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The impact of acute exposure to the glyphosate-based herbicide Templo® on yellowtail tetra fish *Astyanax lacustris*, both before and after treatment through a vertical flow constructed wetland system

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Numerous studies have investigated the genotoxicity of agricultural pesticides and potential alternatives for the treatment of contaminated wastewater. This particular study aimed to evaluate the cyto/genotoxic effects of the glyphosate-based commercial herbicide Templo® on the fish species *Astyanax lacustris*, both before and after treatment with a vertical flow constructed wetland system (VFCW). The micronucleus test (MN), cellular morphological changes (CMC), and comet assay were utilized to assess the herbicide's effects. The *A. lacustris* specimens were exposed to a concentration of 20 µg/L (control group without phytoremediation treatment = GWTP), while another group underwent treatment through phytoremediation (treated group by phytoremediation = TGP) for a duration of 96 hours at the same concentration. The results demonstrated that the herbicide induced MN formation, DNA damage, and various types of CMC in all tested concentrations of *A. lacustris*. Notably, all group analyses yielded significant results ($p < 0.05$). The VFCW system effectively bioremediated the herbicide, achieving a 95% removal rate of the 20 µg/L glyphosate concentration, as confirmed by liquid chromatography-mass spectrometry (UPLC-MS/MS). Therefore, the herbicide Templo® presents a potential risk for genotoxicity and cytotoxicity in aquatic organisms, while the VFCW system has proven to be efficient in treating this herbicide.

Keywords: Aquatic bioindicators, Environmental biomonitoring, Sustainable biotechnology, Pesticide, UPLC-MS/MS.

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Numerosos estudos têm investigado a genotoxicidade de pesticidas agrícolas e potenciais alternativas para o tratamento de águas residuais contaminadas. Este estudo em particular teve como objetivo avaliar os efeitos cito/genotóxicos do herbicida comercial à base de glifosato Templo® na espécie de peixe *Astyanax lacustris*, antes e depois do tratamento com um sistema de wetland construído de fluxo vertical (WCFV). O teste do micronúcleo (MN), as alterações morfológicas celulares (AMC) e o ensaio cometa foram utilizados para avaliar os efeitos do herbicida. Os espécimes de *A. lacustris* foram expostos a uma concentração de 20 µg/L (grupo controle sem tratamento de fitorremediação = GWTP), enquanto outro grupo foi submetido ao tratamento por meio de fitorremediação (grupo tratado por fitorremediação = TGP) por uma duração de 96 horas na mesma concentração. Os resultados demonstraram que o herbicida induziu a formação de MN, danos ao DNA e vários tipos de AMC em todas as concentrações testadas de *A. lacustris*. Notavelmente, todas as análises de grupo produziram resultados significativos ($p < 0,05$). O sistema WCVF biorremediou efetivamente o herbicida, alcançando uma taxa de remoção de 95% da concentração de glifosato de 20 µg/L, conforme confirmado por cromatografia líquida-espectrometria de massas (UPLC-MS/MS). Portanto, o herbicida Templo® apresenta um risco potencial de genotoxicidade e citotoxicidade em organismos aquáticos, enquanto o sistema WCVF provou ser eficiente no tratamento deste herbicida.

Palavras-chave: Bioindicadores aquáticos, Biomonitoramento ambiental, Biotecnologia sustentável, Pesticidas, UPLC-MS/MS.

INTRODUCTION

Brazil, recognized as a global leader in pesticide usage, currently contends with the application of over 1,500 registered commercial agrochemical products (Caldas *et al*., 2019). Although these chemicals play a vital role in safeguarding crops and enhancing food production, their extensive application has resulted in significant and widespread environmental consequences. The runoff and leaching from agricultural fields have led to the introduction of these agrochemicals into various ecosystems, including aquatic environments (Ezeoyili *et al*., 2019).

The repercussions of agrochemical usage extend far beyond the immediate effects, as these chemicals persist in the soil and air due to their bioaccumulation properties and toxicity (Zhou *et al*., 2025). The impact is not confined to target organisms; it also affects non-target species, including aquatic life, plants, mammals, and soil microorganisms (Punniyakotti *et al*., 2024). The need mainly presses this concern for a constant increase in global agricultural production (Hemathilake, Gunathilake, 2022). A study conducted using data from a French fertility clinic revealed that over 55% of sperm samples showed elevated levels of glyphosate in humans. Additionally, this study found evidence of effects on DNA and a correlation between glyphosate concentrations and oxidative stress in seminal plasma, which is a critical factor influencing male fertility (Vasseur *et al*., 2024).

Numerous studies have explored the genotoxicity of agricultural pesticides. The micronucleus test and comet assay have demonstrated their effectiveness as valuable tools for monitoring fish populations (Grisolia *et al*., 2009). These assays exhibit high sensitivity to changes in aquatic habitats and play a crucial role in assessing the potential risks associated with pollution from new chemical substances in water (Tincani *et al*., 2019). Utilizing fish as bioindicators, along with the micronucleus test and comet assay as markers, facilitates the early detection of environmental damage and its subsequent effects on individual organisms, populations, and community health (Flores-Galván *et al*., 2020). The South American fish species *Astyanax lacustris* (Lütken, 1875), commonly known as lambari, is widespread throughout the upper Paraná River basin (Dagosta *et al*., 2024) and is regarded as an effective bioindicator due to its sensitivity to various chemical contaminants found in polluted waters.

In light of the current high levels of water contamination, the demand for effective solutions has become increasingly urgent. Within this context, Constructed Wetlands (CWs) technology stands out as a promising alternative. Designed to replicate the natural processes found in wetlands, CWs provide a comprehensive and viable method for treating contaminated wastewater. They utilize a combination of physical, chemical, and biological processes to effectively remove pollutants, thereby addressing the contemporary need for pre-treatment of effluents prior to their discharge into water bodies (Hassan *et al*., 2021; Kiflay *et al*., 2021).

