ORIGINAL ARTICLE

# A multi-stressor environment impairs the photosynthetic performance of *Virescentia viride-brasiliensis* (Batrachospermales, Rhodophyta)

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**ABSTRACT**

Red algae belonging to Batrachospermales are important contributors to the energy input of lotic ecosystems. Given the importance of these algae for the lotic food web, we tested the photosynthetic performance of a relatively widespread and sensitive species (*Virescentia viride-brasiliensis*) in multi-stressor scenarios. Experiments were performed by exposing algal samples to three nominal concentrations (0.05 mg/L, 0.6 mg/L, and 1.2 mg/L) of tebuthiuron, a commonly used pesticide in Brazil, combined with three projected temperatures due to climate change (21.6ºC, 23.9ºC, and 26ºC). We observed a decrease in photosynthetic yield (Y(II)), regulated energy dissipation (Y(NPQ)), net photosynthetic rate (NPR), and dark respiration rate (DRR), while an increase in non-regulated energy dissipation (Y(NO)) was recorded, all of which indicate stress responses. Furthermore, we observed a dose-dependence relationship in which the negative effects increased with increasing tebuthiuron concentrations and in the scenario with a more severe temperature increase (26ºC). The high sensitivity of *V. viride-brasiliensis* to tebuthiuron highlights its potential bioindicator status because the tebuthiuron concentration accepted as safe for drinking water (0.05 mg/L) was sufficient to decrease its photosynthetic yield. Ultimately, these results show the importance of managing pesticide usage, especially considering the simultaneous occurrence of global warming.

## INTRODUCTION

Freshwater habitats have long been degraded due to human expansion, and the increase in urbanization and industrial and agricultural practices constitute great threats to these environments (Martinuzzi *et al.,* 2014). The expansion of industrial and agricultural areas may affect freshwater ecosystems by limiting stream flows or groundwater stores for supporting irrigation (Scanlon *et al.,* 2007), reducing riparian habitat (Heartsill-Scalley and Aide, 2003), and increasing the amount of sediments, nutrients, metals, and pesticides entering these areas (Tilman *et al.,* 2001; Szöcs *et al.,* 2017; Yadav and Pandey, 2023). Specifically considering pesticides, the unrestricted and widespread use of these substances may ultimately lead them into the water via leaching, run-off, spray drift, or volatilization (Pereira *et al.,* 2009). In water, pesticides may act on non-target species, generating cascade effects and impacts across multiple trophic levels (Lu *et al.,* 2020).

Tebuthiuron (1-(5-tert-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) is a phenyl-urea herbicide commonly used in sugarcane crops (Qian *et al.,* 2017) that acts by inhibiting electron transport in photosystem II after its absorption (Liu, 2010). Although generally applied on the soil, this herbicide has high leaching potential, and water bodies adjacent to such crops may be subjected to residues of this herbicide (Pereira *et al.,* 2009; Madeira *et al.,* 2023). The potential for contamination of water bodies is even more pronounced given that tebuthiuron is resistant to degradation (Oliveira *et al.,* 2023). Due to its mode of action, this herbicide is considered to pose a high environmental risk (Thomas *et al.,* 2020), which has led the European Union to classify it as very toxic (European Chemicals Agency, 2021).

Along with the increasing amounts of contaminants in water, freshwater habitats are also threatened by climate change. The Intergovernmental Panel on Climate Change (IPCC), in its fifth as-

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sessment report, projected that the global mean temperature will increase by at least 2ºC in the following decades, even with strong mitigation efforts (Collins *et al.,* 2013). The increase in temperature may affect freshwater habitats by changing the dynamics and chemical properties of water (Nickus *et al.,* 2010), in addition to affecting the metabolism of organisms and possibly generating cascade effects (Woodward *et al.,* 2010). Temperature is a highly important variable in environments because it is a limiting factor in the adaptative capacity of species (Marotzke *et al.,* 2017) and a strong driver of carbon flow in freshwater environments (Belle *et al.,* 2018). Considering the carbon flow of freshwater habitats, the food web receives energy inputs from sources in the water (autochthonous) and from the surrounding terrestrial vegetation (allochthonous) (Neres-Lima *et al.,* 2017).

