Glifosatın Su Mercimeği (*Lemna minor* L.)'nin Nişasta Birikimine, Klorofil ve Enzim Aktiviteleri Üzerine Etkileri*

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Özet

Bu çalışmada, Su mercimeği (*Lemna minor*) bitkisinin klorofil ve enzim aktivitesi üzerinde glifosat uygulamasının etkisi araştırdık. İlk olarak Erzurum İli'ndeki farklı su kaynaklarından ve tarımsal alanlardaki deşarj sularından *L.minor* bitkisi toplanmıştır. İkinci aşamada *L.minor* iki hafta aklimasyona tabi tutulmuştur ve su mercimeğine laboratuar ortamında 0,48 gL^{-1}; 2,4 gL^{-1}; 4,8 gL^{-1}; 19,2 gL^{-1} konsantrasyonlarında glifosat uygulanmıştır. Deneme sonucunda su örneklerindeki NH_3-N, NO_3^--N, NO_2^--N and PO_4-P değerleri ve bitkideki nişasta oranı (%), klorofil *a* ve klorofil *b* değerleri istatistikî açıdan önemli bulunmuştur ($p<0,05$). Katalaz enzim aktivitesi glifosat uygulamasına bağlı olarak değişim göstermiştir. Klorofil *a* ve klorofil *b* değerleri sırasıyla en yüksek ortalama 0,006 mgL^{-1} ve 0,011 mgL^{-1} olarak kontrol grubunda 14. günde saptanmıştır. Hem klorofil *a* hem de klorofil *b* için en düşük değer (0,000001 mgL^{-1}) 19,2 gL^{-1} glifosat konsantrasyonunda 14. günde bulunmuştur. Nişasta birikimi en yüksek (%11,18) 19,2 gL^{-1} konsantrasyon ve en düşük (% 11,15) 0,48 gL^{-1} konsantrasyon uygulamasında hesaplanmıştır. Bu çalışmaya göre, *L.minor* bitkisinin glifosatın uzaklaştırılmasında doğal bir arıtım yöntemi olarak kullanılabileceği tespit edilmiştir. Bunun yanı sıra glifosatın su mercimekleri üzerine etkilerinin tam olarak anlaşılabilmesi için histoptalojik çalışmalar ile beraber enzim aktivitelerinin araştırılması tavsiye edilmektedir.

Anahtar Kelimeler: Katalaz enzim aktivitesi, klorofil, *Lemna minor*, nişasta.

Effects of Glyposate on Starch Accumulation, Chlorophyll and Enzyme Activity of Duckweed (*Lemna minor* L.)

Abstract

We investigated that effect of glyphosate on chlorophyll and enzyme activity of duckweed (*Lemna minor*) in the study. The experiment consisted of two stages. Firs stage, *L. minor* was collected fresh water and drainage water of agriculture land in Erzurum. At second stage, *L. minor* plants has been acclimated before glyphosate treatment for 2 weeks and these plant sample were exposure with different concentrations of glyphosate (0.48, 2.4, 4.8 and 19.2 gL^{-1}) in laboratory conditions. NH_3^-N, NO_3^-N, NO_2^-N and PO_4^-P concentrations in water sample and starch content (%) of the plant, Chl-a and Chl-b concentration differences at $p<0.05$ were considered as statistically significant. Catalyze enzyme activity exhibited to change depending on glyphosate treatment. Chlorophyll-a and chlorophyll-b were determined the highest mean 0.006 mgL^{-1} and 0.011 mgL^{-1} in the control group on 14. day, respectively. The lowest mean for both of them were found 0.000001 mgL^{-1} at 19.2 gL^{-1} concentrations glyphosate exposure on 14. day. Starch accumulation was calculated to be the highest value (11.18%) at concentrations of 19.2 gL^{-1} and the lowest value (11.15%) at 0.48 gL^{-1} concentrations. According to results of the present study, *L. minor* was found as a natural purification method for the removal of glyphosate. Nevertheless, both histopathology disorder and enzyme activity should be investigated together to understand the effect of glyphosate on duckweed.

Keywords: Catalase enzyme activity, chlorophyll, *Lemna minor*, starch

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INTRODUCTION

In the recent decade, Duckweeds have been investigated as a convenient plant material for ecotoxicological research. A number of researchers have searched the removal of organic and metal contaminants in wastewater with different duckweed species (Appenroth et al., 2010; Leblebici and Aksoy 2011; Mechora et al., 2015; Song et al., 2015; Sree et al., 2015).

