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Ecotoxicity of the Commercial Herbicide Gramocil (Paraquat and Diuron) in Fungal, Invertebrate, Amphibian and Human Cell Models

Efeitos Ecotoxicológicos do Gramocil® (Paraquat e Diuron) em Fungos, Invertebrados, Anfíbios e Modelo Celular Humano

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Abstract

Gramocil® is a commercial herbicide formulation composed of paraquat and diuron. Although banned in several countries, including Brazil since 2020, its environmental persistence remains a matter of concern. This study evaluated the acute toxicity of Gramocil® in four biological systems: *Aspergillus nidulans* (germination and growth), the human hepatic cell line HuH7.5 (viability), *Lithobates catesbeianus* (survival), and *Eisenia fetida* (avoidance behavior). Organisms were exposed to nominal concentrations of 0.01% (~100 mg/L), 0.025% (~250 mg/L), and 0.05% (~500 mg/L). In *A. nidulans*, a significant delay in imbibition was observed at 0.01% and 0.025%, with 94.6% and 99.8% dormant conidia after 2 h, respectively, compared with 84.8% in the control. The 0.05% concentration arrested colony growth and caused lethality over 168 h. In HuH7.5 cells, marked viability loss occurred at all concentrations and exposure times, with values ranging from 20.5% to 25.0% after 24 h, 21.0% to 25.0% after 48 h, and 19.5% to 20.6% after 72 h relative to the negative control. In tadpole assays, 100% mortality occurred within 48 h at 0.025% and 0.05%, and within 72 h at 0.01%, precluding erythrocyte micronucleus analysis. In the terrestrial avoidance test, only 0.05% induced significant avoidance (80%), whereas 0.01% and 0.025% induced 20% and 40% avoidance, respectively. Gramocil® demonstrated high acute multi-model toxicity, indicating environmental and potential human-health risks under the high-exposure scenarios evaluated.

Keywords: Ecotoxicology. Bioindicators. Paraquat. Diuron. *Eisenia fetida*.

Resumo

Gramocil® Gramoci é uma formulação comercial de herbicida composta por paraquate e diuron. Embora proibida em vários países, incluindo o Brasil desde 2020, sua persistência ambiental permanece motivo de preocupação. Este estudo avaliou a toxicidade aguda de Gramocil® em quatro sistemas biológicos: *Aspergillus nidulans* (germinação e crescimento), linhagem celular hepática

humana HuH7.5 (viabilidade), *Lithobates catesbeianus* (sobrevivência) e *Eisenia fetida* (fuga). Os organismos foram expostos a concentrações nominais de 0,01% (~100 mg/L), 0,025% (~250 mg/L) e 0,05% (~500 mg/L). Em *A. nidulans*, observou-se atraso significativo na embebição a 0,01% e 0,025%, com 94,6% e 99,8% de conídios dormentes após 2 h, respectivamente, versus 84,8% no controle. A concentração de 0,05% interrompeu o crescimento das colônias e causou letalidade ao longo de 168 h. Nas células HuH7.5, houve perda acentuada de viabilidade em todas as concentrações e tempos, com valores entre 20,5% e 25,0% após 24 h, 21,0% e 25,0% após 48 h e 19,5% e 20,6% após 72 h, em relação ao controle negativo. Nos ensaios com girinos, ocorreu 100% de mortalidade em até 48 h nas concentrações de 0,025% e 0,05%, e em 72 h a 0,01%, inviabilizando a análise de micronúcleos em eritrócitos. No teste terrestre de fuga, somente 0,05% apresentou evitação significativa (80%), enquanto 0,01% e 0,025% induziram 20% e 40% de evitação. Gramocil® demonstrou elevada toxicidade aguda multimodelo, indicando riscos ambientais e potenciais riscos à saúde humana sob os cenários de alta exposição avaliados.

Palavras-chave: Ecotoxicologia. Bioindicadores. Paraquate. Diuron. *Eisenia fetida*.

1 Introduction

The contribution of agriculture to Brazil's GDP is significant, accounting for approximately 24% (CEPEA, 2023). Agricultural productivity has expanded primarily due to technological progress in machinery, fertilizers, and the use of pesticides, defined as chemical or biological products applied to ward off, eliminate, control pests, or regulate plant growth (Brasil, 1989). Among these, herbicides are particularly concerning due to their widespread use in contemporary agricultural practices and potential for environmental contamination and human exposure (Nogueira *et al.,* 2020; Singh *et al.,* 2020).

