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Genetic Biology

Mutagenicity of Piraí river water – RJ, using *Drosophila melanogaster warts* (*wts*) as a bioindicator

Mutagenicidade de águas do Rio Piraí – RJ, usando *Drosophila melanogaster Warts* (*wts*) como bioindicador

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ABSTRACT

The Piraí River is part of the Paraíba do Sul–Guandu watershed, which supplies water to over 80% of the population of the Rio de Janeiro metropolitan region. Along its course, the aforementioned river passes through a highly industrialized region, where it receives untreated urban sewage. Previous studies conducted with water samples deriving from Piraí River, based on using *Allium cepa* assay, have found cytogenotoxic compounds in them. This study aims to assess the mutagenic potential of genotoxic agents in water, using larvae of the *Drosophila melanogaster Warts* (*wts*) mutant strain as bioindicator. Analyses have investigated the incidence of epithelial tumors in the eyes, head, body, legs and halter of flies subjected to different treatments (positive control - doxorubicin, negative control – distilled water, and two water samples collected from Piraí River). Statistical analysis results have shown that the frequency of epithelial tumors in flies subjected to treatment with water samples was significantly higher than that of the negative control. This finding has evidenced the incidence of mutagenic agents in water and emphasized the importance of adopting sanitation strategies and of supervising industrial waste disposal. *D. melanogaster* Warts model proved to be a useful bioindicator to analyze mutagenic agents found in water samples.

Keywords: Genotoxic; Mutagenesis; Water quality

RESUMO

O Rio Piraí faz parte da bacia hidrográfica Paraíba do Sul-Guandu, que abastece mais de 80% da população da região metropolitana do Rio de Janeiro. Ao longo do percurso, o rio passa por uma região altamente industrializada, recebendo esgoto urbano sem tratamento. Estudos prévios de amostras de água do rio Piraí, realizados por meio do teste *Allium cepa*, demonstraram a presença de compostos citogenotóxicos. Objetivou-se, neste trabalho, avaliar o potencial mutagênico dos agentes genotóxicos presentes na água

utilizando-se larvas da linhagem mutante de *Drosophila melanogaster, Warts (wts*), como bioindicador. As análises avaliaram a presença de tumores epiteliais nos olhos, cabeça, corpo, pernas e halteres, das moscas submetidas aos diferentes tratamentos (controle positivo – doxorrubicina, controle negativo – água destilada, e duas amostras de água coletadas no Rio Piraí). A análise estatística dos resultados demonstrou que a frequência de tumores epiteliais nas moscas submetidas ao tratamento com as amostras de água foi significativamente maior do que no controle negativo, demonstrando a presença de agentes mutagênicos e ressaltando a importância dos serviços de saneamento e fiscalização de despejo de resíduos industriais. Além disso, o modelo de *D. melanogaster Warts* demonstrou ser um bioindicador útil para análise de agentes mutagênicos presentes em amostras de água.

Palavras-chave: Genotóxico; Mutagênese; Qualidade da água

1 INTRODUCTION

The Piraí River is located in Barra do Piraí County, Rio de Janeiro State, Brazil. It receives water transposed from the main river accounting for supplying the metropolitan region of Rio de Janeiro, namely: Paraíba do Sul River. Seventy percent (70%) of the water volume in this river is diverted to Piraí River, in Santa Cecília reservoir; later on, it passes through a series of reservoirs and reaches Guandu River. This entire route accounts for supplying more than 80% of water to Rio de Janeiro metropolitan region; i.e., it represents drinking water supply to more than 9 million people (Araújo et al., 2009; CBH Guandu, 2015; Demanboro, 2015). In this context, the intense anthropogenic activity along the Paraíba do Sul–Guandu stretch exerts significant pressure on the water quality of this system.

Paraíba do Sul-Guandu hydrographic stretch crosses an industrialized region that hosts metallurgical, chemical and food production companies, as well as mining activities; it is exposed to agricultural waste, leaching from landfills and dumps, as well as to runoff from highways and railways. Paraíba do Sul River also receives all untreated urban sewage coming from cities crossed by it. These aggravating factors lead to the vulnerable water treatment carried out by Guandu Water Treatment Plant (Torres et al., 2002; CBH Guandu, 2015).

