





## Article

# Behavioral Parameters of Planarians (*Girardia tigrina*) as Fast Screening, Integrative and Cumulative Biomarkers of Environmental Contamination: Preliminary Results

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**Abstract:** The present study aims to use behavioral responses of the freshwater planarian *Girardia tigrina* to assess the impact of anthropogenic activities on the aquatic ecosystem of the watershed Araguaia-Tocantins (Tocantins, Brazil). Behavioral responses are integrative and cumulative tools that reflect changes in energy allocation in organisms. Thus, feeding rate and locomotion velocity (*p*LMV) were determined to assess the effects induced by the laboratory exposure of adult planarians to water samples collected in the region of Tocantins-Araguaia, identifying the sampling points affected by contaminants. Furthermore, physicochemical and microbiological parameters, as well as the presence of inorganic compounds (dissolved aluminum, total barium, total chloride, dissolved iron, total fluoride, total manganese, nitrates, nitric nitrogen, total sulfate, total zinc) and surfactants, were determined on each specific sampling point. The behavioral biomarkers (feeding rate and *p*LMV) of the freshwater planarians were significantly decreased when organisms were exposed to water samples from four municipalities (Formoso do Araguaia, Lagoa da Confusão, Gurupi and Porto Nacional), sites of the Tocantins-Araguaia hydrographic region—TAHR. Both behavioral biomarkers decreased up to ~37–39% compared to organisms in ASTM medium only. Our results showed that these behavioral biomarkers can be used for fast screening monitoring of environmental samples of freshwater ecosystems, since a decrease in feeding rate and locomotor activity was observed in sites impacted by anthropogenic activities. However, the absence of effects observed in some sampling points does not represent the absence of contamination, since several other classes of contaminants were not determined. In these negative results, the absence of deleterious effects on behavioral biomarkers might only be indicative that the potential presence of contaminants on such sites does not significantly affect the performance of planarians. This fast screening approach seems to be useful to determine contaminated sites in freshwater ecosystems for biomonitoring purposes. This knowledge will help to develop biomonitoring programs and to decide appropriate sampling sites and analysis.

**Keywords:** biomonitoring; planarians; locomotion; feeding rate

## 1. Introduction

Tocantins state (Brazil) is consolidated as the new agricultural frontier of the country, strategically located by environmental conditions and available water resources. In the last few years, Tocantins state has been standing out in the scenario of the national agribusiness of grain production [1], sugar cane [2], melon [3], and watermelon [4]. The increase in agricultural production has facilitated the spread of diseases, pests, and weeds in crops [5,6]. Thus, for achieving high productivity, pesticides and fertilizers have been adopted as the most efficient manner for the short-term control of undesirable organisms [7,8] and have been used in various steps of the production cycle of cultures, whereas such compounds become subject to environmental contamination [9,10]. However, the use of these compounds in agriculture is not unanimous, although its impacts can be considered positive by some segments of society; for others, its impacts are negative, mainly through the environmental point of view [11,12].

In the Tocantins state, agricultural cultivation generally occurs in areas adjacent to aquatic systems (watershed Araguaia-Tocantins). Thus, the aquatic ecosystem can be impacted since the main routes of contamination by xenobiotics, such as metals, in aquatic systems are through runoff, erosion, drift, leaching, and atmospheric deposition [13,14]. Thus, water quality has been influenced by urbanization and modernization, which has brought problems of wastewater disposal and the contamination of surface water, such as lakes [15]. Natural water becomes polluted due to the weathering of rocks, the leaching of soils, and mining processing [16]. The change in land use in agricultural production could increase the use of fertilizers with subsequent leaching to waterways, rivers, and lakes, increasing the risk of eutrophication and the loss of biodiversity [17,18].

Fertilizers have a significant amount of arsenic, chromium, cadmium, lead, zinc, nickel, iron, molybdenum and manganese, which are essential in the healthy growth and maintenance of plants. However, the excess of these metals in sediments of freshwater produces toxic effects to aquatic organisms [19,20]. Heavy metals enter the human body mainly through ingestion, through food, inhalation and dermal contact, such as emissions of waste material in the form of smoke, dust particles, and substance vapors of chemicals from various industrial activities, such as mining and agricultural practices [21].

Exposure to heavy metals can cause their excessive accumulation in body tissues and induce the production of free radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) that lead to an oxidative stress condition. Elevated levels of Zn and Cu can cause various adverse health effects; otherwise, these elements are essential for life and functions of many proteins in the organism. Among the measured elements, arsenic, cadmium, chromium (VI) and nickel are classified as carcinogens [22,23] by the International Agency for Research on Cancer (<https://monographs.iarc.who.int/list-of-classifications>, accessed on 7 April 2021). Copper is not classified as carcinogenic and there is only one publication on Cu as a tumor promoter [24], cited by Taylor et al. (2019) [25].