The CWs are essentially shallow artificial ponds or channels that feature a filter bed and aquatic vegetation to facilitate the removal of various contaminants from effluent (Dotro *et al*., 2017). This sustainable and eco-friendly biotechnology not only proves effective in remediating waters with diverse characteristics but also presents a practical and cost-efficient solution. The benefits of CWs include lower implementation and operational costs, simplified management, and effectiveness in eliminating pollutants such as total suspended solids (TSS), organic load (BOD and COD), and soluble nutrients (Sezerino *et al*., 2018).

Glyphosate-based herbicides are extensively utilized in agriculture, yet they raise significant environmental concerns due to their potential to contaminate water bodies and negatively impact aquatic life. This study aimed to evaluate the health effects of this herbicide on *A. lacustris* both before and after treatment using a vertical flow constructed wetlands (VFCW) system. We employed biomarkers such as the micronucleus test, cellular morphological changes, and the comet assay on erythrocytes. Furthermore, liquid chromatography-mass spectrometry (UPLC-MS/MS) was utilized to assess the effectiveness of the VFCW treatment.

MATERIAL AND METHODS

Pesticide. We utilized a glyphosate-based herbicide, specifically the original Templo® commercial formulation, which is a non-selective systemic herbicide. Its composition comprises: Potassium N-[(hydroxyphosphinato)methyl]glycine (Glyphosate – Potassium Salt - 396.50 g/L; 39.65% m/v), Isopropylammonium N-(phosphonomethyl) glycinate (Glyphosate – Isopropylamine Salt - 295.10 g/L; 29.51% m/v), the acid equivalent of N-(phosphonomethyl)glycine (Glyphosate - 540.00 g/L; 54% m/v),

Monoethylene glycol (13.15 g/L; 1.31% m/v), and other ingredients (584.25 g/L; 58.43% m/v). The herbicide Templo® (Ourofino Agrociência, Uberaba/MG, Brazil, 34118) was procured commercially in the city of Maringá, State of Paraná, Brazil. The following concentrations were tested: 20 µg/L of glyphosate (from glyphosate potassium salt, glyphosate isopropylamine salt, and glyphosate) and 20 µg/L of glyphosate that underwent treatment through the VFCW system utilizing phytoremediation. These concentrations are representative of those typically found in freshwater environments (Disner *et al*., 2011; Annett *et al*., 2014) and fall within the limits established by Brazilian regulations, which permit up to 65 µg/L of glyphosate in Class I water bodies (CONAMA, 2024). Such levels are deemed safe for human consumption (after basic treatment), recreational activities, agricultural irrigation, and the protection of aquatic ecosystems.

Construction of experimental built wetland treatment units with the vertical flow (VFCW). The construction of the VFCW units was guided by recommendations from the manual by Sezerino *et al*. (2018). In this study, two cylindrical high-density polyethylene (HDPE) containers were utilized, each measuring 0.80 m in height and 0.55 m in diameter. The filter composition for the first stage included 0.2 m of crushed stone (25–50 mm), 0.15 m of crushed stone (19–25 mm), and 0.45 m of crushed stone (7–9.5 mm) or gravel. The second stage comprised 0.2 m of gravel (19–25 mm), 0.15 m of gravel (7–9.5 mm), and 0.45 m of sand (1.2–2 mm). The adduction system was made of polyvinyl chloride (PVC) pipes with a diameter of 25 mm, along with necessary connections. Effluent was drained through an adductor system featuring perforations of 8.0 mm in diameter, evenly distributed along its entirety. The drainage pipe was positioned horizontally at the base of the bed, spanning the full diameter of the units. Additionally, the system includes a faucet for dispensing the treated effluent, which is located at the bottom of the reservoir, positioned 10 cm from the bottom (Fig. 1).

The beds were established with *Typha domingensis* at a density of 16 plants per square meter. The propagules were manually collected from a naturally flooded area in a rural setting. Care was taken during the collection process to preserve the rhizomes, which were then carefully transported and transplanted into the experimental units. The residence time of the effluent within the treatment system was 96 h, consisting of 72 h in the first stage and 12 h in the second stage.

Chemicals and reagents. The analytical standard of glyphosate (≥ 99.0%) and HPLC-grade formic acid were sourced from Sigma-Aldrich (Saint Louis, MO, USA). HPLC-grade acetonitrile was procured from JT Baker (San Pedro Xalostoc, State of Mexico, Mexico), while ultrapure water was produced using a Milli-Q® water purification system (Millipore, Billerica, MA, USA). A glyphosate stock solution at a concentration of 1000 µg/L was prepared in water and stored in a light-protected environment at -18°C. This stock solution was then diluted with ultrapure water to yield a 20 µg/L glyphosate solution. Further dilutions of the stock solution produced five aqueous working solutions with concentrations of 2.5, 5.0, 10.0, 50.0, and 100.0 µg/L, which were utilized for preparing the calibration curve. Additionally, a 20 µg/L glyphosate solution was created to assess the efficiency of treatment within the vertical flow constructed wetland.

