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Single Species Algal *(Ankistrodesmus)* Toxicity Tests with Rodeo® and Garlon® 3A

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As the distribution of invasive exotic plants has expanded, the use of herbicides to control these species has also increased. In Washington State, herbicides are frequently used to control aggressive exotic wetland vegetation (Arroll *et al.* 1994). Rodeo® (Monsanto Agricultural Company, St. Louis, MO), a formulation of glyphosate, is being used extensively for the control of purple loosestrife (*Lythrum salicaria*) in Washington State; Garlon®3A (DowElanco, Indianapolis, Indiana) a formulation of triclopyr, is being used experimentally.

Both glyphosate and triclopyr are systemic herbicides. Glyphosate [N-(phosphonomethyl) glycine] is thought to reduce plant growth by inhibiting aromatic amino acid biosynthesis (Jaworski 1972). Triclopyr ([(3,5,6 trichloro-2-pyridinyl)oxy]acetic acid) is a more narrow spectrum herbicide than glyphosate and stimulates abnormal tissue proliferation by mimicking natural plant auxins (Boyall 1983). Under operational application conditions, concentrations of these herbicides in water have been measured at levels of <l µg/mL (Gardner and Grue 1996).

Presently, information on the effects of these compounds on phytoplankton communities is scarce. Studies using single species tests to assess the toxicological effects of glyphosate and triclopyr on algal cell density have provided a range of EC_{50} values depending on the species and test conditions. EC_{50} values from 3 to 590 mg/L have been determined for technical grade glyphosate (Richardson *et al.* 1979, with *Euglena;* Hess 1980, with *Chlamydomonas;* Christy *et al.* 1981, with *Chlorella;* Maule and Wright 1984, with several species of green algae and cyanobacteria) and 11 to 17 mg/L for Garlon 3A and a marine diatom (*Skeletonema costatum,* Cowgill *et al.* 1989). Data on the effects of Rodeo on algae are lacking as is information on the effects of either herbicide formulation on the ecological interactions of algae.

The objective of the present study was to compare the toxicity of the Rodeo and Garlon 3A using single species toxicity tests to calculate cell growth 96-h EC_{50} values for the green alga, *Ankistrodesmus.* Members of this genus are common in water and soil and can grow to such abundance as to form algal blooms (Bold and Wynne 1985). *Ankistrodesmus* has been included in the Annual Book of ASTM

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Standards (American Society for Testing and Materials 1990) as a recommended test organism for assessing adverse effects of test materials on growth of freshwater microalgae. Therefore, the present study not only contributes to our understanding of the effects of herbicides on an environmentally relevant taxa, but also provides information for the comparison of the two herbicides based on standardized toxicity tests.

MATERIALS AND METHODS

Ankistrodesmus was obtained on agar slants from Dr. Frieda Taub, University of Washington, and was grown in 1L of liquid culture of T82 media (ASTM 1990) for at least 14 d prior to testing. Two culture chambers, consisting of 2.6 L glass jars were maintained under constant temperature and light conditions (24° C ± 15% and 2100 lm/cm^2 ± 15% [12 h light: 12 h dark cycle]). Filtered air was bubbled through a glass tube into the cultures to provide gentle aeration and dispersal of the cells. Algal counts and pH readings (using a pH/ORP Controller 3675, Jenco Electronics, LTD.) were conducted twice a week and 0.5L of fresh medium were added weekly to maintain the cultures in a near logarithmic growth state.

To prevent contamination, each culture chamber was autoclaved prior to the introduction of algae and all glassware was acid washed prior to use. All work surfaces were wiped with 75% ethanol and whenever possible, work was conducted in a well ventilated fume hood. Two back-up cultures were maintained adjacent to the liquid cultures on 1% agar petri dishes.

Dose response curves were generated following the procedures described by ASTM (1990). Each test was conducted in an incubator under the same conditions under which the cultures were maintained, except the light intensity was 4300 lm/cm^2 ± 15% (ASTM 1992). Although the ASTM protocol recommends the use of glass bottles, our tests were conducted in 150 mL polyethylene bottles (with loosely fitted caps), because glyphosate chemically binds to glass. The algal solution was prepared by adding 48 mL of fresh medium to 2 mL of algae to obtain a total volume of 50 mL with a concentration of 2 x l0^4 cells/ml.

