Hydrobiologia **188/189**: 353–359, 1989.
M. Munawar, G. Dixon, C. I. Mayfield, T. Reynoldson and M. H. Sadar (eds)
Environmental Bioassay Techniques and their Application.

Bioassays with a floating aquatic plant (*Lemna minor*) for effects of sprayed and dissolved glyphosate

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Key words: duckweed, glyphosate, *Lemna*, phytotoxicity, bioassay, herbicide

Abstract

Macrophytes in forested areas and in prairie wetlands furnish critical habitat for aquatic communities and for several species of birds and mammals. North American agriculture relies heavily on herbicides and these compounds are detected routinely in surface waters of Western Canada. The question is whether these residues have biological meaning. There is surprisingly little literature on the responses of macrophytes to herbicides, or indeed to other chemicals. Previously we have used common duckweed in efforts to detect effects of herbicides and other chemicals. Duckweed clones were developed from local collections and grown axenically. In this study the plants were exposed to glyphosate herbicide either by dissolving formulated Roundup® (Monsanto Canada Inc.) in the culture media or by spraying of the cultures in a laboratory spray chamber. Plant growth was monitored by counting the fronds present on several occasions over a 2-week period following treatment and by taking wet and dry weights of plants after the final counting period. Plant growth, as measured by increased numbers of fronds or increased wet or dry weights was relatively insensitive to glyphosate dissolved in the culture medium. However, the plants were killed by application of glyphosate as a spray.

Introduction

Duckweeds have been used as convenient bioassay organisms for the detection of phytotoxicity since the 1930s. They were among the species used to define the effects of the earliest phenoxy herbicides on plants (Blackman & Robertson-Cunninghame, 1954, 1955). Phenoxy herbicides have since become distributed widely throughout watersheds in central Canada (Gummer, 1980). A recent survey of two small rivers draining agricultural watersheds in western Manitoba revealed the presence of several other herbicides (trifluralin, triallate, bromoxynil, 2,4-D, 2,4,5-T diclofop and dicamba) in addition to phenoxy compounds (Muir & Grift, 1987). While herbicides may seem to be the most obvious application for duckweed bioassays, these plants also respond to other materials such as heavy metals (Nasu & Kugimoto, 1981) and surfactants (Bishop & Perry, 1981).

Glyphosate is being applied in agriculture for weed control and also in forestry for 'conifer release' – the control of young deciduous trees to allow the free growth of conifers in plots being managed for the production of conifers. It is virtually impossible to spray any material over large areas by aircraft, particularly in forestry, without

contamination of surface waters within those areas, and so emergent aquatic vegetation might be exposed both by contact with spray droplets and by contact with herbicide-contaminated water. Duckweeds in laboratory cultures were known to be sensitive to glyphosate in the water, however, the toxicity was reduced by addition of bentonite clay to the water (Hartman & Martin, 1984, 1985). Field trials in which glyphosate was sprayed onto duckweed, however, failed to control the plants (Thayer & Haller, 1985). The difference between bioassay experience and field experience prompted us to question whether the plants might respond differently to glyphosate applied as a surface spray than to glyphosate presented dissolved in the water.

Materials and methods

Duckweed cultures

Stock cultures of a clone common duckweed were cultured from an original collection of plants taken from Lake-of-the-Woods, northwestern Ontario. The clone was obtained by bleaching as described by Hillman (1961), and was maintained in axenic culture using Stewart's (1972) medium with asparagine at 132.1 mg l^{-1} as the source of nitrogen. Plants were grown in 250-ml Erlenmeyer flasks with 100 ml of medium per flasks, in a controlled environment room at 25 °C. Light was provided by General Electric Gro & Sho® lights at about 60 mE m^{-2} s^{-1} with a photoperiod of 16 h light and 8 h dark, essentially as described by Lockhart & Blouw (1980).