Water quality can be assessed by various parameters, such as biochemical oxygen demand (BOD), temperature, conductivity and concentrations of nitrate, phosphorus, potassium and dissolved oxygen. Among many other classes of contaminants, heavy metals are usually of special concern because they might cause poisoning in aquatic animals [15]. At the level of the trophic chain, the most sensitive organisms are those most affected, and in fact, contamination of the aquatic ecosystem, whether due to bioaccumulation or direct exposure, will ultimately lead to the exposure of humans [21,26]. Thus, contamination of aquatic ecosystems has been monitored by analyzing the effects of the presence of contaminants using bioindicator species, which reflects the health state of that specific environment [27,28].

The use of organisms as bioindicators for the evaluation of environmental contamination has been used in several studies, since this strategy offers various advantages concerning the prediction of the flow of contaminants in the populations of the trophic chain [29,30]. Planarians have gained more and more notoriety in ecotoxicology as test organisms with potential for biomonitoring contaminants in freshwater ecosystems, mainly

due to: (i) a useful potential to screen contaminant toxicity and assess the quality of freshwater ecosystems; (ii) several interesting biological characteristics (for example, behavior, regeneration, and reproduction) that can be used to evaluate the sub-lethal and chronic toxicity of environmental contaminants; (iii) some physiological systems that share resemblances to those in mammals (e.g., the nervous system); (iv) being a representative group of invertebrates with wide geographical distribution, functioning as prey and predators; (v) ease of capture and low laboratory maintenance costs [31–34]. Among the freshwater planarians used as test organisms in ecotoxicological bioassays, *Girardia tigrina* (Paludicola, Dugesidae; Girard, 1850) has been used to evaluate lethal and sub-lethal toxicity of xenobiotics and potential biomonitoring [35–37].

Behavioral biomarkers, such as feeding rate and locomotor activity, have been developed as low-cost tools, but with high sensitivity and acceptance as integrative and cumulative responses induced by xenobiotics that are indicative of deleterious cellular alterations and effects at higher levels of biological organization [37]. Moreover, although scarce, the effects of metals on freshwater planarians have been reported in the literature, such as copper's effects on antioxidant defenses in *Dugesia japonica* [38], the genotoxic effects of copper in *Girardia schubarti* [39], and aluminum's neurotoxic effects in *Dugesia estrusca* [40]. Additionally, although not a metal, the effects of chlorine have been reported on the feeding, locomotion, regeneration and reproduction of *Girardia tigrina* [28].

Thus, our present study aimed to determine the usefulness of such biomarkers to assess the environmental contamination of water samples coming from the watershed of Araguaia-Tocantins through the laboratory exposure of adult planarians. These water samples were collected in areas of intense agricultural production and exploitation of mineral resources. In addition, this study also aims to gather information about the behavioral biomarkers and its potential use as fast screening tools that can be adopted to determine important decisions concerning further biomonitoring studies complemented with chemical analysis of different freshwater compartments.

## 2. Material and Methods

### 2.1. Study Area

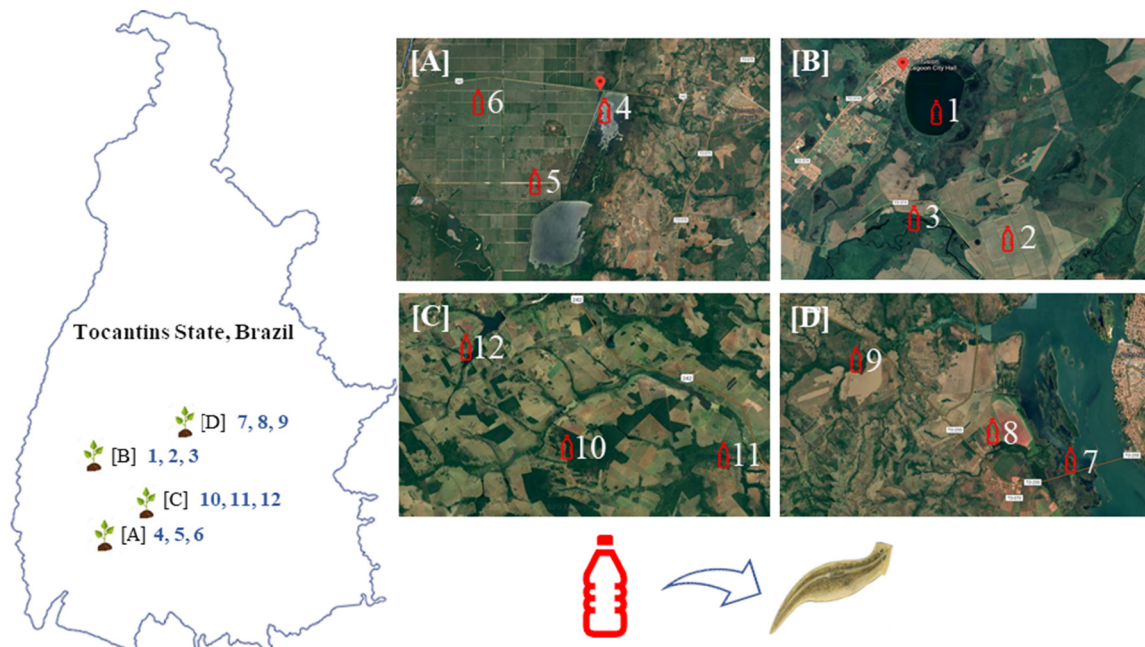
Water samples were collected in four municipalities, Formoso do Araguaia (11°48'02.8" S and 49°37'26.3" W), Lagoa da Confusão (10°50'64.3" S and 49°42'51.0" W), Gurupi (11°51'33.96" S and 48°53'15.14" W) and Porto Nacional (10°47'41.83" S and 49°37'22.854" W), of Tocantins-Araguaia Hydrographic region (TAHR). Those sampling sites represent areas of intense agricultural production and exploitation of mineral resources (Figure 1). Moreover, a local reference site for water sampling was chosen considering a location far from nearby sources of human impact and therefore less contaminated (Figure 1). The sampling period was conducted during the wet season, i.e., January to February.

