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Brief Report

Assessing the Effect of Glyphosate Toxicity on *Lemna minor* in Different Temperature Regimes

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Abstract: Temperature-dependent chemical toxicity has become a crucial issue taking into consideration that lakes, especially shallow waterbodies, are impacted by climate change worldwide. In this study, we are looking for an answer to what extent standard ecotoxicity assays being performed under constant and relatively low temperatures are capable of predicting the chemical risk posed by pesticides. *Lemna minor* test plants were exposed to glyphosate in concentrations in the range of 25, 50, 100, 200, and 400 µg/L at temperatures 10, 15, 20, 25, 30, and 35 °C. Two peaks appeared when growth inhibition was assessed; lower concentrations elucidated higher inhibition, at 20 °C, while higher concentrations were found at a higher temperature of 30 °C. The toxic effect experienced at 20 °C indicates that reported PNEC values cannot be sufficient to protect non-target aquatic species in certain environmental scenarios. In addition to growth inhibition, phytotoxicity was also assessed based on peroxidase (POD) concentrations. In general, POD showed greater sensitivity, already showing a response at the lowest temperature tested, 10 °C. Decreased POD activity was detected in the temperature range of 10–30 °C, most probably indicating damage to cell and plasma membranes.

Keywords: *Lemna minor*; climate change; elevated water temperature; chemical hazard

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1. Introduction

Lakes worldwide are impacted by climate change, shallow lakes being the most vulnerable [1]. In recent decades, the increase in annual water temperature was estimated to be about 0.15–0.3 °C per decade in 24 European lakes [2].

Climate change is expected to alter the environmental fate and behaviour of pesticides. Increased temperature will most likely increase volatilization and speed up degradation; on the other hand, in warmer scenarios, pesticide application might grow [3].

As standard ecotoxicological tests use pre-set and relatively low temperatures, the following question arises: to what extent can these tests predict toxicological response to a certain chemical in waters with elevated temperatures? In the literature, contradictory data are available; some authors report an increase and others a decrease in toxicity at higher temperatures [4]. Several studies have already dealt with the temperature dependence of the effect of chemicals on aquatic organisms, which is often explained using the concept of Q10 (the doubling of the metabolic rate as a result of a 10 °C increase in temperature). The point of this is that as the temperature rises, the rate of metabolism increases, thus increasing the uptake and distribution of toxins [5–7]. Moreover, as the temperature rises, the solubility of oxygen in water decreases, which indirectly affects the response of organisms to toxic substances [8]. On the other hand, higher temperatures also enhance biochemical detoxification and the removal of chemicals [9].

The glyphosate-based formulation [N-(phosphonomethyl)glycine, CAS No. 1071-83-6] (in the following: glyphosate) that we used is a systemic, broad-spectrum herbicide, which has become the most frequently used herbicide globally [10]. The mode of glyphosate's

action is blocking the shikimic acid pathway by inhibiting enolpyruvyl shikimic phosphate (EPSP) synthase, resulting in stunted growth, leaf malformation, loss of green coloration, tissue damage, and finally, death [11]. Considering that glyphosate is a non-selective herbicide, it affects both weeds and crops [12]. Based on the research of Radwan and Fayez, the application of glyphosate, even at low doses, caused chlorosis and curling of the leaf edges on peanut plants, and significantly increased POD and CAT activity, which indicates oxidative stress [13]. However, its use is considered safe, as microorganisms decomposed it into phosphate and carbon dioxide within a short time when it entered into the soil [12].

During a large-scale monitoring program between 1990 and 2015, traces of pesticides found in surface waters were examined in Hungary. The results brought a startling realization, according to which pesticide residues were found in detectable quantities even in the majority of nature conservation or recreation areas [14]. Extremely high concentrations of pesticides found in the environment of industrial plants (in water paths flowing from artificial ponds and basins) are a particular concern [15].

