Bull. Environ. Contam. Toxicol. (1994) 53: 120–126

Environmental Contamination and Toxicology

Effects of Atrazine, Isoproturon, and Mecoprop on the Macrophyte *Lemna minor* and the Alga *Scenedesmus subspicatus*

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Received: 26 August 1992/Accepted: 16 January 1994

The use of herbicides is increasing world-wide as the need for selective weed control becomes more important. The UK Ministry of Agriculture, Fisheries and Food data base (*D Thompson, pers. comm.*) indicates that approximately 50% of approved biocides in the UK are herbicides.

Fast, sensitive test procedures for assessing potential herbicidal impacts on non-target species within the freshwater environment are therefore essential. Algal toxicity tests have been used in the UK until now to assess phytotoxicity, and internationally approved standard methods are available (e.g. International Standards Organisation 1989). However, recent studies (Taraldsen 1990) have questioned the relevance of algal test data in assessing phytotoxic impacts on higher vascular plants and some studies (Garten 1984; Thomas 1986) have indeed shown that EC_{50} (growth) results gained with algae and higher plants can differ by as much as a factor of five hundred. For these reasons, the UK pesticide registration authority now also requires a *Lemna* macrophyte test to be conducted with herbicides, although a standardized guideline is not yet available.

This study compared the toxicity of three herbicides, atrazine, isoproturon and mecoprop to a freshwater green flagellate alga, *Scenedesmus subspicatus*, and a macrophyte plant, the common duckweed *Lemna minor*. One objective of the work was to assist the development of a practical guideline for routine *Lemna* tests.

L. minor was chosen as the representative macrophyte because it is widespread in the UK, small, easy to handle and culture, and reproduces asexually at a fast rate. Three different end points were identified for the *L. minor* test and these were used to compare the sensitivity of the technique with that of the International Standards Organisation (ISO) algal bioassay.

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MATERIALS AND METHODS

The test compounds were chosen to represent three different herbicide groups. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a systemic triazine used to control annual grasses and broad-leaved weeds. It is absorbed principally through the roots but also by the foliage and it inhibits photosynthetic electron transport and interferes with other enzymic processes. Isoproturon (1,1-dimethyl-3-(4-isopropylphenyl)-urea) is a systemic urea-type herbicide used to control annual grasses and broad-leaved weeds, it is absorbed by the roots and leaves and inhibits photosynthetic electron transport. Mecoprop (2-[(4-chloro-o-tolyl)oxy] propionic acid) is an aryloxyalkanoic acid used for post-emergent control of broad-leaved weeds, it is absorbed by the leaves with translocation to the roots where it interferes with hormonal processes (Royal Society of Chemistry 1991). Technical grade atrazine (98% purity) and isoproturon (98% purity) were obtained from Ciba-Geigy Agrochemicals, Whittlesford, Cambridge, UK and technical grade mecoprop (98% purity) was obtained from Aldrich Chemicals Ltd., Gillingham, Dorset, UK.

In the macrophyte study the nominal test concentrations based on active ingredient were: 28, 56, 112, 224 and 448 $\mu g\ L^{-1}$ for atrazine and isoproturon and 0.5, 0.9, 1.8, 3.6 and 7.2 $mg\ L^{-1}$ for mecoprop.

L. minor plants were taken from an established laboratory culture started in 1988 from a single plant taken from an Essex pond. Test solutions were made up in Steinbergs nutrient medium (Kwan and Smith 1990) which was buffered to pH 7.0 with 0.1 M NaOH. Five double fronded *Lemna* colonies were exposed to 200 mL of test solution in 250-mL beakers; four replicate vessels were used for each test concentration. The test vessels were held in a water bath maintained at a temperature of 25 ± 1°C. Continuous illumination of 3500 lux was provided by four cool white fluorescent tubes suspended 12 cm above the test vessels. Solutions were renewed every 2 da and the test was terminated after 10 da.

Three parameters were measured: chlorophyll a content, fresh weight, and frond production. Chlorophyll a was determined by crushing the *Lemna* plants with a mortar and pestle in 10 mL of acetone, this was refrigerated for 24 hr and then gently centrifuged at 2000-3000 rpm for 10 minutes to remove the cellulose material and the supernatant tested using the method outlined by Lorenzen (1967). Total fresh weight in each test container was determined at the end of the exposure period for fresh blotted-dry plants. Frond counting was carried out daily and included all daughter fronds as long as they protruded from the natural outline of the parent frond.

The EC_{50} values were calculated using the SAS Institute non linear model procedure. The model fitted was based upon that described by Barton *et al.* 1993. The EC_{50} values were compared using the t-test.