Because industrial, domestic and agricultural waste types change water quality, several organisms are directly, or indirectly, affected by them at different trophic chain levels. Polluting agents can affect the food chain and harm several species. In addition, they account for the aggravating factor of becoming a public health issue, since they carry compounds with mutagenic, cytotoxic and/or genotoxic potential that, oftentimes, are not ruled out during water treatment processes (de Assunção and Pesquero, 1999; Nielsen and Rank, 1994; Oliveira et al., 2011; Kasper et al., 2018). Given these potential impacts on biota and public health, it is essential to employ experimental approaches capable of detecting and quantifying genotoxic effects associated with water quality.

Previous analyses carried out at the Plant Genotoxic Activity Laboratory (LAGeP – UFRRJ, Seropédica, Brazil), based on using *Allium cepa* assay, as described by Sacramento et al. (2020), detected cytogenotoxicity in water samples deriving from Paraíba do Sul River. These samples were collected at four different points downstream of the transposition region in Barra do Piraí County. It is important to note that, in addition to the previously mentioned sources of contamination, according to the Mid-Paraíba do Sul Basin Committee (CBH-Médio Paraíba do Sul, 2017), only 60% of the sewage generated in Barra do Piraí is collected, and this fraction remains untreated. Consequently, approximately 1.229 million m^3 of untreated sewage are discharged annually into the river within the municipality.

The detected genotoxic potential indicates incidence of components that can affect the genetic material in different ways, such as by interfering in mitotic spindle fibers or in cell enzymes' performance. These effects may be transient and subject to repair. However, some of these components can directly affect the DNA sequence and cause permanent changes in it; consequently, it is seen both as genotoxic and mutagenic (Dearfield et al., 2002). It is important to conduct analyses based on using bioindicators to assess the mutagenic potential of components found in water. In this

regard, the use of sensitive and well-characterized model organisms represents an effective strategy for detecting mutagenic effects in environmental samples.

Drosophila melanogaster is an insect species belonging to Order Diptera, Family Drosophilidae, which is commonly used as model in studies conducted in different fields, such as those associated with toxicological genetics. Its manipulation requires low maintenance cost, and its genome presents approximately 77% homology with genes linked to different diseases affecting humans (Adams et al., 2000; Fernández-Moreno et al., 2007; Kim et al., 2011; Nepomuceno, 2015; Chifiriuc et al., 2016; Larkin et al., 2021).