In our study, the phytotoxicity of glyphosate (GLP) was assessed at different temperatures following the protocol of the standard *Lemna* sp. growth inhibition test [16]. *Lemna* sp. have been widely applied as representatives of non-target species (e.g., [17]) as GLP is well-known to affect non-target aquatic organisms [18]. GLP's effects have been well documented for this test organism [19]. Lewis and Thursby (2018) found duckweed to be amongst the species most sensitive to glyphosate [20]. Duckweeds are also representative test species, as they float on the surface. Lake surface water temperatures in many lakes worldwide show clear warming trends [21]. As GLP is highly water-soluble, water contamination by leaching into nearby waterbodies is a growing problem [22].

In addition to the growth rate and its inhibition, and the end-points originally described by OECD guidelines, peroxidase levels were also measured. Camp and Buchwalter (2016) highlight the importance of assessment of sublethal endpoints in acute toxicity assays when temperature-dependent toxicity is to be evaluated [23]. In the study by Passardi et al. (2005), peroxidases showed the strongest response in comparison to other biomarkers such as superoxide dismutase or glutathione reductase levels [24]. POD was found to be the most sensitive end-point in heavy-metal (CuO)-stressed *Lemna minor* (Linnaeus, 1753) [25]. In the comparative study by Radwan and Fayez (2016), POD showed the strongest response to GLP treatment of antioxidant enzymes [13]. POD activity measured in white mustard (*Sinapis alba*) seedlings treated with PAH extract showed a correlation with the amount of PAH accumulated by the plant [13].

The protocols used to assess the ecotoxic effect accurately describe the incubation temperature, which is advantageous from the point of view of comparability, but it is based on an average temperature that varies due to global climate change. The aim of our study is to learn about the effect of glyphosate on a wide temperature spectrum on the commonly used *Lemna minor* test subject, whereby in addition to growth inhibition, we also observe the POD enzyme activity.

2. Materials and Methods

A local clone of *L. minor* was isolated from plants collected in Tapolca stream, Hungary (46°51′01.6″ N 17°25′17.4″ E), and maintained as stock culture in Steinberg medium (OECD 2006). The collected plant material was identified by Dr Katalin Hubai, and voucher specimens were deposited at the Herbarium of the Department of Limnology, University of Pannonia, Veszprém. Prior the experiments, plants were acclimated for one month according to guidelines, at room temperature, in the prescribed growth medium and illuminated with ~8500 lux in a 16/8 light cycle.

Taifun® Forte (360 g/L glyphosate [isopropylamine salt], Adama Hungary Ltd., Budapest, Hungary) was used. It is recommended for arable lands as well as in horticulture and viticulture, for both mono- and dicotyledonous plant control. The different test concentrations were prepared from a stock solution by dissolving the commercial product in deionized water.

A range of nominal concentrations within 25–400 µg/L were selected (25, 50, 100, 200 and 400 µg/L), based on the study of Silva et al. [26]. This range covers the 112 µg/L PNEC value suggested for Italy [27]. Clean Steinberg medium was used as the negative control. A static test was performed with no renewal of the test medium, as herbicide application is normally a single event [28].

Some 15 healthy colonies consisting of 2 fronds each were placed in 150 mL Erlenmeyer flasks. Controls and test vessels were places randomly in an incubator (Velp Refrigerated Incubator FOC 225I), where they were repositioned every day to minimise the influence of spatial differences in light intensity or temperature. Tests were performed in accordance with the protocol defined by OECD (2006). The only significant diversion from the protocol was the temperature regime; the OECD protocol defines the standard temperature 24 ± 2 °C, while in our study, incubation temperatures were as follows: 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. The tests at different temperatures were performed in the same incubator one after the other, always at only one temperature at a time. Frond and colony numbers and the appearance of the colonies were recorded at the end of the exposure, after 7 days. Specific growth rates were calculated following the formula given by OECD guidelines:

$$\mu = \frac{\ln F_t - \ln F_0}{t} \quad (1)$$

where μ is the growth rate, F_t is the number of fronds after 7 days, F_0 is the initial number of fronds, and t is the exposition time (7 days).

$$Ir\% = \frac{\mu_k - \mu_t}{\mu_k} \times 100\% \quad (2)$$

where $Ir\%$ is the growth inhibition, μ_k is the growth rate of the control, and μ_t is the growth rate of the sample.