Chemical analysis was undertaken for atrazine. Samples were extracted with dichloromethane and analysed using a Hewlett Packard 5890 Series 1 gas

chromatograph with nitrogen/phosphorus detection (Department of the Environment, UK 1986). Two sets of atrazine solutions were sampled, each test concentration being analysed immediately after it was made up and again when it was discarded after 2 da.

One set of isoproturon solutions was also analysed, at zero and two days, again using a dichloromethane extraction followed by a reversed phase HPLC technique with a 25 cm C18 column and UV detection at 230 nm (Williams *et al.* 1991).

No chemical analysis was conducted for mecoprop; however it has a half-life in aerobic aqueous conditions of 7-10 da (Howard *et al.* 1991). Therefore, little degradation would have been likely to occur over the 2-da solution renewal period.

All EC_{50} values were calculated on the basis of nominal concentrations.

The *S. subspicatus* algal test was carried out in accordance with the ISO (1989) protocol. The culture of *S. subspicatus* was obtained from the Culture Centre of Algae and Protozoa, Institute of Freshwater Ecology, Cumbria, UK and was maintained in the medium outlined in the ISO protocol. Tests were conducted on 100 mL of test solution in 250-mL Erlenmeyer flasks stoppered with a cotton wool plug. Each herbicide was dissolved in the test medium to give the following nominal test concentrations; 28, 56, 112, 224 and 448 $\mu g\ L^{-1}$ for atrazine and isoproturon and 80, 93, 107, 121 and 135 $mg\ L^{-1}$ for mecoprop. Concentration ranges were selected on the basis of an initial range finding test. Tests were run in a constant temperature room held at 20°C with constant illumination of 1200-1400 lux supplied by four fluorescent tubes suspended 80 cm above the test solutions. Test vessels were placed on an orbital shaker set at 100 revs/min.

At the start of the test sufficient algae were innoculated into each test solution to give a cell density of 10^4 cells mL^{-1}. Cell density was measured daily over a 4- da exposure period with a Varian DMS100 spectrophotometer. Cell numbers were derived from a calibration curve previously determined for this species, of absorbance at 690 nm against cell numbers.

The EC_{50} values were calculated as described previously for *Lemna*.

RESULTS AND DISCUSSION

The results in Table 1 indicate that isoproturon is slightly more toxic than atrazine to *L. minor* and that both substances are at least two orders of magnitude more toxic than mecoprop ($p<0.05$). For atrazine there is no major difference in sensitivity between any of the three endpoints used to measure toxicity. However frond number was not as sensitive a measure as the other two for isoproturon but was a more sensitive measure than fresh weight for mecoprop ($p<0.05$).

The results in Table 2 show that atrazine has a similar toxicity to isoproturon for *S. subspicatus* and that they are both at least three orders of magnitude more toxic than mecoprop.

Table 1. Toxicity of atrazine, isoproturon and mecoprop to *L. minor*.

Herbicide	Endpoint	10-da EC_{50} ($\mu g\ L^{-1}$)	95% Confidence Limits
Atrazine	Frond number	56	45-67
"	Fresh Weight	60	48-70
"	Total chlorophyll	62	50-74
Isoproturon	Frond number	40	37-43
"	Fresh Weight	33	30-35
"	Total chlorophyll	31	29-34
Mecoprop	Frond number	5147	3822-6473
"	Fresh Weight	7352	6131-8573
"	Total chlorophyll	6228	4173-8285

Table 2. Toxicity of atrazine, isoproturon and mecoprop to *S. subspicatus*.

Herbicide	Endpoint	96hr EC_{50} ($\mu g\ L^{-1}$)	95% Confidence Limits
Atrazine	Cell Production	21	14-27
Isoproturon	"	21	15-27
Mecoprop	"	102660	97642-108678

Table 3. Analytical results for atrazine.

Nominal Concentration ($mg\ L^{-1}$)	Measured Concentration ($\mu g\ L^{-1}$)			
	Day 0 New soln.	Day 2 Used soln.	Day 2 New soln.	Day 4 Used soln.
Control	1	0.5	0.46	-
28	47	34	44	31
56	66	52	-	-
112	168	131	160	74
224	312	248	336	238
448	576	323	587	217

Table 4. Analytical results for isoproturon.

Nominal Concentration ($mg\ L^{-1}$)	Measured Concentration ($\mu g\ L^{-1}$)	
	Day 0 New solution	Day 2 Used solution
Control	1.1	1.2
28	29.8	28.6
56	53.8	57.0
112	108.5	123.5
224	200.0	214.0
448	370.0	424.0

The analytical results (Tables 3 and 4) show that atrazine was more prone

to degradation under the test conditions than was isoproturon. Atrazine concentrations fell by up to 50% over the two day exposure period, which suggests that test solutions should be renewed daily for less stable toxicants.