However, only about 0.1% of applied herbicides reach target organisms, while more than 95% affect non-target organisms (Kaur *et al.,* 2023; Meena *et al.,* 2020). This imbalance occurs because herbicides penetrate the soil during application on crops and are transported by runoff, eventually reaching water bodies and becoming potential pollutants (Lopes; Albuquerque, 2018). In addition, soil quality is fundamental to the sustainability of the planet, as it also affects other natural resources (Lucas *et al.,* 2025).

The effects of the widespread application of herbicides in agriculture have serious repercussions for both the environment and human health. These impacts are closely linked to the loss of biological diversity, causing damage to wildlife and vegetation, as well as contributing to soil, air, and water pollution. Thus, it is crucial to assess potential effects on non-target organisms and the environment. Future research should prioritize improving methods to assess the environmental risks of herbicides, which involves assessing their impacts on soil health, water quality, beneficial organisms, and ecosystems (Parven *et al.,* 2025).

Gramocil® is a commercial herbicide containing the active ingredients diuron and paraquat. It is used to control broad-leaved weeds and annual grasses in various crops such as citrus, banana, cotton, coffee and sugarcane as well as no-till areas of soy, cotton, corn and wheat. However, it is important to note that herbicides containing paraquat have been banned in Europe since 2007 due to their extreme toxicity to human health (Pizzutti *et al.,* 2016), and this prohibition has been extended to Brazil since September 2020 (Brasil, 2020).

While the individual toxicities of technical-grade paraquat and diuron have been investigated, studies assessing the whole commercial formulation Gramocil® remain limited. Formulations contain surfactants and adjuvants that can facilitate tissue penetration and amplify toxicity relative to analytical standards. In this context, this study aimed to evaluate the acute toxicity of the commercial herbicide formulation Gramocil® across four distinct experimental models representing different environmental compartments and a human cell line: the soil-colonizing filamentous fungus *Aspergillus nidulans*, bullfrog tadpoles (*Lithobates catesbeianus*), the terrestrial earthworm *Eisenia fetida*, and the human hepatoma cell line HuH7.5.

The fungus *Aspergillus nidulans* was chosen as a eukaryotic microbial model of decomposers, possessing well-characterized genetics and synchronized conidial germination kinetics (d'Enfert, 1997). Tadpoles of *Lithobates catesbeianus* were selected as freshwater aquatic bioindicators due to their permeable cutaneous membrane and vulnerability at the water-sediment interface (Rios *et al.,* 2017). The earthworm *Eisenia fetida* is a standardized terrestrial bioindicator highly sensitive to xenobiotics in soil matrices (ABNT, 2011; Filipi *et al.,* 2023; Teles *et al.,* 2024). Finally, human hepatoma cells (HuH7.5) were used as an *in vitro* model for xenobiotic cytotoxic screening, since hepatic tissue represents a primary site of chemical bioactivation and clearance (Conte *et al.,* 2022).

2 Material and Methods

2.1 Herbicide solution preparation

The agrochemical Gramocil® was prepared at concentrations of 0.01%, 0.025%, and 0.05% in sterile distilled water. The resulting suspensions were filtered through 0.22 µm Millipore filters and stored in amber bottles until use. The selected concentrations corresponded to approximately 100, 250, and 500 mg/L, respectively. These concentrations were selected based on preliminary dose-range tests and ecotoxicological studies involving paraquat or diuron. Although herbicide concentrations in surface waters are generally reported in the low microgram-per-liter range, higher short-term concentrations may occur locally after accidental spills, tank-washing discharges, or direct runoff from treated areas. Therefore, the tested range was intended to investigate the effects of acute exposure to Gramocil® under high-exposure scenarios. Environmental monitoring and runoff studies have reported diuron concentrations of up to 2,849 µg/L (2.849 mg/L) in surface-water runoff after

