Bull. Environ. Contam. Toxicol. (2006) 76:883–890
© 2006 Springer Science+Business Media, Inc.
DOI: 10.1007/s00128-006-1001-3

Bulletin of Environmental Contamination and Toxicology

Comparative Sensitivity of Green Algae to Herbicides Using Erlenmeyer Flask and Microplate Growth-Inhibition Assays

Ž. Pavlić,[1] B. Stjepanović,[1] J. Horvatić,[2] V. Peršić,[2] D. Puntarić,[1] J. Čulig[1]

[1] Department of Ecology, Institute of Public Health, Mirogojska c. 16, HR-10000 Zagreb, Croatia
[2] Department of Biology, Josip Juraj Strossmayer University, Lorentza Jaegera 9, HR-31000 Osijek, Croatia

Received: 20 May 2005/Accepted: 8 March 2006

Algae and aquatic plants have a major role in the environmental conditions of stagnant and flowing waters, being important in the uptake, storage, release and deposition of nutrients (Fairchild et al. 1998). Much of the research has focused on the phytoplankton. Algae have been shown to be sensitive to a wide range of contaminants. Thus, algae have been recommended for use in regulatory testing (Halling-Sørensen et al. 1996). The potential effects of herbicides on aquatic primary producers are particularly important, and need to be studied in ecotoxicological experiments (Ma 2001). Due to the high costs of chronic toxicity testing with algae there have been several approaches to develop and establish miniaturized bioassays using algae (Eisentraeger et al. 2003).

The objective of this study was to evaluate the sensitivity of the freshwater green algae *Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus* and *Chlorella kessleri* to selected herbicides using standard Erlenmeyer flask and miniaturized microplate algal growth inhibition assays and to compare sensitivity among populations of these three green algae to the herbicides metribuzin, alachlor and isoproturon.

MATERIALS AND METHODS

The algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak, CCAP 278/4, formerly called *Selenastrum capricornutum* (ISO/TC N 182, 1999) and *Desmodesmus subspicatus* (Chod.) Hegew. & Schmidt, CCAP 276/22, formerly called *Scenedesmus subspicatus* (Hegewald 2000), were obtained from the Culture Centre of Algae and Protozoa (Ambleside, Cumbria, UK), while *Chlorella kessleri* Fott et Novak, strain Larg/1 was supplied by the Culture Collection of Autotrophic Organisms (Trebon, Czech Republic) and cultivated at the Department of Biology, Faculty of Education, J.J. Strossmayer University of Osijek. All algae were cultured under the same lighting (138 $\mu E\ m^2 s^{-1}$ intensity; 16h : 8h light:dark cycle; cool-white fluorescent bulbs) and temperature conditions (23 ± 2°C).

Analytical grade metribuzin (PESTANAL® - 4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2-4-triazin-5-one; CAS 021087-64-9; purity 99.8%), alachlor (PESTANAL® - 2-chloro-2',6'-diethyl-n-(methoxymethyl)acetanilide; CAS 15972-60-8; purity 99.9%) and isoproturon (PESTANAL® - CAS 34123-59-6; purity

Correspondence to: Ž. Pavlić

99.9%) from Sigma-Aldrich Laborchemikalien GmbH, Riedel-de Haën, Seelze, Germany, were used. Herbicide solutions (100%) were serially diluted using additional medium to obtain five concentrations from 10 - 200 μg L^{-1} (for metribuzin), 10 - 1000 μg L^{-1} (for alachlor) and 10 - 100 μg L^{-1} (for isoproturon). Herbicide test solutions were added on day 0 of each experiment. Three replicates *per* treatment and six replicates for controls were used.

All experiments were conducted with two different methods. The first method was the International Organization for Standardization (ISO) method (ISO 8692 1989) (Table 1). All control and treatments flasks, were incubated for three days (72 h) at 23 ± 2°C under a shaking procedure of 110 min^{-1} (Innova 4340, New Brunswick Scientific, New Jersey, USA) and exposed to overhead light, intensity 138 μmol m^{-2} s^{-1} to ensure exponential algal growth. Every 24 h the algal density was quantified using spectrophotometer measurements at 750 nm wavelength on a Lambda 14P (Perkin Elmer, Norwalk, Connecticut, USA). The pH value in all samples was measured at the beginning and at the end of experiment using a glass electrode (pH 526 WTW, Weilheim, Germany).