### 2.2. Water Analysis

The sampling, storage and transport of the water samples to the laboratory were carried out following the specifications of the sampling of surface waters according to the National Water Agency [41]. Then, samples were subsequently sent to the Conagua Ambiental laboratory for analysis (Goiás State, Brazil—<https://conaguaambiental.com.br/wp/>, accessed on 15 July 2020) and also used in the laboratory for exposure of planarians.

For the analysis of the water samples, they were collected in borosilicate glass bottles and PET containers for the first use, containing the necessary preservatives (Conagua Ambiental protocols—<https://conaguaambiental.com.br/wp/>, accessed on 15 July 2020) to avoid the deterioration of the sample. The collection was carried out at a depth no greater than 30 cm. Later, the flasks were sealed and transported to the laboratory at a temperature of 4 °C. The water samples for the exposures were collected and transferred at 4 °C and expected until the sample reached the temperature for conducting the experimental tests, 22 ± 1 °C, without the addition of additives.

Analyses were carried out on the water samples to determine chemical, physical and biological parameters following the methods of the Environmental Protection Agency and Standard Methods for the Examination of Water and Wastewater [42].



**Figure 1.** Map of Tocantins-Araguaia hydrographic region (TAHR), Brazil. (A) Formoso do Araguaia, 4 (Taboca), 5 (small irrigation channel), 6 (large volumes of water in the channel); (B) Lagoa da Confusão, 1 (lake), 2 (water channel), 3 (Urubu river); (C) Gurupi, 10 (Dam Brejo Pantanal), 11 (Santo Antônio river), 12 (Reference, dam with crystal clean water); (D) Porto Nacional, 7 (Tocantins river), 8 (large volumes of water in the channel), 9 (small irrigation channel); adapted from Google Maps and Google Earth (2020). 🚰 = Water collection sites. Water collected in each site was used to expose adult planarians (*G. tigrina*) in the laboratory.

### 2.3. Planarians

The specimens of *G. tigrina* used in the bioassays were obtained from the University of Sao Paulo and maintained in laboratory cultures in the Federal University of Tocantins, Tocantins State-Brazil, since 2014. American Standard Test and Materials (ASTM) hard water was used as a culturing medium [43]. Organisms were maintained in controlled conditions in plastic boxes ( $30 \times 18 \times 10 \text{ cm}^3$ ), containing 1 L of ASTM medium, at  $22 \pm 1 \text{ }^\circ\text{C}$ , in the dark and constant aeration. The planarians were fed with bovine liver (*ad libitum*) twice a week, followed by medium renewal two hours after the beginning of feeding activity, except the 96 h prior to exposure and the entire exposure period.

#### Development and Design Test of Behavioral Responses

The planarians have their eating behavior altered according to the chemical composition of the water in the natural environment [44]. Despite this, we did not determine the hard water in the different water samples studied. In this way, we used ASTM hard water as a control treatment, since the planarian culture is maintained in this culture medium and we were able to determine the number of salts (sodium hydrogen carbonate, magnesium sulfate, potassium chloride, and calcium sulfate) available to the organisms. For example, calcium ions in the aquatic environment are considered to be indispensable for the feeding behavior of planarians, and exposure to higher levels of calcium ions enhanced the feeding behavior, showing that there was a good correlation between the concentration of calcium ions and the responsiveness of planarians to foods [44]. In addition, the amount of calcium salt can be up to fifty times the amount of potassium salt without unfavorable results for



planarians [45]. On the other hand, in the collection of water samples in the field, it was not possible for us to determine an uncontaminated reference point to adopt as a control (in addition to the control with ASTM medium).

