THE USE OF VARIOUS BIOMARKERS OF THE BIOINDICATOR *Lemna minor* TO ESTIMATE THE TOXICITY OF TWO HERBICIDES WITH DIFFERENT MODE OF ACTION

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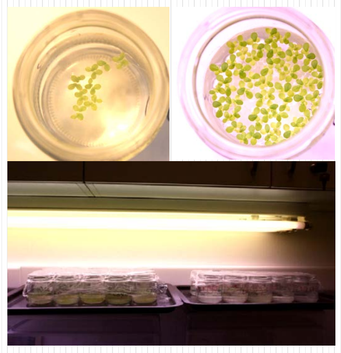

Background

Bioindicators: species used to monitor the health of an environment or ecosystem.

Biomarker: a change induced by a contaminant in the biochemical or cellular components of a process, structure or function that can be measured in a biological system. A main concept of this approach is based on the correlation of the effects of a contaminant at different levels of structural organization (enzyme activity, cells, tissues, organisms, et al.).

Oxidative stress biomarkers: Toxic effects of pollutants often depend on their capacity to increase the cellular levels of reactive oxygen species (ROS). When ROS levels production exceeds antioxidant defences, cells experience oxidative stress. The mechanism underlying most assays employing biomarkers of oxidative stress that are in use is related to the change in the activity of the ROS defences system. Antioxidant enzymes, like glutathione reductase (GR), guaiacol peroxidase (G-POD) and superoxide dismutase (SOD) are often modified in response to cellular oxidative stress. Enzymatic tests have proved to be suitable for monitoring the effects of pollutants on organisms.

Toxicity test parameters

- ***Lemna minor*:** a fast growing duckweed plant regularly used as a bioindicator in ecotoxicological dose-response studies (OECD Guidelines 221, 7 days test)
- **Toxicants:** Herbicides [active substances (technicals)]
  - Metribuzine: triazinone, photosystem II inhibitor
  - Tritosulfuron: sulfonylurea, amino acid synthesis inhibitor
- **Measurements:** growth (frond number), activity of glutathione reductase (GR), guaiacol peroxidase (G-POD), superoxide dismutase (SOD), total proteins.
- **Days:** day 1, day 3 and day 5 (GR, G-POD, SOD, total proteins), day0-day 7 (growth)
- **Concentrations:**

Table 1: The concentrations in mg/l of the active substances used in the trials, based on the results of the pre-tests (Tables 2 and 3).

Active substance	Concentration (mg/l)			
	C1	**C2**	**C3**	**C4**
Metribuzine	0.380	0.095	0.055	0.030
Tritosulfuron	0.100	0.060	0.030	0.010

Table 2: Pre-test results of metribuzine. The estimation of the Effective Concentrations (EC) in mg/l (EC were estimated with SPSS).

Metribuzine		
Effective concentration	**Estimate**	**95% c.l.**
EC_{100}	0.376	0.237-0.819
EC_{75}	0.095	0.073-0.133
EC_{50}	0.054	0.041-0.070
EC_{25}	0.031	0.021-0.041

Table 3: Pre-test results of tritosulfuron. The estimation of the Effective Concentrations (EC) in mg/l (EC were estimated with SPSS).

Tritosulfuron		
Effective concentration	**Estimate**	**95% c.l.**
EC_{100}	0.602	0.160-39.760
EC_{75}	0.066	0.032-0.376
EC_{50}	0.027	0.013-0.070
EC_{25}	0.011	0.003-0.021

Materials and Methods

- The toxicity of the two herbicides was assessed by growth inhibition tests in *Lemna minor* based on standard OECD protocols. Growth (number of fronds) was measured daily from day 0 until day 7 in the 4 different concentrations as well as in control. Three replicates were used in each treatment.
- Experimental conditions: 24h light, 25^{0}C
- Different treatments were used for enzyme extraction (for the measurement of the 3 enzymes and total proteins). For each of the 3 different days (day 1, day3, day 5), and for each concentration (C0, C1, C2, C3, C4) 3 replicates were used (15 extractions/day).
- *Lemna minor* plants were homogenized in liquid nitrogen and 150 mg of each replicate was used for the enzyme extraction.
- The responses of Glutathione reductase (GR), guaiacol peroxidase (G-POD) and superoxide dismutase (SOD) and total proteins were measured spectrophotometrically using a Microplate reader. The data from the above trials were statistically analyzed by applying 2-factorial ANOVA, with and 5 concentrations and 3 time periods as experimental factors and levels respectively (5×3). Significance of the main factors and interactions was assessed at three confidence levels (0.05, 0.01 and 0.001). When the combination or the interaction was significant, means were separated by applying the Duncan's multiple range test ($p \leq 0.05$). The STATISTICA software package (STATISTICA for Windows 8.0, Tulsa, OK, USA) was used to perform statistical analysis.