In tropical regions, autochthonous energy may play an important role in sustaining the aquatic food web, with many studies describing the relevance of benthic algal communities as one of the main primary producers (Branco *et al.,* 2017; Neres-Lima *et al.,* 2017). Moreover, these organisms contribute to species richness, increase habitat complexity, and stabilize the substrate, in addition to playing a relevant role in the interface of abiotic and biotic components of the food chain (Downes *et al.,* 2000; Branco *et al.,* 2010). A significant part of stream benthic algae with macroscopic growth, the so-called macroalgae (sensu Sheath and Cole, 1992), is formed by species of red algae (Rhodophyta) [ca. 20% of the total, according to Necchi Júnior (2016)]. Most stream red macroalgae present photosynthetic characteristics of shade-adapted algae because there is a higher abundance of these organisms in waters with low radiation, low flow speed, and, in general, low to moderate nutrient levels, so they are possibly indicators of good water quality (Branco *et al.,* 2017).

Thus, considering the environmental risks associated with the exposure of non-target organisms to herbicide residues and the increase in temperature related to climate change, coupled with the relevance of Rhodophyta species for primary production in lotic habitats, the present study aimed to evaluate the photosynthetic performance of the gametophytes of *Virescentia viride-brasiliensis* Necchi Júnior, Agostinho & Vis (Batrachospermales, Rhodophyta) after being subjected to different concentrations of tebuthiuron along with temperature increases projected by two IPCC scenarios. Considering the previous descriptions of the shade-adapted traits of these algae and their higher occurrence in sites with good water quality, we hypothesize that temperature increases and the presence of tebuthiuron may individually generate negative effects on the photosynthetic performance of *V. viride-brasiliensis* and this multi-stressor condition may ultimately amplify such a negative effect.

## METHODS

### Experimental design, sampling, and preparation of algal specimens

The experimental design proposed for the present study consisted of exposing *Virescentia viride-brasiliensis* samples to treatments with three different concentrations of tebuthiuron-based herbicide and three temperatures calculated from scenarios of global warming proposed by the IPCC, singly and in binary combination (so-called multi-stressor treatments). Responses of specific photosynthetic parameters were tested by applying two analytical techniques: dissolved oxygen evolution and chlorophyll *a* fluorescence.

Samples of *V. viride-brasiliensis* were collected from the Pari river, located in the Cervo River microbasin, in the western region of the state of São Paulo, Brazil (22°38’33.0 “S 50°12’14.8 “W). Algal samples were taken to the laboratory in transparent vials containing river water and then cleaned to remove sediment and possible epiphytes and invertebrates. After cleaning, samples of 150 mg (±10 mg) were prepared with five replicates (n=5) for each multi-stressor treatment. Weighing was performed using an analytical scale (Shimadzu AUW320).

First, the 150 mg samples were transferred to 150 mL Erlenmeyer flasks containing 100 mL of Bold’s basal medium (BBM) (Watanabe, 2005) without herbicide and placed inside B.O.D. incubators (Nova Ética, model 411 / FDP355) under the temperature determined for each experimental treatment (described below), constant irradiance (140 µmol.m$^{-2}$s$^{-1}$), and 12 h/12 h photoperiod (light/dark cycle) for 24 h for acclimation. After the 24-hour acclimation period, the BBM of the samples for treatments with exposure to tebuthiuron were replaced with BBM containing the concentrations of the active ingredient of tebuthiuron as specified for each treatment. The exposure period of the samples lasted 7 days, and the media were renewed on the third and fifth days to avoid nutrient depletion and to keep a steady concentration of the active ingredient (Oliveira *et al.,* 2016).