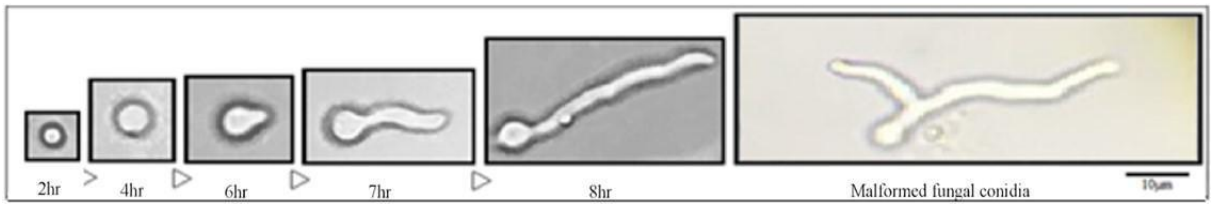
natural rainfall events. Although this concentration is substantially lower than those tested in the present study, it supports the potential for off-site transport of diuron from treated areas to adjacent aquatic environments (USEPA, 2003). Accordingly, the nominal concentrations tested in the present study (100, 250, and 500 mg/L) were approximately 35-, 88-, and 175-fold higher, respectively, than this reported maximum environmental concentration. Therefore, these concentrations should not be interpreted as representative of typical ambient surface-water levels. Rather, they were selected as a high-dose screening range to characterize acute hazards associated with the commercial formulation under accidental-release or point-source exposure scenarios. This design prioritizes the identification of acute toxic potential across multiple biological models instead of estimating toxicity at environmentally realistic exposure levels.

2.2 Toxicity assays with *A. nidulans*

We used the biA1methG1 haploid strain of *A. nidulans*, provided by Professor Carmen Lucia M. S. C. Rocha of the State University of Maringá-UEM. Liquid complete medium and solid complete medium were used and prepared according to Pontecorvo *et al.* (1953).

For germination tests, conidia from five-day-old colonies grown in solid complete medium at 37 °C were collected from colonies and suspended in 0.01% (v/v) of Tween 80. Conidia suspensions were filtered through glass wool, counted in a Neubauer chamber and inoculated into either liquid complete medium (control) or exposed to Gramocil® treatments (0.01%, 0.025%, 0.05%). For incubation, 100µL of each suspension was transferred to moist microscopy chamber slides and incubated at 37 ºC for up to 8 hours. Every 2 hours, germination in three slides of each condition was assessed using an optical microscope. At each reading time, approximately 300 conidia were counted and the percentages were calculated for each of the germination phases (dormant conidia, swollen, with germinating bulge and with germination tube) (Figure 1). After 8 hours, survival was estimated by considering only conidia with germ tubes as viable and an estimate of malformed germinated conidia was made as well (Conte *et al.,* 2022; Mosmann, 1983).

Figure 1 - Developmental stages of *Aspergillus nidulans* conidia



Source: the authors.

Conidial malformations were identified where the first germ tube bifurcated, a second tube

began formation prematurely or at an angle other than 180º, or when more than two tubes were formed, in addition to other observed alterations (Figure 1). Cells were counted through an optical microscope with a 400x final magnification, capturing images with a Canon EOS Rebel 3TI camera equipped with an SLR/DSLR adapter NDPL-2 (2x), for binocular microscopes. The Fiji J software was used to measure conidia. This experiment was performed in triplicate across 3 independent tests.

2.3 Colony growth and development assay with *A. nidulans*

Conidia were collected with five days of growth in solid complete medium at 37ºC and inoculated for the test of diameter and general appearance of the colony in the center of petri dishes containing solid complete medium and solid complete medium plus herbicide solution 0.01%, 0.025% and 0.05%. Then, the plates were incubated at 37 ºC for 7 days (168 hours). Every 24 hours, the diameter of the colonies, observation of the growth and shape of the colony, edges and surface and color of the reverse of the plate were determined. With these data, the growth rate of each colony with and without the herbicide was estimated. This experiment was done in triplicate in 3 independent trials.

2.4 Cytotoxicity assay with HuH7.5 cells

Human hepatoma HuH7.5 cells (HuH7.5) were cultured in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, in 25-cm² culture flasks (Invitrogen - Carlsbad, CA, USA) and incubated in a CO_2 incubator at 37ºC with 5% CO_2.

Cells were seeded into 96-well microplates at a density of 1×10^4 cells per well in 100 µL of complete medium and allowed to adhere for 24 h. Wells containing culture medium without cells were used as blank controls. After cell adhesion, medium was aspirated and replaced with 100 µL of complete medium containing Gramocil® at 0.01%, 0.025%, or 0.05%. The negative control (CO-) received a fresh complete medium, while the positive cytotoxic control (CO+) was treated with methyl methanesulfonate (MMS, 500 µM). Cells were incubated for 24, 48, and 72 h. Each condition was evaluated in three independent biological experiments, each performed on separate experimental occasions using independently prepared cell cultures and treatment solutions. Within each independent experiment, each treatment and control was assessed in triplicate wells, which were considered technical replicates. Thus, each condition was represented by three biological replicates and nine individual absorbance measurements overall.

