movement of water accelerates the loss process, particularly due to the high conversion rate to CO_2 that is 4.7 $gLday^{-1}$.[42]

The high capacity of naphthalene volatilization may be an alternative explanation to low DNA damage in treated animals. Even knowing that the naphthalene is quickly absorbed, it is possible due to the high conversion rate and contaminant loss that the exposure time might have been insufficient to cause DNA damage to be observed by the comet assay.

Naphthalene water insolubility was overcome by its dilution in ethanol for posterior use in experiments. This can lead to an association between two types of different chemical substances and may lead to a difficulty in interpreting the results. Due to some controversial data, it is difficult to know whether a compound interferes with the action of the other, increasing or decreasing its activity on individuals or facilitating their elimination.

Another way the results can be explained as suggested by Santos[43] is that ethanol increases the metabolic rate of the organisms. Thus, by increasing the metabolic rate, removal of naphthalene would be facilitated explaining lower damage values than the control itself with ethanol. However, this mechanism only works when ethanol was combined with another reagent, since when ethanol is alone the damage may be higher.

Compared to the solvent control (ethanol), the data scores were similar to the pure negative control, or even higher, as in the case of *G. brasiliensis* erythrocytes. Some information about ethanol may explain these results. Ethanol contributes primarily to the generation of free radicals, which means that there is an increase in lipid peroxidation, and this could contribute to an increased DNA damage.[44]

The high damage in solvent control, especially in *G. brasiliensis* erythrocytes can be explained also by Santos[43] as a result of an increase in ammonia excretion rate caused by ethanol. Thus, damage can be observed because of its side effects. When there is a large amount of ammonia in water, it can cause more DNA strand breakages in the cells exposed. The same study indicated that the naphthalene has the opposite effect, that is, reduces the excretion of ammonia, thereby confirming that the lowest scores on the respective negative control.

The decrease of observed damage in many of the exposed groups compared to control may be due to the type of organisms used in the study. Fish quickly capture lipophilic organic contaminants, such as naphthalene, from the environment and have a variety of mechanisms to protect them against deleterious effects.[43] Such effects may include the elimination even before the metabolism, which seems to be responsible for further damage. Furthermore, the use of native species of fish in these types of studies is extremely important because of the inherent susceptibility and adaptations of these organisms. In addition, the exposure time also seems to be informative in the experiment for analysis of damage caused by metabolites, we believe that an extended exposure time should be applied. This was demonstrated by Ramsdorf[45] for *R. quelen* and *Astyanax sp.*, where the concentration of 3.0 mgL^{-1} naphthalene showed most of the DNA damage, in PMNT and comet assay of erythrocyte, detected after 28 days of exposure to contaminant.

From the results of HPA accumulation in the bile, it can be seen that only in *A. lacustris* there was an accumulation, with difference in almost all concentrations tested, except in the lowest (0.005 mgL^{-1}) in comparison with control group. Melancon and Lech[12] reported that after aquatic acute exposure to naphthalene the bile of fish contained hundred times more naphthalene and their metabolites than water. Roubal[46] found in a study with naphthalene that the largest percentage of the accumulated compounds in the gallbladder are metabolites. It is possible that due to the short period of exposure the time for metabolism was insufficient, suggesting difference between the sensitivity or elimination and metabolism capacity of *G. brasiliensis* compared to *A. lacustris*. Empirical behavioral distinction between the two species, one of the Characidae family (*A. lacustris*) and one of the Cichlid family (*G. brasiliensis*), may be responsible in part for different results, Characidae usually presents lower displacement and swimming and Cichlids are more agile, these behavior suggests that Ciclidae metabolism is less likely to accumulate higher concentrations of pollutants in the liver in a short period of time.

The increase in hepatic GST activity in fish after exposure to contaminants is common and has been reported in several studies, for example, the exposure to soluble fraction of diesel fuel,[47] Butiltin,[48] Rondup®[49] and exposure to sediment collected in polluted sites.[50]

The increase of GST activity has been associated with a defensive adaptation of an organism against a variety of organic compounds in the environment.[51] GST is involved in detoxification and excretion of xenobiotics and their metabolites.[52] This higher activity may be induced by pro-oxidants and/or electrophilic compounds as an antioxidant response.[53] The results in both species showed an increased GST activity in organisms exposed to naphthalene. This is a further indication that fish are metabolizing and modifying the compound probably to an hydrophilic form in order to facilitate excretion. Moreover, the pronounced activity of the enzyme leaves no doubt about the contaminant was available to fish. It is possible to suggest the protective antioxidant action of liver enzyme is responsible for the minor damage to the genetic material of the liver cells.

When only the solvent control is taken into consideration an increased GST activity can be verified. Thus, ethanol also seems to be responsible for the activation of detoxification enzymes. Ethanol is also a xenobiotic compound for fish and its chemical properties provide their entry into cells

probably resulting in higher enzymatic activity. In the case of *A. lacustris* only the highest naphthalene concentration was different from the solvent control. This shows that if the contaminant concentration is too low, other factors may interfere with the analysis, what makes very difficult to distinguish which compound is associated with some effect on GST activity. Biochemical biomarkers have fast response and they are the first to present changes,[45] because they are unspecific and potentially sensitive to any toxic substance to the body.

The experimental conditions applied in this work demonstrated that both *A. lacustris* and *G. brasiliensis* presented similar susceptibility to naphthalene exposure. Comet assay analyses found that naphthalene is not genotoxic under certain conditions. However, the toxicity must be considered due to an induction of the GST activity. The activity of this enzyme may have been responsible for less damage to liver tissue compared to other analyzed tissues. Toxicity was also evidenced by fish disorientation in aquaria under concentrations above 0.3 mgL^{-1}.

Low naphthalene concentrations in the water can stimulate apparent benefits as less DNA damage, and this is a compensatory response to an imbalance of homeostasis. Naphthalene as well as their metabolites can accumulate in the bile of exposed fish. It was verified higher accumulation of HPA with two rings in the specie *A. lacustris.*

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