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Response of *Lemna minor* to Sodium Chloride and a Statistical Analysis of Continuous Measurements for EC50 and 95% Confidence Limits Calculation

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Aquatic plants are receiving increased attention for use in toxicity tests. Of these *Lemna minor* (duckweed) offers advantages of a wide distribution in ponds and lakes throughout the United States (Hillman 1961), ease in culturing and a free-floating habit that exposes them to both surface active and hydrophobic compounds as well as to dissolved compounds. An important part of routine toxicity testing is maintenance of a quality control program which includes reference toxicant tests. Data from these tests are used in control charts or precision tables to monitor the sensitivity of test organisms.

The purposes of this work were to evaluate NaCl as a reference toxicant for *Lemma* and to define statistical procedures for analysis of the plant dry weight (biomass) measurements resulting from toxicity tests. Dry weight was the measurement selected on which to base the point estimate because it is reported to be more accurate (Cowgill *et al.* 1989), more reproducible and less time consuming (ASTM 1988) than counts of plants or fronds. The criteria used to evaluate NaCl as a reference toxicant were; 1) a concentration-dependent response and 2) a reciprocal response between growth and concentration.

Procedures for calculation of an EC50 and associated Confidence Limits (CL) are readily available for quantal (descrete) data such as mortality counts of fish or invertebrates (Stephen 1977; EPA 1985). Such procedures are, however, not recommended (Stephen 1977) for continuous data such as dry weight measurements. In fact, quantal and continuous data require different statistical methods (Finney 1955). Other problems and view-points regarding application of statistical techniques to analysis of quantal and non-quantal data have been discussed but not resolved (Adams *et al.* 1985). It has been suggested that point estimates of EC50s (EPA 1989) or "appropriate linear or nonlinear regression

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techniques" to calculate EC50s and CL (ASTM 1988) are appropriate for dry weight data but calculation procedures are not given. Others suggest the use of inverse estimation for calculation of EC50s and 95% CL for both counts and dry weight measurements (Cowgill *et al.* 1989). The use of straight-line graphical interpolation has been recommended (Walsh *et al.* 1987), but this method does not permit calculation of CLs. This report presents a method for calculation of an EC50 and associated CLs from continuous data.

MATERIALS AND METHODS

Lemna was cultured and tested in Hoagland's medium (ASTM 1988) modified by the omission of sucrose, yeast extract and bactotryptone, adjusted to pH 5.0 and incubated at 24 ± l°C with a 24 hr light period of 3100 to 3700 Lux.

Tests were conducted according to methods outlined by ASTM (1988). Briefly, Baker-analyzed grade NaCl was dried overnight at 60°C weighed to ± 0.001 g and dissolved in 100 mL of medium contained in 400-mL beakers. The concentrations used were 0 (control), 2.59, 4.32, 7.2 and 12.0 g/L; all with four replicates. Eight plants with three fronds each were removed from culture, rinsed once in deionized water (DW) and distributed to the beakers one replicate at a time. Four additional replicates were included to estimate the dry weight of the inoculum which was subtracted from final dry weight values. All beakers were covered with a watch glass and placed into the incubator according to a random number assignment which was changed daily. After seven days the plants were rinsed once in deionized water, placed in tared aluminum weigh boats and dried for 20 to 24 hr at 56 to 60°C to a constant weight.

Data analysis utilized MINITAB software programs Arithmetic, Plotting and Regression (Ryan *et al.* 1985).

RESULTS AND DISCUSSION

Dry weight values from experiments in which plants were exposed to concentrations of NaCl or a control solution (medium only) are shown in Table 1. The mean values show the reciprocal relationship between growth and concentration of NaCl.

Dry weight values were transformed to arcsin $\sqrt{P}$, in radians, where P is proportional inhibition (P=(control-test)/control) which is appropriate for the conversion of proportional data to the normal distribution (Zar 1984). A simple linear regression of arcsin $\sqrt{P}$ of dry weight (Y) on log NaCl concentration (X) resulted in coefficients of correlation of 0.953 to 0.990 (significant, $P < 0.05$) for the four data sets indicating that the response to NaCl was concentration-dependent. The regression yielded error terms (e_i) obtained from the

Table 1. The dry weight of *Lemna* grown in four experiments at four concentrations of NaCl and a control, with four replicates at each concentration.

NaCl,g/L	Dry Weight, g					
	Experiment				Mean	Std. Dev.
	1	2	3	4		
0.00	0.0393	0.0403	0.0386	0.0287	0.0365	0.0046
0.00	0.0397	0.0374	0.0441	0.0311		
0.00	0.0344	0.0333	0.0424	0.0340		
0.00	0.0395	0.0321	0.0393	0.0299		
2.59	0.0301	0.0266	0.0365	0.0228	0.0305	0.0043
2.59	0.0323	0.0282	0.0381	0.0238		
2.59	0.0317	0.0321	0.0349	0.0295		
2.59	0.0277	0.0304	0.0345	0.0288		
4.32	0.0239	0.0179	0.0239	0.0189	0.0210	0.0032
4.32	0.0209	0.0171	0.0235	0.0152		
4.32	0.0224	0.0194	0.0282	0.0219		
4.32	0.0210	0.0209	0.0223	0.0178		
7.20	0.0124	0.0095	0.0133	0.0107	0.0119	0.0015
7.20	0.0121	0.0100	0.0128	0.0100		
7.20	0.0123	0.0115	0.0140	0.0121		
7.20	0.0113	0.0138	0.0141	0.0104		
12.0	0.0048	0.0043	0.0064	0.0061	0.0058	0.0008
12.0	0.0063	0.0053	0.0070	0.0048		
12.0	0.0059	0.0064	0.0067	0.0064		
12.0	0.0052	0.0056	0.0053	0.0056		