Table 1. Experimental conditions for algae in Erlenmeyer flask growth-inhibition and miniaturised algal growth-inhibition assays.

Condition	Erlenmeyer flask growth-inhibition assay	Miniaturised algal growth-inhibition assay
Temperature (°C)	23 ± 2	23 ± 2
Light source	Cool-white fluorescent	Cool-white fluorescent
Light intensity (μE m^2s^{-1})	138	138
Photoperiod	Continuous white light	Continuous white light
Test chamber	250 mL Erlenmeyer flasks	250 μL 96 wells microplate
Test volume (mL)	100	0.2
Final concentration (cell density)	1×10^4 cells mL^{-1}	1×10^4 cells mL^{-1}
Test type	Static, non renewal	Static, non renewal

The second method was a miniaturized growth bioassay according to Lukavský (1994) in immunological plates with suspended algal cultures of 0.2 mL $well^{-1}$. Imunological plates polystirene FB type, 9 x 12 cm and 96 wells of 250 μL were used. Test samples (100 μL) were added to each well and replicated six times in a column. The algae stock solution (100 μL) was added to each microplate well to obtain a concentration of 10^4 cells mL^{-1}. The plates were closed with lids and cultivated in a glass box, with a temperature of 23 ± 2°C, CO_2 concentration 2 - 3% (v/v). The box was placed under fluorescent lamps with 138 μmol m^{-2} s^{-1} light intensity (Table 1). In all samples pH value was measured at the beginning and at the end of each experiment. The increase in algal biomass was measured at 750 nm, after three days, with Labsystems Multiscan MS spectrophotometer that was connected to a personal computer by the Genesis program. Reference tests were

carried out with potassium dichromate ($K_2Cr_2O_7$) in both methods.

ANOVA and Student Newman-Keuls multiple-range test were employed to determine if treatments were significantly different from each other. Results were deemed significantly different at the level $p \leq 0.05$. EC_{50} values with 95% confidence limits were estimated by the linear regression of the probit of percentage growth on log dose of herbicides. In this study, EC_{50} values are the concentrations of herbicides, derived by the method of calculation "comparison of areas under growth curves", which results in a 50% growth reduction relative to the control values at 72 h (ISO 8692 1989). All results are presented as mean ± S.D. Statistical analysis was performed using standard statistical packages (STATISTICA® for Windows).

After data processing, the EC50s were translated into toxic units (TU) according to the formula: TU = [1/EC50] × 100 (Isidori et al. 2000). Because of the lack of a standard classification system to express the degree of toxic hazard, the arbitrary (log) toxicity scale was used, which ranks the toxicity in five classes: class 0 (non toxic) TU = 0; class 1 (slightly toxic) TU = <1; class 2 (toxic) TU = 1-10; class 3 (very toxic) TU = 11-100 and class 4 (extremely toxic) TU = >100 (Isidori et al. 2000).

RESULTS AND DISCUSSION

Comparing the two methods, the Erlenmeyer flask and microplate algal growth assays, the herbicide EC_{50} values ranged from 12 to 995 μg L^{-1} among the three chemicals tested, with considerable variation across species and chemicals (Table 2). Analysis of variance of ranked data was used to statistically compare relative algal sensitivity and chemical toxicity. The herbicides showed significant differences in relative toxicity ($p < 0.05$) across the species tested. The triazine herbicide (metribuzin) was more toxic than the acetanilide herbicide (alachlor) (Figs. 1 and 2), with the exception of the *P. subcapitata* and *C. kessleri* responses to alachlor, which were the two most sensitive responses measured. In both methods, 50% growth inhibition was caused by alachlor concentration of 12 to 15 μg L^{-1} in *P. subcapitata* and 22 to 32 μg L^{-1} in *C. kessleri* (Table 2).