### Determination of temperature scenarios

The calculations of experimental temperatures were based on the procedures described by Vilas Boas *et al.* (2019). The average measured temperature (Mt) in the streams of the Cervo River microbasin was used as a reference value, to which the temperature increases predicted by two specific IPCC scenarios for tropical regions were added. The first scenario tested was RCP 4.5, with a projected maximum temperature increase of 2.3ºC, while the second scenario was RCP 8.5, with a projected maximum temperature increase of 4.4ºC (Collins *et al.,* 2013).

Temperature measurements were taken in ten streams using a multiparameter probe (HORIBA U-50), and the Mt obtained was 21.6ºC (*Tab. S1*). In tropical regions, freshwater algae are more abundant during the winter due to the lower rainfall regime, and in winter, the highest stream temperatures are found at 16:00 h (Vilas Boas *et al.,* 2019). Therefore, measurements for calculating Mt were carried out in June between 15:00 h and 17:00 h. By adding the maximum increase projected for both IPCC scenarios to Mt, the temperature of the experimental RCP 4.5 and RCP 8.5 scenarios were set to 23.9ºC and 26ºC, respectively. In addition, Mt (21.6ºC) was used as the control.

### Determination of nominal concentrations of tebuthiuron

We tested three nominal concentrations of tebuthiuron. The first concentration corresponded to the maximum concentration allowed by the North American Environmental Protection Agency in water bodies (U.S. Environmental Protection Agency, 1988). The second concentration was the recommended dosage of an application of tebuthiuron (Combine®500 SC) for sugarcane crops. The third was taken as the ‘worst case scenario’, with twice the recommended dosage of application on clayish soils. Nominal concentrations were 0.05 mg/L (named Tl - Tebuthiuron low), 0.6 mg/L (Tm - Tebuthiuron medium), and 1.2 mg/L (Th - Tebuthiuron high), while the control (Ct) had no herbicide.

### Multi-stressor treatments

For the multi-stressor treatments, combinations of the IPCC scenarios and concentrations of tebuthiuron were made. A total of

12 multi-stressor treatments were performed, coded by the following acronyms: Mt-Ct, Mt-Tl, Mt-Tm, and Mt-Th; 4.5-Ct, 4.5-Tl; 4.5-Tm, and 4.5-Th; 8.5-Ct, 8.5-Tl, 8.5-Tm, and 8.5-Th (see the topics Determination of temperature scenarios and Determination of nominal concentrations of tebuthiuron for acronyms).

## Experimental analyses

Chlorophyll *a* fluorescence analysis and dissolved oxygen evolution were performed to assess the photosynthetic response of *Virescentia viride-brasiliensis* (Vilas Boas *et al.,* 2019; Vilas Boas and Branco, 2022).

The analysis of photosynthetic responses using the chlorophyll *a* fluorescence was performed using a Diving-PAM fluorometer (Walz, Effeltrich, Germany) and following the standardized methodology for similar studies with stream macroalgae (Vilas Boas *et al.*, 2019; Oliveira *et al.*, 2021; Vilas Boas and Branco, 2022). After a 30-minute acclimatization period in the dark, the samples were positioned on the tip of the optical fiber and then evaluated using the "Induction Curve" function (Schreiber *et al.*, 1995). Before recording the actual induction curve, a single saturation pulse of 2000 µmol photons $m^{-2} s^{-1}$ was applied for the assessment of F0, Fm, and Fv/Fm after dark adaptation. After a delay of 30 s, actinic illumination (285 µmol photons $m^{-2} s^{-1}$) was turned on, and 12 pulses of saturating light (2000 µmol photons $m^{-2} s^{-1}$) lasting 0.8 s each were applied at 15 s intervals (Oliveira *et al.*, 2016).

Based on the data produced by these curves, the following photosynthetic parameters of each sample submitted to each treatment were evaluated (Klughammer and Schreiber, 2008; Cosgrove and Borowitzka, 2011; Oliveira *et al*., 2016): i) quantum yield of photochemical energy conversion in PS II (YII), ii) quantum yield of regulated non-photochemical energy loss in PS II (YNPQ), and iii) quantum yield of non-regulated non-photochemical energy loss in PS II (YNO).