Exposures to dissolved glyphosate

Glyphosate (N-(phosphonomethyl)glycine) was used as the formulated commercial herbicide Roundup® (Monsanto Canada Inc.), supplied at 356 g l^{-1} of the isopropylamine salt of glyphosate. Plant cultures were treated with glyphosate by dissolving the herbicide in the culture medium. For exposures to dissolved glyphosate the concentrations used ranged from 10^{-7} M to 10^{-3} M of glyphosate as the isopropylamine salt. Exposures were conducted in 125-mL Erlenmeyer flasks to which sterilized culture medium and glyphosate were added before the addition of 10 representative fronds of duckweed from the stock clone. Five flasks were set up for each exposure concentration, and there were also five untreated controls.

Exposures to sprayed glyphosate

For application of a spray to the surface of the plants a laboratory sprayer was used consisting of a spray nozzle which moved along a rigid track at a rate and pressure selected by the operator. The sprayer was calibrated using dyes to allow delivery of any desired quantity of material to the surface of the cultures. The application rate used was that recommended for field control of annual weeds up to 15 cm high, namely 2.25 l of formulated Roundup® per ha, or approximately 800 g of active ingredient per hectare (Monsanto Canada Inc.). This rate of application to the surface of the dishes would produce, on complete mixing, a concentration of about 2.34×10^{-5} M glyphosate in the culture medium. Exposures were conducted by placing sufficient duckweed fronds in deep Petri dishes with a surface area of 0.00785 m^2 and containing 117.8 ml of culture medium. After spraying, the exposed plants were allowed to stand in the spray apparatus for 6, 12, or 24 hr without disturbance, and then 10 apparently normal fronds were selected from each dish and transferred to 125-ml Erlenmeyer flasks where they were grown in clean culture medium. (There was no visible injury to plants at the time the samples of 10 fronds were removed). The standing times in the apparatus were required; preliminary experiments showed that removal of the sprayed cultures immediately after spraying resulted in loss of toxicity, presumably by washing glyphosate deposits off the plants into the medium.

Controls were treated in the same way as sprayed cultures except that the dish covers were

left in place. There were 10 replicates for each exposure.

Plant responses to treatments

The numbers of fronds were counted several times over a 2-week period following exposure, and growth curves were plotted. Following the final count, cultures were drained, blotted, weighed, then air dried to constant weight at 95 °C and re-weighed.

Statistical procedures

Data describing plant growth response to dissolved glyphosate were fitted to the following model, at each level of glyphosate concentration.

$$Ln \quad (\text{frond} \quad \text{number}) = \beta_0 + \beta_1(\text{time}) + \beta_2(\text{time})^2 + E_i$$

Plant growth response to fixed glyphosate concentration with variable exposure time data were also fitted to this model, and considered at each level of exposure time. Plant 'doubling times' were estimated as a function of the coefficients of the above model, and were calculated as of the ninth day after treatment started. The physical interpretation of the model parameters may be described as follows: β_0 is the intercept, and is proximate to the natural log of the frond number at the outset of the experiment; β_1 is the instantaneous rate of culture growth at time zero; β_2 is the deceleration of growth over time, and E_i is the error component.

Plant weight response to dissolved, as well as sprayed, glyphosate was fitted to a simple linear model with exposure time as the independent variable and plant weight the dependent variable. Results based on plant dry weight coincided with those based on plant wet weight.

Analysis of variance, regression, and covariance analysis were used to analyze the data, employing contrasts and simultaneous multiple comparisons (Duncan) to determine differences between treatment levels (SAS, 1986).