Data indicated significant differences ($p < 0.05$) in sensitivity to herbicides. In both methods, *P. subcapitata* was more sensitive (12 – 41 μg L^{-1} with average value of 29.08 μg L^{-1}) than *C. kessleri* (22 – 60 μg L^{-1}; average 38.50 μg L^{-1}) and *D. subspicatus* (27 – 995 μg L^{-1}; average 378.33 μg L^{-1}) to study herbicides, except for the *D. subspicatus* response to isoproturon who showed higher sensitivity than *P. subcapitata* and *C. kessleri* (Fig. 2).

Toxicity results in toxic units for tested herbicides are shown in Figure 3. According to these results, metribuzin and alachlor had "slightly toxic" effect (Class 1) only on *D. subspicatus* whereas all three tested herbicides exerted a "toxic" effect (class 2) on *P. subcapitata* and *C. kessleri*. Interspecies algal sensitivities were lower for the urea herbicide (isoproturon) and triazine herbicide (metribuzin) than for to the acetanilide herbicide (alachlor).

Table 2. Median effective concentrations (EC50s) of three herbicides tested with three species of algae and Erlenmeyer flasks growth inhibition assay (method 1), and miniaturized algal growth inhibition assay (method 2).

		Pseudokirchneriella subcapitata	*Desmodesmus subspicatus*	*Chlorella kessleri*
Metribuzin	method 1	22.5 ± 2.29 d	155.0 ± 10.5 f	26.0 ± 3.00 d
	method 2	37.0 ± 2.00 cd	180.0 ± 10.5 e	37.0 ± 3.61 cd
Isoproturon	method 1	41.0 ± 4.27 cd	27.0 ± 3.61 d	54.0 ± 3.61 cd
	method 2	47.0 ± 3.05 cd	33.0 ± 2.08 cd	60.0 ± 3.61 c
Alachlor	method 1	12.0 ± 2,00 g	880.0 ± 43.0 b	22.0 ± 5.29 d
	method 2	15.0 ± 2.52 g	995.0 ± 16.7 a	32.0 ± 4.58 cd

All units are given in μg L^{-1}. Results are expressed as mean ± SD. Values marked with different letters are significantly different according to Newman-Keuls test, $P < 0.05$.

Differences across chemical classes were most probably due to the single mode of action of the ureas and triazines (inhibition of photosynthesis at photosystem II) (Fairchild et al. 1998) as opposed to the multiple modes of action of the acetanilides (inhibition of cell division and of numerous biosynthetic reactions), which are not fully understood (Fairchild et al. 1998).

Our results demonstrated that there was a different response to various herbicides among the three tested algae and that the sensitivity of these algae exposed to alachlor varied by nearly two orders and to metribuzin by one order of magnitude. Wang and Freemark (1995) found that no species could be identified as "always being the most sensitive or always the least sensitive". The sensitivity varies not only among toxicants, but also among taxonomic groups and species. Investigations using different algal species as test organisms have shown that algae vary greatly in their response to chemicals. Different sensitivity of green algae to the compounds could induce species shifts within communities (Tadros et al. 1994; Ma and Liang 2001).

The results of regression analysis showed significant accordance between the conventional flask method and microplate bioassay method (the correlation coefficient, $r = 0.9956$) and lesser toxic responses in microplates than in flask assay for tested algae and herbicides (Fig 4). These results are in agreement with those reported by Rojíčková et al. (1998) who suggested that higher EC_{50} values were obtained probably due to higher ratio of volume to surface area of tested solution in microplates.

Microplate algal assays offer many advantages over Erlenmeyer flask tests in reducing laboratory resources. Using the 96-well microplate algae growth-inhibition assay, far more samples, more parallels and more concentrations of samples can be

