THE USE OF THE BIOMARKERS CHLOROPHYLLS AND CAROTENOIDS, FOR THE INTERPRETATION OF THE EFFECTS IN *LEMNA MINOR* AFTER EXPOSURE OF TWO HERBICIDES WITH DIFFERENT MODE OF ACTION

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EXTENDED ABSTRACT

The impact of herbicides in aquatic vascular plants is often investigated in toxicity laboratory studies. *Lemna minor* is a fast growing aquatic vascular plant regularly used as a bioindicator in ecotoxicological dose-response studies. Chlorophylls and carotenoids are among the most commonly used biomarkers when monitoring the effects of toxicants. For this study we chose two herbicides, tritosulfuron and metribuzin, with different modes of action. Tritosulfuron is a sulfonylurea herbicide, an amino acid synthesis inhibitor. Metribuzin, on the other hand, is a triazinone herbicide, a photosystem II inhibitor.

In the study we investigated changes in content of photosynthetic pigments, in particular chlorophylls a, b and carotenoids, caused by tritosulfuron and metribuzin in *Lemna minor*. We also evaluated these changes compared to growth rate (based on frond number) endpoint on this species. The toxicity of the two herbicides was assessed by growth inhibition tests in *Lemna minor* based on standard OECD protocols (7 day test). Growth inhibition, caused by the two herbicides, was measured daily from day 0 until day 7. The four effect concentrations chosen (EC_{100}, EC_{75}, EC_{50}, EC_{25}), were evaluated from pre-tests. The amounts of chlorophyll a and b as well as carotenoids were measured spectrophotometrically on days 1, 3 and 5 after exposure.

Obtained results showed that adverse effects on *Lemna minor*, based on growth inhibition caused by the herbicides, were not clearly connected with a decrease in chlorophyll a, b and carotenoids content. After exposure to various concentrations of tritosulfuron, *Lemna minor* chlorophyll and carotenoid content showed a decrease which was more intense as the time of exposure increased. For metribuzin, although there was a decrease in pigment content in high concentration treatments on day 3, on day 5 there was no evident effect in pigment concentrations in any treatment. On the other hand, growth rate was reduced with the increase of the concentration of both herbicides on days 3 and 5; growth rate differed statistically between all concentration treatments, except the lowest concentration.

We can conclude that for both herbicides growth rate is a more sensitive endpoint to measure the toxicity than pigments. Moreover, tritosulfuron (the amino acid biosynthesis inhibitor) affects pigment concentration more promptly and more intensely than metribuzin (the photosystem II inhibitor) in concentrations causing similar growth inhibition. Further research on the ways that chlorophylls and carotenoid are affected by these two herbicide categories is necessary.

Keywords: Growth inhibition, Chlorophyll, Carotenoid, *Lemna minor*, herbicide, biomarker

1.INTRODUCTION

One of the main factors that negatively affect not only the agricultural environment but also ecosystems, human and animal health, is the existence of residues of plant protection products. The addition of chemicals foreign to the ecosystems entails significant dangers and hence, it is studied extensively by researchers. One of the scientific sectors that study this subject is ecotoxicology. More particularly, ecotoxicology deals with the effects of xenobiotic factors in different ecosystems (Arapis, 1998).

One of the xenobiotic factors whose ecotoxicological effects are studied is the plant protection products. Although they are applied to particular crops, they often affect other ecosystems as well. The fate and behavior of plant protection products are processes of various biotic and abiotic parameters. Regardless of the manner in which they are applied, plant protection products are involved in the processes of absorption, transfer, removal, transformation and their degradation in the environment. Leaching of plant protection products into deeper soil layers can pollute underground aquifers. Plant protection products can also be transferred with spray clouds, leaching and surface water runoff (rivers, lakes, seas). All the above can affect aquatic organisms. For the study of the risks associated with the use of plant protection products on non-target organisms, and the environment, and for marketing authorization for new plant protection products, toxicity tests and studies of the fate of the environment and the behavior of plant protection products are required (specific EU directives, among which 91/414/EC for plant protection products, as well as international organization directives-from WHO and FAO). The purpose of these studies is, among others, data selection regarding toxicology in humans and the effects on non-target organisms and the environment (Ziogas and Markoglou, 2007).

