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

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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.

MATERIALS AND METHODS

Lemna minor: aseptically cultivated in Erlenmeyer flasks in Steinberg medium, pH 6.8

Experimental conditions: 24h light (photon flux density of 60-90 μmol/m^2/s), 24^{0}C

Toxicants: Herbicides [active substances (technicals)]

- Metribuzine: triazinone, photosystem II inhibitor
- Tritosulfuron: sulfonylurea, amino acid synthesis inhibitor

Concentrations:

- 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
- All herbicide concentrations shown are nominal concentrations (Table 1). The experiments were conducted under static conditions.
- Controls: Plants without addition of toxicant.

Measurements:

- **Growth inhibition**, calculated based on the average specific growth rate of Lemna minor according to the number of fronds. 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).
- **Chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoids.**

  Lemna minor plants were frozen in liquid nitrogen and ground to powder. **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. The supernatant was transferred to a 3ml cuvette. 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. The quantitative determination of Chla, Chlb and carotenoids was calculated in accordance with the equations described by Lichtenthaler:

  $C_a = 13.36A_{665} - 5.19A_{649}$
  $C_b = 27.43A_{649} - 8.12A_{665}$
  $C_{x+c} = (1000A_{470} - 2.13C_a - 97.64C_b)/209$

  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.
- **Days:** day 1, day 3 and day 5 (chlorophyll a, b, carotenoids), day0-day 7 (growth)

RESULTS

- Growth rate inhibition of the four concentrations relative to control is presented in Tables 2 and 3. Chla, Chlb and carotenoid content in *Lemna minor* frond tissue, are presented in Tables 4 and 5. 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.
- 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.
- 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.
- Chlorotic symptoms on days 3 and 5 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.
- Chla was the pigment most affected by both herbicides compared to the other two, as can be seen in Tables 4 and 5.
- Pigments on day 1 of exposure, although in all herbicide concentrations are lower than the ones of the control, they do not differ statistically from the control group, thus following the same pattern as the growth rate. On day 3, Chla decreased at the three higher concentrations of tritosulfuron and metribuzin, On day 5, Chla decreased even more at the three higher concentration of tritosulfuron but, metribuzin treated plants, on the other hand, showed no changes in Chla on day 5 for all concentrations. These findings are in accordance with the visual observations of chlorotic symptoms.
- Chlb and carotenoids changes compared to control follows the same pattern as Chla, but Chlb and carotenoids were less affected than Chla in both herbicide treatments for the relevant time period.

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

Table 2: The results of tritosulfuron concentrations tested in % of growth inhibition (number of Lemna fronds) compared to the control. EC_{50} 51 μg/l (95% c.l.: 36-71). NOEC 30 μg/l.

Tritosulfuron		
Concentrations	mg/l	% Inhibition
C1	100	84.86
C2	60	64.90
C3	30	7.77
C4	10	4.35

Table 3: The results of metribuzine concentrations tested in % of growth inhibition (number of Lemna fronds) compared to the control. EC_{50} 78 μg/l (95% c.l.: 74-83). NOEC 30 μg/l.

Metribuzin		
Concentrations	μg/l	% Inhibition
C1	380	98.49
C2	95	65.19
C3	55	30.02
C4	30	4.52

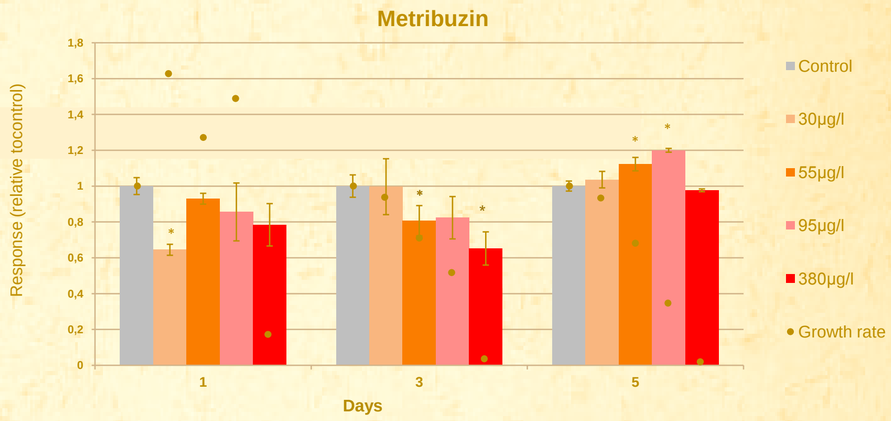

Figure 1. Chla content (columns, n=3) at various concentrations on 3 different days for metribuzin treatments. Filled symbols represent the specific growth rate. All responses are given relative to the controls as mean ± standard deviation. Statistically significant differences compared to control are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

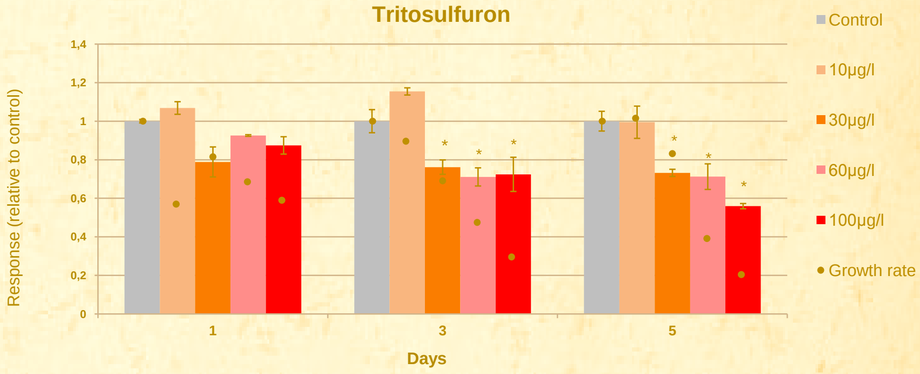

Figure 2. Chla content (columns, n=3) at various concentrations on 3 different days for tritosulfuron treatments. Filled symbols represent the specific growth rate. All responses are given relative to the controls as mean ± standard deviation. Statistically significant differences compared to control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

Table 4: 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.

Metribuzin Concentration (μg/l)	DAY 1			DAY 3			DAY 5		
	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids mg/g FW (% of control)	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids mg/g FW (% of control)	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids 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 5: 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.

Tritosulfuron Concentration (μg/l)	DAY 1			DAY 3			DAY 5		
	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids mg/g FW (% of control)	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids mg/g FW (% of control)	Chla mg/g FW (% of control)	Chlb mg/g FW (% of control)	Carotenoids 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)

DISCUSSION-CONCLUSIONS.

- The 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.
- For both herbicides growth rate is a more sensitive endpoint than pigments. It is more representative of the toxicity of tritosulfuron and metribuzin in *Lemna minor,* since it is affected by the various herbicide concentrations (statistically significant different). And it is prompt, since we have a clear picture of herbicide toxicity from day 3 of exposure. Therefore, pigments are not recommended for routine toxicity screening for these herbicides in this species.
- 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.

«This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund»

European Union European Social Fund
OPERATIONAL PROGRAMME EDUCATION AND LIFELONG LEARNING Investing in knowledge society
MINISTRY OF EDUCATION, LIFELONG LEARNING AND RELIGIOUS AFFAIRS MANAGING AUTHORITY
NSRF 2007-2013 programme for development EUROPEAN SOCIAL FUND
Co- financed by Greece and the European Union