Individual *G. tigrina* ( $0.83 \pm 0.11$  cm in total length) were exposed ( $n = 10$  per condition) during 96 h to 20 mL of 12 water samples, on Petri dishes ( $\varnothing = 4.6$  cm), including the reference site (Figure 1) and a laboratory control with ASTM water only. The exposed organisms were maintained at  $22 \text{ }^\circ\text{C} \pm 1$  under dark conditions and no food was provided. After four days exposure, planarians from each condition were transferred to clean medium and locomotor velocity and feeding rate were determined as post-exposure analysis.

Briefly, planarians were individually transferred ( $n = 10$  per condition) to new crystalizing dishes containing 20 mL of ASTM medium, and twenty live larvae of *Chironomus xanthus* (six days old) were released on each plate. Then, the feeding rate per hour was calculated, analyzing the number of larvae consumed per planarian at the end of 12 h.

To determine the locomotor velocity ( $p$ LMV), planarians were placed in a plate ( $\varnothing$  35 cm) with 200 mL of ASTM hard water and an adhered millimeter paper (grid lines spaced 0.1 cm apart) below. The  $p$ LMV was measured individually, after scoring the number of crossed grid lines for each planarian for 2 min (taking as reference the crossing of the planarian head) according to the methodology already adopted by our research group [28,35,36]. There was no mortality of planarians during the experimental assay.

#### 2.4. Statistical Analysis

For  $p$ LMV and feeding rate data, we used one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons in order to determine differences between sampling and reference sites and the laboratory control. Prior to ANOVA, the homogeneity of variances of data was assessed using the Brown–Forsythe test and the normality of data by using the D'Agostino and Pearson test. Locomotion data were transformed ( $Y = \text{rank}(Y)$ ) for the correction of homogeneity of variance. Statistical analysis was performed using the software GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, San Diego, CA, USA).

### 3. Results

#### 3.1. Sampling Sites

Disturbing concentrations of aluminum were found in the surface water samples at points 2, 3, 7, 10 and 11—at these points, concentrations of 0.10, 0.13, 1.02, 0.27 and  $0.38 \text{ mg L}^{-1}$  of aluminum were detected, respectively (Table 1—concentrations higher up to 10 times the limit acceptable). For these points already indicated, the pH was variable, obtaining values of 5.44, 7.1, 7.25, and 6.97, respectively.

The water sample collected at site 1 (Lagoa da Confusão lake) affected the planarian locomotion, decreasing to 36.18%; in this sample, concentrations below the acceptable limit of inorganic compounds (total fluoride, total manganese, nitrates, nitric nitrogen) were found (Table 1). The water sample collected at site 2 (water channel, Lagoa da Confusão) altered the  $p$ LMV of the planarian, decreasing when compared to the control. In this sample, concentrations above the permissible limit of inorganic compounds (dissolved aluminum, dissolved iron) were found (Table 1), as well as an acid pH of 5.44, affected the feeding rate and  $p$ LMV of the planarian.

#### 3.2. Feeding Rate

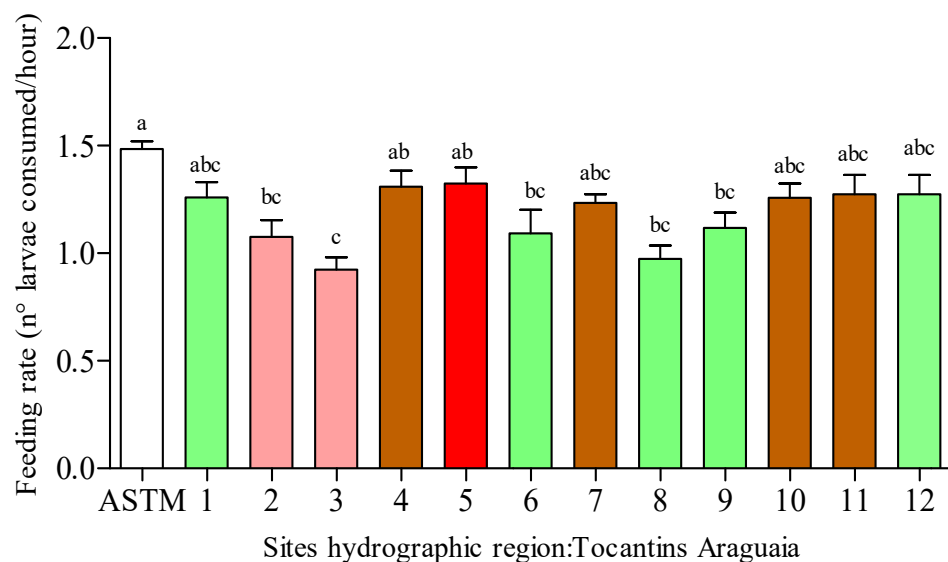
Planarians exposed in the laboratory to water samples from the Tocantins Araguaia hydrographic region caused the alteration of their post-exposure feeding rate. Significant differences were found in sampling sites when compared with the ASTM condition in the laboratory ( $p < 0.0001$ , Figure 2). Planarians, when exposed to water samples from site 3 (Urubu river—Lagoa da Confusão), site 8 (large volumes of water in the channel—Porto Nacional), site 2 (water channel—Lagoa da Confusão), site 6 (large volumes of water in the channel—Formoso do Araguaia), and site 9 (small irrigation channel—Porto Nacional),