Duckweed absorbs organic substances by roots to prevent the growth of algae. The growth of duckweed depends upon the nutrient as ammonium and phosphate forms. Ammonium is important source for duckweed. Duckweed is preferred because of rapid growth rate, removal of high levels of nutrient removal and low fiber and high protein content (Landolt 1986; Körner et al., 1998). It is also used to improve the quality of the water from the facultative lagoons or stabilization ponds (Tchobanoglous and Burton 1991; Yılmaz et al. 2005). Phytotoxic and genotoxic in *Lemna gibba* were affected under metal exposure (Cakmak, 2012). Obermeier et al. (2015) reported that *Lemna* sp. might be used as a tool for phytoremediation of low-level contamination with metals and organic xenobiotics but some authors recommend a more detailed analysis of the development of the oxidative burst following copper exposure and of the enzymatic metabolism of pethoxamide in order to elucidate the extent of its removal from water.

Herbicide is the most common method used as weed control in the agricultural field. Chemical drugs for example herbicide, pesticide damaged plant and animal lives in these areas (Pérez et al., 2011). Ayoola (2008) determined increase of oxidative stress and death of fish larvae depending on the ratio of increased concentration of the herbicide.

Glyphosate (N- (phosphonomethyl) glycine) is a broad-spectrum systemic herbicide used to kill weeds, especially annual broadleaf weeds and grasses known to compete with commercial crops grown around the globe. It was discovered to be herbicide by Monsanto chemist John E. Franz in 1970 (Franz, 1970). The research conducted in Brazil was indicated that glyphosate [N- (phosphonomethyl) glycine] in the rice fields of the waste water of the aquatic ecosystem input simultaneously take place with the fish breeding season and thus posed a potential hazard to aquatic life (Giesy et al. 2000; Primel et al., 2005). For instance, Topal et al. (2015) have emphasized that glyphosate was negative effect on the antioxidant system and energy metabolism of juvenile rainbow trout.

Starch is a type of energy source stored mainly in tubers or seeds. Duckweed only has fronds as its dominant starch storage organ. The starch content in duckweed varied sharply in the different populations and growth periods of duckweed, which demonstrates the quality of duckweed biomass and needs careful management. Except for *Lemna aequinoctialis* and *Lamna punctata,* which maintained a lower starch content, all groups accumulated higher starch contents in the last stage. Negative relationship between starch content and growth rate was observed. At the same time, starch content was also negatively correlated with N and P contents (Xiao et al., 2013).

Catalyse (CAT) is a ubiquitous antioxidant enzyme that degrades hydrogen into water and oxygen (Iwase et al., 2013). Peroxidase (HRP) is a hemoprotein catalyzing the oxidation by hydrogen peroxide of a number of substrates such as ascorbate, ferrocyanide, cytochrome C and the leuco form of many dyes. While all peroxidise isozymes appear to catalyze the same reaction, the individual isozymes may differ markedly in physic-chemical and kinetic properties (Shannon et al., 1996; Resmankova and Sirova, 2007).

An increase in nutrient input is causing changes of ecosystem processes. Understanding of these change is important for both basic knowledge and management strategies of lotic and lentic ecosystems (Resmankova and Sirova, 2007). The aim of this study was to determinate the effect of herbicide on starch accumulation, chlorophyll quantitative and enzyme activity of *L.minor*.

MATERIAL and METHODS

Plant cultivation and glyphosate treatment

L.minor was collected from different fresh water and drainage water sources of agricultural land in Erzurum (Turkey). Our working sites were coordinated to 39°40.355'N-41°01.020'E. The plan was cultivated as descript before (Cirik et al., 2011). Plant was acclimatized before glyphosate treatment for 2 weeks under conditioning chamber (25±1°C, 350 μmolm^{2}s^{-1} PAR, 14-h photoperiod). After acclimatization, approximately 300 g of fresh mass was transferred 3 L glass-container in a modified Swedish Standard (SIS) growth medium (OECD, Annex A). The medium was replaced weekly. Plant was treated with different concentration of glyphosate (0.48, 2.4, 4.8 and 19.2 gL^{-1}) and it was set up with three replicates for each concentrations (for 2 weeks).