The net photosynthetic rate (NPR) and dark respiration rate (DRR) were measured by changes in the dissolved oxygen concentrations using the light and dark bottle technique (Littler and Arnold, 1985; Thomas, 1988) and following the same procedures described by Vilas Boas *et al.* (2019). For dark respiration rate experiments, five algal samples of each experiment and controls were transferred to 110 mL glass bottles wrapped in aluminum foil (dark bottles) and incubated for 1 h in the dark at the same temperature conditions in which they were maintained during the experiments. For the NPR experiments, the same samples were transferred to 110 mL glass bottles with 98.5% transparency (light bottles) and incubated for 1 h at the same conditions in which they were maintained during the experiments. Measurements of the initial (before incubation period) and final (after incubation period) concentrations of dissolved oxygen were obtained using a YSI model 5100 oximeter (Yellow Springs, USA) equipped with a self-stirring probe. From the data obtained with these measurements, calculations of the photosynthetic and dark respiration rates were performed using the following formulas proposed by Littler and Arnold (1985): NPR = [(F) - (I)] * V / IT / DW and DRR = [(I) - (F)] * V / IT / DW where NP: net photosynthetic rate; DR: dark respiration rate; (F): final concentration of dissolved oxygen after the incubation period; (I): initial concentration of dissolved oxygen before the incubation period; V: volume (liters) of medium in the bottle; IT: incubation time; and DW: dry weight.

## Statistical analysis

Differences in the values of photosynthetic parameters among the different multi-stressor treatments were identified with two-way ANOVA tests followed by the Scott–Knott multiple comparison test (Scott and Knott, 1974). To perform the two-way ANOVA and Scott–Knott tests, chlorophyll *a* parameters, NPR, and dark respiration rate were used as dependent variables, while the factors of multi-stressor treatments (tebuthiuron concentrations and IPCC scenarios) were used as independent variables. The statistical software Sisvar 5.8 (Ferreira, 2011) was used.

# RESULTS

Significant differences between the treatments and control were found for all photosynthetic parameters and all treatments (Tabs. S2 and S3). Considering the chlorophyll *a* fluorescence parameter, the Y(II) values were significantly lower ($p$<0.001, F = 25.11) in all treatments with exposure to tebuthiuron compared to the control. In the Mt scenario, Tl, with 0.05 mg/L of tebuthiuron, showed a reduction of 14% in the Y(II) values compared to the control, while the Y(II) values of Tm and Th Y(II) were 91% and 99% lower than control, respectively (Fig. 1, *Tab. S2*). In the other tested IPCC scenarios, the Y(II) values were reduced in all treatments compared to the control as well. Thus, the reduction in Y(II) in the RCP 4.5 scenario was of the order of –57%, –96%, and –77% for Tl, Tm, and Th, respectively, while the reduction in RCP 8.5 was of the order of –78%, –87%, and –92% for Tl, Tm, and Th, respectively. No significant effect of the temperature factor was observed for Y(II) (Fig. 1, *Tab. S2*).

The Y(NPQ) parameter was only affected by the tebuthiuron concentrations, and the IPCC temperature scenarios had no effect, as detected in the two-way ANOVA tests. Y(NPQ) values were significantly lower ($p$<0.001, F = 13.91) in the Tl, Tm, and Th treatments compared to the control (–29%, –56%, and –62%, respectively, for the Mt; –19%, –25%, and –58% for RCP 4.5; and –39%, –54%, and –69% for RCP 8.5; Fig. 2, *Tab. S2*). For Y(NO), the three treatments exposed to tebuthiuron showed a significant increase ($p$<0.001, F = 34.84). In the Mt scenario, Tl, Tm, and Th showed Y(NO) values 22%, 57%, and 62% higher than the control, respectively (Fig. 3, *Tab. S2*). For the RCP 4.5 scenario,