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay, according to the protocol suggested by Mosmann (1983), with modifications. The culture medium was replaced with 100 µL of unsupplemented medium containing 0.167 mg mL^{-1} MTT reagent. Plates were incubated for an additional 4 h, after which the MTT-containing medium was discarded and 100 µL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance was measured at 492 nm using a microplate reader (Thermo Plate).

Cell viability was calculated as the ratio between the mean absorbance of each treated condition and the mean absorbance of the negative control, expressed as a percentage. For each independent experiment, the mean of the three technical replicate wells was calculated before statistical analysis; therefore, the experimental unit for inferential analysis was the independent biological experiment (n=3), not the individual well. Results are presented as mean ± standard deviation of the three independent experiments.

2.5 Micronucleus test with *L. catesbeianus*

The experimental protocol was approved by the Ethics Committee on the Use of Animals of the State University of Western Paraná (CEUA; Protocol No. 20.594.198-3). Tadpoles of *Lithobates catesbeianus* (Gosner stages 26–39; mean body mass approximately 3.5 g), obtained from a single commercial spawning batch, were acclimated for 7 days in 5-L aquaria containing dechlorinated tap water under controlled conditions (22 ± 2 °C, 12:12-h photoperiod, continuous aeration, and fish pellets provided every 48 h).

Following acclimation, tadpoles were assigned to an untreated negative control or to Gramocil® exposure groups at 0.01%, 0.025%, and 0.05%. Each treatment and the control were represented by one 5-L aquarium containing five tadpoles as five technical replicas. Water quality parameters, including pH (6.5–7.5), dissolved oxygen (>5 mg L^{-1}), and temperature (22 ± 2 °C), were measured every 48 h using a Horiba U-52 multiparameter probe. The pH was adjusted when necessary to maintain the specified range. Tadpole survival was assessed every 24 h, and individuals were considered alive when swimming activity was observed after gentle stimulation with a glass rod. Dead individuals were removed at each observation, and water volume was adjusted, when necessary, to maintain approximately 1 L of test solution per remaining animal.

The planned micronucleus protocol was based on the methodology of Gauthier et al. (2004). At the end of exposure (14 days), surviving tadpoles were to be anesthetized and euthanized by immersion in 5% benzocaine solution for approximately 10 min. After confirmation of death, caudal blood was to be collected, smeared onto clean and dry glass slides, and fixed in methanol for 1 h. The

slides were then to be air-dried at room temperature, fixed in 96% ethanol for 30 min, dried at room temperature for 12 h, and stained with 20% Giemsa solution prepared in phosphate buffer for 20 min.

For each tadpole, 1,000 erythrocytes were to be examined under a light microscope using a 100× oil-immersion objective to determine the frequency of micronuclei and other nuclear abnormalities, including segmented, binucleated, blebbed, and reniform nuclei. The mean number and percentage of micronuclei and other nuclear abnormalities were planned to be calculated for each control and treatment group.

In the current study, due to 100% mortality in all Gramocil®-exposed groups within 48–72 h, the planned erythrocyte micronucleus analysis could not be performed because no live animals remained for blood collection. Consequently, this component of the study had to be interpreted as an acute survival screening assay and not as a complete assessment of genotoxicity.

2.6 Escape test with *E. fetida*

Adult *Eisenia fetida* earthworms with well-developed clitella (body mass 300–600 mg) were used in a behavioral avoidance test conducted according to ISO 17512-1 (ABNT, 2011; Campelo *et al.,* 2022; Gonçalves *et al.,* 2015). Tropical Artificial Soil (TAS) consisted of 70% sieved fine quartz sand, 20% kaolin clay, and 10% dried coconut fiber. Soil moisture was adjusted to 60% of the maximum water-holding capacity.

Rectangular polypropylene test chambers with perforated lids were used. The external surfaces were covered with aluminum foil to prevent light exposure and maintain homogeneous internal conditions. Each chamber was divided into two equal compartments by a removable plastic divider. One compartment received 250 g of control soil (TAS moistened with filtered water), whereas the other received 250 g of test soil moistened with Gramocil® at 0.01%, 0.025%, or 0.05%. A positive control containing boric acid (1 g H_3BO_3 kg^{-1} dry soil) and a dual negative control, in which both compartments contained untreated TAS, were also included. After removal of the divider, 10 earthworms were placed along the central line separating the two soil sections and allowed to move freely. Each treatment condition was evaluated in duplicate chambers (two independent experimental units), with 10 earthworms per chamber.