For measuring peroxidase activity and total protein content, frozen leaves were homogenised in ice cold mortar with phosphate buffer (50 mmol/L, pH 7), 1 mmol/L EDTA and 0.5 mmol/L PMSF. After 20 min centrifugation at 15,000× g at 4 °C, the supernatant was collected and TMB peroxidase activity (A 654) was measured according to the method of Imberty et al. (1984), with minor modifications [29]. The oxidation rate was estimated by measuring the absorbance change in A 654 nm, and enzyme activity in the U/mL extract was measured.

3. Results and Discussion

3.1. Growth Inhibition

The growth rate of the control was highest at 30 °C and lowest at 15 °C, starting at 10 °C, respectively: 3.026, 2.948, 3.456, 3.521, 3.704, and 3.026. Figure 1 and Table 1 show the growth inhibition recorded at different temperatures. The highest inhibition in the case of the higher concentrations applied (400 and 200 µg/L) was experienced at 30 °C. On the contrary, the two lower concentrations applied (100 and 50 µg/L) triggered the highest inhibition at 20 °C. No inhibition was experienced at lower or higher temperatures. At the lowest temperature tested (10 °C), no growth was experienced.

Table 1. The growth inhibition induced by each glyphosate concentration at the given temperatures.

Concentration	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
25 µg/L	1.51	−4.36	14.23	0.26	10.34	−3.95
50 µg/L	1.51	−4.84	18.69	13.24	10.19	−1.92
100 µg/L	−0.25	0.55	16.87	10.69	14.03	0.25
200 µg/L	−2.38	0.00	11.80	15.16	21.74	1.77
400 µg/L	−4.60	11.25	12.44	18.42	23.64	4.20