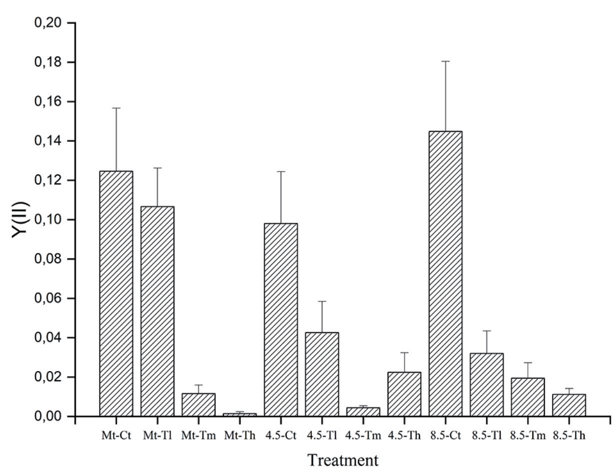


**Fig. 1.** Effective quantum yield of photosystem II (Y(II)) mean values ± SD (n=5) for *Virescentia viride-brasiliensis* after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios. Mt, mean of measured temperatures.

Y(NO) was 19%, 28%, and 41% higher than the control for Tl, Tm, and Th, respectively. Lastly, in the RCP 8.5 scenario, Y(NO) values were 38%, 57%, and 56% higher than the control, respectively. The values of Y(II), Y(NPQ), and Y(NO) in treatments Tm and Th were also significantly different from the values of these parameters in Tl. Y(II) values of Tm and Th were lower than Tl (–89% and –98%, respectively, for the Mt; –89% and –47% for RCP 4.5; and –39% and –65% for RCP 8.5; Fig. 1, *Tab. S2*). Y(NPQ) values for Tm and Th were also lower than Tl (–38% and –46%, respectively, for the Mt; –7% and –47% for RCP 4.5; and –25% and –49% for RCP 8.5; Fig. 2, *Tab. S2*). Lastly, Y(NO) values were higher in Tm and Th compared to Tl (+28% and +23%, respectively, for the Mt; +7% and +18% for RCP 4.5; and +13% for RCP 8.5; Fig. 3, *Tab. S2*).

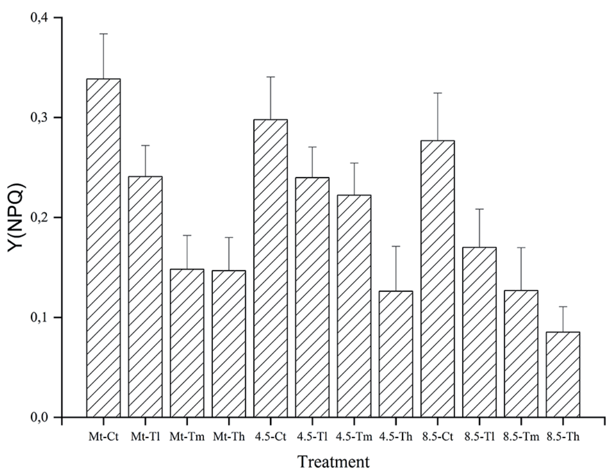


**Fig. 2.** Regulated non-photochemical energy loss in photosystem II (Y(NPQ)) mean values ± SD (n=5) for *Virescentia viride-brasiliensis* after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios.

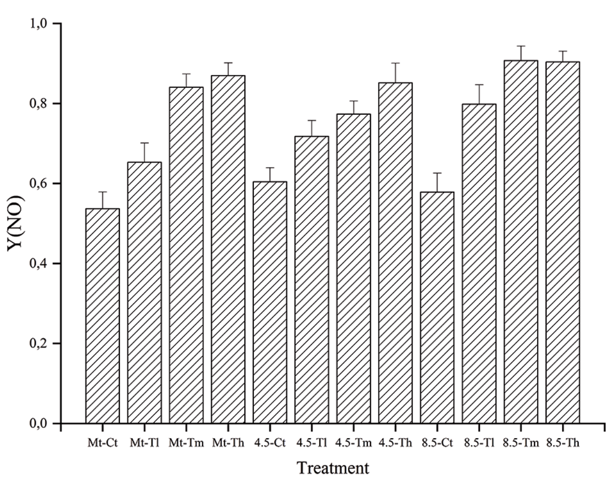


**Fig. 3.** Non-regulated non-photochemical energy loss in photosystem II (Y(NO)) mean values ± SD (n=5) for *Virescentia viride-brasiliensis* after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios.