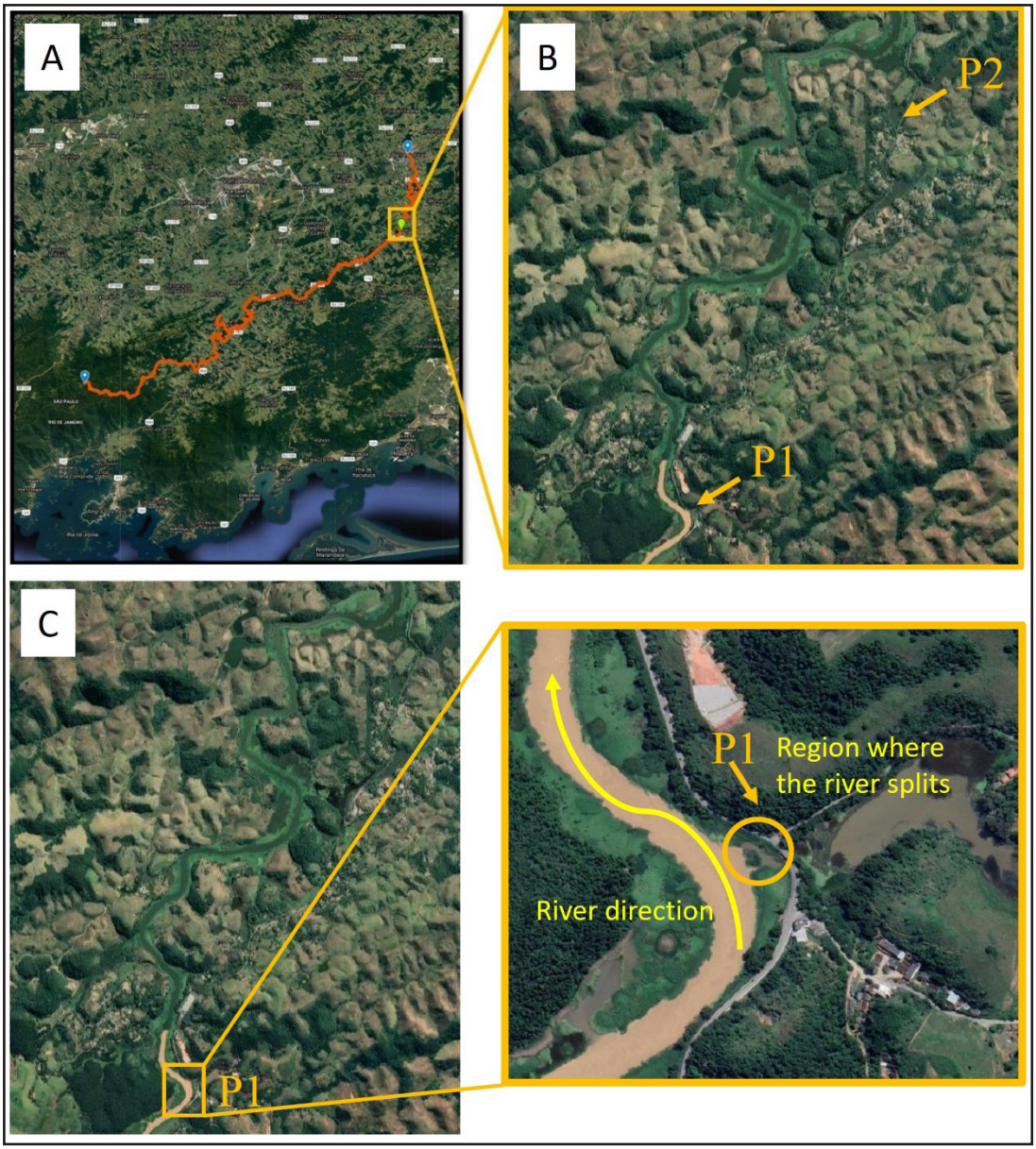
D. melanogaster mutant lineage *Warts* (*wts*) carries the *wts* gene in heterozygosis. This name was given to it due to the shape of epithelial tumors presented by this lineage when the *wts* gene is mutated or deleted. The *wts* gene is a tumor suppressor gene, homologous to the LATS1 gene (Large tumor suppressor kinase 1) found in humans (Nishiyama et al., 1999; Nepomucemo, 2015), which encodes a serine/threonine kinase enzyme (Justice et al., 1995; Xu et al., 1995). The group of serine/threonine kinase enzymes accounts for converting extracellular stimuli into a wide range of cell responses to help controlling cell proliferation, programmed cell death (apoptosis), cell differentiation and embryonic development (Cross et al., 2000; Cargnello and Roux, 2011; Manoharan et al., 2018). In addition, *wts* gene loss or inactivation leads to lack of control over both the amount and the direction of cell proliferation in progress, as well as to warts, or tumors, formation in adult individuals (Justice, et al., 1995). Tumors develop throughout the body of the fly and can be readily observed under light microscopy, thereby rendering this strain a sensitive and efficient bioindicator for the assessment of mutagenicity in both isolated compounds and complex mixtures (Nepomuceno, 2015)

Thus, the aim of the current study was to assess the incidence of mutagenic agents in water samples deriving from Piraí River, based on using *Drosophila melanogaster* mutant lineage *Warts* (*wts*) as bioindicator.

2 MATERIAL AND METHODS

2.1 Water samples collection

Figure 1 – Satellite image showing the water sample collection points in Piraí River



Source: Authors' private collection – The red line in the first image (A) represents Piraí River course, which starts in São Paulo State and ends in Rio de Janeiro State. Collection points 1 and 2 in image B are highlighted by yellow arrows. The third image (C) highlights the enlarged region of point 1, which shows the river flow direction (in yellow); the circle represents the section where the river splits

The selection of water collection points was based on previous research carried out by Sacramento et al. (2020), in which four sampling points were selected

downstream of the transposition site. These points were randomly chosen, with intervals of approximately 2 km between them, and analyses were conducted during both the dry and rainy seasons. The two points presenting the highest cytogenotoxicity level, in both seasons, were selected for the present study, namely: Point 1 (P1), which was located at geographic coordinates 22°35'05.7" S and 43°50'12.4" W; and point 2 (P2), at geographic coordinates 22°33'06.3" S and 43°49'05.0" W (Figure 1). In the present study, sampling was conducted during the rainy season.