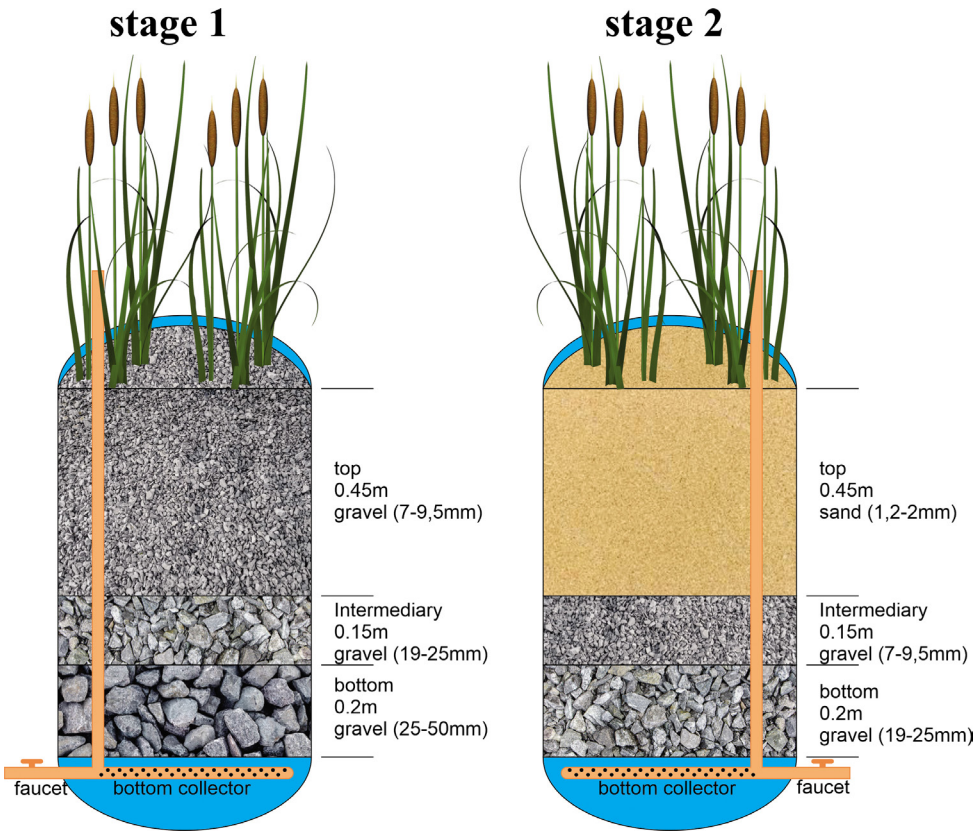

FIGURE 1 | Scheme of an experimental VFCW unit.

UPLC-MS/MS. Chromatographic analysis of glyphosate was performed using an ACQUITY UPLC H-Class system coupled with a triple quadrupole mass spectrometer Xevo-TQD equipped with a Z-spray™ electrospray ionization source (Waters, Milford, MA, USA). Samples (2 µL) were injected onto the ACQUITY UPLC® BEH C18 reverse-phase column (2.1 × 50 mm, 1.7 µm) acquired from Waters (Milford, MA, USA). The solvent system consisted of 0.1% formic acid in water (90%, solvent A) and acetonitrile (10%, solvent B). Elution was performed in isocratic mode at a flow rate of 0.4 mL min^-1 with a total run time of 4 min (glyphosate retention time of 1.1 min) at 30 ± 1°C. Glyphosate determination was performed in negative electrospray ionization mode (ESI-) with MS conditions as follows: capillary voltage 2.5 kV, cone voltage 25 V, extractor voltage 3.0 V, source temperature 130°C, and desolvation temperature 400°C. Nitrogen was produced by a high-purity nitrogen generator (Peak Scientific®, model NM32LA, Renfrewshire, Scotland) and used as cone gas and desolvation gas with flow rates of 25 and 450 L h^-1, respectively. Argon (99.9%, White Martins, Rio de Janeiro, Brazil) was used as collision gas at a pressure of 3.00 × 10^-3 mbar for MS/MS, and the collision energy for monitored transitions (m/z 168.1 → 81 and m/z 168.1 → 150) was 12 eV. MassLynx™ 4.1 software (Milford, MA, USA) was used for instrument control, data acquisition, and processing.

Experimental design. Adult individuals (males and females) of *A. lacustris*, with a mean weight of 9±12 g and length of 8±12 cm, were obtained from a local breeding facility. The fish were acclimatized in aquaria with dechlorinated water at room temperature, constant aeration, a natural photoperiod (12:12 h light/dark cycle), and fed with specific small fish feed once daily (Basic Alcon® Fish Food, Camboriú/SC, Brazil) for 10 days in the Sectorial Vivarium for Fish Keeping and Experimentation at the Universidade Estadual de Maringá (UEM) in Maringá, Paraná State, Brazil. Voucher specimens were deposited in the Fish Collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, municipality of Maringá, Paraná State, Brazil, as *Astyanax lacustris* (NUP 25442).

Three groups/ conditions were tested, with four fish per group, and all experiments were performed in triplicate; in total, 36 fish were used in the study. The number of individuals per aquarium was established according to the body mass/water volume ratio, which should not exceed 0.5–2 g/L (CONCEA Guide, annex I, fish). The control group fish were maintained in dechlorinated water. The other two groups of fish were exposed to glyphosate (glyphosate potassium salt, glyphosate isopropylamine salt, and glyphosate) one being at a concentration of 20 µg/L (group without treatment by phytoremediation = GWTP) and the other, at the same concentration, but was treated through the VFCW system using phytoremediation (treated group by phytoremediation = TGP). In all cases, the total volume of the aquarium was 10 L. The sample concentration was not disclosed to prevent interference with the observer's bias in the final evaluation.

After four days of exposure, the fish were removed from the aquarium (one at a time), and their blood was collected to analyze micronucleus test, cellular morphological changes, scanning electron microscopy, and comet assay. Afterward, the fish were removed from the aquarium (one at a time) and bathed in an anesthetic solution: clove oil, 5 ml, diluted with ethyl alcohol, 20 ml, as per Inoue *et al.* (2005). Then, 1 ml of this solution was added per L of water. The animal was only manipulated after it failed to respond to physical stimulus, denoting death from anesthetic overdose (Svobodová, Vykusová, 1991). Afterward, they were fixed in commercial absolute alcohol, recorded with number and origin, and stored in glass vials from the UEM/NUPELIA laboratory collection as a control.