In order to evaluate the consequences of the use of plant protection products and toxic xenobiotic substances on the environment, suitable organisms (bioindicators), such as birds, mammals, beneficial insects, bees, soil organisms, aquatic organisms (fish, crustaceans, algae, aquatic plants and sediment organisms) and non target plants areutilised. In the last few years the evaluation of the hazard of xenobiotic particles of plant protection products on ecosystems has been studied with the use of biomarkers.

A biomarker is an indicator signaling an event or condition in a biological system or sample, giving measure of exposure, effect or susceptibility. Such an indicator may be a measurable chemical, biochemical, physiological, behavioral or other alteration within an organism (IUPAC). Examples of such changes are changes in enzyme activity, changes in hormones and in protein levels as well as changes in chlorophylls and carotenoids.

Lemna minor is a fast growing aquatic vascular plant regularly used as a bioindicator in ecotoxicological dose-response studies. In this study *Lemna minor* was used to assess the toxicity of two herbicides with different modes of action. The toxicity was studied with the use of different endpoints. According to the OECD 221 protocol for *Lemna* sp. growth inhibition testing, one of the recommended endpoints is the *Lemna* frond number-growth rate based on frond number. Chlorophyll and carotenoid content are some of the biomarkers often used as endpoints as well (Cedergreen and Streibig, 2005). The above mentioned endpoints were evaluated as far as their results are concerned.

2.MATERIALS AND METHODS

Lemna minor was collected from an artificial pond of the Agricultural University of Athens campus. The plants were disinfected by immersion in 0.5% (v/v) NaClO solution for 4 min and then rinsed with distilled water, according to OECD 221 protocol. They were aseptically cultivated in Erlenmeyer flasks in Steinberg medium, pH 6.8, and maintained at 24^0C, under continuous illumination (photon flux density of 60-90 μmol/m^2/s) provided by cool white fluorescent lamps. Both the flasks and the medium were autoclaved and sealed with cotton wool. Plants were transferred weekly to new media under aseptic conditions.

Treatments were performed with two herbicides with different modes of action. Technical material of both herbicides was used. Metribuzin (technical grade, 95.3% purity) is a photosystem II inhibitor, which belongs to the chemical class of triazinones. Tritosulfuron (technical grade, 99.59% purity) is an amino acid synthesis inhibitor, which belongs to the chemical class of sulfonylureas. All herbicide concentrations shown are nominal concentrations. The experiments were conducted under static conditions. Herbicide stock solutions were prepared in methanol, with all the solutions prepared just before the experiments were begun. Herbicide stocks were added to plant test medium to obtain the highest test concentration. This solution was then diluted using additional medium to obtain the other concentrations; four treatment concentrations per herbicide. Plants without addition of toxicant were used as controls.

At the beginning of the trial (day0), *Lemna minor* plants were transferred to 1000ml beakers containing culture medium and the dissolved herbicides concentrations, found in Table 1. These concentrations were chosen based on pre-tests, according to which, the chosen concentrations cause 100%, 75%, 50%, 25% and 0% inhibition of growth. Growth inhibition was calculated based on the average specific growth rate of *Lemna minor* according to the number of fronds. More specifically, the average specific growth rate was calculated according to the following equation:

$$\mu_{ij} = (\ln(N_j) - \ln(N_i))/ t$$

where μ_{ij} is the average specific growth rate from time i to j, N_j is the number of fronds at time j, N_i is the number of fronds at time i and t is the time period from i to j.

Percent inhibition of growth rate was calculated according to the following equation:

$$\%I_r = [(\mu_c-\mu_T)*100]/\mu_c$$

where I_r is the percent inhibition in the average growth rate, μ_c is the mean value for μin the control and μ_T is the mean value for μof each treatment. The duration of the test is 7 days (from day 0 to day 7) according to the OECD 221 protocol.