Results-Conclusions

Table 4: The results of metribuzine concentrations tested in % of growth inhibition (number of Lemna fronds) compared to the control. EC_{50} 0.078 mg/l (95% c.l.: 0.074-0.083). NOEC 0.03 mg/l.

Metribuzine		
Concentrations	**mg/l**	**% Inhibition**
C1	0.380	98.49
C2	0.095	65.19
C3	0.055	30.02
C4	0.030	4.52

Table 5: The results of tritosulfuron concentrations tested in % of growth inhibition (number of Lemna fronds) compared to the control. EC_{50} 0.051 mg/l (95% c.l.: 0.036-0.071). NOEC 0.03 mg/l.

Tritosulfuron		
Concentrations	**mg/l**	**% Inhibition**
C1	0.100	84.86
C2	0.060	64.90
C3	0.030	7.77
C4	0.010	4.35

Metribuzine

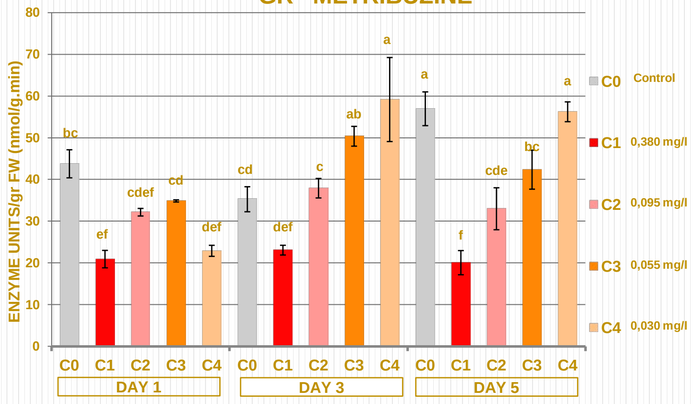

Figure 1: The activity of GR in nmol substrate converted to product per minute (U or units) per gram fresh weight (nmol/g.min) after metribuzine exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<1‰.

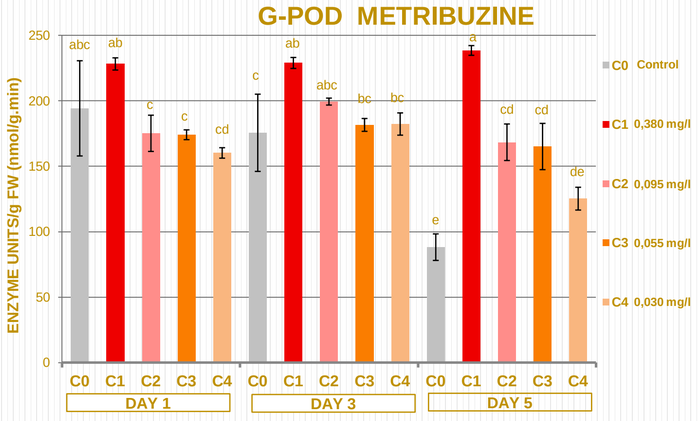

Figure 2: The activity of G-POD in nmol substrate converted to product per minute (U or units) per gram fresh weight (nmol/g.min) after metribuzine exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<0.05.

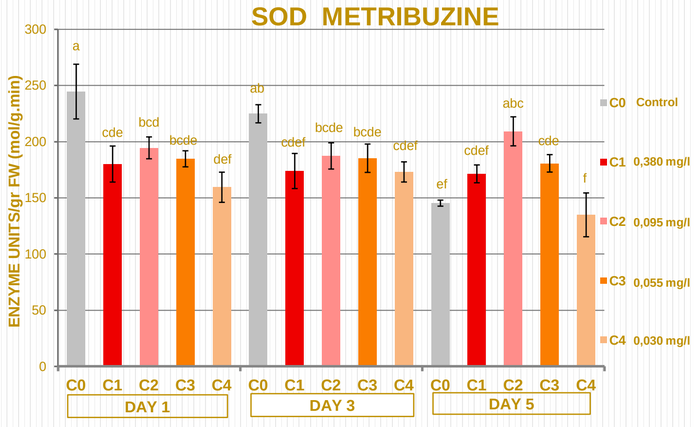

Figure 3: The activity of SOD in mol substrate converted to product per minute (U or units) per gram fresh weight (mol/g.min) after metribuzine exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<0.01.