Scanning electron microscopy (SEM). For the analysis of blood samples using scanning electron microscopy, three individuals were randomly selected for each concentration of the herbicide Templo®. The blood samples were fixed in a solution with 2.5% glutaraldehyde and 0.1M PBS at pH 7.4 for 24 h, kept at 7°C. After this, the material went through a dehydration process with varying increasing concentrations of alcohol to dehydrate the blood cells (7.5%, 15%, 30%, 50%, 70%, 90% and 100%). Afterward, the blood samples were critically dried (critical point) using a Leica CPD030 dryer and coated with gold using a Shimadzu IC-50 dryer. Analyzes were performed using a Quanta 250-Fei scanning microscope at the Microscopy Center of the Research Support Complex (COMCAP) at the UEM, as described by Gigliolli *et al.* (2015).

The height of the sample concerning the detector was 10 mm, with an accelerating voltage of 15.00 KV and a spot size of 3.0. During image capture, the xT Microscope microscope interface program was used. The concentration of the sample was not

disclosed to prevent interference with the observer's bias in the final evaluation; it was not known what concentration that sample was in so that it would not interfere with the observer's bias in the final evaluation. Qualitative assessments of changes in blood samples were carried out, focusing on capturing images of cytoplasmic morphological changes.

Micronucleus test and cellular morphological changes in erythrocytes. The micronucleus (MN) and cellular morphological changes (CMC) in the erythrocytes test were performed based on the description by Hooftman, Raat (1982). After anesthesia of the animals, blood was extracted from the caudal vein with a heparinized syringe. A sample (approximately 10 µl) was dripped onto a sanitized glass slide, and the smear was performed with the aid of another slide. The dripped slide was kept at room temperature, drying for at least 12 h. Afterward, they were fixed with immersion in absolute ethanol for 20 min. Staining was performed for 10 min with 5% Giemsa solution diluted in phosphate buffer (pH 6.8). Then, the slides were washed in distilled water, left to dry naturally, and kept in closed boxes until analysis under microscopy. The slides were analyzed by optical microscopy under 1000 × magnification. The MN count and the study of CMC were performed on 2,000 red blood cells per fish.

Comet assay. The comet assay was conducted according to Speit, Hartmann (1999) with modifications according to Ferraro *et al*. (2004). A sample (approximately 10 µL) of blood was collected from the tail vein of each fish with a heparinized syringe and diluted in fetal calf serum, 1 mL. Slides were prepared for microscopy using this cell suspension, 10 µL, added to low-melting-point agarose at 37°C, 120 µL, followed by incubation in lysis solution (1 mL of Triton X-100, 10 mL of DMSO, and 89 mL of stock lysis solution consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 2.5 M NaOH and 1% N-lauryl sarcosinate). The slides were then kept in the dark for 1h at 8C.

After lysis, slides were placed in the electrophoresis tank, covered in buffer solution (0.3 N NaOH, 1 mM EDTA, pH > 13) in a cold environment at 8C for 20 min to unwind the DNA. Electrophoresis was performed for 20 min at 25 V, 300 mA, field strength 1V/cm; slides were neutralized (0.4 M Tris) for 10 min, fixed in absolute ethanol for 10 min, and then stained with ethidium bromide (2 µg/mL), and covered with coverslips for analysis, after drying for approximately 12 h. Analyses were performed blind to test conditions under an epifluorescence microscope, with image capture at 400× magnification, counting 100 nucleoids per fish. DNA damage was visually classified into four classes based on DNA migration: 0 (no apparent damage), 1 (slight damage), 2 (medium damage), 3 (large damage), and 4 (maximum damage).

Statistical analysis. Statistical analyses were performed separately using the Kolmogorov-Smirnov normality test. Micronucleus (MN) and cellular morphological changes (CMC) data were obtained by the One-way ANOVA test and followed by Tukey's post-test. For the Comet Assay test, data were received by the One-way ANOVA test and followed by the Bonferroni post-test. The significance level adopted was 5%, and the results were expressed as mean ± standard error. The graphics were executed in the GraphPad Prism 9 program.

RESULTS

A total of 39 MN and 3085 CMC were identified in the individuals tested, with those from the GWTP group displaying a greater number of abnormalities compared to the TGP group (Tab. 1). Nevertheless, when analyzing all combined alterations statistically, the TGP group still showed significant abnormalities in comparison to the negative control (Fig. 2A). Various alterations in the erythrocytes were documented, including cytoplasmic vacuoles (Figs. 3A, 4B, C), micronuclei (Fig. 3B), crenated erythrocytes (Figs. 3B, 4H), notches (Fig. 3B), nuclear fragmentation (Fig. 3B), tear-drop shapes (Figs. 3C, 4F), lobed forms (Fig. 3D), blebbing (Fig. 3D), macronuclei (Fig. 3E), immature erythrocytes (Figs. 3F, 4E), elliptocytes (Figs. 3F, 4D), anucleate cells (Fig. 3G), cytoplasmic constriction (Fig. 4G), and binucleate cells (Fig. 3H). The Comet Assay revealed statistically significant DNA damage in both the GWTP and TGP groups when compared to the control group (Tab. 2; Fig. 2B).

TABLE 1 | Absolute numbers for alteration in *Astyanax lacustris* erythrocytes. GWTP = Group without treatment by phytoremediation; TGP = Treated group by phytoremediation.