Determination of starch contention

Plant material (200 mg fresh weigh) was homogenised in 4mL 18% (w/v) HCL. The suspension was shaken for 60 min at 5 °C and centrifuged for 20 min at 5000 g. An aliquot was mixed with the same volume of Lugol's solution (0.5% w/v KI and 0.25% w/v I_2 in water) and measured at 605 nm and 530 nm. The calorimetric technique used was based on the method of Magel (1991) and the amount of starch per fresh weight (%) was calculated by using formula S=[Cs x Vol (extr) x 100]/FW, S: starch (%), Cs: A_{605}/(0.07757 x P+4.463), Vol (extr): volume of the plant extract (ml), FW: fresh weight (mg) (Appenroth et al., 2010).

Determination of chlorophyll analysis

Chlorophylls *a* and *b* were measured from each glyphosate concentration. We homogenized 0.1 g of each groups were extract in 10 ml 100% acetone solution in the dark for 4 days. After centrifugation at 10.000xg for 10 min, absorbances were taken 663 and 645nm. Chlorophyll *a* and *b* rates were calculated in each group according to (Smith et. al., 1988). Equations:

Chl-a= [12.7 (A_{663})-2.69 (A_{645})] x (V/1000 x G)

Chl-b= [22.9 (A_{645})-4.68 (A_{663})] x (V/1000 x G)

where A: absorbance (nm), V: 10 ml G: plant weight (g).

Catalyse enzyme activities

Plants samples (0.5 g) were homogenized in solution of 3 ml potassium phosphate (pH 7.8). The homogenised sample was centrifuged for 4 °C and 25 min at 14000 rpm.

Reaxion mixtures were prepared with both 50 mM buffer and 15 mM H_2O_2 (290 μL) and contained 10μl supernatant. Catalyse activity (CAT) was measured by spectrophotometrically the change in absorbance 240 nm (Aebi, 1984).

Water sampling analyses

Water samples were collected on day 7 and 14. The nesslerise method was applied to the water samples to determine the concentration of ammonia nitrogen (NH_3-N), calculated colorimetrically using the Nessler reactive reagent at a wavelength of 410 nm The nitrite nitrogen (NO^-_2-N) was determined by diazotizing with sulfanilamide and coupling with N-1-naphthylenediamine dihydrochloride to form a color azo dye; colorimetric measurement was then performed by spectrophotometer at 520 nm. In the nitrate nitrogen (NO_3^--N) analysis, after the reaction between nitrate ion and brucine, the absorbance of the yellow color was determined spectrophotometrically at 420 nm and total orthophosphate PO_4-P as molybdate-reactive phosphorus. (Anonymous, 1995). Total hardness, Ca-hardness and alkalinity were measured by titrimetric method and total suspended solid matter was analyzed according to the American Public Health Association (Anonymous, 1995).

Statistical analysis

The statistical analyses were used with Multivariate. IBM SPSS program was used for statistical analysis. The significance of the difference between variability data and validity result was determined by least significant difference *LSD* test. Differences at $p<0.05$ were considered to be statistically significant. (Kesici and Kocabaş, 2007).

RESULTS and DISCUSSION

L. minor was collected from freshwaters in agriculture field and the plant was treated with different concentrations of glyphosate to containing 0.48, 2.4, 4.8, 19.2 gL^{-1}. These doses were detected by calculating both primarily experimental doses and used in the agriculture field. Chlorophyll-a, chlorophyll-b, ammonium-nitrogen, nitrite-nitrogen, nitrate-nitrogen and total orthophosphate values were significantly different among the each treatment ($p<0.05$). The whole sampling of the exposure to four glyphosate concentrations after 7 days showed little mortality but death of almost all samples especially exposure 19.2 gL^{-1} concentration after 14 days (Fig 1) occured. Previous studies found that the growth the duckweed, as measured by increased numbers of fronds or increased wet or dry weights was relatively insensitive to glyphosate dissolved in the culture medium (Lockhart et al., 1989).