2.2 Mutant lineages

Two mutant lineages (*wts* and *mwh*) carrying genetic markers warts (*wts*, 3-100) and multiple wing hairs (*mwh*, 3-0.3) were used in the herein conducted test. They were kindly provided by researchers from the Cytogenetics and Mutagenesis Laboratory of the University Center of Patos de Minas (UNIPAM, Patos de Minas, Brazil).

Because the 3-100 mutant allele is lethal in homozygosity, in the *wts* lineage, it is maintained in the presence of *TM3* chromosome balancer, *Sb1*, which is featured by short, thick hairs. The *mwh* allele in the *mwh* lineage 3-0.3 can be maintained in homozygosity (Nepomuceno et al., 2015). These lineages were crossed to generate the heterozygous individuals employed in the experiments.

2.3 Maintenance of *Drosophila melanogaster* mutant lineages

Drosophila melanogaster mutant lineages were kept in bio-oxygen demand incubator, at 25ºC, on average, in the Multidisciplinary Laboratory of the Genetics Department of UFRRJ's Biological and Health Sciences Institute. To ensure the availability of a sufficient number of individuals for the experiments, routine and continuous subculturing procedures were performed. The investigated lineages were kept in glass vials filled with proper culture medium, which comprised distilled water, biological yeast (*Saccharomyces cerevisiae*), sugar, salt, corn flour, wheat flour, hydrochloric acid

and nipagin. A total of 400 females from the *wts*/TM3Sb1 lineage and 300 males from the *mwh/mwh* lineage were used to perform the crosses.

2.4 Test focused on detecting epithelial tumor in *Drosophila melanogaster* (*wts* Test)

Virgin females belonging to the *wts*/*TM3Sb1* lineage and males belonging to the *mwh*/*mwh* lineage were allowed to mate to obtain *wts* +/+ *mwh* larvae (Vartak et al., 2015).

Males and females were placed in flasks filled with the previously described medium, where they remained for 48 hours, for mating purposes. After this period was over, they were transferred to flasks filled with their own laying medium, which comprised biological yeast (*Saccharomyces cerevisae*) and sugar, where females deposited their eggs for 8 hours. A single female can lay up to 75 eggs in one day, and it results in 500 eggs within a 10-day period-of-time, in total (Resh and Cardé, 2009). After the 8-hour period was over, both male and female individuals were discarded and the aforementioned flasks filled with laying medium were kept in the incubator. Heterozygous larvae *(wts*+/+*mwh*) found in the medium reached the third larval stage within approximately 72 hours, when they were ready to be subjected to the treatments. They were washed in reverse osmosis water and collected with the aid of a fine-mesh sieve. Heterozygous individuals were used because they provide a permissive background for the expression of somatic genetic events, such as mutation, mitotic recombination, and loss of heterozygosity, enabling the detection of both genotoxicity (via *mwh*) and tumor formation (via *wts*).

2.5 Larval treatment

Third-instar larvae were placed in previously labeled glass vials, each containing 3 g of dehydrated potato flakes. For each treatment (negative control, positive control, and water samples collected from two sites of the Piraí River), two vials were prepared. Subsequently, 10 mL of the water sample collected at each analyzed site was added to the respective vials. The negative control consisted of the addition of

10 mL of distilled water to the medium, while the positive control consisted of 10 mL of 0.4 mM doxorubicin. Next, approximately 150 larvae were introduced into each vial and subjected to chronic exposure, in order to simulate environmental exposure conditions, for approximately 48 hours. After this period, individuals that reached the adult stage within the following 72 hours were collected, placed in vials containing 70% ethanol, and separated according to treatment.

2.6 Analyzing flies and counting tumor clones

D. melanogaster specimens were transferred from bottles filled with 70% ethanol to glycerin-filled watch glasses to be analyzed in a stereoscopic microscope in order to better assess epithelial tumor clones in their eyes, head, body, legs and halter. It was done to guarantee that the observed changes could be unequivocally classified.