Alteration	Negative control	GWTP	TGP
Micronucleus	0	33	6
Notched	14	211	145
Binucleate	0	71	39
Macronucleus	4	92	55
Nuclear fragmentation	0	510	224
Elliptocity	4	308	249
Bebbled	9	109	49
Lobed	0	108	26
Cytoplasmic vacuole	0	543	311
Crenated erythrocyte	0	130	98
Tear drop shape	0	64	67
Immature erythrocyte	4	132	130
Anucleate	0	46	21

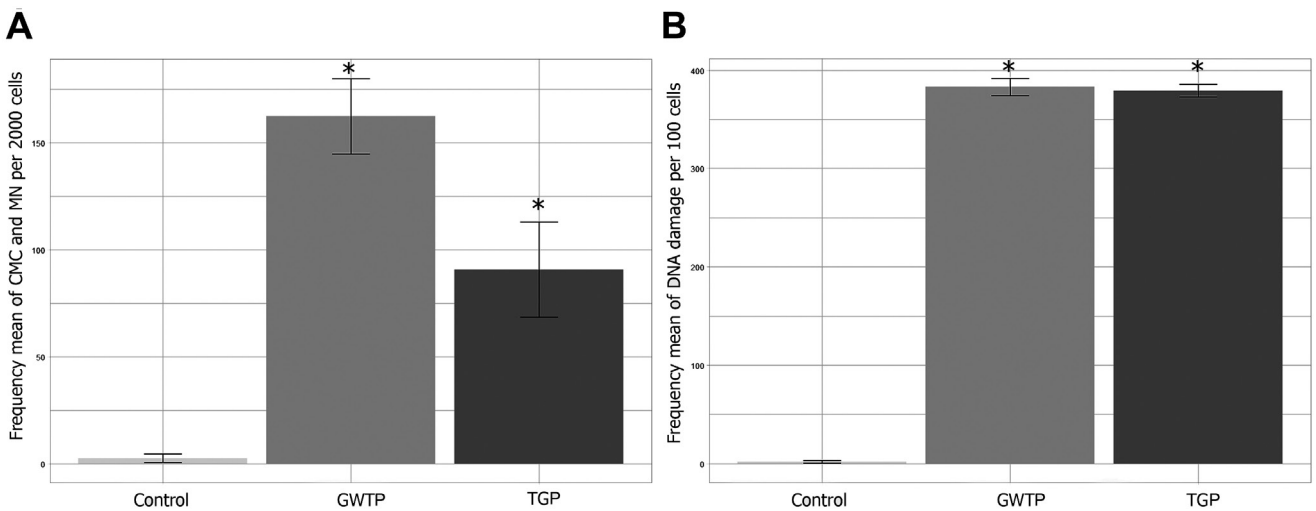

FIGURE 2 | Graphs with the averages of the changes found in the analysis of (**A**) cellular morphological changes (CMC) and micronuclei (MN); (**B**) comet assay. The bars show the standard deviation. In total, 36 fish were used in the analyses. *Significant statistical ($p < 0.05$) change when compared to the control group. GWTP = Group without treatment by phytoremediation; TGP = Treated group by phytoremediation.

TABLE 2 | DNA damage score of *Astyanax lacustris* erythrocytes in Comet Assay. GWTP = Group without treatment by phytoremediation; TGP = Treated group by phytoremediation.

Damage	Negative Control	GWTP	TGP
0	1178	0	0
1	20	0	1
2	2	32	36
3	0	192	175
4	0	989	988