Table 1. The concentrations in μg/l of the two active substances used in the trial, based on the results of the pre-tests

Active substance	**Concentrations in μg/l**			
	C1	**C2**	**C3**	**C4**
Metribuzin	380	95	55	30
Tritosulfuron	100	60	30	10

At the beginning of the experiment 12 repetitions of each treatment (4 concentrations per active substance and control) with 50 Lemna fronds each were prepared for each herbicide (total of 120 observations). *Lemna minor* plants were photographed daily in order to estimate the total frond number, which was counted by using digital macro photography combined with image analysis software (Image-Pro Plus Version 3.1). On days 1, 3 and 5 of herbicide exposure chlorophyll and carotenoid content were determined for all concentrations and controls of both herbicides (3 replicates per treatment). Plants, without their roots, were dried on a paper towel and transferred to a ceramic mortar, where they were frozen in liquid nitrogen and ground to powder with a pestle. On a four decimal balance 40mg of ground fronds per replicate were used for chlorophyll and carotenoid extraction performed with 95% ethanol. 1ml of ethanol was added and the homogenate was centrifuged (15 min, 4000 RPM, 4°C in JP SELECTA Centrifuge). The supernatant was transferred to a 3ml cuvette and a second extraction was performed again with1 ml of 95% ethanol. After the centrifugation the supernatant was transferred to the 3ml cuvette as well. The extraction was performed three times in total. All the work was carried out at 4 ^{0}C. The absorbance of pigment extract was measured at wavelengths of 665, 649 and 470 nm with a spectrophotometer (HITACHI U-1100). The quantitative determination of chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoids was calculated in accordance with the equations described by Lichtenthaler (Lichtenthaler, 1987). More particularly, chlorophyll and carotenoid content was calculated using the following equations:

$C_a = 13.36A_{665} - 5.19A_{649}$
$C_b = 27.43A_{649} - 8.12A_{665}$
$C_{x+c} = (1000A_{470} - 2.13C_a - 97.64C_b)/209$
where C_a, C_b, and C_{x+c} is the content (in μg/ml) of Chla, Chlb and total carotenoids, respectively, and A_{665}, A_{649} and $A_{470}$is the absorbance at 665, 649 and 470nm.According to the chlorophyll content of the extract, the chlorophyll content per gram fresh weight was calculated.

3.RESULTS

The toxicity of both herbicides on the growth rate of *Lemna minor* at the end of day 7 was estimated as mentioned above. The control average specific growth rate was 0.3040 for metribuzin and 0.2994 for tritosulfuron, both above the limit for test validation in the average specific growth rate of 0.275d^{-1}(OECD 221 protocol); for this reason the test is valid.

All data were analysed statistically with one-way analysis of variance (ANOVA) followed by Student's t-test, using JMP-10 software. P values less than 0.05 were considered significant.

The growth rate inhibition of the four concentrations, from the highest to the lowest concentration, on day 7 compared to control were 98.49%, 65.19%, 30.02%4.52%and 84.86%, 64.90%, 7.77% and 4.35% for metribuzin and tritosulfuron respectively. Growth rates relative to the controls on days 1, 3 and 5 are shown in Figures 1 and 2. Growth rate based on frond number was reduced with the increase of the concentration of both herbicides on days 3 and 5 and growth rate differed statistically between all concentration treatments, with the exception of the lowest concentration. On day 1 growth rate does not seem to be affected by either herbicide. Only the treatment with the highest concentration of metribuzin shows a low growth rate when compared to all other concentrations and control. Despite the fact that for both herbicides the inhibition on growth rate caused by the two highest concentrations on days 3 and 5 was intense, chlorotic symptoms were evident only

for tritosulfuron at the two highest concentrations. On day 7, chlorotic symptoms were evident in *Lemna minor* plants treated with both herbicides, but fronds treated with tritosulfuron were completely discolored.