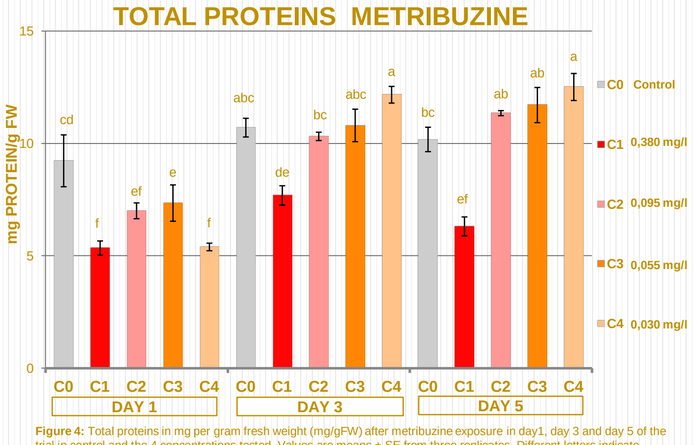

Figure 4: Total proteins in mg per gram fresh weight (mg/gFW) after metribuzine exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<1‰.

Tritosulfuron

C1 > C2 > C3 > C4
C0 Control

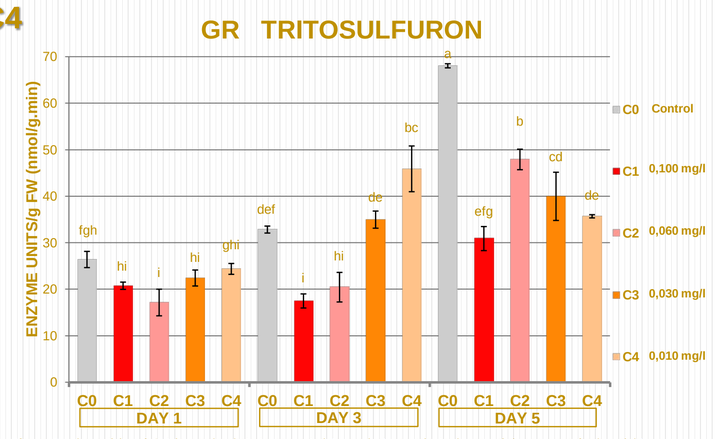

Figure 5: The activity of GR in nmol substrate converted to product per minute (U or units) per gram fresh weight (nmol/g.min) after tritosulfuron exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<1‰.

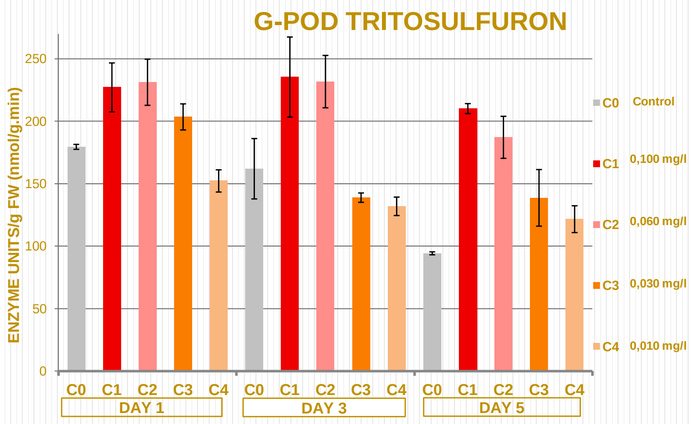

Figure 6: The activity of G-POD in nmol substrate converted to product per minute (U or units) per gram fresh weight (nmol/g.min) after tritosulfuron exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates.