This result was unexpected as atrazine is usually more persistent but this could be attributable to greater photodegradation or removal via macrophyte bioaccumulation. Analysis of degradation products and plant tissue is recommended for future studies.

Isoproturon, however, was reasonably stable over the 2-da exposure period and in each case became slightly more concentrated. This may be due to the evaporation (approximately 10%) that occurred between solution changes.

Atrazine and isoproturon were approximately two times more toxic to *Scenedesmus* compared to *Lemna* ($p<0.05$). However mecoprop was approximately sixteen times more toxic to *Lemna* than to *Scenedesmus*.

Frond counting is preferred over the other methods since it is easier to carry out and in most cases appears to be of similar sensitivity to the other endpoints. However, by examining other endpoints it is possible to gain more information on a toxicant's mode of action, e.g., an inhibition of chlorophyll content would suggest that the toxicant exhibited herbicidal activity by interfering with chlorophyll production whereas an inhibition in root or frond growth would indicate that these were the areas where the herbicidal activity was focussed.

It is apparent from these results and from previous studies (Garten 1984; Thomas 1986) that algal test results alone may not be sufficient to assess toxicants for their potential adverse effects on aquatic vascular plants. Garten and Frank (1984) found that a standard algal test was more sensitive than terrestrial vascular plants in approximately 50% of tests when assessing a range of herbicides, so it is likely that similar limitations apply when assessing toxicity of herbicides to aquatic macrophytes.

Certain groups of herbicides have modes of action that affect higher plants much more severely than algae. Dicamba 2,4-D and picloram, for example, are up to one thousand times more toxic to a range of higher terrestrial plants than to the alga *Selenastrum capricornutum* (Garten 1984). Each of these herbicides acts as an auxin-like growth regulator and the difference in toxicity is explained by the fact that algal growth is not controlled by auxins. Also, herbicides such as dalapon, naptalam and propham that are absorbed by or have their primary effect upon the roots have also been shown to be up to one hundred times more toxic to higher plants than algae (Garten 1984). For these groups of herbicides it is clear that algal tests are not sufficiently sensitive screening tools.

L. minor bioassays may have advantages over algal tests when assessing the effects of brief exposures. Concentrations of the herbicide atrazine of up to

275 μg L^{-1} have been detected in run-off water from agricultural land (Southwick 1990) which is roughly five times the *Lemna* 10-da EC_{50} calculated in the present study. Isoproturon has also been found at concentrations up to 17.2 μg L^{-1} in agricultural run-off water (Williams *et al.* 1991) which is near to the 10-da EC50 of 30 μg L^{-1}. These high concentrations are normally short lived but *Lemna* could be easily used as a bioassay to assess the impacts of such run-off events or of spillages. The surface micro-layer of water bodies can also contain high concentrations of chemicals (Wu *et al.* 1980 found concentrations of atrazine of between 150 and 8850 μg L^{-1} in the surface micro-layer of an estuary) and *Lemna* is an ideal species to use for investigating this phenomenon since it is a surface dwelling plant.

Other methods using *Lemna* species do exist (US EPA 1985) and tend to be comparable to those used in this study in most aspects but the present approach has advantages in important areas. The American methods (US EPA 1985) recommend the use of Hoagland's medium, the pH of which ranges from 4.5 to 5.5. The low pH of this medium would present problems in tests upon herbicides which have half-lives of only a few hours in the lower pH range (e.g., the sulphonylureas). In static renewal test procedures in which the test medium is renewed every other day it is essential that this problem is avoided by the use of media of a more neutral nature. Medium hardness has also been shown to greatly reduce the sensitivity of the *Lemna* test (M. Kirby, unpubl. data), especially when investigating metal toxicity. Steinberg's medium as used in this study is easily buffered to pH 7.0 unlike Hoagland's medium whose salt constituents begin to come out of solution when it is buffered to neutrality and is also a much 'softer' medium than Hoagland's, i.e., 42 and 700 mg L^{-1} as $CaCO_3$ respectively. Therefore Steinberg's seems a more appropriate and versatile medium to use.

The results of this study suggest that the *Lemna* bioassay should be used to complement algal tests in assessing the phytotoxicity of possible environmental pollutants, especially those with herbicidal activity. Tests using *Lemna* also offer greater potential than algal and other macrophyte tests in assessing brief and surface microlayer exposures. The methods developed for this study show promise as the basis of a standardized guideline for *Lemna* tests.

Acknowledgments. The author would like to thank Mr A Turnbull and Mr C Allchin for their analytical support. Miss L Howell for statistical analysis of the data. Also Mr J Thain and Dr P Matthiessen for their support and guidance in the study and Mrs D Murray and Mrs A Mills for helping to get it onto paper.

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