Figure 1. *L.minor* was showed growing during study period. (a) plant acclimation for 7 days, (b) glyphosate treatment on 7 days, (c) glyphosate treatment on 14 days

L.minor was cultured in experimental condition and exposed to glyphosate to contain 0.48, 2.4, 4.8, 19.2 gL^{-1} concentrations, and chlorophyll, starch accumulation and enzyme activity were observed. The survivor rate reduced when glyphosate dose increased but

these did not stop on starch accumulation and enzyme activity for instant, the highest starch accumulation was calculated at concentrations of 19.2 gL^{-1} on day 14. According to Xu et al. (2011) this situation was due to the starch accumulation in duckweed plants to trigger under nutrient starvation stress. However, chlorophyll decreased in all treatment groups compared to control group was observed. Previous studies have observed that on nitrogen- or phosphate-deficient medium, vegetative growth of duckweed was quickly reduced and eventually ceased. (Xiao et al., 2013).

Chlorophyll-a and chlorophyll-b were determined to be the highest mean 0.006 mgL^{-1} and 0.011 mgL^{-1} for the control group on day14, respectively. The lowest mean for both of them were found 0.000001 mgL^{-1} at 19.2 gL^{-1} concentrations glyphosate exposure on day 14. The control groups survived and created new plant period of 14 days. In week 7, the significant decrease of photochemical efficiency in both 4.8 gL^{-1} and 19.2 gL^{-1} was observed but concentration of 2.4 gL^{-1} glyposate increased in comparison to the other group (Fig 2). The high value of chlorophyll-a was calculated in control groups on days 7 and 14. However, after glyphosate treatment was observed plant died and a decreased level of chlorophyll *a* and *b* were determined. *L.minor* increased in growth medium on 7 days albeit NH_3-N and NO_3^--N values decreased on day 14. Nitrate can be used by duckweed, was constantly found in the duckweed culture pond for growing (Xu et al., 2011). Furthermore plant was exposured to glyposate, absorbed through roots and effective on actively growing plants, but on day 14 chlorophyll-a value was reduced. Results of studies with other toxic substances were found similar data for example *L.minor* was exposed to 1 and 10 mgL^{-1} Se treatment. A concentration of 1 mg Se did not affect photochemical efficiency, while higher concentrations of selenite (10 mgL^{-1} Se) are toxic for duckweed plants (Mechora et al., 2015). Different Lemnaceae species expressed different sensities to the CuNP suspension and cupper nitrate also were showed limitation for grow rate of these species (Song et al., 2015).

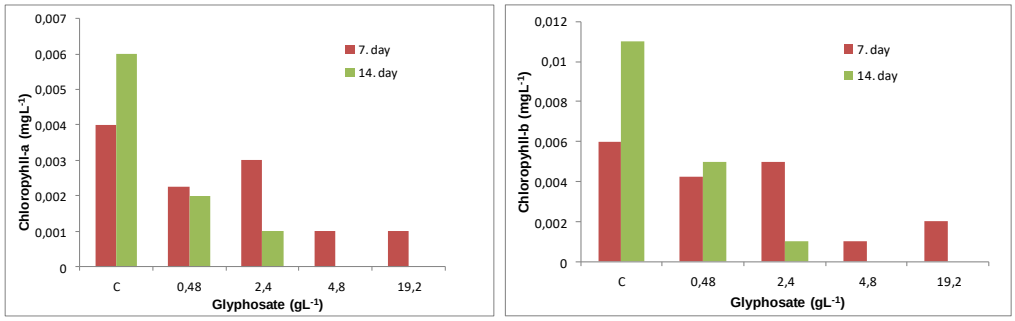

Figure 2. Influence of glyposate on the chlorophyll *a* and *b* in duckweed

In the present study, the influence of different concentrations of glyphosate on starch accumulation was statistically significant ($p<0.05$). The starch accumulation was found to be the highest value at concentrations of 19.2 gL^{-1} (11.18%) and the lowest value at 0.48 gL^{-1} dose (11.15%) (Fig 3). The glyphosate treatment on starch accumulation was not significant both control groups and at concentrations of 0.48 gL^{-1}. During the first seven-day period was observed not only increase of plant growth rate but reduce of starch accumulation were observed. Starch percentage increased fast during nutrient starvation (Tao et al.,2013). Sree et al. (2015) reported relationship between starch accumulation in

plants and Co^{+2} treatments, because in 4 days period Co^{+2} content increased with starch accumulation but reduced of relative growth rate.