Observations were carried out separately. Epithelial tumor counts were based on the incidence of changes observed in the aforementioned regions, as well as on the total number of changes per fly, in each tested sample and in the control experiments (Magalhães et al., 2017).

2.7 Statistical analysis

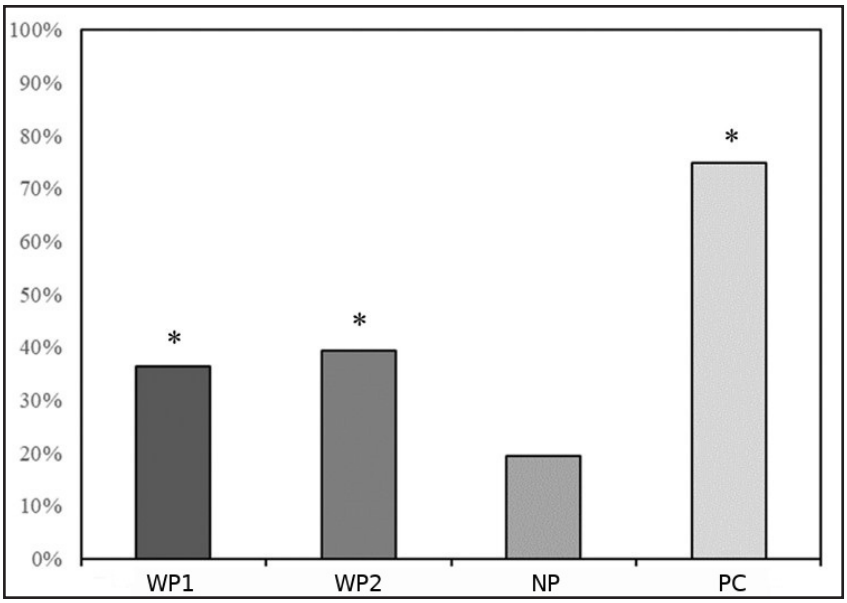
Statistical differences in the frequency of tumor clones observed between the tested samples and the controls were assessed using non-parametric Mann-Whitney U test, at a 5% significance level, using Past software, version 4.0. The statistical analysis of tumor burden per individual in each treatment, was performed using Kruskal-Wallis test, followed by Dunn's multiple comparisons test, also at 5% significance level.

3 RESULTS AND DISCUSSION

Two hundred (200) flies were assessed in each of the 4 treatments, and it totaled 800 analyzed individuals. At least one tumor was observed in 39 individuals

in the negative control (19.5%); in 150 individuals in the positive control (75%); in 73 individuals (36.5%) deriving from collection point 1 (WP1); and in 79 (39.5%) individuals deriving from collection point 2 (WP2) (Figure 2). The frequency of individuals with at least 1 tumor in both collection points (1 and 2) and in the positive control was significantly higher than that observed for the negative control (Mann-Whitney test, $p<0.001$). It is important to note that the occurrence of a small percentage of tumors in individuals from negative control is expected and may result from spontaneous mutations or environmental factors that generate basal stress.

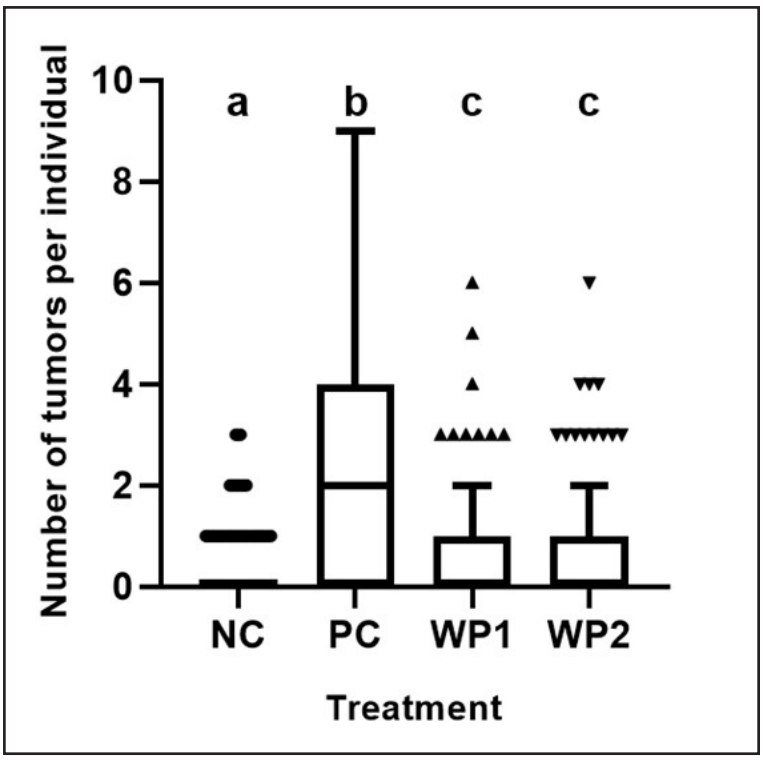
Figure 2 – Rate of *D. melanogaster* individuals presenting, at least, one tumor in any body part, in each treatment