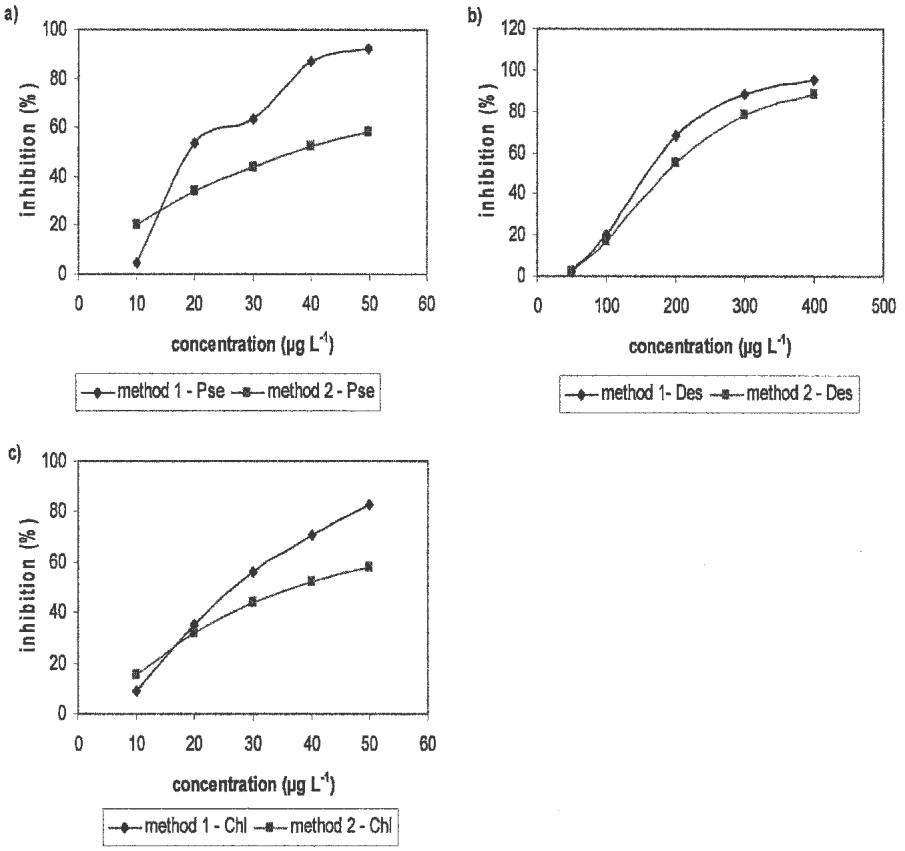

Figure 1. Inhibition of metribuzin for: a) Pse (*Pseudokirchneriella subcapitata)*, b) Des (*Desmodesmus subspicatus)* and c) Chl (*Chlorella kessleri)* with conventional (method 1) and miniaturized algal assays (method 2).

tested with less work and incubation room. Because of these advantages, there is a need for continued research and development of the miniaturized bioassay that will greatly facilitate the use of algae data in environmental risk assessment.