relationship; $e_i = Y_i - \hat{Y}$, where $\hat{Y}$ is fitted by the regression model and Y_i are the data points. The e_i were tested for two assumptions important in linear regression; 1) normal distribution of e_i and 2) the variance of e_i is independent of X. Analysis by the Kolmogorov-Smirnov test for goodness of fit to a normal distribution (Zar 1984) confirmed that the error terms for all four data sets were distributed normally ($P > 0.10$), thereby satisfying assumption one. A plot (not shown) of e_i against log NaCl concentration revealed that the variance was not stable but varied inversly with NaCl concentration. This trend was apparent in the values for standard deviation shown in Table 1.

To identify a means of stabilizing the variance and satisfying assumption two, a weighting approach (Neter *et al.* 1985) was tested in which weights of log NaCl and $(\log NaCl)^2$ were evaluated. Variances weighted with $(\log NaCl)^2$ were most constant over all levels of NaCl. Therefore, the four data sets were analyzed by weighted $[(\log NaCl)^2]$ least square regression analysis using the model:

(1) $\arcsin \sqrt{P}$ dry weight $= \beta o, + \beta_1(\log NaCl) + e_i$

There was a highly significant regression effect (P=0.000) for all four data sets, indicating a linear relationship between arcsin of dry weight of *Lemna* and the log of the concentration of NaCl in which they were grown (Table 2).

Table 2. Regression equations and selected statistics from the weighted least square regression of arcsin $\sqrt{P}$ of dry weight (Y) on the log concentration of NaCl (X) with weights = $(\log X)^2$ for the four experiments in Table 1.

Exp.	Equation	$S^2(b_1)$	MSE	F	P
1	Y=0.0435 + 1.06 log X	0.00144	0.00062	779.99	0.000
2	Y=0.0836 + 1.02 log X	0.00413	0.00177	252.12	0.000
3	Y=-0.0503 + 1.14 log X	0.00281	0.00121	463.77	0.000
4	Y=0.0087 + 1.05 log X	0.00593	0.00255	185.70	0.000

The EC50s and associated 95% CLs were calculated by inverse prediction (Neter *et al.* 1985) using the regression equations in Table 2:

(2) $$\hat{X} = (Y_o - b_o)/b_1$$

The variances of the EC50s were calculated using the equation:

(3) $$S^2(\hat{X}) = (MSE/b_1^2)(1 + 1/n + (\hat{X} - \bar{X})^2 / \Sigma(X_i - \bar{X})^2)$$

The approximate 95 % CLs were calculated from the following equation:

(4) $$\hat{X} \pm t(1-\alpha/2, n - 2)S(\hat{X})$$

The approximation for the CLs was verified by the relationship:

(5) $$[(t(1 - \alpha/2, n - 2))^2 MSE]/[b_1^2 \Sigma(X_i - \bar{X})^2] < 0.1$$

Where :

$\hat{X}$ = EC50 = log NaCl
Y_o = 0.785398 (50% growth inhibition: arcsin $\sqrt{P}$ with P = 0.5)
b_o = intercept
b_1 = slope
t = Students-t statistic = 2.145
α = 0.05
n = sample size = 16 for each experiment
$S(\hat{X})$ = standard error of the EC50
$\bar{X}$ = mean of log X values = 0.74632
$\Sigma (X_1 - \bar{X})^2$ = $MSE/S^2(b_1)$
$S^2(b_1)$ = variance of b_1
MSE = mean square from the weighted regression

Results of calculations on data from the four experiments are shown in Table 3.

Table 3. The EC50s (g NaCI/L) and approximate 95 % CLs for four experiments in which *Lemma* was exposed to NaCl for seven days. Values are decoded from log concentration.

Experiment	EC50	95% CL		Validity of CL*
		Lower	Upper	
1	5.00	4.44	5.64	0.006
2	4.88	3.95	6.02	0.018
3	5.39	4.62	6.30	0.010
4	5.50	4.30	7.03	0.025

* Use of the approximate CL is appropriate when this value is less than 0.1.

The resulting EC50s are similar in value; 4.88 to 5.50 g/L with a coefficient of variation of 5.8%) indicating a consistent response to NaCl.

These findings indicate that stable variance measures can be obtained for separate experiments. The variance was inversly proportional to concentration of NaCl and weighting is applied inversly with variance, therefore weights of $(\log NaC1)^2$ were applied (by computer subcommand) as multipliers to the model (equation 1) resulting in the weighted regression equations shown in Table 2.

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