Source: Authors' private collection – * Mann-Whitney Test, based on using $p \leq 0.05$ as significance level, by taking into consideration differences against negative control (distilled water).

In addition to tumor incidence, tumor burden per individual was analyzed to better characterize treatment effects (Figure 3). A significant difference among groups was observed (Kruskal-Wallis test, $p< 0.05$). The number of tumors per individual in both collection points and in the positive control was significantly higher than that observed for the negative control. No significant difference was observed between WP1 and WP2, suggesting similar effects between these treatments.

Figure 3 – Tumor burden per individual in each treatment



Source: Authors' private collection – Data are presented as median and interquartile range (box), with whiskers representing 1.5×IQR (Tukey). Individual data points are shown. Different letters indicate statistically significant differences between groups (Kruskal–Wallis test followed by Dunn's multiple comparisons test, $p < 0.05$).

Table 1 – Frequency of epithelial tumors observed in *D. melanogaster* (*Warts*) specimens exposed to water deriving from two points in Piraí River, Barra do Piraí County, Rio de Janeiro State, Brazil

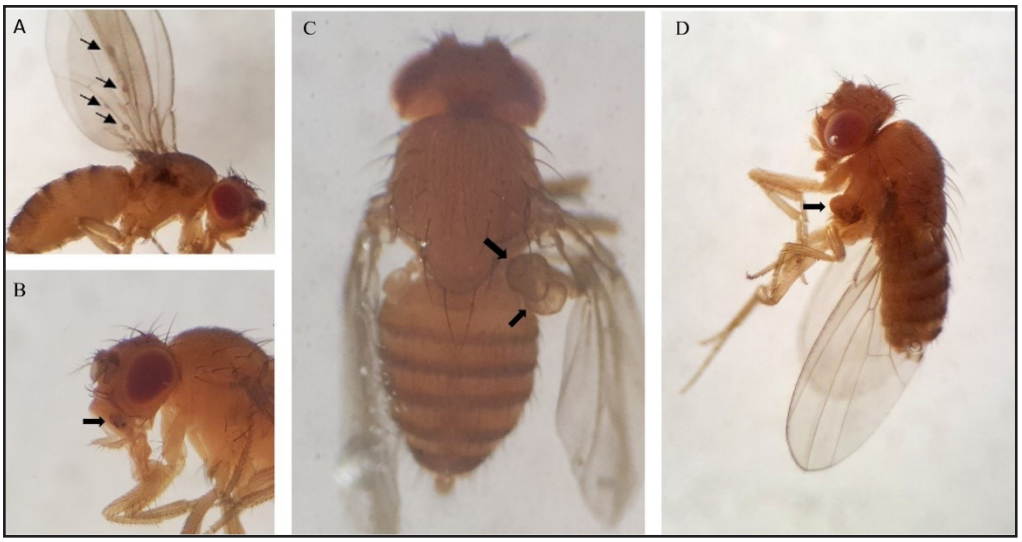
Treat	N flies	Frequency of observed tumors (total of tumors)						
		Eye	Head	Wing	Body (t + a)	Leg	Halter	Total
NC	200	0,01 (2)	0,01 (2)	0,105 (21)	0,125 (25)	0,01 (2)	0,01 (2)	0,265 (53)
Dox	200	0,04 (8)*	0,16 (32)*	1.31 (262)*	0,4 (80)*	0,235 (47)*	0,065 (13)*	2,21 (442)*
WP1	200	0,015 (3)	0,11 (22)*	0.07 (14)	0,345 (69)*	0,02 (4)	0,005 (1)	0,565 (113)*
WP2	200	0,025 (5)	0,085 (17)*	0,12 (24)	0,395 (79)*	0,04 (8)	0,005 (1)	0,665 (133)*