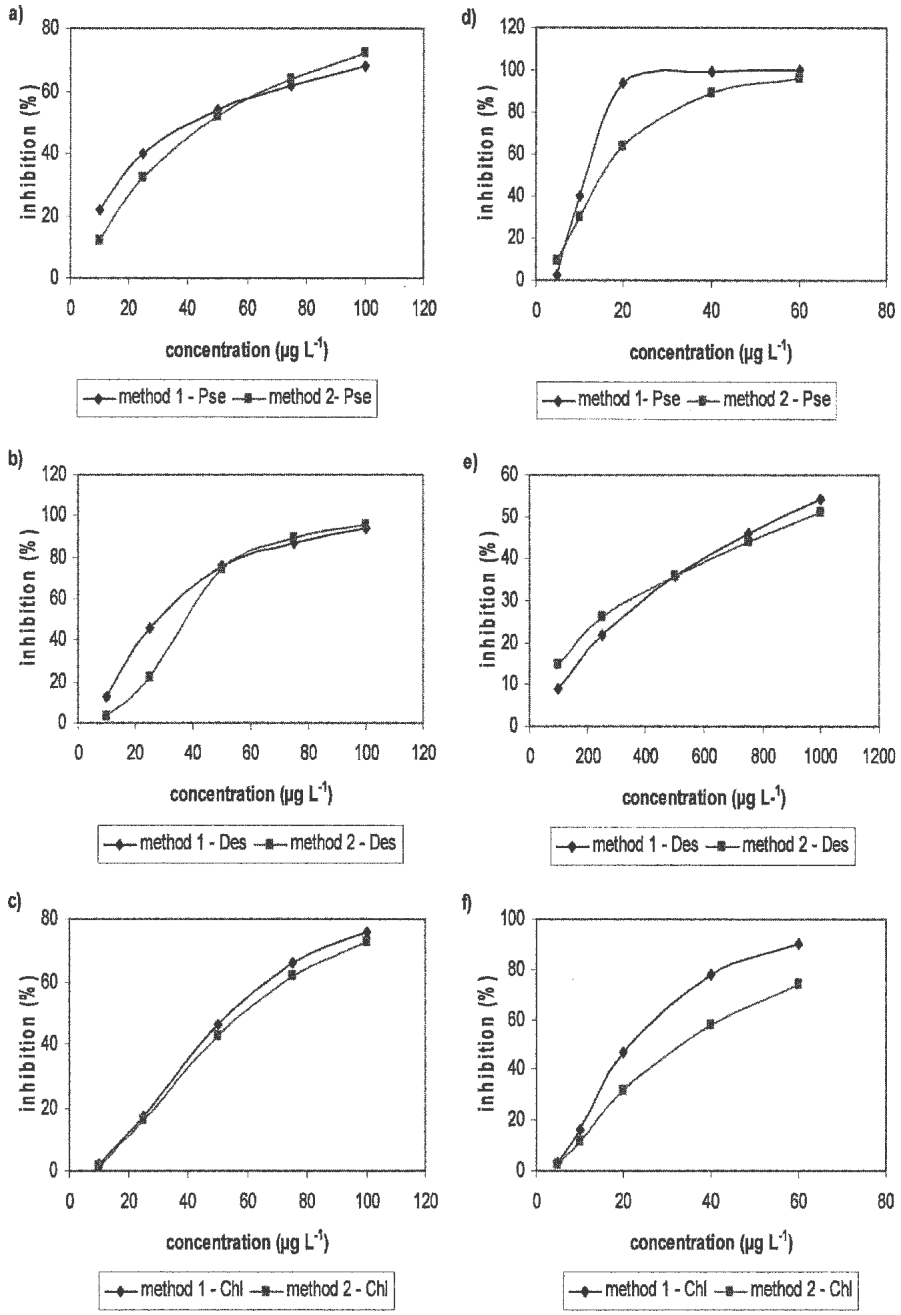

Figure 2. Inhibition of isoproturon (a-c) and alachlor (d-f) for Pse (*Pseudokirchneriella subcapitata)*, Des (*Desmodesmus subspicatus)* and Chl (*Chlorella kessleri)* with conventional (method 1) and miniaturized algal assays (method 2).

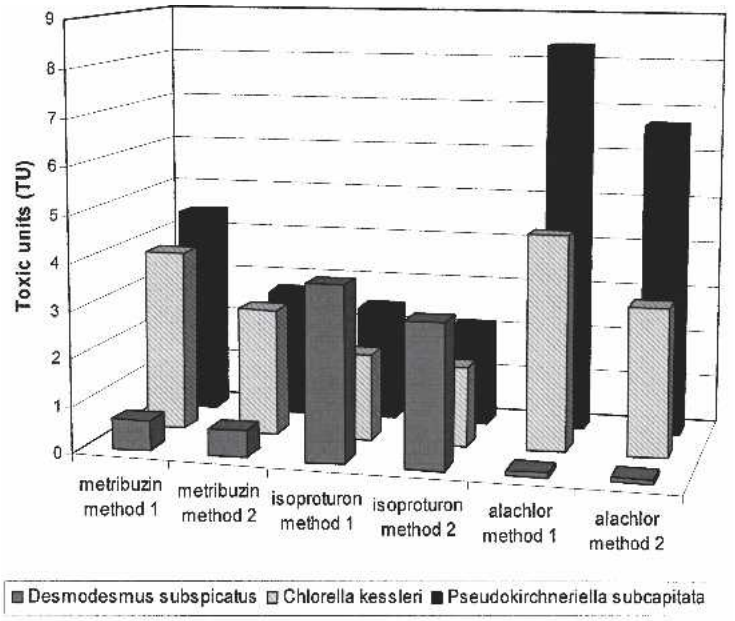

Figure 3. Toxicity results visualized in toxic units (TU) of metribuzin, isoproturon and alachlor for *P. subcapitata*, *D. subspicatus* and *Ch. kessleri* with conventional (method 1) and miniaturized algal bioassays (method 2) .