showed a significantly decreased feeding activity of 38, 34, 28, 26 and 25%, respectively, when compared to the ASTM control condition (Figure 2).

**Table 1.** Analysis of surface water samples collected at eleven sampling sites (1–11) and the reference (12) site in Tocantins.

| Test                      | AL      | LQ    | Unit                 | Sites of Hydrographic Region Tocantins Araguaia |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
|---------------------------|---------|-------|----------------------|---|---|---|---|---|---|---|---|---|----|----|----|------|------|------|------|------|------|------|------|-----|------|------|-----|
|                           |         |       |                      | 1   | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |      |      |      |      |      |      |      |      |     |      |      |     |
| Biochemical oxygen demand | 5       | 0.2   | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Dissolved oxygen          | >5      | 0.1   | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Turbidity                 | 100     | 0.21  | NTU                  |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Acidity/alkalinity        | 6.0–9.0 | 0.1   | pH                   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total dissolved solids    | 500     | 0.05  | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Cyanobacterial density    | 50,000  | 1     | Cel mL <sup>-1</sup> |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Dissolved aluminum        | 0.1     | 0.004 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total barium              | 0.7     | 0.005 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total chloride            | 250     | 0.5   | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total chlorine            | 0.01    | 0.01  | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Dissolved iron            | 0.3     | 0.04  | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total fluoride            | 1.4     | 0.04  | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total manganese           | 0.1     | 0.007 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Nitrates                  | 10      | 0.1   | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Nitric nitrogen           | 1       | 0.001 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total sulfate             | 250     | 0.11  | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Total zinc                | 0.18    | 0.007 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
| Surfactants               | NR      | 0.001 | mg L <sup>-1</sup>   |   |   |   |   |   |   |   |   |   |    |    |    |      |      |      |      |      |      |      |      |     |      |      |     |
|                           |         |       |                      |   |   |   |   |   |   |   |   |   |    |    |    | 0.74 | 0.61 | 0.68 | 0.76 | 0.64 | 0.63 | 0.64 | 0.62 | 0.7 | 0.61 | 0.59 | <LQ |

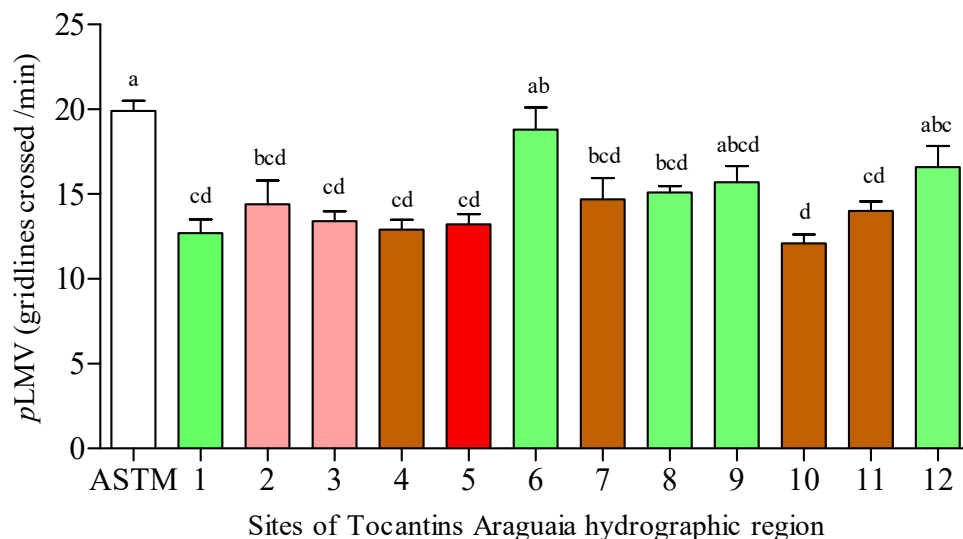
Note: ■ Very high concentrations; ■ High concentrations; ■ Above acceptable limits; ■ Acceptable limit (AL); ■ <Limit of Quantitation (LQ); ■ Limit not recorded. Sites of TAHR: Lagoa da Confusão (1–3); Formoso do Araguaia (4–6); Porto Nacional (7–9); Gurupi (10–12). The coloration for the results is expressed in comparison to the acceptable limit for surface waters (AL), these colors are associated with the graphs. AL: In agreement with the National Environment Council (Conselho Nacional do Meio Ambiente—CONAMA), BRAZIL-Resolution No 357, of 17 March, 2005 [46]. Provides for the classification of bodies of water and environmental guidelines for their classification, as well as establishing the conditions and standards for the discharge of effluents, and other measures.