The chambers were maintained in complete darkness at 22 ± 2 °C for 48 h. At the end of exposure, the divider was reinserted and the number of organisms present in each compartment was recorded. Avoidance was calculated according to Equation 1, where: n_C is the number of worms found in section B (control soil); n_T is the number of worms found in section A (test soil) and N is the total

number of worms (sum of replicates per sample).

$$A(\%) = \frac{n_C - n_T}{N} \times 100 \quad (1)$$

The test chamber, rather than each individual earthworm, was considered the experimental unit. Individual earthworms within the same chamber were not treated as independent replicates. An avoidance response greater than 60%, corresponding to more than 80% of organisms located in the control soil, was interpreted as indicating limited habitat function in accordance with ISO 17512-1.

2.7 Statistical analysis

For conidial germination, the proportions of conidia observed in each developmental stage (dormant, swollen, germinating bulge, and germ tube) were analyzed using a Generalized Linear Model (GLM) with binomial distribution and logit link function to evaluate the effects of Gramocil® treatment, exposure time, and their interaction. This model was selected because the response variable consisted of proportions derived from conidial counts. Additionally, mean percentages for each germination stage were compared between treatment and control groups using a two-way ANOVA followed by Tukey's post-hoc test, to provide pairwise comparisons at the evaluated exposure times. Final mortality and malformation means were compared among treatments and the control using one-way ANOVA followed by Tukey's post-hoc test. For the colony growth and development assay, fungal growth was analyzed by two-way ANOVA considering treatment and time as factors, followed by Tukey's post-hoc test. Results were expressed as means with the standard error of the mean (SEM) as a measure of dispersion.

For the cytotoxicity assay with HuH7.5 cells, cell viability determined by the MTT assay was analyzed using two-way ANOVA, with Gramocil® concentration and exposure time as factors. Dunnett's post-hoc test was used to compare each treatment with the negative control at each exposure time.

For the *Lithobates catesbeianus* bioassay, the Kruskal–Wallis test was initially planned for the micronucleus and nuclear-abnormality endpoints. However, this analysis was not performed because all Gramocil®-exposed tadpoles died before blood collection, preventing the collection of micronucleus data. Given the small sample size (five tadpoles per group housed in a single aquarium) and the lack of independent aquarium-level replication, inferential hypothesis testing was not applied to mortality data. Instead, tadpole mortality was reported descriptively as cumulative percentages at each 24-h observation interval and at the end of exposure.

For the *Eisenia fetida* avoidance test, the distribution of earthworms between control and Gramocil®-treated soil compartments was analyzed using a one-tailed Fisher's exact test against the expected 50:50 distribution. Each chamber was considered the experimental unit and replicate chambers were analyzed separately; individual earthworms within the same chamber were not treated as independent replicates. An avoidance response greater than 60%, corresponding to more than 80% of organisms located in the control soil, was interpreted as indicating limited habitat function according to ISO 17512-1.

All statistical analyses were performed using GraphPad Prism 8.0 and Action Stat 3.0.0 software. Statistical significance was set at $p<0.05$, and the significance threshold for the initially planned Kruskal–Wallis analysis was $\alpha=0.05$.

3 Results and Discussion

The multi-model assessment demonstrated that the commercial formulation Gramocil® exhibits pronounced toxicity across microbial, vertebrate, invertebrate, and cellular test systems.

A central aspect of this formulation is the co-presence of paraquat and diuron. While paraquat acts as a redox-cycling agent that diverts electrons from NADPH and produces superoxide radicals, hydrogen peroxide, and lipid peroxidation cascades, diuron acts primarily as an inhibitor of photosystem II in primary producers. However, diuron is also known to disrupt cellular respiration and mitochondrial function in heterotrophic systems by acting as an inhibitor of the mitochondrial respiratory chain (Dragone *et al.,* 2015)**.** Although specific reactive oxygen species (ROS) and apoptotic markers were not quantified in the present study, the literature supports the hypothesis that the simultaneous presence of diuron and paraquat, combined with commercial adjuvants and surfactants that increase cuticular and cellular permeability, may generate additive or synergistic cytotoxic effects, contributing to the severe membrane damage and rapid energy depletion observed here.