Results and discussion

Plant growth response to dissolved glyphosate

The mean (geometric) numbers of fronds produced in cultures exposed to glyphosate dissolved in the culture medium are plotted in Fig. 1. Coefficients for the model describing the growth curves are given in Table 1, and these show that the model provided a good description of the growth, as judged by r^2 values exceeding 0.97. There was no inhibition of frond production at concentrations of 10^{-4} M or lower, however, culture growth was eliminated at 10^{-3} M, hence the threshold for phytotoxicity was somewhere in the range between 10^{-4} M and 10^{-3} M. Indeed there was a small but significant enhancement of frond production at 10^{-4} M and 10^{-5} M, presumably

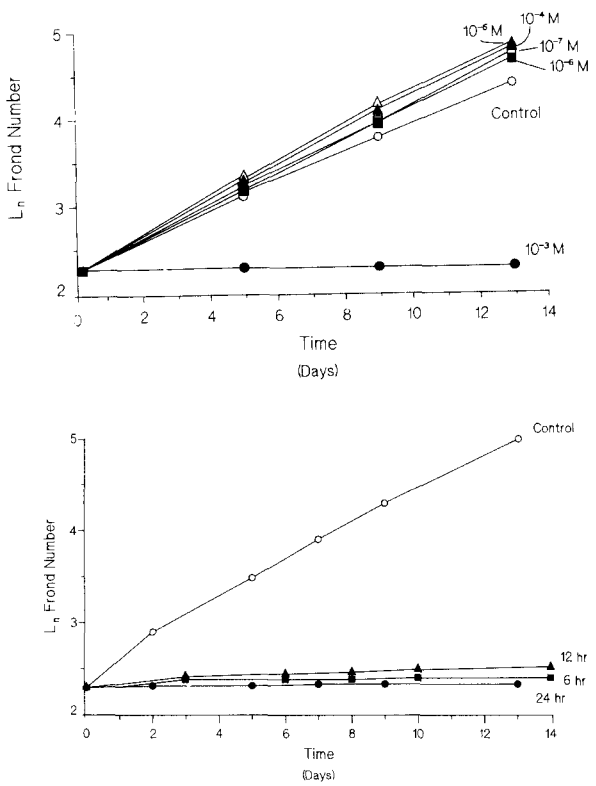

Fig. 1. Numbers of fronds present in duckweed cultures exposed to several concentrations of glyphosate dissolved in the culture medium (above) and exposed to a surface spray at 800 g active ingredient per ha and allowed to stand undisturbed for varying periods in the spray chamber (below).

Table 1. Coefficients for growth equation[1] for different concentrations of glyphosate dissolved in the culture medium.

Glyphosate concentration (M)	β_0	β_1		β_2	r^2	Doubling time (days)
0 (Control)	2.29	0.197	b	−0.00211	0.979	4.7
10^{-7}M	2.26	0.239	ab	−0.00350	0.994	4.3
10^{-6}M	2.26	0.229	ab	−0.00314	0.992	4.4
10^{-5}M	2.26	0.265	a	−0.00483	0.993	4.4
10^{-4}M	2.26	0.249	a	−0.00397	0.986	4.3
10^{-3}M	2.30	0.003	–	−0.00012	ns[2]	–

Slopes β_1 followed by the same letter failed to differ at the $\alpha = 0.01$ probability level.

[1] L_n (frond number) = $\beta_0 + \beta_1$(time) + β_2(time)^2 + E_i

[2] Since there was no growth at 10^{-3}M glyphosate there was no significant relationship between time and frond number, so that r^2 and doubling times were not meaningful. Doubling times for other exposures were calculated arbitrarily at day 9.

illustrating the phenomenon of hormesis (Stebbing, 1982).

In these exposures the plants were somewhat less sensitive than in studies reported previously with glyphosate. Jaworski (1972) found that glyphosate at 10^{-3} M inhibited completely the growth of *Lemna gibba*, that growth was significantly (75%) inhibited at 10^{-4} M, and essentially unaffected at 10^{-5} M. Cooley & Foy (1986) reported that 10^{-4} M glyphosate reduced growth of *Lemna gibba* to about one third of untreated controls while 10^{-5} M had no effect. Hartman & Martin (1984) used a bioassay technique similar to that reported here and found growth of *Lemna minor* reduced by about one third at only 2 mg l^{-1} (approximately 10^{-5} M) with growth almost eliminated at 10 mg l^{-1}. While the plants used here were relatively insensitive to glyphosate, they are not insensitive to other herbicides. For example, the cultures used here were comparable to those from another laboratory in their sensitivity to terbutryn. We obtained a growth reduction at a starting concentration of terbutryn of 3.16×10^{-8} M (about 8 μg l^{-1}, Lockhart *et al.*, 1983), and Bahadir & Pfister (1985) reported growth reduction at a concentration which started at 30 μg l^{-1} and fell to 10 μg l^{-1} over a 1-week exposure period. Richardson (1985) has reviewed a variety of bioassays with glyphosate.