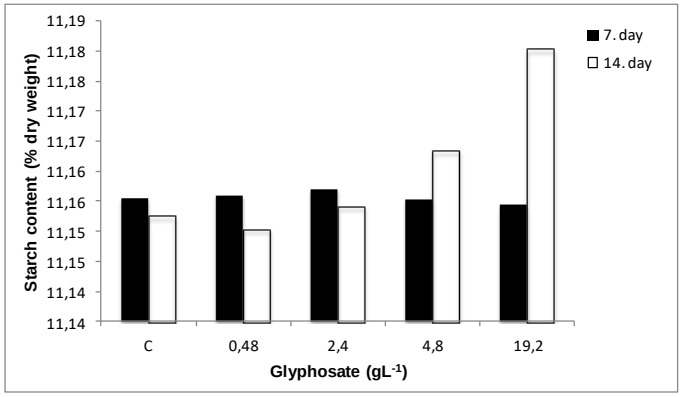

Figure 3. The amount of starch content in duckweed, different concentration of glyphosate

Glyphosate is not only toxic matter but organic matter thus starch content has been negative effected glyphosate treatment for 7 days period. After this period, plant was under toxin stresses and growing was blocked and plant give reaction to toxin so starch accumulation was increased. Under nutrient starvation condition, starch content of duckweed was substantially increased and the total amount of starch tripled after 10 days of starch accumulation (Xu et al., 2011). Salt stress was exposed on to ten of 34 duckweed clones and salinity stress was influent than nutrient limitation and heavy metal stress in level of starch accumulation in these clones (Sreeet al., 2015).

One of the effect on starch accumulation is NH_4–N and PO_4–P values. The search of 300 m^2duckweed pond (a total amount of 9.07 kg NH_4–N and 0.85 kg PO_4–P were removed) was found starch continent reached 29.8% after duckweed grown in well water for eight days (Xu et al., 2011). Glyphosate molecule formulae is consists of NH and P and this research showed that NH_3-N and PO_4-P value were the highest on 7 days but starch content was lowest. It seemed that there was a negative relationship between starch content and N and P content (Xiao et al., 2013).

Catalyse enzyme activity in all treatment were higher at the sampling date (Fig 4). Catalyse enzyme activity exhibited change depending on glyphosate treatment. This enzyme showed higher concentration on 14 days in exposure 0.48 gL^{-1} and 19.2 gL^{-1}, and its values were 1.65 mgL^{-1} and 3.8 mgL^{-1}, respectively. Even though, the enzyme concentrations on day 7 in exposure 4.8 mgL^{-1} concentrations and control group, were 0.5 gL^{-1} and 0.7 gL^{-1}, respectively. Glyphosate is the herbicide which is stress on plant growing mechanism and this stress trigger to enzyme activity. *L.minor* contains 2.4 gL^{-1} concentrations the most effective glyphosate toxin and its catalase enzyme activity was increased. According to Kielak et al. (2011), glyphosate may inhibit the chlorophyll synthesis by reducing the formation of the daminolevulinic acid (ALA) as a porphyrin precursor. On the other hand, porphyrin is an integral part of some antioxidative enzymes such as CAT, APX and cytochromes as well.

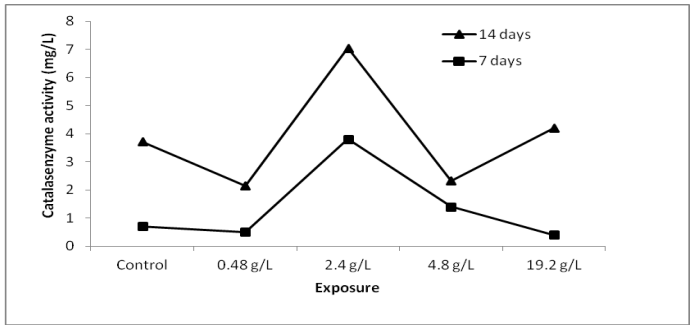

Figure 4. Catalase enzyme activity in duckweed, different concentration of glyposate

The influence of different glyphosate treatment on water quality parameters was investigated for period of 14 days. The highest NH_3-N value was determined at concentrations 19.2 gL^{-1} and 4.8 gL^{-1} in 7 days, the lowest value was detected at concentrations 19.2 gL^{-1} in 14 days. The highest and lowest values of NO_3^--N were between 224.73 ± 63.19 mgL^{-1} and 139.50 ± 61.51 mgL^{-1}. The highest and lowest values of NO_2^--N values were estimated at a dose 19.2 gL^{-1} not only on day 7 (0.17 ± 0.00 mgL^{-1}) but also on day14 (0.01 ± 0.00 mgL^{-1}). The highest PO_4-P was observed at a dose 2.4 gL^{-1} (0.23±0.00 mgL^{-1}), while the lowest PO_4-P was found at a concentrations 4.8 gL^{-1} (0.00±0.00 mgL^{-1}) (Fig 5).