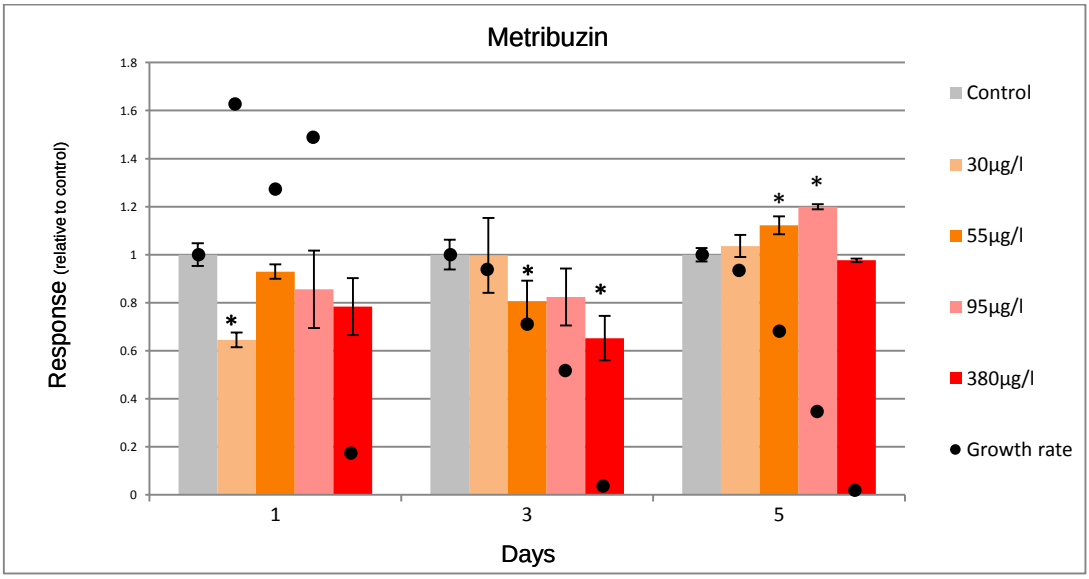

Figure 1. Chla content (columns, n=3) at various concentrations on 3 different days for metribuzin treatments. Filled symbols represent the specific growth rate. All responses are given relative to the controls as mean ± standard deviation. Statistically significant differences compared to control are marked with an asterisk.

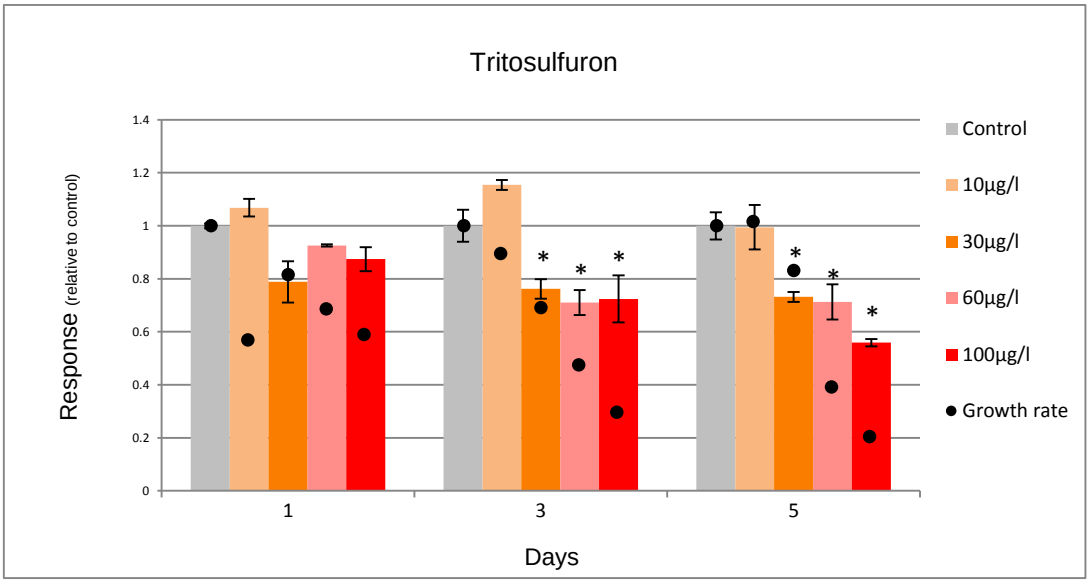

Figure 2. Chla content (columns, n=3) at various concentrations on 3 different days for tritosulfuron treatments. Filled symbols represent the specific growth rate. All responses are given relative to the controls as mean ± standard deviation. Statistically significant differences compared to control are marked with an asterisk.