* Mann-Whitney Test conducted based on using $p \leq 0.05$ as significance level, by taking into consideration differences against negative control (distilled water). Treat - Treatment; NC – negative control; DOX - doxorubicin; WP1 – Water point 1; WP2 – Water point 2; N flies – Number of flies analyzed; Body (t + a) – Body (thorax + abdomen).
Source: Authors (2024)

As shown in Table 1, the number of tumors observed in each body part of specimens in the sampled points and in the controls was taken into account for statistical analysis purposes. The total number of epithelial changes observed in flies subjected to the treatment conducted with water samples collected in two different points in Piraí River, was significantly higher than that observed for negative control flies.

Epithelial changes herein observed in the assessed flies show tumors' size, number and body segment variations, as shown in Figure 4.

Figure 4 – Epithelial tumors found in *Drosophila melanogaster* (*Warts*) flies



Source: Authors' private collection – (A) tumors in the wing of an individual subjected to doxorubicin (positive control); (B) tumor in the mouthparts of a fly subjected to water sample deriving from collection point 1; (C) Major change in the right halter (individual subjected to water deriving from collection point 2); (D) individual exposed to water deriving from collection point 2, presenting a large tumor in its body.

Flies treated with doxorubicin recorded higher frequency of tumors in all analyzed regions. Doxorubicin is a widely investigated and used mutagenic and carcinogenic agent capable of affecting topoisomerase II, which is a DNA gyrase with intense activity in cell proliferation processes (Kaiserová et al., 2006). Specimens exposed to doxorubicin, at a concentration of 0.4mM, were expected to show frequency of tumors significantly different ($P \leq 0.05$) from that of the negative control (Babudri et al, 1984). This difference reinforces doxorubicin's potential use as positive control and validates results reported in the literature (Vasconcelos, 2016).

Flies subjected to treatment with water samples only recorded significant difference in the frequency of tumors in body parts, such as head and thorax + abdominal region, in comparison to the negative control. The thorax + abdominal region recorded the largest number of tumors, as also observed in other studies (Freitas et al., 2014; Bontempo and Orsolin, 2017; Rocha et al., 2018). According to Sidorov et al. (2001), the incidence of more tumors in this region is explained by their larger number of cells and longer cell proliferation period. However, the higher frequency of tumors in the head, thorax, and abdomen regions may also be explained by differences in proliferative activity and in the dynamics of gene expression, particularly involving the Hippo/Wts pathway. The Hippo pathway controls organ size by promoting cell growth and proliferation while inhibiting apoptosis (Irvine and Harvey, 2015). The *Warts* gene is one of the tumor suppressor genes involved in this pathway, and its loss leads to increased proliferation and reduced apoptosis (Tumaneng et al., 2013). Furthermore, different tissues may exhibit variations in the regulation of this pathway, since Hippo signaling is controlled by multiple upstream inputs, many of which depend on the local physical environment of the cells (Pan et al., 2018).

Based on a study previously performed by our research group (Sacramento et al., 2020), water cytogenotoxic potential was confirmed through *Allium cepa* assay applied to samples collected in both analyzed collection points. Meristematic cells of plant roots grown in water samples have shown larger number of nuclear changes, such as bud formation, as well as large and multiple nucleoli, among other anomalies. The herein observed effects on *D. melanogaster* mutant lineage *Warts* enabled inferring that, in addition to the previously detected genotoxic potential, the assessed water samples had sufficient amounts of mutagenic components to damage the investigated genome, given the higher incidence of tumors in the epithelium of individuals exposed to water. The detection of water genotoxicity using two bioindicators—one plant and one animal—reinforces the reliability of the obtained results and suggests that the mechanisms involved in mutation generation are conserved among eukaryotes.