The test was initiated by adding one of the two herbicide formulations at different concentrations (0, 5, 20, 80, 320, 1280, or 5120 µg active ingredient/ml media) to the test chambers or the same volume of distilled water to the controls. Extra bottles of each of the seven herbicide concentrations were prepared for measuring the pH at the beginning, middle, and end of each test using a destructive sampling technique. These bottles were subsequently eliminated from the study. Algal density was measured by microscopically counting a subsample of cells from each test chamber using a hemocytometer. Two samples were counted from each bottle and two counts were conducted for each sample. The test was conducted for 10 d based on preliminary evidence suggesting that, under experimental conditions, the algal growth curve becomes asymptotic between Days 10 and 12 (Arroll 1995). Algal counts were conducted every day for the first 4 d and then every other day through Day 10.

In order to differentiate between the direct toxic effects of the Rodeo formulation and the effects of associated changes in pH, this test was repeated using three of the

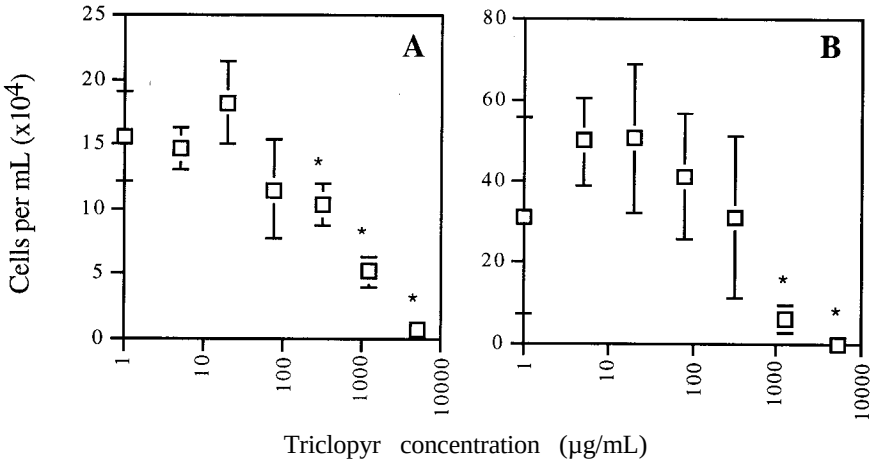

Figure 1. Density of *Ankistrodesmus* cells exposed to concentrations of Garlon 3A: (A) Number of cells 96 h following addition of Garlon 3A; (B) number of cells 10 d following addition of Garlon 3A. Error bars indicate one standard deviation. Control values (triclopyr concentration = 0) are given on the y axis at x = 1. * = values significantly different from controls.

concentrations (20, 320 and 5120 µg/mL) and adjusting the initial pH of the culture following addition of the herbicide to 7.0 using 0.05 M NaOH.

The concentrations that produced a statistically significant effect on algal density were determined using Dunnett's procedure for multiple comparisons (Dunnett 1955; $p \leq 0.05$ unless noted otherwise). A probit analysis (Gelber *et al.* 1985) was used to derive the 96-h EC_{50} and the 95% confidence limits. We used the PROBIT procedure in SAS®, which uses maximum likelihood to calculate the regression parameter estimates (SAS 1989).

The probit model was

$$p = \Pr(\textit{living cells}) = F(b_0 + b_1 \times \ln(\text{concentration})), \text{ where}$$

$$F(x) = \int_{-\infty}^{x} e^{-z^2/2} / \sqrt{2\pi}\, dz \text{ (the normal link [SAS 1989])}$$

and *living cells* is the ratio of cells living in the treatment sample to the total in the control sample.