Chla, Chlb and carotenoid content in *Lemna minor* frond tissue, after exposure to the herbicides for days 1, 3 and 5, are presented in Tables 2 and 3. The average pigment contents of control, on fresh weight basis, are within the range found for *Lemna minor* in other studies (Hou et al, 2007). In Figures 1 and 2 the Chla content and specific growth rate is presented, relative to control, at various concentrations at different time periods for the herbicides tritosulfuron and metribuzin. Chla was the pigment most affected by both herbicides compared to the other two, as can be seen in Tables 2 and 3, which is in compliance with other studies (Geoffroy et. al., 2004).

Table 2. The toxic effects of metribuzin on photosynthetic pigment contents in *Lemna minor* on days 1, 3 and 5 of exposure. Values represent means ± SE (n=3). Statistically significant differences compared to control are marked with an asterisk.

Concentration (µg/l)	Day 1			Day 3			Day 5		
	Chla mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)	**Chla** mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)	**Chla** mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)
0	0.489±0.027 (100)	0.223±0.013 (100)	0.152±0.007 (100)	0.595±0.021 (100)	0.261±0.009 (100)	0.162±0.005 (100)	0.459±0.017 (100)	0.203±0.009 (100)	0.141±0.005 (100)
30	0.315±0.018* (64.44)	0.155±0.009* (69.49)	0.096±0.004* (63.09)	0.593±0.053 (99.64)	0.291±0.024 (111.27)	0.165±0.012 (101.84)	0.476±0.026 103.59	0.245±0.013 (120.76)	0.149±0.003 (106.14)
55	0.455±0.017 (92.94)	0.222±0.007 (99.66)	0.132±0.005 (86.95)	0.480±0.023* (80.68)	0.241±0.013 (92.30)	0.136±0.007 (83.93)	0.516±0.022 (112.25)	0.256±0.011 (126.26)	0.152±0.005 (107.94)
95	0.419±0.093 (85.56)	0.203±0.045 (91.28)	0.120±0.027 (79.12)	0.490±0.038 (82.33)	0.235±0.016 (90.11)	0.132±0.008 (81.33)	0.551±0.006 (119.97)	0.259±0.004 (127.84)	0.159±0.001 (112.77)
380	0.384±0.068 (78.39)	0.185±0.033 (83.20)	0.108±0.019* (71.53)	0.388±0.021* (65.17)	0.186±0.009* (71.10)	0.114±0.006* (70.30)	0.449±0.004 (97.63)	0.201±0.005 (99.15)	0.135±0.002 (95.61)

Table 3. The toxic effects of tritosulfuron on photosynthetic pigment contents in *Lemna minor* on days 1, 3 and 5 of exposure. Values represent means ± SE (n=3). Statistically significant differences compared to control are marked with an asterisk.

Concentration (µg/l)	Day 1			Day 3			Day 5		
	Chla mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)	**Chla** mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)	**Chla** mg/g FW (% of control)	**Chlb** mg/g FW (% of control)	**Carote-noids** mg/g FW (% of control)
0	0.381±0.007 (100)	0.176±0.004 (100)	0.124±0.012 (100)	0.409±0.035 (100)	0.177±0.018 (100)	0.121±0.013 (100)	0.418±0.029 (100)	0.187±0.010 (100)	0.144±0.010 (100)
10	0.407±0.018 (106.84)	0.183±0.009 (103.90)	0.133±0.007 (107.72)	0.472±0.013 (115.43)	0.217±0.013 (121.97)	0.143±0.001 (118.84)	0.416±0.048 (99.44)	0.184±0.018 (98.34)	0.131±0.004 (90.88)
30	0.300±0.045 (78.85)	0.138±0.017 (78.60)	0.099±0.015 (80.74)	0.311±0.026* (76.17)	0.143±0.012 (80.38)	0.115±0.012 (95.44)	0.306±0.011* (73.18)	0.141±0.005* (75.65)	0.116±0.003 (80.14)
60	0.352±0.002 (92.56)	0.166±0.001 (94.49)	0.114±0.002 (92.98)	0.291±0.027* (71.08)	0.134±0.013* (75.54)	0.111±0.014 (91.89)	0.298±0.038* (71.22)	0.149±0.024 (79.91)	0.131±0.010 (90.49)
100	0.333±0.026 (87.43)	0.162±0.012 (92.74)	0.103±0.009 (83.22)	0.296±0.020* (72.41)	0.138±0.012* (77.78)	0.112±0.004 (92.69)	0.234±0.008* (55.91)	0.117±0.008* (62.81)	0.111±0.003* (76.73)