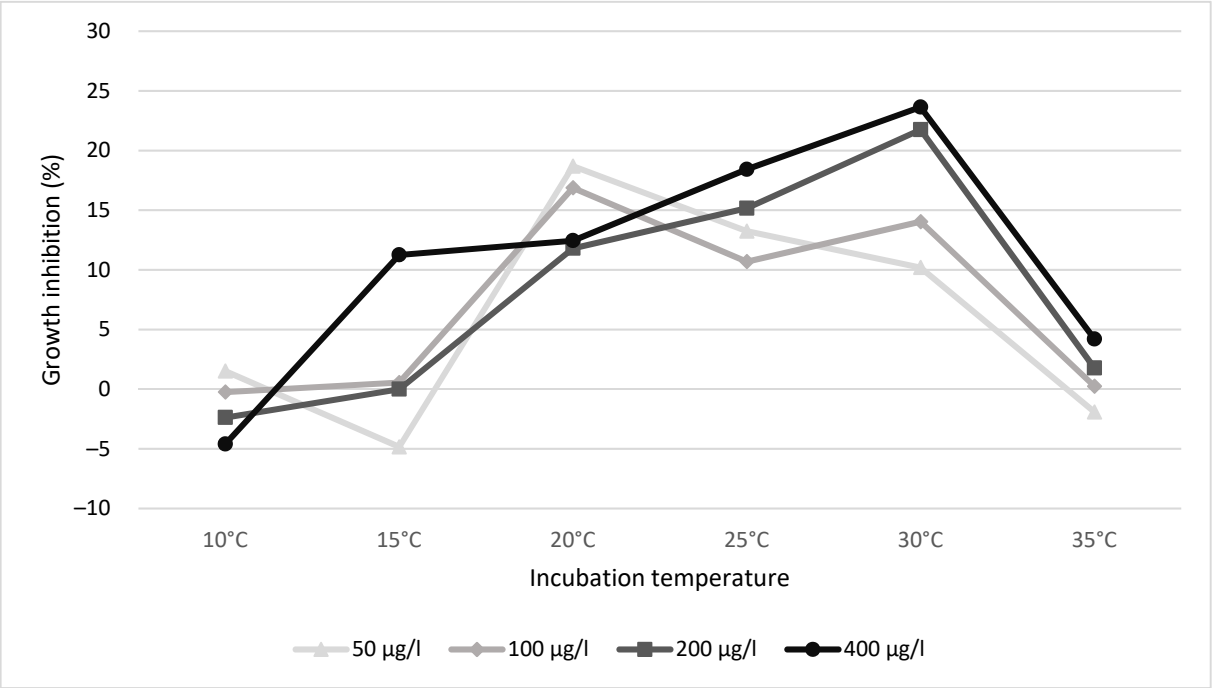

Figure 1. Growth inhibition expressed as a % of control at different temperatures.

Basiglini et al. (2018) assessed the phytotoxic effects of treated wastewater samples, on duckweed both under winter and summer conditions. Under winter conditions, no growth was reported either in the control or in the sample [30]. Seasonal variation in the growth rates of duckweed is a normal phenomenon [31]. Van Der Heide et al. (2006) measured the growth rate of *L. minor* at temperatures between 15 and 33 °C. The highest growth rate was experienced at 24 °C. Significantly lower rates were found at the two lowest temperatures, and modestly reduced growth rates at the two highest [32].

In the study of Rosenkrantz et al. (2013), herbicide flupyrsulfuron-methyl toxicity decreased by a factor of 2 at a lower temperature of 15 °C in comparison to 24 °C. The explanation given was that the growth rate of the plants was reduced at the lower temperature [33].

Photosynthetic activity is a temperature-dependent process, although some controversy surrounding this exists in the literature. Some authors report decreases but others increases in photosynthetic rates as a result of warming, most likely depending on species [34]. Inhibition of photosynthetic processes is generally responsible for phytotoxic effects, which in turn determine biomass [35]. Mateos-Naranjo and Perez-Martin (2013) exposed *Bolboschoenus maritimus* to sub-lethal doses of glyphosate herbicides, and found a significant decrease in biomass at higher concentrations, most likely due to a decrease in the net photosynthetic rate. Overall, GLP had negative impact on photochemical (PSII) apparatus [36]. Gomes et al. (2017) reported that glyphosate acid and its formulation roundup negatively affected the activities of enzymes associated with the mitochondrial electron transport chain in *Dimorphandra wilsonii* [37]. Da Silva Santos (2020) found that glyphosate decreased chlorophyll a and b contents and the photosystem II (PSII) photochemical activity, and also promoted oxidative stress [38]. Concentration-dependent induction of antioxidant enzymes such as peroxidase, catalase, ascorbate peroxidase, and superoxide dismutase was reported by Radwan and Fayez [13].

At higher temperatures, biochemical detoxification and elimination mechanisms will most likely increase, thereby lowering the toxic risk of pesticides [26]. This might explain why growth inhibition reduces at temperatures above 20 °C in cases of lower concentrations, i.e., 50 and 100 µg/L. However, it seems that the toxic effect(s) caused by higher concentrations (200 and 400 µg/L) cannot be counteracted.

It should be noted that the lower concentrations tested, 50 and 100 µg/L, trigger the highest growth inhibition at 20 °C. On one hand, this suggests that the aforementioned 112 µg/L PNEC value defined for Italy cannot be protective enough for non-target plants. A temperature of 20 °C was found to be optimal for growth in the study by Paolacci et al. [39]. Additionally, 20 °C is a relevant environmental scenario in temperate lakes; for example, the average temperature in Lake Balaton (Hungary) during summer is 22.5 °C (and on the hottest days, 26–28 °C) [40].

Environmental risk seems to appear at a high water temperature (30 °C) and high GLP concentration (in the range of 200–400 µg/L), and may be environmentally relevant, for example, to South America, where extremely high GLP concentrations were reported (such as 2777 µg/L in Colombia or 10,500 µg/L in Argentina) [41].

3.2. Peroxidase Activity

In general, chemical stress might trigger the overproduction of reactive oxygen species (ROS) in plants [42]. POD, an antioxidant defense enzyme, plays a major role in overcoming oxidative stress [43].