Recent study of the dissipation of glyphosate residues from forest ponds treated by several passes of an aircraft emitting glyphosate at a rate of 0.89 kg ha^{-1} has shown that maximum residues were under 200 mg l^{-1}, (Goldsborough, 1989) and so the duckweed cultures would not be sensitive to these concentrations of dissolved glyphosate. An earlier study of glyphosate sprayed at 3.3 kg ha^{-1} over a forest stream resulted in peak residues in the first 3 hr after spraying of only about 2.7 mg l^{-1} (Newton *et al.*, 1984).

Plant weight responses to dissolved glyphosate

The effect of dissolved glyphosate on frond growth in terms of wet and dry weights is shown in Table 2. The same conclusions follow from frond weights as from growth in frond numbers. Concentration of glyphosate at 10^{-4} M or lower did not reduce wet or dry weights below controls, while 10^{-3} M obviously did.

The low toxicity of glyphosate in the water is not surprising in view of the high water-solubility of this herbicide (1.2%, Weed Science Society, 1983). The tendency of a compound to accumulate in duckweed is related to its octanol/water partition coefficient (Lockhart *et al.*, 1983) which is inversely proportional to its water solubility (Chiou *et al.*, 1977). Given its high water solubility, glyphosate would have little tendency to

Table 2. Mean wet and dry weights of duckweed fronds after 21 days in cultures exposed to a range of starting concentrations of glyphosate dissolved in the culture medium.

Glyphosate (M)	Wet Weight (mg) ± S.D.		Dry Weight (mg) ± S.D.	
0	236 ± 33	a	23.9 ± 5.5	c
10^{-7}M	253 ± 18[1]	a	22.8 ± 1.6[1]	c
10^{-6}M	265 ± 22	a	22.3 ± 0.7[1]	c
10^{-5}M	280 ± 34	a	25.4 ± 1.1	c
10^{-4}M	256 ± 13	a	22.4 ± 2.4	c
10^{-3}M	7.6 ± 1.9	b	0.3 ± 0.1	d

[1] Mean of four cultures.
Figures are arithmetic means of 5 cultures except as noted.
Means followed by different letters are statistically different at the $\alpha = 0.01$ probability level using Duncan's test.

partition from the water to the plants, and it would also wash off the plants easily prior to penetration. Indeed users are warned that rainfall occurring 6 hr after treatments of weeds with glyphosate may reduce its effectiveness, and heavy rainfall within 2 hr may wash glyphosate off leaves (Weed Science Society, 1983).

Plant growth response to sprayed glyphosate

The application of glyphosate as a spray to the surface of cultures was more toxic to the plants than was adding glyphosate to the culture medium. Figure 1 shows the effect on frond production of spraying commercial Roundup® at the rate recommended for control of weeds up to 15 cm high (2.25 l formulated product per hectare = 801 g isopropylamine salt of glyphosate ha^{-1}). Whether the plants were left in the exposure chamber for 6, 12, or 24 hr made little difference; in all cases the subsequent growth of the cultures was essentially zero, while untreated plants (which were left standing covered in the spray apparatus 24 hr after treatment) grew normally. The theoretical concentration in the water if all the glyphosate deposited were mixed throughout the volume of medium in the dish was 2.3×10^{-5} M. This concentration was too low to cause measurable reductions in culture growth (Fig. 1). The plants must have experienced an exposure greater than that available through mixing of glyphosate with the water. Presumably the plants intercepted droplets of spray and were exposed through deposit contact, perhaps with some additional exposure through the water.