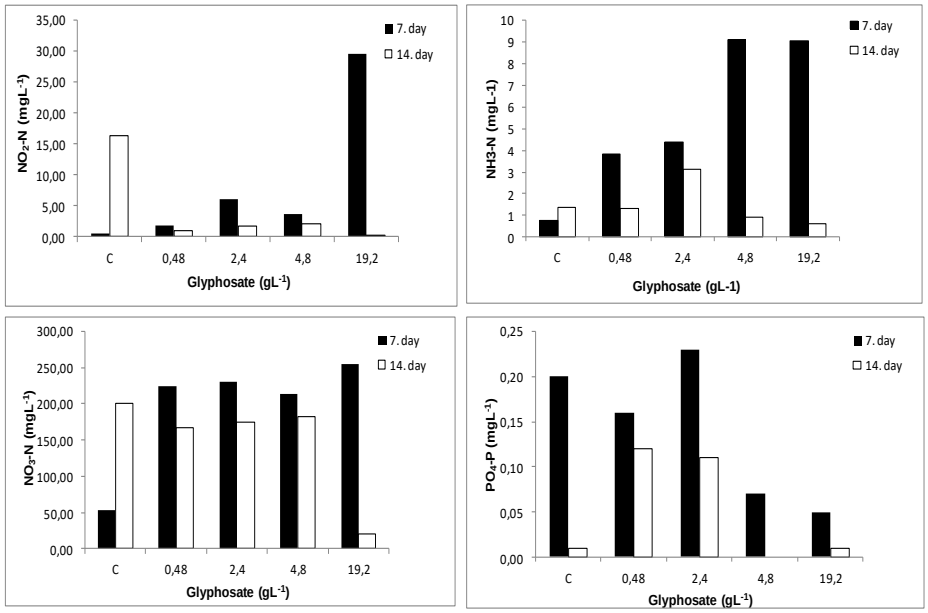

Figure 5. The changes in NH_3-N, NO_3-N, NO_2-N and PO_4-P concentration with different glyphosate concentration during periods of 7- 14 days.

Duckweed is an important plant for removing organic material in the water and studies also demonstrate better performance of nitrogen and phosphorus removal in the planted wetland systems (Sims et al., 2013). Phosphorus also is removed from the water by chemical precipitation and sludge removal (Smith and Moelyowati, 2001). In this experiment, nitrogen enrichment (NH_3-N, NO_3^--N and NO_2^--N) concentrations in the

water of whole groups on day 7 were lower than NH_3-N concentrations in the water at 19.2 gL^{-1} dose on day 14. A reduction in the PO_4-P concentration was observed on day 14. Leblebici and Aksoy (2011) reported that nitrate, phosphate and sulphate concentration in water decreased with treatments in *L.minor* and *S.polyrhiza*. According to Ge et al. (2012) *L. minor* efficiently recovered the nitrogen and phosphorus nutrients in the SL wastewater. 100% of the NH_4-N, 75.0% of the NO_3-N (trace) and 74.8% of the PO_4-P in the SL wastewater were removed by the duckweed after 18 days of culture. *L. gibba* is a useful reference that this species is affected by representative environmental concentrations of glyphosate found in water bodies of agroecosystems of the Pampa's plain (Sobrero et al., 2007). Hence, this results show that duckweed is even more effective in a range of wastewater applications.

Using glyphosate in agricultural areas is dangerous for carcinogenic effects on humans according to WHO (World Health Organisation). Herbicide applications have negative effects not only on directly to human health but also with discharging to aquatic ecosystems. Aquatic plants seem to be better protected by the larger distances to the sprayed fields required for potentially toxic herbicides, by adsorption of some of the drift by bank vegetation 29 and probably also by dilution of the herbicides in water (Cedergreen and Streibig, 2005). To conclude, *L. minor* was found as a natural purification method for the removal of glyphosate. Histopathologic disorder and enzyme activity should be researched together to understand the effect of glypohosate on duckweed.

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