Contamination of the hydrographic stretch between Paraíba do Sul and Guandu Rivers, which includes Piraí River, can be explained by the industrialization of its surrounding areas, which host companies that operate in different sectors, such as metallurgy, chemical products, pharmaceuticals and slaughterhouses. Waste deriving from these companies, as well as untreated urban sewage and agricultural chemicals, are indiscriminately released into waterways and dump contaminants in both water and sediments (Pfeiffer et al., 1986; Torres et al., 2002; CBH Guandu, 2015). The main constituents of these effluents include heavy metals (e.g., As, Br, Cd, Cr, Cu, Fe, Pb, Mn, Sb, Hg, Ni, Ag, Tl, U, and Zn), nitrogenous compounds such as ammonia, nitrates, and nitrites, organic solvents, as well as a wide range of other organic and inorganic substances. Several studies have demonstrated the cytotoxic, genotoxic, and mutagenic potential of these residues (Dutta and Singh, 2018; Bhat et al., 2019; Sabeen et al., 2020; Jayawardena et al., 2021). Furthermore, features presented by the two locations in Piraí River, where water samples were collected from, may have influenced the mutagenic effects caused by the collected samples.

The first collection point was located after Ribeirão das Lajes dam, in a section where water arrives at high volume and where the river bends and splits, as shown in Figure 1 (C). Elznicová et al. (2019) have investigated pollution hotspots along a stretch of Ploučnice River, Czech Republic, where the point accounting for the highest concentration of deposits resembled collection point 1 stretch in Piraí River. The aforementioned authors assessed the concentration of more than 30 elements, among them pollutants, such as barium, nickel, lead and uranium. Lithogenic elements like iron, silicon, aluminum and zirconium, whose natural origin is associated with soil and rocks, were also assessed. High concentrations of these elements along the river were associated by these authors with mining activity performed on its banks, which is an issue also observed along Paraíba do Sul-Guandu hydrographic stretch.

A considerable number of small boats, canoes and sterndrives were anchored at the location where Point 2 water samples were collected in. Sterndrives are small

boats whose engine is positioned in the hull, almost always at the end of the boat. According to Asplund (2000), the problem with these equipment types lies on the fact that they can add metals and chemical products to the water column. A certain amount of fuel entering the engine is discharged without burning and released into the water. Two-stroke engines can emit from 25% to 30% of their unburned gas and oil mix into the water. These pollutants affect water pH and its dissolved oxygen level, and it can affect the ecosystem of rivers or lakes. Polycyclic Aromatic Hydrocarbons (PAHs), which belong to a class of contaminants that slowly degrade in the environment and that harm human health, among other organisms, stand out among pollutants released by the incomplete burning of fuel and combustion residues (IARC, 2010). PAHs have mutagenic, carcinogenic and teratogenic potential, besides harming human health. These contaminants cause cytogenotoxic damage in humans by damaging the main cell macromolecules, such as lipids, proteins and nucleic acids (da Silva Junior et al., 2021).

It is important to highlight that the tumors observed in the analyses performed do not result from the activity of a single component, but rather from a complex mixture of all components present in the water samples collected at the indicated sites, including possible synergistic interactions among them.

4 CONCLUSIONS

The assay employed to detect epithelial tumors in *Drosophila melanogaster* specimens of the mutant *Warts* lineage proved effective in identifying agents with mutagenic characteristics in water samples collected at two distinct points along the Piraí River (Piraí Municipality, Rio de Janeiro State, Brazil). This finding reinforces its effectiveness as a bioindicator in water sample analysis, as it is a rapid, low-cost method, sensitive to different classes of agents, with tumors that can be easily detected using a stereoscopic microscope. Furthermore, the evolutionary conservation between the

warts gene and the human *LATS1* gene supports the use of this assay in detecting compounds with carcinogenic effects.

The results obtained corroborate previous studies and confirm the need to investigate strategies to reduce the impacts generated by industrial activities and the discharge of untreated sewage, among other practices that contribute to pollution in the Paraíba do Sul–Guandu hydrographic stretch. Firstly, the presence of compounds with genotoxic and mutagenic potential may affect the development of organisms at different trophic levels, thereby compromising the food chain. Secondly, such residues, including organic components of industrial and pharmaceutical origin, as well as heavy metals, may not be fully removed during water treatment processes, posing risks to human health.

Overall, this study underscores the need to expand basic sanitation services and strengthen the regulation of industrial effluent discharges, as well as emphasizing the importance of using this model for the regular monitoring of water bodies, particularly those that contribute to public water supply.

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