3.1 Conidia germination assay and growth and development of *A. nidulans*

Germination of dormant *A. nidulans* conidia occurs in three stages and in this order: activation and isotropic growth (imbibition), nuclear division and polarized growth (bud and germinated) (Table 1). Each of these phases is controlled by phase-specific genes that regulate the events related to germination and the time at which these modifications should take place.

Table 1 – Stages of germination, malformations and mortality in conidia of *A. nidulans*, after 8 hours of treatment with Gramocil® (0.01%, 0.025% and 0.05%) and control.

			2h (%)	4h (%)	6h (%)	8h (%)
Control	**Stages of germination**	**Dormant**	84.76[a]	18.49[a]	1.60	0.14
		Swollen	15.24[a]	81.27[a]	1.62	0.11
		Button	0[a]	0.24[a]	4.82	0
		Germinated	0[a]	0	91.96	93.83
		Malformations	-	-	-	5.92[a]
		Mortality	-	-	-	0.25[a]
Gramocil® 0.01%	**Stages of germination**	**Dormant**	94.55[b]	23.92[b]	2.27[a]	0.35[a]
		Swollen	5.45[b]	75.76[b]	3.27[a]	0.13[a]
		Button	0[a]	0.32[a]	4.72[a]	0.25[a]
		Germinated	0[a]	0[a]	89.74[a]	86.8[a]
		Malformations	-	-	-	12.47[b]
		Mortality	-	-	-	0.48[a]
Gramocil® 0.025%	**Stages of germination**	**Dormant**	99.75[b]	81.78[b]	83.5[b]	24.76[b]
		Swollen	0.25[b]	18.22[b]	14.43[b]	74.62[b]
		Button	0[a]	0[a]	2.07[b]	0.62[a]
		Germinated	0[a]	0[a]	0[b]	0[b]
		Malformations	-	-	-	-
		Mortality	-	-	-	99.38
Gramocil® 0.05%	**Stages of germination**	**Dormant**	99.75[b]	83.42[b]	63.03[b]	59.38[b]
		Swollen	0.25[b]	16.58[b]	36.27[b]	40.38[b]
		Button	0[a]	0[a]	0.7[b]	0.24[a]
		Germinated	0[a]	0[a]	0[b]	0[b]
		Malformations	-	-	-	-
		Mortality	-	-	-	99.76

[a,b] Different letters indicate statistical differences when comparing control versus treatment.
Source: research data.

The results show a delay of the imbibition phase of conidia treated with Gramocil® when compared to the control, demonstrating that Gramocil® herbicide exposure significantly delayed or inhibited germination in a dose-dependent manner. There were 94.55%, 99.75% and 99.75% of dormant conidia after two hours of incubation with Gramocil® at concentrations of 0.01%, 0.025% and 0.05%, respectively, compared to 84.76% in the control group.

The delay persisted after four hours, reaching 84% of dormant conidia at the concentration of 0.05% of Gramocil®.

This delay in the imbibition process remained after six hours of treatment at concentrations of 0.025% and 0.05% of Gramocil®, while at 0.01% the conidia developed similarly to the control after six and eight hours. In contrast, at concentrations of 0.025% and 0.05%, almost 100% of the conidia remained in the dormant or swollen phases even after eight hours of exposure.

To assess whether this delay could be overcome over longer periods, conidia were grown in solid medium in the presence of Gramocil® at the same concentrations. Colonies of *A. nidulans* developed at the concentration of 0.025% with a marked delay in growth and reduced final size, while

at 0.05% no colony developed, even after seven days of treatment (168 hours) (Table 2).

Table 2 – Mean total growth (cm) of the colony of *A. nidulans* after 168 hours in control and pesticide Gramocil® at different concentrations

Hours	Control	Gramocil® 0.01%	Gramocil® 0.025%	Gramocil® 0.05%
0-24	0.4 ± 0.1^{a}	0.2 ± 0.1^{b}	0.3 ± 0.4^{b}	0^{b}
0-48	1.0 ± 0.2^{a}	0.8 ± 0.1^{b}	0.5 ± 0.1^{b}	0^{b}
0-72	1.8 ± 03^{a}	1.2 ± 0.1^{b}	0.6 ± 0.0^{b}	0^{b}
0-96	2.7 ± 0.1^{a}	1.7 ± 0.2^{b}	0.9 ± 0.0^{b}	0^{b}
0-120	3.5 ± 0.1^{a}	2.2 ± 0.1^{b}	1.0 ± 0.0^{b}	0^{b}
0-144	4.0 ± 0.2^{a}	2.6 ± 0.3^{b}	1.2 ± 0.0^{b}	0^{b}
0-168	4.1 ± 0.2^{a}	2.9 ± 0.5^{b}	1.3 ± 0.1^{b}	0^{b}

^{a,b}Different letters indicate statistical difference when compared control versus treatment.
Source: research data.