Figures 2–6 show the concentration–effect relationships of GLP concentrations and POD activity levels at different temperatures between 10 and 35 °C. Peroxidase activity is expected to show concentration-dependent increase, as reported by most studies, while no response has also been observed in cases of low levels of contamination [44]. On the contrary, high levels of treatment might trigger a significant decrease [42,45,46]. It may be assumed that cell and plasma membranes are damaged, and the plants' ability to counteract toxic stress is reduced. The sensitivity of plants, however, might be species-specific; in the study by Wang et al. (2015), atrazine triggered a decrease in the POD activity of *Scirpus tabernaemontani* in a concentration-dependent manner, but applying the same concentrations resulted in an increase in the enzyme activity in *Acorus calamus* [47].

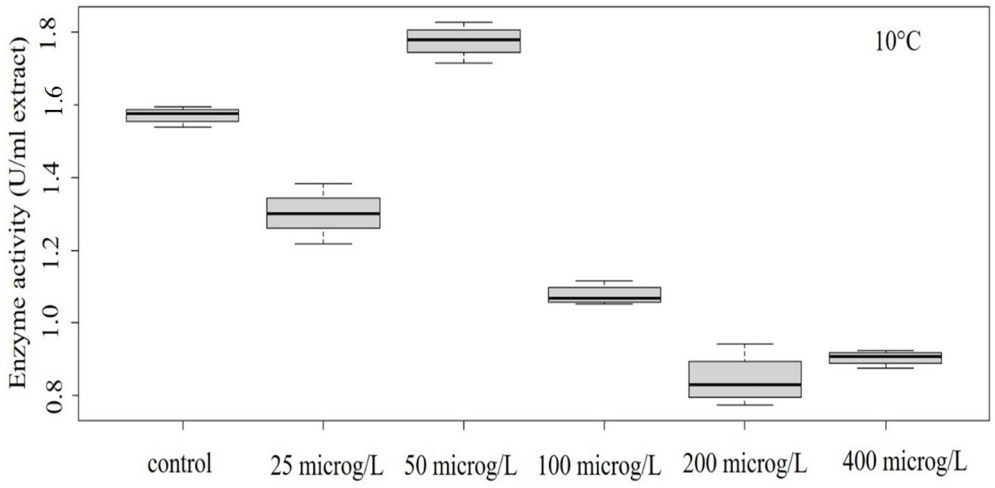

Figure 2. Concentration–effect relationship of enzyme activity at 10 °C.

In our study, decreased POD activity was detected in the temperature range of 10–30 °C (Figures 2–5). At 10 °C, the effect on enzyme activity caused by GLP was significant (ANOVA: Df = 5; F = 364.7; $p = 1.13 \times 10^{-12}$) (Figure 2). Significant effects could be also determined in the case of treatment performed at 15 °C (ANOVA: Df = 5; F = 5.776; $p = 0.00608$) (Figure 3) and at 25 °C (ANOVA: Df = 5; F = 28.52; $p = 0.00000289$) (Figure 4).

At 30 °C, the enzyme activity showed significant differences at different concentrations (ANOVA: Df = 4; F = 4.804; $p = 0.0121$) (Figure 5). At 35 °C, significant differences in enzyme activity were still obtained (ANOVA: Df = 5; F = 5.096; $p = 0.00977$) (Figure 6); however, a biphasic response was experienced (an increase in the protective enzyme concentration at lower concentrations, but a decrease at higher concentrations, indicating serious damage).