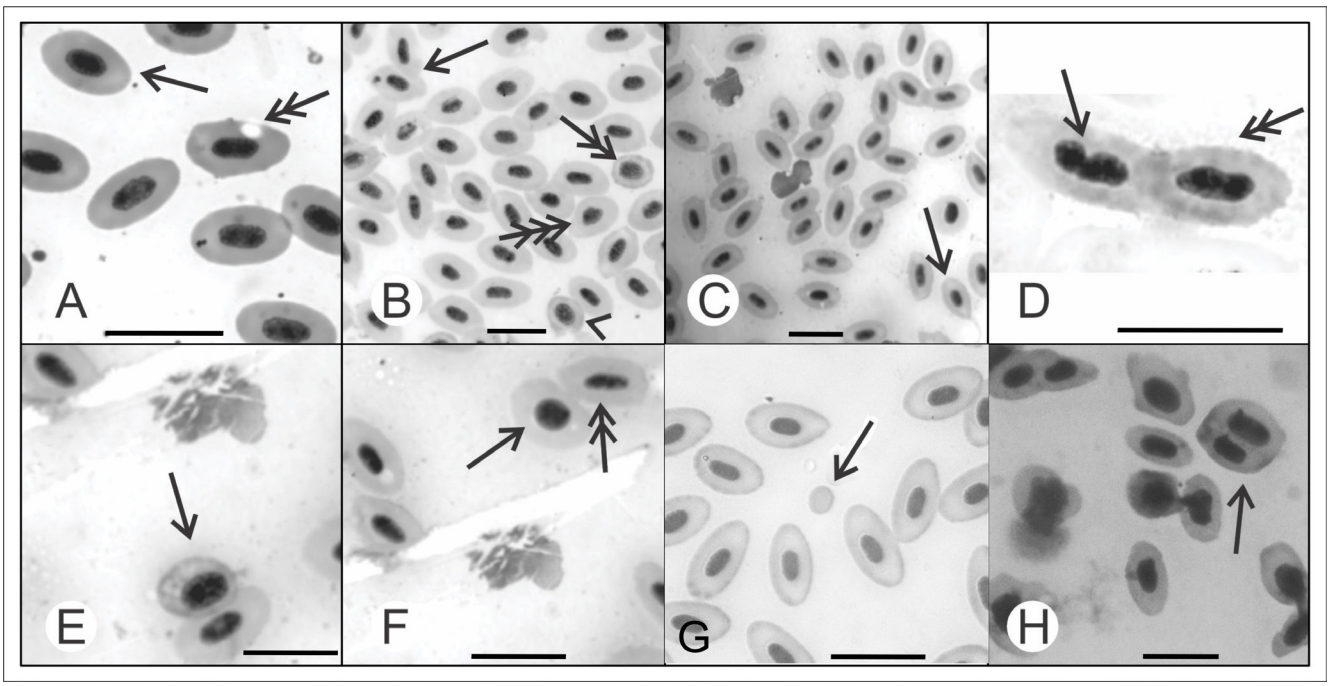

FIGURE 3 | Photomicrograph of *Astyanax lacustris* erythrocytes exposed to herbicide Templo® and stained with Giemsa. (**A**) Normal erythrocytes (single arrow) and cytoplasmic vacuole (double arrow), (**B**) Micronucleus (single arrow) and Crenated erythrocyte (double arrow), Notched (triple arrow) Nuclear fragmentation (arrowhead) (**C**) Tear drop-shaped erythrocyte (**D**) Lobed (single arrow), Blebbed (double arrow), (**E**) Macronucleus (single arrow), (**F**) Immature erythrocyte (single arrow) Elliptocyte (double arrow), (**G**) Anucleate, (**H**) Binucleate (single arrow). Magnification: 1000X. Scales bars = 5 µm.

Prior to initiating the water analysis, glyphosate fragmentation was performed to select the relevant fragments documented in the literature (Fig. 5). Following this, the water analysis was conducted using the UPLC-MS/MS technique, which yielded an R^2 value of 0.9926 for the glyphosate calibration curve, represented by the equation y = 7639.9x - 9111.7. This technique underscored the efficacy of the treatment conducted with the vertical flow constructed wetland; the glyphosate concentration in the solution was measured at 20 µg/L (Fig. 6A) and reduced to 1 µg/L after treatment (Fig. 6B).

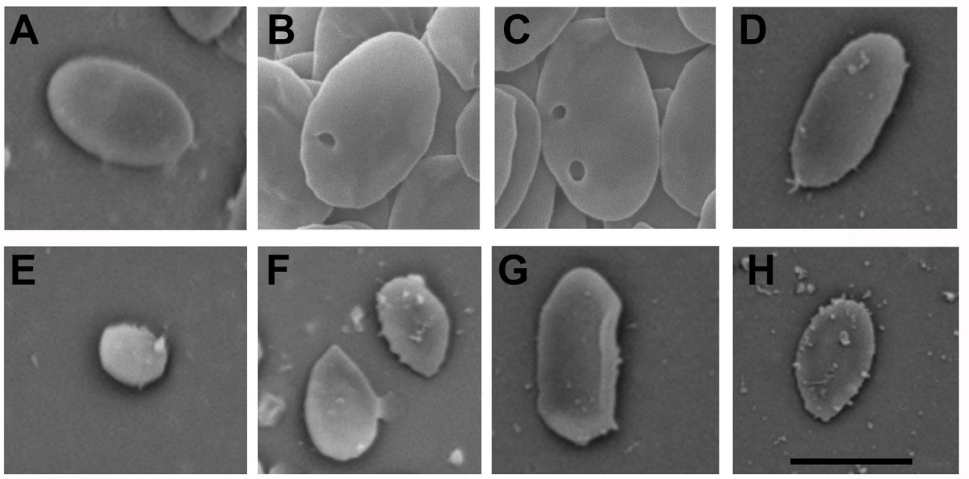

FIGURE 4 | Scanning Electron Microscopy of *Astyanax lacustris* erythrocytes exposed to herbicide Templo®. (**A**) Normal erythrocyte; (**B**) and (**C**) Cytoplasmic vacuole; (**D**) Elliptocyte; (**E**) Immature erythrocyte; (**F**) Tear-Drop; (**G**) Cytoplasmic constriction; (**H**) Crenate erythrocyte. Scales bar = 5 µm.