The results for colony growth presented in Table 2 show that Gramocil® at 0.01% and 0.025% caused significant delays in fungal colony development, whereas the concentration of 0.05% was lethal. Morphological analysis revealed that colonies grown at concentrations allowing development exhibited similar characteristics to the control, including dark green coloration and white edges.

In *A. nidulans*, conidial germination relies on early mitochondrial activation, cell wall biosynthesis, and polarized actin cytoskeleton assembly (d'Enfert, 1997). The observed arrest at the dormant and swollen phases, together with bifurcated germ tubes, strongly suggests that Gramocil® interferes with metabolic energy production and apical growth coordination.

3.2 Assay with HuH7.5 cells

All tested concentrations (0.01%, 0.025% and 0.05%), at all evaluated time points (24, 48 and 72 h), showed significantly lower mean absorbance values compared to the negative control, indicating cytotoxicity (Table 3). Cell viability was markedly reduced from the lowest concentration and shortest exposure time, with values dropping below 26% after 24 hours, 25% after 48 hours, and 21% after 72 hours. A dose-dependent reduction in viability was observed at 24 and 48 hours, with increasing concentrations further inhibiting mitochondrial activity. Additionally, a time-dependent effect was evident at all concentrations, as viability progressively decreased from 24 to 72 hours.

Table 3 - Percentages of cell viability of liver cells treated for 24, 48 and 72 hours with the concentrations (%) of the agrochemical Gramocil®

Groups	HuH7.5		
	VC [%]		
	24 h	48 h	72 h
CO-	100.00	100.00	100.00
CO+	39.20*	29.67*	21.84*
0.01%	25.00*	24.79*	20.45*
0.025%	25.69*	21.13*	19.50*
0.05%	23.68*	20.96*	20.55*

CO-: Negative Control, CO+: Positive Control. *Results differ statistically from the negative control ($p<0.05$).
Source: research data.

In HuH7.5 cells, the rapid reduction of MTT formazan crystals indicates profound impairment of mitochondrial succinate dehydrogenase activity within 24 h. While literature reports LC50 and EC50 values for analytical-grade paraquat in mammalian liver cells in the range of 50–200 μM (~13–50 mg/L) (Conte *et al.,* 2022), the whole formulation tested here at 100–500 mg/L provoked massive cell death (>75%), consistent with severe acute cytotoxicity.

3.3 Aquatic lethality in *L. catesbeianus* tadpoles

At 48 h, 100% mortality of *Lithobates catesbeianus* tadpoles occurred in the 0.025% and 0.05% groups, and 80% in the 0.01% group. By 72 h, 100% mortality was reached in all herbicide-treated tanks, whereas no mortality occurred in the control tank. Because no individuals survived past 72 h in any treatment group, erythrocyte micronuclei and nuclear abnormalities could not be evaluated. These findings confirm the extreme acute vulnerability of anuran larvae to direct immersion in Gramocil® at runoff concentrations, preventing the expression of sublethal mutagenic endpoints and precluding any statistical inference for this assay (Table 4).

Table 4 - Accumulated mortality rate (%) of tadpoles exposed to different concentrations (%) of agrochemical Gramocil®

Sample	24 hours	48 hours	72 hours
CO-	0	0	0
0.01%	0	80	100
0,025%	0	100	100
0.05%	0	100	10

CO-: Negative Control.
Source: research data.