**Figure 2.** Feeding rate (number of *C. xanthus* larvae consumed per hour) of *G. tigrina* after 96 h exposure with water samples from Tocantins-Araguaia Sites Hydrographic Region: Lagoa da Confusão (1–3); Formoso do Araguaia (4–6); Porto Nacional (7–9); Gurupi (10–12); Reference (12). A laboratory control with ASTM only was also used to expose planarians. Values represent the mean ( $\pm$  SEM),  $n = 10$ . Different letters indicate significant differences between study sites when  $p < 0.05$ . Colors used in graph bars are associated with analysis of water performed: ■ very high concentrations, ■ high concentrations, ■ above acceptable limits, ■ acceptable limit.

### 3.3. Planarian Locomotor Velocity (*p*LMV)

The post-exposure locomotion velocity (*p*LMV) of *G. tigrina* was significantly affected ( $p < 0.0001$ ) in planarians after 96 h exposure to water samples from site 10 (Dam Brejo Pantanal-Gurupi), site 1 (lake—Lagoa da Confusão), site 4 (Taboca-Formoso do Araguaia), site 5 (small irrigation channel—Formoso do Araguaia), site 3 (Urubu river), site 11 (Santo Antônio river), site 2 (water channel), site 7 (Tocantins river) and site 8 (large volumes of water in the channel), showing decreases of 39, 36, 35, 34, 33, 30, 28, 26 and 24%, respectively, when compared to the control condition using ASTM only (Figure 3).



**Figure 3.** Planarian locomotor velocity (*p*LMV) of *G. tigrina* (gridlines crossed/min), after 96 h of exposure to water samples of Sites of Tocantins-Araguaia Hydrographic region: Lagoa da Confusão (1–3); Formoso do Araguaia (4–6); Porto Nacional (7–9); Gurupi (10–12); Reference (12). A laboratory control with ASTM only was also used to expose planarians. Values represent the mean ( $\pm$ SEM),  $n = 10$ . Different letters indicate significant differences between study sites when  $p < 0.05$ . Colors used in graph bars are associated with analysis of water performed: ■ very high concentrations, ■ high concentrations, ■ above acceptable limits, ■ acceptable limit.

## 4. Discussion

Alternative methodologies involving invertebrates, including freshwater planarians, have been awakened worldwide. In this context, the effects observed on the feeding rate and *p*LMV of *G. tigrina* are considered sensitive and relevant parameters from the point of view of environmental monitoring of different contaminants. Additionally, these endpoints may be linked to changes in higher levels of biological organization, as they are the result of a series of biochemical and physiological processes caused by environmental stress [47–49].

The results of our study showed the alteration of various physical and chemical parameters and the presence of various heavy metals in water samples that altered the behavior of the planarian (Table 1). We observed that the behavioral results of *G. tigrina* were shown to be sensitive to the TAHR water samples, as a possible result of synergistic or antagonistic interactions of the physical-chemical parameters of each sample, illustrating that these are important integrative and cumulative ecotoxicological parameters with ecological relevance. The biomarkers of environmental contamination evaluated showed differences in sensitivity depending on the water sample location in TAHR. For *p*LMV, significant differences were observed when planarians were exposed to water samples from most of the sampling points of TAHR (sampling points 1, 2, 3, 4, 5, 7, 8, 10, 11—Figure 3). On the other hand, the feeding rate of planarians was affected when organisms were exposed to less contaminated sites (2, 3, 6, 8, 9—Figure 2).

Despite the fact that planarians' locomotion is achieved mainly by gliding (ciliary beating), the locomotor behavior is related to the proper functioning of the nervous system,

since muscle contraction can be employed [50]. In addition, it is important to note that the feeding activity of planarians is dependent on their locomotor ability to capture prey, and also related to the nervous system functioning, such as movement and muscle action, the perception of chemical stimuli, as well as the extra-corporal digestion of prey [49,51,52].