For the probit analysis, the 96-h EC_{50} for each herbicide was where

$$\Pr(\textit{living cells}) = 0.5.$$

The confidence limits were calculated as

$$\hat{x}_1 = (F^{-1}(p) - \mathbf{x}^{*\prime}\mathbf{b}^{*}) / b_1 \text{ (SAS 1989), where}$$

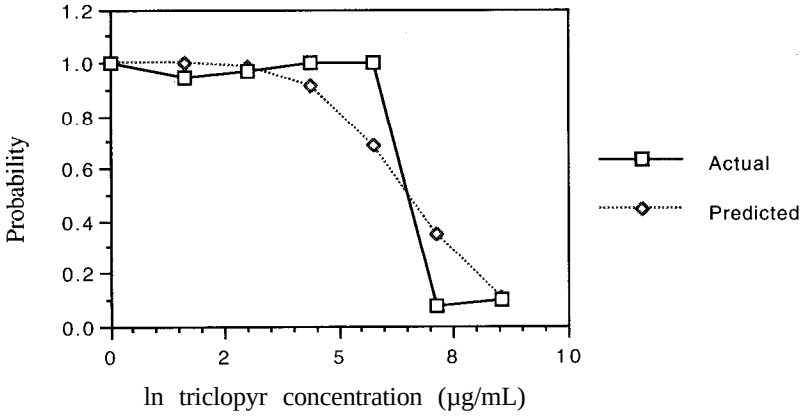

Figure 2. Dose-response relationship of *Ankistrodesmus* and Garlon 3A (values are 96-h data with unadjusted pH).

F = the normal cumulative distribution
x* = vector of independent variables excluding the first one
b* = vector of parameter estimates excluding the first one
b_1 = parameter estimate of the independent variable.

A separate probit analysis was performed on the data for: Garlon-unadjusted pH; glyphosate-unadjusted pH; glyphosate-adjusted pH.

RESULTS AND DISCUSSION

Cell density 96 h following exposure to the three highest Garlon 3A concentrations (320, 1280, and 5120 µg/mL) was significantly lower than controls (Fig. 1A). Ten d after the herbicide was added, there were no significant differences between the controls and the cultures exposed to 320 µg/mL (Fig. 1B). At the higher concentrations (1280 and 5120 µg/mL), the number of algal cells was still significantly lower than the controls.

The probit analysis yielded the following regression model for the Garlon 3A concentrations: p = F(4.13 - 0.63 x ln(concentration)) [significant at Pr >χ^2 = 0.0001]. The 96-h EC_{50} value for Garlon 3A was calculated to be 692 (±480) µg/mL (Fig. 2).

Ninety-six h following the addition of Rodeo, algal density at exposures ≥80 µg/ml was significantly lower than controls (Fig. 3A). By Day 10, the cultures exposed to 80 µg/mL glyphosate were no longer significantly different from controls (Fig. 3B). However, at the three highest concentrations (320, 1280, and 5120 µg/mL), there were still significantly fewer algal cells.

The probit analysis yielded the following regression model for the Rodeo concentrations: p = F(2.72 - 0.63 x ln(concentration)) [significant at Pr > χ^2 = 0.0001]. The 96-h EC_{50} for Rodeo was calculated to be 74 (±47) µg/mL.