In *L. catesbeianus* tadpoles, the 100% mortality observed within 48–72 h aligns with the

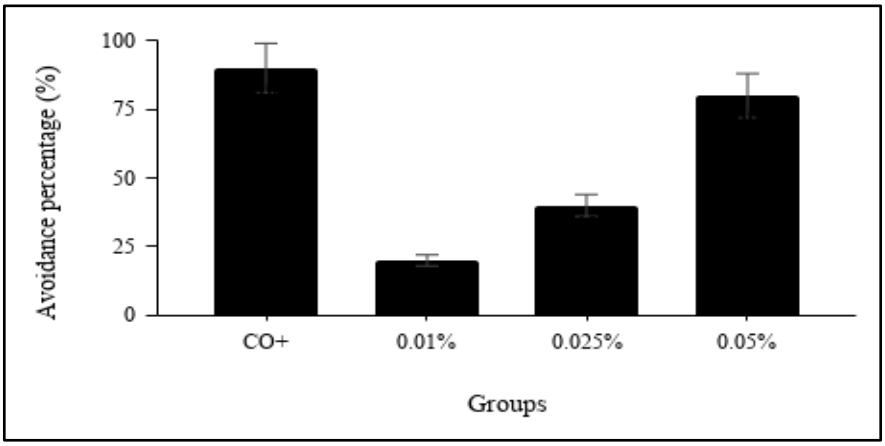
known acute sensitivity of amphibians to bipyridylium and phenylurea compounds. In previous literature, acute 96-h LC50 values for paraquat in anuran larvae have been reported between 1.5 and 15 mg/L (Rios *et al.,* 2017), whereas our tested concentrations (100–500 mg/L) far exceeded lethal thresholds, leading to rapid systemic collapse before cytogenetic alterations (micronuclei) could be evaluated.

3.4 Escape test with *E. fetida*

In the dual-control soil containers, earthworm distribution was 55% vs. 45%, fulfilling the validity criterion of the ISO 17512-1 standard (within 40%–60%). Boric acid (positive control) induced 80% avoidance (90% in control soil vs. 10% in test soil; $p < 0.01$, Fisher's exact test), confirming test sensitivity.

For Gramocil®, avoidance rates were 20% at 0.01%, 40% at 0.025%, and 80% at 0.05% (Figure 2). Under ISO 17512-1 guidelines, a habitat is considered altered/toxic when avoidance exceeds 60%, and an avoidance rate ≥ 80% indicates complete loss of soil habitat function. Thus, while 0.01% and 0.025% prompted behavioral migration without reaching the regulatory toxicity threshold, the 0.05% concentration exhibited severe soil toxicity.

Figure 2 - Avoidance percentage (%) in *Eisenia fetida* exposed to boric acid (CO+) and different concentrations of Gramocil®



Source: research data.

For *E. fetida*, sensory chemoreceptors enable earthworms to detect xenobiotics in soil pore water (Filipi *et al.,* 2023). Avoidance exceeded the 60% threshold only at 0.05% (~500 mg/L), indicating that at lower concentrations (0.01%–0.025%), adsorption of paraquat to clay minerals and organic matter in Tropical Artificial Soil may reduce immediate bioavailability, buffering behavioral repulsion.

It is noteworthy that the tested nominal concentrations (0.01% to 0.05%, corresponding to 100–500 mg/L) are three to five orders of magnitude higher than typical environmental background

concentrations in surface water bodies, which are commonly below 1 μg/L. Thus, our findings model acute, catastrophic contamination events, such as direct agricultural runoff, improper disposal, or accidental tank discharge, rather than chronic environmental exposure.

Consequently, because of this narrow high-dose concentration range and the resulting total mortality in tadpole and cell assays, standard regulatory benchmarks such as LC50, EC50, NOEC, and LOEC could not be derived. Furthermore, the concentrations used were nominal, lacking analytical verification (e.g., HPLC-MS) to assess paraquat and diuron breakdown or to confirm the exact adjuvant composition of the formulation.

4 Conclusion

The commercial formulation Gramocil® exhibited pronounced acute toxicity across the four biological systems evaluated at concentrations of 0.01%, 0.025%, and 0.05%. The formulation suppressed conidial germination and colony growth in *A. nidulans*, induced severe loss of mitochondrial viability in human hepatoma HuH7.5 cells, caused rapid total mortality in *L. catesbeianus* tadpoles (precluding micronucleus assessment), and triggered significant soil habitat avoidance in *E. fetida* at 0.05%.

These findings document the acute multi-system hazards of the whole commercial formulation under high-concentration runoff or accidental discharge scenarios. Given that paraquat was already officially banned in Brazil in 2020 due to its toxicological profile, future studies should focus on environmental residue monitoring in vulnerable watersheds, evaluation of chronic sublethal concentrations (μg/L) to establish NOEC/LOEC values, and the analytical differentiation of synergistic contributions between diuron, paraquat, and surfactant adjuvants.

Data availability statement

The data that support the findings of this study are available from the corresponding author, M.S.B.C, upon reasonable request.

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