The lower sensitivity of the feeding test, compared to *p*LMV, may be related to the short exposure time and period of feeding post-exposure. Although future studies are needed to elucidate this, we hypothesized that a longer exposure period of planarians (>96 h) and a shorter post-period of feeding (<12 h) would better reflect the increased energy required for maintenance and defense mechanisms [53]. Although in our study planarians have been exposed to TAHR water samples for 4 days (96 h static exposure), there still seems to be no consensus on the specific number of days planarians should be exposed in static or semi-static conditions—usually from 8 days of exposure up to 14 days [35–37].

Feeding tests reported in the most recent scientific literature generally occur for a period of 3 h, when organisms are exposed to a specific contaminant [35–37]. According to our results, it seems that the feeding activity of planarians is dependent on the locomotor activity and feeding requirement of exposed planarians: (i) planarians exposed to water of less contaminated sites showed a decreased locomotor activity and probably a low requirement of food; (ii) planarians exposed to highly contaminated water also showed a decreased locomotor activity, but an increased requirement for food to deal with deleterious effects of contaminants. Therefore, those differences seem to be more evident in feeding tests with long post-exposure periods.

Various studies have reported the toxicological effects of one or more compounds in organisms [37,54]. However, studying the synergism or antagonism that may occur with the presence of contaminants and environmental parameters (pH, dissolved oxygen, turbidity) in organisms is complex [55]. Prolonged exposure to these metals can cause cell death and alterations in DNA, lipids, proteins, enzymes, and homeostasis of calcium and sodium ions [56]. For example, 40 mg L<sup>-1</sup> Fe<sup>3+</sup> has been shown to cause 100% mortality in *Dugesia japonica* at 25 °C; additionally, the low temperature could slow down the effect of Fe<sup>3+</sup> on planarian toxicity, and at a suitable temperature, the toxic effects of Fe<sup>3+</sup> on planarian can be accelerated [57].

Heavy metals, such as, Zn, Cu, and Fe, are necessary for the proper functioning of various enzymes and proteins. However, when the same metals accumulate above the threshold, they become toxic. This induces the generation of reactive nitrogen and oxygen species (RNS; ROS), which results in the peroxidation of lipids in the plasma membrane [23,58]. Reports show heavy metal bioaccumulation in organisms through food chains [59,60]. It has been shown that the enzymatic activity of superoxide dismutase and catalase can play an important role on glutathione peroxidase in the antioxidant defense system of *Dugesia japonica* in response to exposure to copper [38]. Another study conducted with *Girardia schubarti* suggests that copper could modulate the genotoxic effects associated with the exposure of complex mixtures in the environment [39]. Furthermore, in our work we found concentrations of up to 0.88 mg L<sup>-1</sup> of dissolved iron with the temperature in the Tocantins state fluctuating between 30 and 38 °C. In the field conditions, temperatures above 27 °C could cause mortality and slow movements in *G. tigrina*; consequently, the species releases mucus to change the surrounding pH of its external environment to maintain its physiological function [23].

High levels of dissolved aluminum (point 7), total chlorine (points 4 and 11), and zinc (point 5) were found in water samples of TAHR, which may be associated with the intensive and periodic use of fertilizers and agricultural correctives (in the case of aluminum), whereas it is known that freshwater planarians can present different responses to heavy metals. The high toxicity of aluminum, if compared to chromium and cadmium, causes uncoordinated writhing in exposed planarians, suggesting that neurotoxic effects were produced in intact and regenerating *Dugesia estrusca* [40]. Furthermore, chlorine plays a key role in disinfecting drinking water and wastewater; on the other hand, it represents a



risk to the freshwater ecosystem when used for a longer period [28]. These authors report the chronic effect of chlorine on *Girardia tigrina* with observed effect concentrations (LOEC) of 210 µg/L for feeding and *p*LMV, using the same methodology of our assays, as well as LOEC of 168 µg/L for head regeneration and 263 µg/L for reproduction (fertility and fertility). This is worrying from an environmental point of view, since at the sample of point 11 of TAHR, we found concentrations of total chlorine of 70 µg/L—the same order of magnitude as the LOEC values reported by Rodrigues Macêdo and co-authors [28]. On the other hand, zinc exposures have been reported to cause oxidative damage in aquatic organisms, for example, in the shrimp *Atyaephyra desmarestii*, while the feeding rate of amphipod *Echinogammarus meridionalis* was severely reduced with zinc exposures [60]. The authors report that a decrease in feeding rates reduces the total energy available, and the processes of metabolism and detoxification are also likely to be reduced.