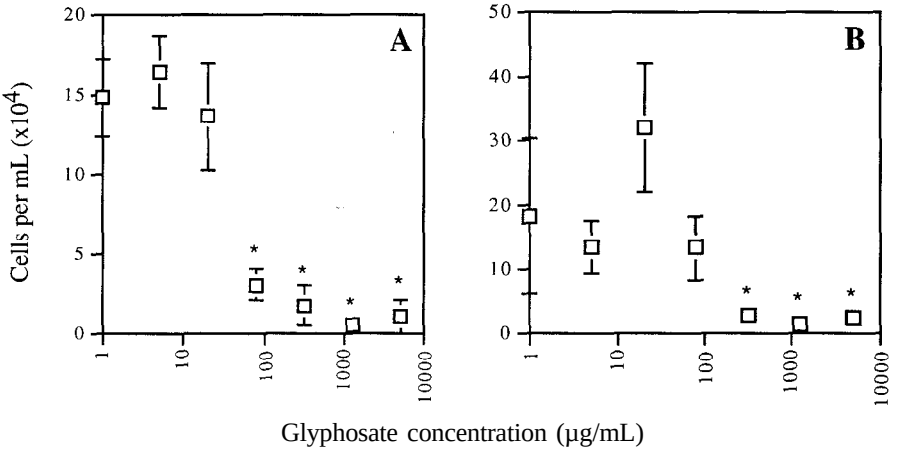

Figure 3. Density of *Ankistrodesmus* cells exposed to concentrations of Rodeo: (A) Number of cells 96 h following the addition of Rodeo; (B) number of cells 10 d following the addition of Rodeo. Error bars indicate one standard deviation. Control values (glyphosate concentration = 0) are given on the y axis at x = 1. * = values significantly different from controls.

Both herbicides caused a decline in the pH of the media (Fig. 4). However, the effect of Rodeo was much greater than Garlon 3A. At the highest herbicide concentration (5120 µg/mL), the initial pH of the Garlon cultures dropped from 7.0 to 6.0, whereas the pH of the Rodeo cultures dropped from 7.0 to below 4.5.

We repeated the Rodeo test under neutral pH conditions and recalculated the 96-h EC_{50} value. The probit analysis yielded the following regression model: p = F(3.61 - 0.6 x ln(concentration)) [significant at Pr > χ^2 = 0.0012]. With adjusted pH, the 96-h EC_{50} value for Rodeo increased to 412 (±511) µg/mL (Fig. 5), suggesting that the observed dose response relationship was influenced by the low pH environment. Significant decreases in algal density were detected only at the 320 and 5120 µg/mL at both 96 h and 10 d, and the difference in the toxicity of the two herbicides to *Ankistrodesmus* was less pronounced.

The results of our study indicate that when herbicide-induced changes in pH are not adjusted for, the toxicity of Rodeo to *Ankistrodesmus* was 4.5 times greater than that of Garlon 3A (96-h EC_{50}s = 74 and 692 µg/mL, respectively). However, these results may not be consistent under field conditions (e.g., different pH). Toxicity of herbicides to aquatic organisms depends on the ability of the toxic material to diffuse through the media and permeate cell membranes. Water quality conditions which influence the stability or solubility of herbicides may have substantial effects on their toxicity (Lockhart *et al.* 1989). This has been demonstrated for glyphosate in several studies which showed toxicity varied with water quality conditions (Folmar *et al.* 1979, temperature and pH; Hartman and Martin 1984, suspended sediment; Wan *et al.* 1987, water type).

Maule and Wright (1984) calculated the 96-h EC_{50} of glyphosate to a variety of algal

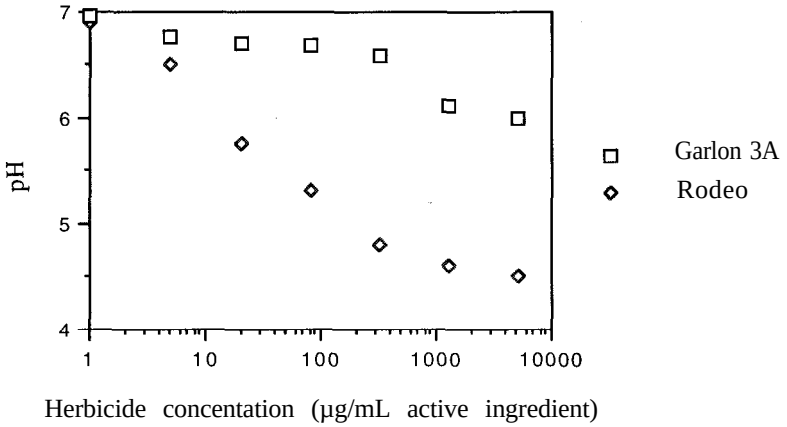

Figure 4. Effects of herbicide concentration on pH. Control values (herbicide concentration = 0) are given on the y axis at x = 1.