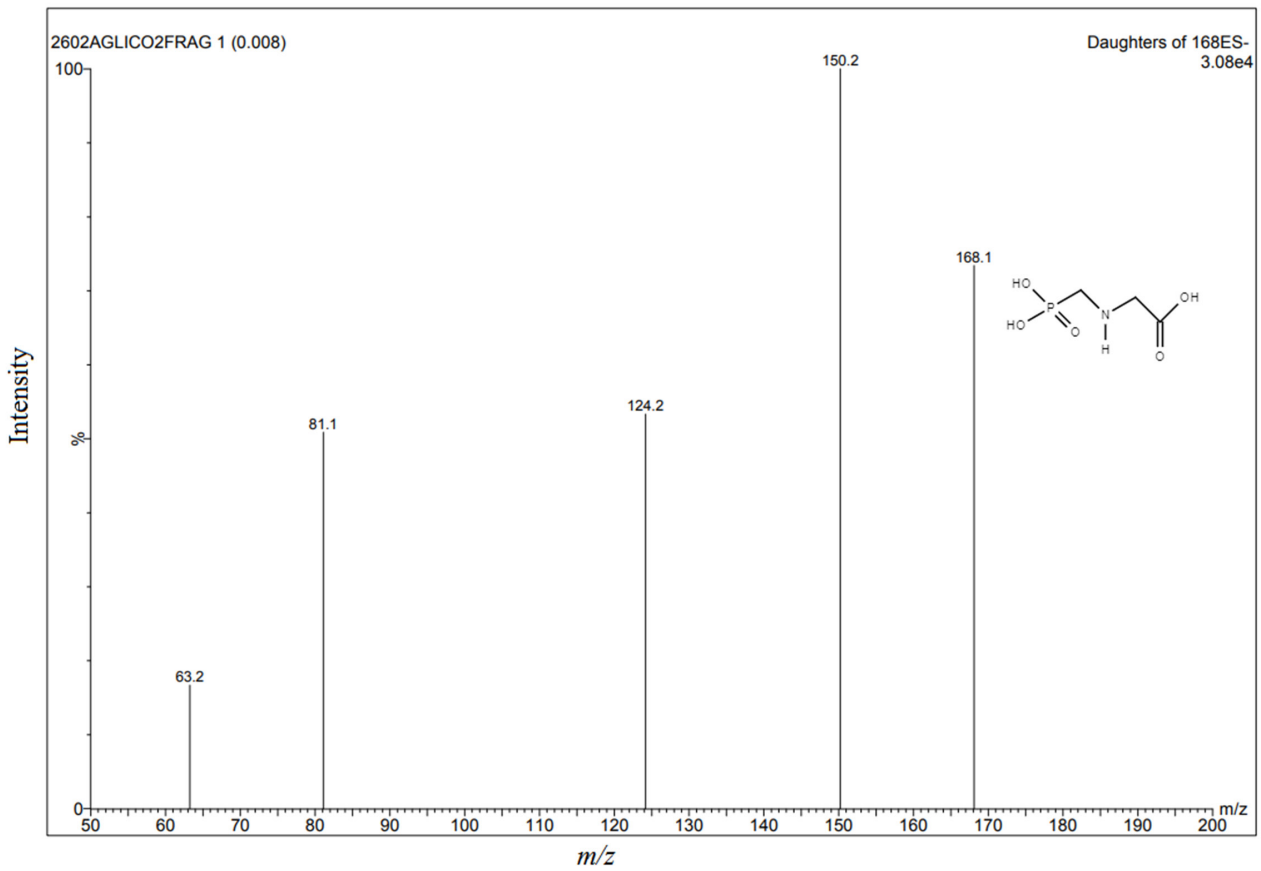

FIGURE 5 | Glyphosate fragmentation.

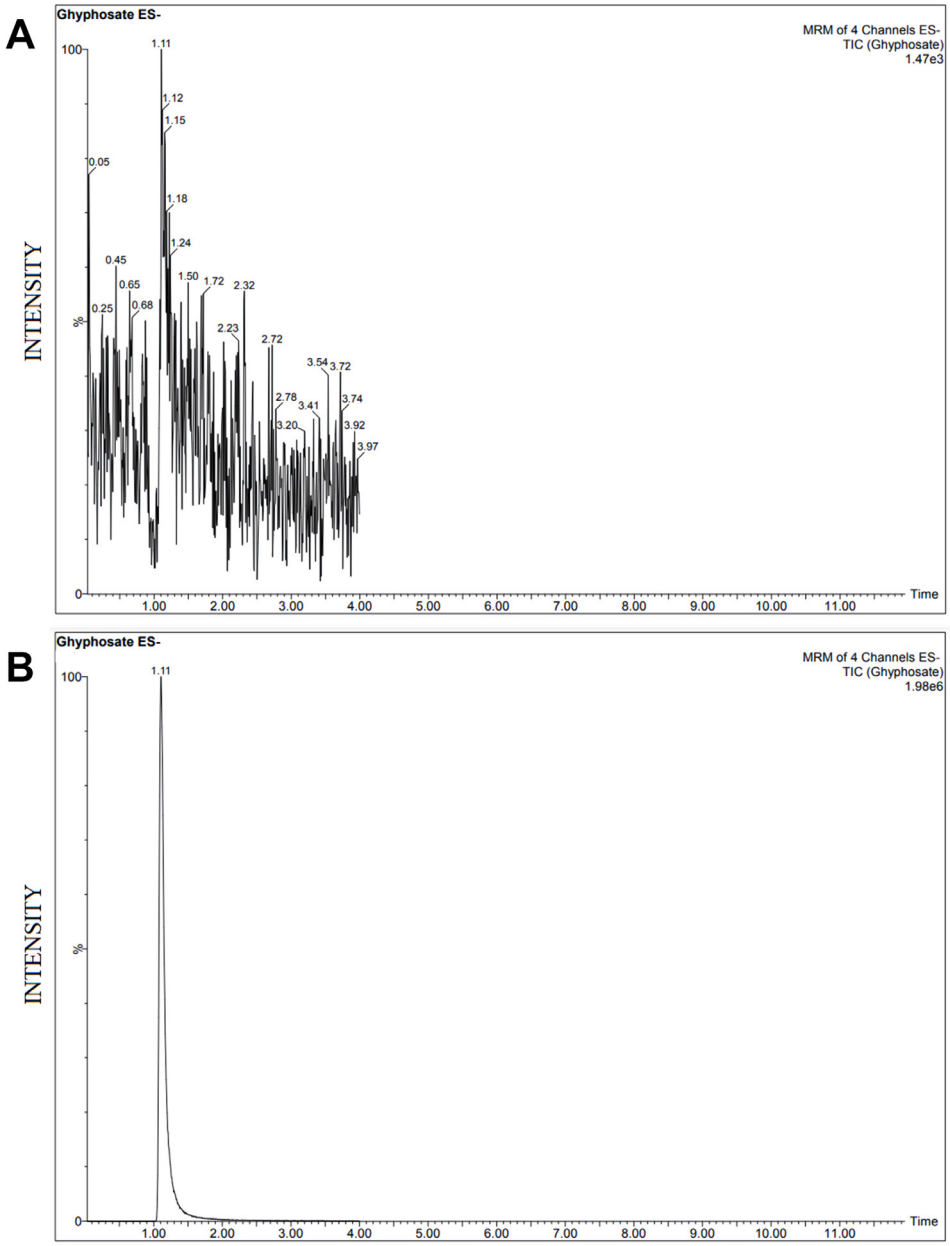

FIGURE 6 | Glyphosate retention time before (**A**) and after treatment by VCFW (**B**).

DISCUSSION

Acute exposure to the herbicide Templo® has produced significant findings, revealing statistically significant damage to the cells of *A. lacustris*. This damage encompasses the presence of micronuclei and various alterations in cell morphology. These results emphasize the cytotoxic and genotoxic potential of this contaminant, even at concentrations below the permissible limits (up to 65 µg/L) set by Brazilian legislation for class I freshwater environments (CONAMA, 2024).