We did not measure oxygen levels during experimental feeding and locomotion tests, since the exposure occurred in 96 h in a shallow, flat vessel (Petri dish). In spite of this, the results of the analysis of the samples collected in the field revealed low concentrations of dissolved oxygen, mainly in the water samples of points 5 and 11. Although we cannot say that the effects on the feeding of planarians are related to low concentrations of oxygen, we speculate whether the effects observed in *p*LMV could be related, among other factors, to low oxygen concentrations in samples 5 and 11. It has been reported that low respiration rates can affect the physiological performance of freshwater invertebrates and have been associated with changes in behavior [37,61,62]. During moderate stress, maintenance costs increase to meet the additional energy demands for stress protection and damage repair, or metabolism and/or food assimilation is affected by the stressor. As a result, the aerobic range decreases [53,63]. Increases in temperature (as occur in TAHR-tropical climate) can lead to an increased metabolic rate and O<sub>2</sub> consumption by aquatic organisms and the consequent production of reactive oxygen species (ROS) [64,65]. For example, studies in *Girardia dorocephala* and *Schmidtea mediterranea* show that as the temperature increases, oxygen consumption will also increase [66].

Studies in *G. tigrina* show that it decreases *p*LMV when exposed to xenobiotics, for example, *p*LMV displayed a dose-dependent negative correlation with scopolamine concentrations from 0.001 to 1.0 mM, and a further increase in scopolamine concentration to 2.25 mM did not further decrease *p*LMV [67]. In another study, galantamine showed high anticholinesterasic activity when compared to the other drugs, with a reduction in *p*LMV, presenting screw-like movement and hypokinesia, with a *p*LMV of 65 crossed lines during 5 min [68]. The *p*LMV seems to be a good indicator when exposed to xenobiotics, as shown by experiments carried out where, as the concentration increases, the locomotion speed of the planaria decreases [35–37,54]. This is also demonstrated in our study, where *p*LMV decreases in local (2–5, 7, 10–11) sites with the presence of xenobiotics.

It should be considered that alterations in the behavior of the planarian can affect the food chain. The stability of a species has been shown to be highest when it is at the top of the food chain, and lowest when it is just below the top level, and it exhibits a switching pattern at intermediate levels [69]. Consequently, we could mention that the decrease in the feeding rate and the *p*LMV could be a consequence of the reallocation of energy to the stress response and the maintenance of homeostasis in planarian.

The study of these biomarkers of behavior in the planarian suggests that the trophic chains in the freshwater ecosystems of the TAHR are being altered, since these organisms play a fundamental role in these ecosystems. Likewise, the results of this study alert us to the need to monitor the consequences of anthropic actions and agricultural pressure in the state of Tocantins—Brazil. At the same time, behavioral tests used with planarians seem to be fast screening tools that contribute to the biomonitoring of freshwater systems and further conservation measures to be taken in the Tocantins-Araguaia hydrographic system. In sum, the tributaries of the Tocantins-Araguaia hydrographic region, comprising the municipalities of Formoso do Araguaia, Lagoa da Confusão, Gurupi, and Porto Nacional, presented contamination by heavy metals and other pollutants, which caused deleterious

effects in behavioral responses of the freshwater planarian *G. tigrina*. Finally, this research provides an important approach to assess the ecological effects of contaminants in lotic ecosystems, using the behavior of planarians to assess water quality in tropical aquatic systems subjected to anthropic pressure.

We aimed to use a behavioral tool that could predict deleterious effects of water samples from the environment even without knowing the physical or chemical properties of water. This would help to identify the impacted hotspots in a fast and cost-effective way. Obviously, further biomonitoring would be necessary on those hotspots for the identification of the environmental problems. Briefly, our goal was to use a behavioral biomarker to assess the sublethal effects of water contamination. The use of a behavioral test presents the advantage of being cumulative and integrative, i.e., it is caused by insufficient energy allocation for behavior due to its use for other processes altered by stress conditions, and that might be a chemical compound, an abiotic factor, or even the conjugation of several factors.

## 5. Conclusions

The development of agriculture in the hydrographic Tocantins-Araguaia region contributes to the increased concentrations of metals, causing deleterious effects on benthic organisms, such as *G. tigrina*, that potentially affect various organizational levels of the trophic chain.

The exposure in the laboratory of planarians to water of different sampling points in the hydrographic region Tocantins-Araguaia caused a reduction in the feeding rate and *p*LMV. The use of these cumulative and integrative behavioral biomarkers showed high sensitivity to contaminants and abiotic factors of water samples.

These results emphasize the importance of evaluating biomarkers of environmental contamination, using the behavioral parameters of *G. tigrina* as fast screening tools in order to help the development and ecological relevance of biomonitoring programs in contaminated watersheds.

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