Pigments on day 1 of exposure do not seem to be affected by herbicides, thus following the same pattern as the growth rate, with the exception of the treatment with the lowest concentration of metribuzin in which Chla concentration is lower when compared to all other concentrations and control. On day 3,Chladecreased around 30% from the control at the

three higher concentrations of tritosulfuron and decreased 35% at the highest concentration of metribuzin, and around 20% at the other two highest concentrations. On day 5, Chla had a 45% decrease in the highest concentration of tritosulfuron and a 30% decrease in the next two highest concentrations, whereas the lowest concentration did not differ from the control, like on days 1 and 3. Metribuzin treated plants, on the other hand, showed no changes in Chla on day 5 for all concentrations.

Chlb changes compared to control follows the same pattern as Chla, but Chlb was less affected than Chla in both herbicide treatments for the relevant time period. Reduction reached up to38% for tritosulfuron treatment on day 5 and 29% for metribuzin treatment on day 3. Chlb was the least affected pigment in the case of metribuzin, whereas carotenoids were the least affected pigments in the case of tritosulfuron. The results need to be confirmed in additional experiments with these herbicides.

Carotenoids reduction after herbicide treatment also follows the same pattern as Chla. Their maximum reduction was 24% for tritosulfuron on day 5 and 30% for metribuzin on day 3.

4.DISCUSSION-CONCLUSIONS

After exposure to various concentrations of tritosulfuron, *Lemna minor* chlorophyll and carotenoid content showed a decrease which was more intense as the time of exposure increased. This is in accordance with the visual observations of chlorotic symptoms. Additionally, the growth rate based on frond number was more sensitive in measuring the toxicity of tritosulfuron in *Lemna minor* since it was affected by the various herbicide concentrations (statistically significant different), and ranged, relative to control, from almost zero to 1.02 (approximately the same as the control).On the other hand, chlorophylls and carotenoids showed statistically significant differences only with the control. Additionally, the information that the growth rate gives us is prompt, since we have a clear picture of herbicide toxicity from day 3 of exposure.

For metribuzin treatments chlorophyll and carotenoid content did not respond as expected within the 5 days of exposure. Although there was a decrease in their content in high concentration treatments on day 3, on day 5 there was no evident effect in any treatment. This lack of response on day 5 in pigment content at nearly no growth concentrations for the photosystem II inhibitor metribuzin was noteworthy. This is also mentioned in studies with other photosystem II inhibitors (Cedergreen et al, 2007). The reason for this lack of response could be the exposure time. This argument is enhanced by the fact that chlorotic symptoms were evident at the end of exposure, on day 7. Nevertheless, growth end point based on growth rate was, as in tritosulfuron treatments, more representative of the toxicity of metribuzin in *Lemna minor.* In conclusion, for both herbicides growth rate is a more sensitive endpoint than pigments. Therefore, pigments are not recommended for routine toxicity screening for these herbicides in this species. Research must also explore the ways that the *Lemna* species reacts to oxidative stress and how, although growth is inhibited, chlorophyll and carotenoid concentration is not adversely affected in all cases.

The lack of response of the growth rate and pigments for both herbicides on day 1 is probably due to the fact that the growth rate and pigments are not rapidly affected by the herbicides in all concentrations. Thus, it is suggested that other biomarkers that are affected primary to growth by photosystem II herbicides and amino acid herbicides, such as chlorophyll a fluorescence and total proteins respectively, should be investigated.

Finally, it can be concluded that tritosulfuron, affects pigment concentration more promptly and more intensely than metribuzin in concentrations causing similar growth inhibition. This is probably due to the different mode of action of these two herbicides. Tritosulfuron inhibits the biosynthesis of the amino acids valine, leucine and isoleucine, and consequently it inhibits protein biosynthesis. Metribuzin inhibits the electron transport of photosystem II. Although chlorophylls take part in photosynthesis, it seems that the inhibition of amino acid-protein biosynthesis affects chlorophyll content more rapidly than the inhibition of photosynthesis. Further research on the ways that chlorophylls and carotenoid are affected by these two herbicide categories is necessary.

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