Numerous fish species have been utilized in studies concerning glyphosate contamination, and they are recognized as effective bioindicators for this xenobiotic. In *Colossoma macropomum*, the glyphosate-based herbicide GLI-UP® was found to increase the frequency of DNA damage and the presence of micronucleated erythrocytes (Aranha, 2013). In *Prochilodus lineatus*, two studies confirmed the genotoxic potential of a formulated herbicide, which adversely affected DNA integrity in the fish. One study noted significant effects after just 6 h of exposure to a substantially higher concentration of 10 mg/L of the glyphosate-based herbicide Roundup® (Cavalcante *et al*., 2008), while another observed impacts over up to 96 h of exposure at concentrations of 1 and 5 mg/L of the glyphosate-based herbicide Roundup Transorb® (Moreno *et al*., 2014).

Amid the escalating concerns regarding the presence of xenobiotics in freshwater and treated water, researcher is presenting promising solutions. Various methods for removing glyphosate from freshwater and drinking water have been explored, each demonstrating potential effectiveness. Rúbio *et al*. (2016) utilized granular activated carbon derived from dendê coconut shells, which were impregnated with silver (Ag) and copper (Cu), achieving a 66.1% removal rate for a 20 mg/L glyphosate concentration. Similarly, Yamaguchi *et al*. (2019) investigated activated carbon loaded with manganese (Mn) and iron (Fe), which emerged as a highly effective adsorbent for glyphosate removal from aqueous solutions. In another study, Melo, Naval (2023) pulverized activated carbon and attained approximately 60% removal of glyphosate. Beyond activated carbon, alternative materials have also been employed for glyphosate elimination from water, including a boron-doped diamond anode (Lima *et al*., 2021) and selected plant species in artificial wetlands (López-Chávez *et al*., 2021).

An alternative method for water treatment is the use of wetlands, which has proven effective in addressing various types of wastewater. Studies have demonstrated their capability in treating textile effluents (Silva, 2023), handling glass industry discharges (Gholipour *et al*., 2020), removing pharmaceuticals (Ranieri *et al*., 2011; Ávila *et al*., 2014; Chen *et al*., 2016), and eliminating various nitrogen compounds from wastewater (Vymazal, 2005; Vymazal, Kröpfelová, 2011; Ayaz *et al*., 2012).

The bioremediation of the herbicide Templo® using the VFCW system has not only demonstrated remarkable efficiency but also proven to be reassuringly effective. UPLC-MS/MS analysis confirmed the VCFW system's capability to treat effluent contaminated with glyphosate, detecting only 1 µg/L of the herbicide in the effluent from TGP's VCFW system. The VFCW system successfully achieved a 95% removal rate of glyphosate from an initial concentration of 20 µg/L. Consequently, the VCFW system represents an ecologically sound and sustainable solution, effectively addressing water pollution caused by glyphosate.

The UPLC-MS/MS technique is a sensitive and precise method for detecting and quantifying a variety of substances. Previous studies have utilized this technique to reveal the concentrations of numerous xenobiotics, including agricultural chemicals such as atrazine, glyphosate, and 2,4-D (Luzzi *et al*., 2024), as well as pharmaceuticals like paracetamol, caffeine, omeprazole, and dexamethasone (Gaffney *et al*., 2014; Castro-Pastrana *et al*., 2021). Additionally, it has been employed to detect antibiotics in milk (Magon *et al*., 2018) and acrylamide in coffee (Galuch *et al*., 2019).

The MN and CMC tests were performed with the highest scientific rigor to ensure the accuracy and validity of the results. The findings revealed that the TGP individuals

displayed statistically significant cyto/genotoxic responses in comparison to the negative control. Of particular concern is that even a minimal concentration of 1 µg/L of glyphosate (TGP) could trigger these responses, thereby posing potential health risks to those tested.

The comparable DNA damage and nuclear alterations observed between the groups can be attributed to the high sensitivity of the bioindicator and biomarkers employed. The fish species *A. lacustris* serves as a highly responsive bioindicator, capable of detecting damage even at minimal residual concentrations. Consequently, the positive findings in the remediated group indicate the persistence of trace elements, yet these do not undermine the overall effectiveness of the remediation method. The high sensitivity of both the comet assay and micronucleus test has been corroborated in various studies, which demonstrate that even low concentrations of contaminants can elicit genotoxic responses (Mitchelmore, Chipman, 1998; Bücker *et al*., 2006; Frenzilli *et al*., 2009), often exhibiting dose-dependent damage (Zhang *et al*., 2012). Considering that glyphosate can cause cumulative or prolonged effects that were not entirely neutralized during the remediation period in the present study, further studies could be conducted to propose a longer treatment period for this contaminant to better mitigate these effects.

In light of the troubling findings, it is crucial for environmental protection agencies to meticulously reassess the regulations governing glyphosate-based herbicides in freshwater ecosystems. The current allowable concentration of 65 µg/L significantly exceeds the level of 1 µg/L, which has been shown to cause detrimental effects on *A. lacustris*. Therefore, a more stringent regulatory approach is essential to safeguard both public health and the environment.

Thus, the research has shown that the herbicide Templo® has harmful effects on cellular, nuclear, and DNA levels, posing potential risks of genotoxicity and cytotoxicity in aquatic organisms. However, the study also underscored the effectiveness of the VFCW system in bioremediation, demonstrating its capability to treat the herbicide efficiently. Consequently, the VFCW system emerges as a viable alternative for the purification of contaminated wastewater across various applications. Furthermore, the current allowable concentration of 65 µg/L significantly surpasses the threshold of 1 µg/L, which has been found to have detrimental effects on *A. lacustris*.

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The Ethics Committee approved all procedures for using Animals in Research (CEUA - UEM), license number 131410181022.

COMPETING INTERESTS

The authors declare no competing interests.

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