Plant weight responses to sprayed glyphosate

The spray deposit effectively eliminated plant weight increases (Table 3) at all three standing periods just as it inhibited frond production (Fig. 1). These results confirm the susceptibility of the plants to sprayed glyphosate. In early experiments with the track sprayer, plants were removed from the apparatus immediately after spraying, and the spray had no effect, presumably because even gentle movement of the dishes caused some mixing of plants and medium and washed glyphosate deposits off plants surfaces into the medium where there was insufficient herbicide to affect growth. This suggests that glyphosate would be relatively ineffective on duckweed in field settings where mild wave action would have the same effect as our early removal of dishes from the sprayer. In fact, glyphosate at application rates up to 6.7 kg ha^{-1} was ineffective in controlling growth of duckweed in outdoor experimental trials (Thayer & Haller, 1985). Apparently 6 hr of undisturbed standing after spraying was long enough to allow sufficient glyphosate to penetrate the plants and exert its phytotoxic action.

Table 3. Wet and dry weights of duckweed fronds after 13 or 14 days in cultures exposed to glyphosate applied as a surface spray of commercial Roundup® at 800 g ha^{-1}.

Treatment	Standing time (hr)	Wet Weight (mg) ± S.D.		Dry Weight (mg) ± S.D.	
Unsprayed control	24	225 ± 31	a	14.5 ± 1.9	c
Sprayed	6	12.1 ± 1.5	b	2.3 ± 0.3	d
Sprayed	12	17.6 ± 8.1	b	2.4 ± 0.8	d
Sprayed	24	11.0 ± 1.4[1]	b	1.9 ± 0.4[1]	d

[1] Mean of nine cultures.
Cultures were allowed to stand undisturbed in the spray apparatus for 6, 12, or 24 hr after spraying before sub-cultures were taken for measurement of effects over a 13- or 14-day observation period in untreated medium.
Figures are arithmetic means of 10 cultures except as noted, with standard deviations.
Means followed by different letters are statistically different at the $\alpha = 0.01$ probability level using Duncan's test.

Conclusions

Glyphosate is inherently phytotoxic to duckweed, but the plants are relatively insensitive to glyphosate in the water, probably because glyphosate would have little tendency to partition from water to plants and hence the dosage experienced by the plants would be less than for a more hydrophobic compound. Following spray applications deposit contact may pose a greater risk to emergent aquatic vegetation than contamination of the water. Non-target emergent vegetation would be expected to suffer damage if the deposit were not washed off within some time less than six hours. With sprayed glyphosate phytotoxicity may be expressed more appropriately in terms of spray rates than in conventional units of concentrations in the water.

Acknowledgements

The Department of Plant Science, University of Manitoba, kindly allowed the use of its laboratory-scale track sprayer for the spray exposures.

References

Bahadir, M. & G. Pfister, 1985. A comparative study of pesticide formulations for application in running waters. *Ecotoxicol. Envir. Saf.* 10: 585–590.

Bishop, W. & R. Perry, 1981. Development and evaluation of a flow-through growth inhibition test with duckweed (*Lemna minor*). In: D. Branson & K. Dickson (Eds.), *Aquatic Toxicology and Hazard Assessment: Fourth Conference.* American Society for Testing and Materials, Philadelphia. Special Technical Publication 737. pp. 421–435.

Blackman, G. & R. Robertson-Cuninghame, 1954. Interactions in the physiological effects of growth substances on plant development. *J. Exp. Bot.* 54: 184–203.

Blackman, G. & R. Robertson-Cuninghame, 1955. Interrelationships between light intensity, temperature, and the physiological effects of 2 : 4-dichlorophenoxyacetic acid on the growth of *Lemna minor*. *J. Exp. Bot.* 6: 156–176.

Chiou, C., V. F. D. Schmedding & R. Kohnert, 1977. Partition coefficient and bioaccumulation of selected organic chemicals. *Envir. Sci. Technol.* 11: 475–478.