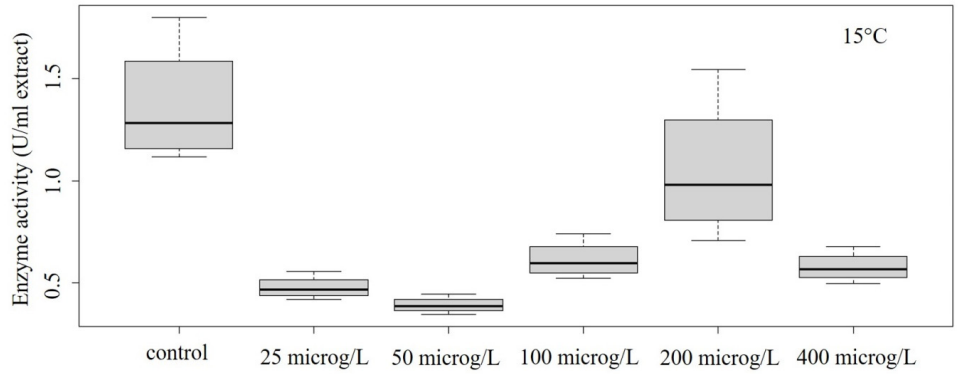

Figure 3. Concentration–effect relationship of enzyme activity at 15 °C.

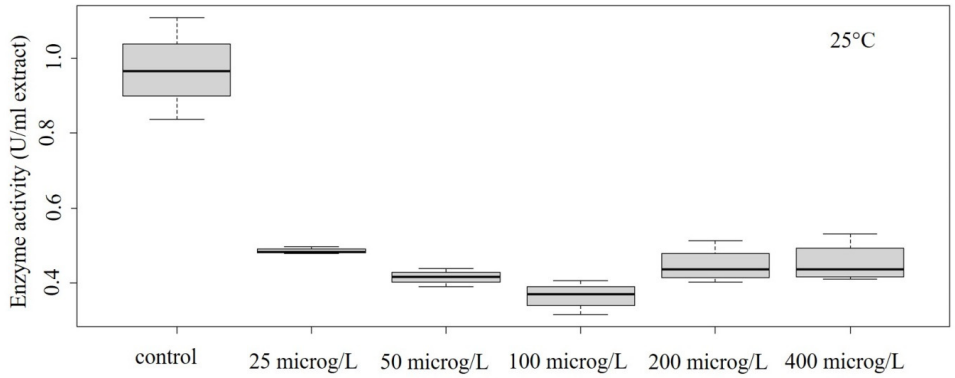

Figure 4. Concentration–effect relationship of enzyme activity at 25 °C.

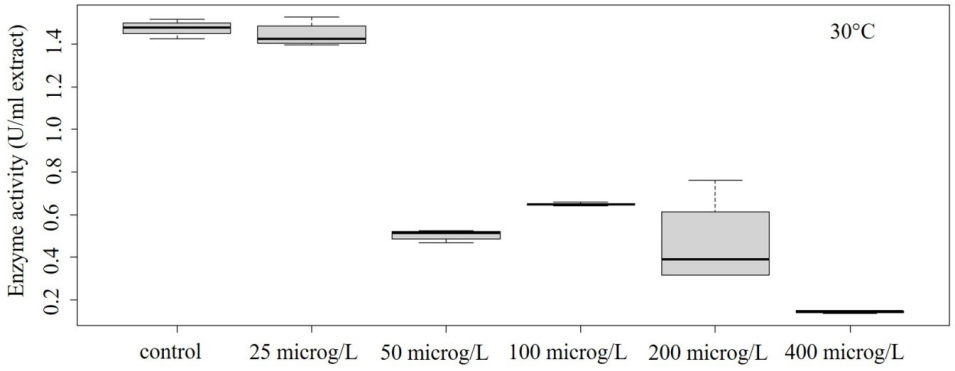

Figure 5. Concentration–effect relationship of enzyme activity at 30 °C.