For the oxygen evolution analysis, the two-way ANOVA tests revealed that the NPR was significantly reduced by tebuthiuron ($p<0.05$, F = 3.11) and temperature ($p<0.05$, F = 3.91) factors, as well as the interaction ($p<0.01$, F = 4.09) between these two factors. The NPR of *V. viride-brasiliensis* was significantly lower with the interaction of the temperature and tebuthiuron concentrations, especially in the RCP 4.5 scenario with Tm (with a reduction of 79% compared to the same treatment in the Mt) and with Th (–66% compared to the same treatment in the Mt; Fig. 4, *Tab. S3*). The dark respiration rate (DRR) was significantly lower t ($p<0.001$, F = 5.67) in all treatments compared to the control. DRR values at Mt treatment were 73%, 82%, and 91% lower than the control for Tl, Tm, and Th, respectively (Fig. 5, *Tab. S3*). For the RCP 4.5 scenario, values were 74%, 95%, and 95% lower than

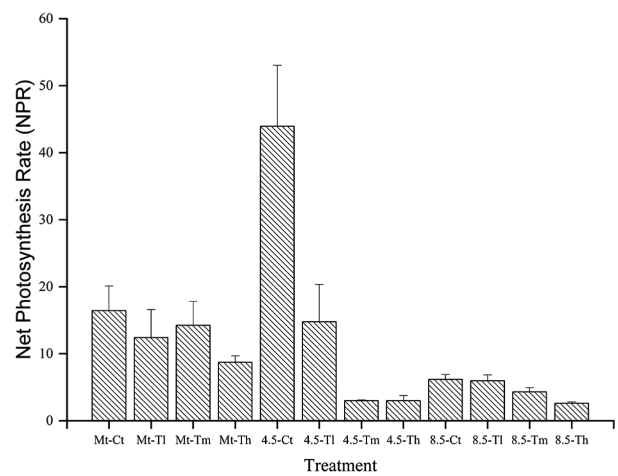


**Fig. 4.** Net photosynthetic rate (NPR) mean values ± SD (n = 5) for *Virescentia viride-brasiliensis* after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios.

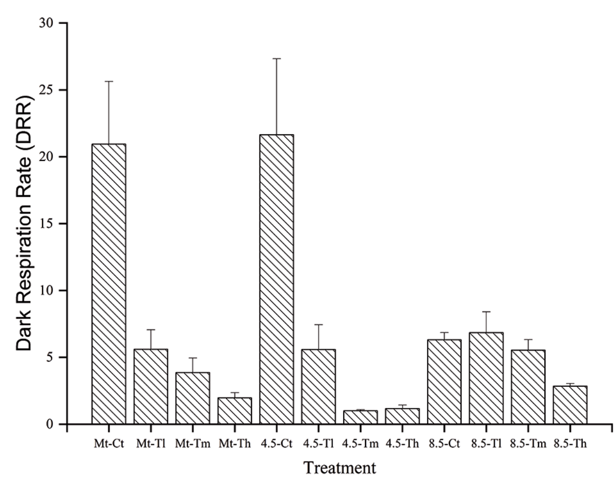


**Fig. 5.** Dark respiration rate (DRR) mean values ± SD (n=5) for *Virescentia viride-brasiliensis* after 7 days exposure to different tebuthiuron concentrations and temperature increase scenarios.

the control for Tl, Tm, and Th, respectively. In the RCP 8.5 scenario, Tl showed an increase in respiration rate of 8% compared to the control, while Tm and Th showed reductions of 12% and 55%, respectively (Fig. 5, *Tab. S3*).