Cooley, W. & C. Foy, 1986. Effects of SC-0224 and glyphosate on inflated duckweed (*Lemna gibba*) growth and EPSP-synthetase activity from *Klebsiella pneumoniae*. *Pestic. Biochem. Physiol.* 26: 365–374.

Goldsborough, L. & A. Beck, 1989. Rapid dissipation of glyphosate in small forest ponds. *Arch. Envir. Contam. Toxicol.*, in press.

Gummer, W., 1980. Pesticide monitoring in the prairies of Western Canada. In: B. Afghan & D. Mackay (Eds.). *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment*, pp. 345–372. Plenum Press, New York.

Hartman, W. & D. Martin, 1984. Effect of suspended bentonite clay on the acute toxicity of glyphosate to *Daphnia pulex* and *Lemna minor*. *Bull. Envir. Contam. Toxicol.* 33: 355–361.

Hartman, W. & D. Martin, 1985. Effects of four agricultural pesticides on *Daphnia pulex*, *Lemna minor* and *Potomogeton pectinatus*. *Bull. Envir. Contam. Toxicol.* 35: 646–651.

Hillman, W., 1961. The *Lemnaceae*, or duckweeds, a review of the descriptive and experimental literature. *Bot. Rev.* 27: 221–287.

Jaworski, E., 1972. Mode of action of N-phosphonomethylglycine: Inhibition of aromatic amino acid biosynthesis. *J. Agric. Food Chem.* 20: 1195–1198.

Lockhart, W., B. Billeck, B. deMarch & D. Muir, 1983. Uptake and toxicity of organic compounds: Studies with an aquatic macrophyte (*Lemna minor*). In: W. Bishop, R. Cardwell, and B. Heidolph (Eds.), *Aquatic Toxicology and Hazard Assessment: Sixth Symposium*, American Society for Testing and Materials, Philadelphia. ASTM STP 802. pp. 460–468.

Lockhart, W. & A. Blouw, 1980. Phytotoxicity tests using *Lemna minor*. In: E. Scherer (Ed.). *Toxicity Test for Freshwater Organisms*, Can. Dep. Fish. Oceans, Winnipeg. Spec. Pub. Fish. Aquat. Sci. No. 44. pp. 119–130.

Monsanto Canada Inc. *Roundup Liquid Herbicide by Monsanto.* 26 pp.

Muir, D. & N. Grift, 1987. Herbicide levels in rivers draining two prairie agricultural watersheds (1984). *J. Envir. Sci. Health*, B22: 259–284.

Nasu Y. & M. Kugimoto, 1981. *Lemna* (duckweed) as an indicator of water pollution. I. the sensitivity of *Lemna paucicostata* to heavy metals. *Arch. Envir. Contam. Toxicol.* 10: 159–169.

Newton, M., K. Howard, B. Kelpsas, R. Danhaus, C. Lottman & S. Dubelman, 1984. Fate of glyphosate in an oregon forest ecosystem. *J. Agric. Food Chem.* 32: 1144–1151.

Richardson, W., 1985. Bioassays for glyphosate. In: E. Grossbard & D. Atkinson (Eds.). *The Herbicide Glyphosate*, pp. 286–298. Butterworths.

SAS Institute Inc., Cary, North Carolina, 1986. *SAS Systems for Linear Models.*

Stebbing, A., 1982. Hormesis – the stimulation of growth by low levels of inhibitors. *Sci. Tot. Envir.* 22: 213–234.

Stewart, G., 1972. The regulation of nitrite reductase level in *Lemna minor* L. *J. Exp. Bot.* 23: 171–183.

Thayer D. & W. Haller, 1985. Effect of herbicides on floating aquatic plants. *J. Aquat. Pl. Man.* 23: 94–95.

Weed Science Society of America, Champaign, Illinois, 1983. *Herbicide Handbook*, fifth edition. pp. 258–263.