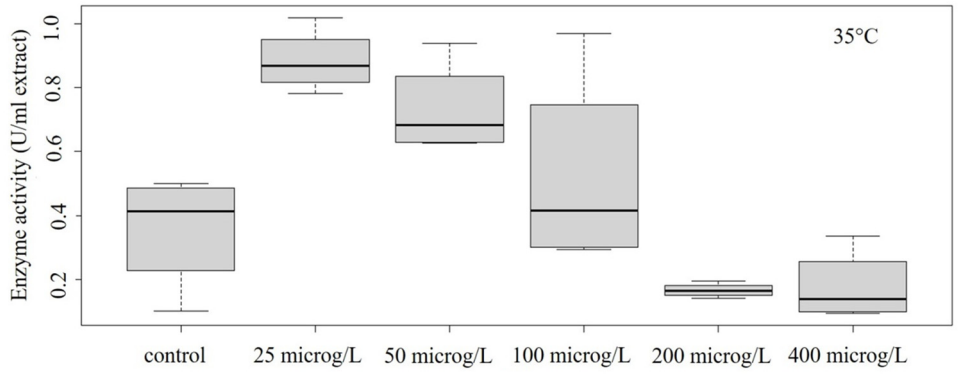

Figure 6. Concentration–effect relationship of enzyme activity at 35 °C.

A biphasic response of G-POD was observed in the study of Teisseire and Vernet [48]. *Lemna minor* test plants were treated with the phenylurea herbicide diuron. An increase in enzyme activity was experienced during the first part of the exposure, but later, a significant decrease was observed. Hu et al. (2018) found a significant increase in POD enzyme activity in *L. minor* after 10 µM Cu treatment, while a concentration of 20 µM decreased POD activity ($p < 0.05$) [49]. A similar biphasic response was reported in the study of Pouresmaeil et al. (2022), when the potential herbicidal effect of Moldavian dragonhead (*Dracocephalum moldavica* L.) essential oil was investigated [50]. Lower levels of potential damage can be attributed to faster metabolic processes, similar to growth inhibition [26].

GLP has been widely reported to increase ROS production, leading to oxidative damage [51]. Our results support that POD activity reflects oxidative stress even at very low concentrations. Smedbol et al. (2018), however, reported that only the highest glyphosate (500 and 1000 mg/L) concentrations triggered changes (increase) in peroxidase activity when a commercial GBH formulation, Factor540®, was tested on freshwater phytoplankton species. Comparison might suggest the increased sensitivity of *L. minor* either as test species or as a dominant member of freshwater associations [52].

Enzyme activity was found to be a more sensitive endpoint in comparison to biomass reduction when the phytotoxic effects caused by the herbicide trichloroacetate were evaluated. Geoffroy et al. (2004) also found antioxidant enzyme activity a more sensitive end-point than biomass when *L. minor* test plants were exposed to the herbicide flumioxazin [53].

It should be noted that predicted temperature increases will have a general effect on the toxicity of different chemicals used in aquatic environments. Moraies et al. (2023) demonstrated the synergistic effect of increased temperature and endocrine disrupters in coastal habitats [54]. Predictions suggest that the combined effects of pesticides and elevated temperatures will be present on different trophic levels, affecting whole communities, and will be most severe in cases of heatwaves [55]. Higher temperature regimes will most likely result in a higher prevalence of agricultural pests, which in turn require higher dosages of pesticides [56].

4. Conclusions

The phytotoxicity of glyphosate (GLP) was assessed using the *Lemna* sp. growth inhibition test in different temperature scenarios. In addition to growth inhibition, peroxidase activity was also measured. Growth inhibition showed a clear concentration- and temperature-dependent pattern. The lower concentrations tested, 50 and 100 µg/L, triggered the highest growth inhibition at 20 °C, while in cases of higher concentrations being applied (400 and 200 µg/L), the highest inhibition was experienced at 30 °C. No inhibition was experienced at lower or higher temperatures. In general, the growth rate of the plants is reduced at lower temperatures, while at higher temperatures, biochemical detoxification and elimination mechanisms will most likely increase. On the other hand, POD activity already produced aresponse at the lowest temperature tested, 10 °C. Decreased POD activity was detected in the temperature range of 10–30 °C, most likely indicating damage to cell and plasma membranes. In general, POD seems to be a more sensitive end-point in detecting the phytotoxic effects of GLP in different temperature scenarios.

Author Contributions: All authors contributed to the study conception and design. Conceptualization was performed by B.E.-V. and N.K.; data collection and analyses were performed by B.E.-V., K.H. and T.-A.S. The first draft of the manuscript was written by N.K. and all authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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