## DISCUSSION

Both chlorophyll *a* fluorescence and dissolved oxygen analyses showed clear trends in the negative effects of tebuthiuron on the photosynthetic performance of *V. viride-brasiliensis*. It seems that the negative effect of tebuthiuron on the photosynthetic efficiency of *V. viride-brasiliensis* is related to the fact that this herbicide binds to the D1 protein, forming the triplet state of chlorophyll, which, in turn, can react with oxygen. The reactive oxygen species (i.e., singlet oxygen) that are formed ultimately is capable of damaging photosystem II (Krieger-Liszkay, 2005).

Considering the IPCC scenarios, only the data for Y(NO) and NPR showed that the smaller increase in temperature projected by the RCP 4.5 scenario would not be harmful to *V. viride-brasiliensis* because no differences were observed. In the absence of tebuthiuron, the rise of 2.3ºC resulted in an increase in the NPR values (Fig. 4, *Tab. S3*). However, for the RCP 8.5 scenario, Y(NO) values were significantly higher than those of the Mt treatment, showing that an increase of 4.4ºC in temperature would be stressful for this species (Fig. 3, *Tab. S2*). Even though few parameters were affected by temperature and no statistical difference was found between RCP 8.5 and Mt in the NPR, the Y(NO) results showed an important negative response to this IPCC scenario. Similar results were found by Vilas Boas *et al.* (2019), with the gametophyte of other species of Batrachospermales (*Sirodotia delicatula*) showing increased Y(NO) values at temperatures above 27ºC. Regarding the interaction between factors in the NPR analysis, while the RCP 4.5 scenario had higher NPR values than Mt when no tebuthiuron was present, its addition caused a significant drop in this parameter, reaching values below those of the Mt for the Tm and Th treatments. Although tebuthiuron is toxic in any scenario, these responses suggest that in the most severe IPCC scenario, the negative effects of herbicide can be intensified, supporting our hypothesis.

In comparison with other freshwater macroalgae species subjected to the same stressors, *V. viride-brasiliensis* stands out due to its pronounced susceptibility to tebuthiuron. Tests with two species of green algae, *Nitella microcarpa* var. *wrightii* and *Oedogonium* sp., have also shown a relevant reduction in their photosynthetic yield when exposed to tebuthiuron; however, significant results were only observed when these algae were subjected to higher concentrations of the herbicide (Vilas Boas and Branco, 2022, 2024). The sensitivity of this species to low concentrations of tebuthiuron also highlights the potential status of this species as a bioindicator of good water quality (Stancheva and Sheath, 2016). Although we cannot state that the effect produced by the lowest concentration would eradicate the algae from sites with tebuthiuron, higher concentrations caused severe damage to the species, with reductions of 50-99% in the effective quantum yield (Y(II)). Therefore, this organism, and presumably other sensitive species, might disappear from waters containing concentrations of this pesticide of ≥0.6 mg/L. Additionally, the lifetime advisory concentration of tebuthiuron recommended by the United States Environmental Protection Agency (US-EPA; i.e., 0.05 mg/L) had significant negative effects on the photosynthesis of *V. viride-brasiliensis*. This finding suggests that, although this concentration may be considered safe for drinking water to humans, it can represent significant environmental risks.

Ultimately, these results show the importance of properly controlling pesticide usage, especially when reference values defined as safe for human consumption might be deleterious on non-target organisms. Climate change may also cause unforeseen effects when organisms are subjected to multi-stressor scenarios, highlighting the importance of updated guidelines and studies on this subject.

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*Online supplementary material:*
*Tab. S1. Temperature values (ºC) and GPS coordinates of 10 streams in the Cervo River microbasin.*
*Tab. S2. Two-way analysis of variance results for Y(II), Y(NO), and Y(NPQ).*
*Tab. S3. Two-way analysis of variance results for net photosynthetic Rate (NPR) and dark respiration rate.*