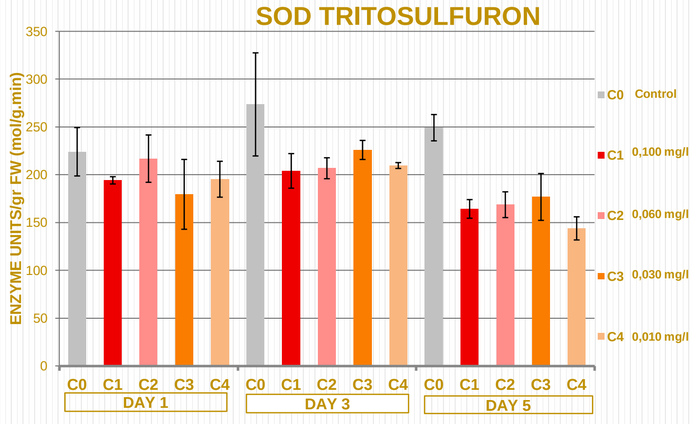

Figure 7: The activity of SOD in mol substrate converted to product per minute (U or units) per gram fresh weight (mol/g.min) after tritosulfuron exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates.

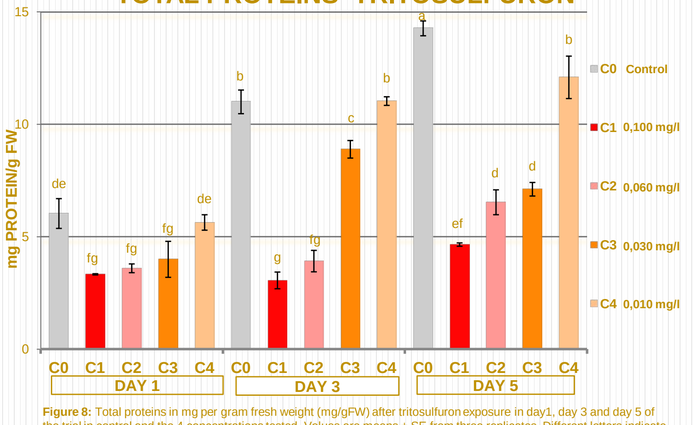

Figure 8: Total proteins in mg per gram fresh weight (mg/gFW) after tritosulfuron exposure in day1, day 3 and day 5 of the trial in control and the 4 concentrations tested. Values are means ± SE from three replicates. Different letters indicate significantly different values at P<1‰.

Results-Conclusions

- Figures 1-8 show enzyme activities of Glutathione reductase (GR), guaiacol peroxidase (G-POD) and superoxide dismutase (SOD) as well as total proteins in both herbicides in all concentrations, control and on the 3 different days.
- GR showed the best results compared to G-POD and SOD. It was the only enzyme that had statistically significantly different results between different concentrations on all 3 days in both herbicides. The decrease in GR activity with the increase of the concentration of the toxicants, instead of an increase to prevent oxidative stress and the fact that within the five days of the exposure there is no recovery in the enzyme activity compared to the control, indicate the intense stress caused by the herbicides.
- G-POD and SOD gave statistically significantly different results between different concentrations on all 3 days only for metribuzine. For tritosulfuron G-POD gave a statistically significant difference between the means of all days of control. C3 and C4 with the means of all days of the two highest concentrations (C1. C2). SOD for tritosulfuron also gave a statistically significant difference between the means of all days of control and the means of all days of all concentrations.
- Total proteins also had statistically significantly different results between different concentrations on all 3 days for both herbicides. Particularly for tritosulfuron the results on all three days were very succinct, showing a gradient reduction of total proteins from control to the highest concentration. This could possibly be explained by the fact that tritosulfuron inhibits the synthesis of amino acids.
- As for the sampling days, day 5 is the one that showed a clearer trend in most of the parameters tested. This is in agreement with the macroscopic results based on frond number calculation. In future tests day 5 could be chosen as the one to measure antioxidant enzymes.
- Between the two herbicides, metribuzine gave better results in all parameters tested except total proteins. There could be many reasons for this phenomenon. One of the factors that could have led to this is the different mode of action of the two herbicides. The mode of action plays an important role in the stress caused as well as in the antioxidative mechanisms triggered. Another element that plays a role regarding the differences observed is the fact that the growth inhibition in the concentrations chosen for metribuzine had a more regular scale when compared to tritosulfuron (Tables 4 and 5). and thus metribuzine is expected to give a clearer picture of the parameters tested.
- In order to acquire a better understanding of the findings, further investigation is needed as far as the action of antioxidant enzymes, their normal levels, and their role in physiological processes and enzymatic defence systems in general.
- For a better understanding of the ecotoxicological risk assessment other biomarkers (e.g. chlorophylls) should also be measured at the same time intervals.

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