species and concluded that the herbicide was "relatively non-inhibitory to microalgae." They found *Chlorococcum hypnosporum* to be the most sensitive alga tested (EC_{50} = 68 µg/mL) and *Chlorella pyrenoidosa* to be the least sensitive (EC_{50} = 590 µg/mL). Hess (1980) reported that 1000 µg/mL of glyphosate resulted in a *Chlamydomonas* cell population growth rate that was 30% of the controls. However, in neither of these studies was the pH of the test environment reported, nor was an attempt made to compensate for changes in water quality parameters associated with the addition of the herbicides. A growth rate EC_{50} value of 17.7 µg/mL was calculated for *Chlorella sorokiniana* (Christy *et al.* 1981). Christy and co-workers reported pH values that ranged from 6.15 in the controls to 5.05 at the highest glyphosate concentration (591 µg/mL). In the present study, the range of pH values was noticeably larger (7.00 in the controls to 4.70 at 320 µg/mL glyphosate). This difference may be related to differences in the buffering capacity of the media or due to an enhanced acidity of the Rodeo formulation as compared to technical grade glyphosate.

Very little information is available on the effects of Garlon 3A or triclopyr on phytoplankton. Garlon 3A was reported to be slightly toxic ($10 < EC_{50} < 100$ µg/mL) to the marine diatom, *Skeletonema costatum* (Cowgill *et al.* 1989). The EC_{50} value calculated in the present study (692 µg/mL) is substantially greater. This may reflect a difference in sensitivity of the test organisms or a difference in the water quality conditions under which the tests were conducted. The pH of our Garlon 3A test was 6.04-7.00, whereas Cowgill and co-workers reported pH ranging from 7.70-9.00.

Standardized *in vitro* toxicity tests have been used to identify those herbicides which have the greatest potential for causing detrimental environmental effects in the field. However, the results presented here indicate that it is difficult to predict the effects of herbicides in the field by extrapolating from data obtained under defined *in vitro* conditions. Calculated EC_{50} values can vary depending on the experimental conditions of the study, and therefore, the toxicological ranking of chemicals, such as Rodeo and Garlon 3A, may change depending on the water quality conditions of the test.

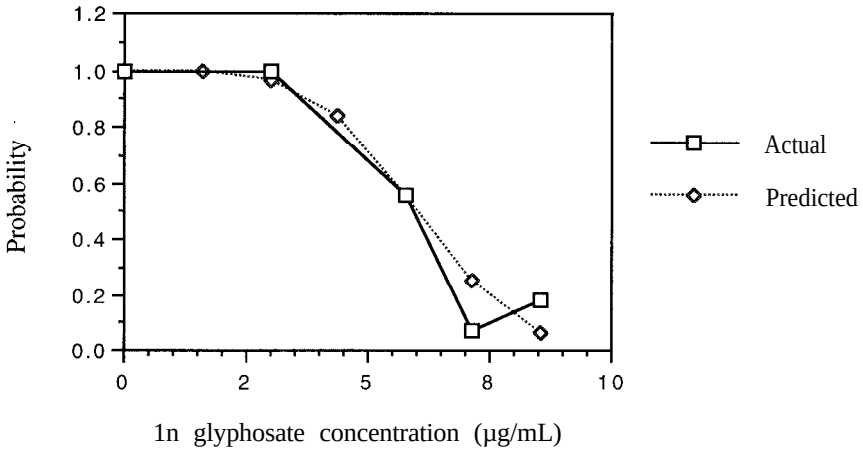

Figure 5. Dose-response relationship of *Ankistrodesmus* to Rodeo. Values indicate 96-h data with initial pH adjusted to 7.0.

Under the conditions used for the control of purple loosestrife by Gardner and Grue (1996), field application of Garlon 3A or Rodeo was not associated with changes in the *in situ* pH. This could in part reflect a higher buffering capacity of the wetland water as compared to the algal media, but more likely, it is probably due to the low concentrations of the herbicides (1µg active ingredient /mL) in the wetland following the application. Although in the present study, laboratory data obtained without a pH adjustment indicate that the phytotoxicity of Rodeo is significantly greater than that of Garlon 3A, a significant change in pH would not be anticipated under field conditions and the toxicity of Rodeo to *Ankistrodesmus* would be expected to be similar, if not lower than Garlon 3A.

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