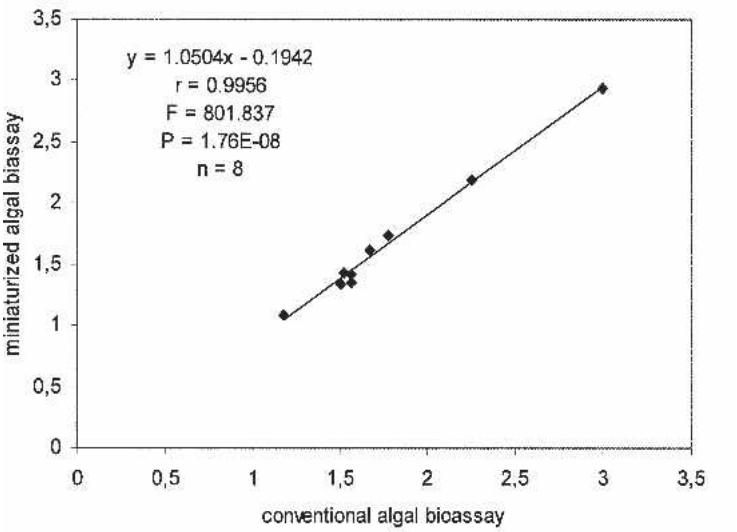

Figure 4. A comparison of conventional and miniaturized algal bioassays based on linear regression of the $\log_{10}$ values of EC_{50} for tested algae and herbicides.

REFERENCES

Eisentraeger A, Dott W, Klein J, Hahn S (2003) Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays. Ecotoxicol Environ Saf 54:346-354

Fairchild JF, Ruessler DS,Carlson AR (1998) Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metolachlor. Environ Toxicol Chem 17:1830-1834

Halling-Sørensen B, Nyholm N, Baun A (1996) Algal toxicity tests with volatile and hazardous compounds in air-tight test flasks with CO_2 enriched headspace. Chemosphere 32:1513-1526

Hegewald E (2000) New combinations in the genus *Desmodesmus (Chlorophyceae, Scenedesmaceae*). Algolog Stud 96:1-18

Isidori M, Parrella A, Piazza CML, Strada R (2000) Toxicity screening of surface waters in southern Italy with Toxkit microbiotests. In: Persoone G, Janssen C, De Coen W (eds) New Microbiotests for Routine Toxicity Screening and Biomonitoring. Kluwer Academic/Plenum Publishers, New York, pp 289-293

ISO 8692 (1989) Water quality – Freshwater algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*. International Organization for Standardization, Geneve, Switzerland

ISO/TC 147/SC 5/WG N 182 (1999) Nomenclature *Selenastrum capricornutum*. International Organization for Standardization, Geneve, Switzerland

Lukavský J (1994) Miniaturized algal growth bioassay. In: Aubrecht G, Dickand G, Prentice C (eds) Monitoring of Ecological Change in Wetlands of Middle Europe. Slimbridge, UK. Staphia 31:151-156

Ma J (2001) Differential sensitivity among populations of two green algae *Scenedesmus obliquus* and *Chlorella pyrenoidosa* to 30 herbicides. Bull Environ Contam Toxicol 68:275-281

Ma J, Liang W (2001) Acute toxicity of 12 herbicides to the green algae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. Bull Environ Contam Toxicol 68:347-351

Rojíčková R, Dvořáková D, Maršálek B (1998) The use of miniaturized algal bioassay in comparison to the standard flask assay. Environ Toxicol Water Qual 13:235-241

Tadros JS, Philips J, Patel H, Pandiripally V (1994) Differential response of green algal species to solvents. Bull Environ Contam Toxicol 52:333-337

Wang W, Freemark K (1995) The use of plants for environmental monitoring and assessment. Ecotoxicol Environ Saf 30:289-301