LETTERS

Brine Shrimp "Bioassay" Problems

There are two major problems with the recent brine shrimp exercise of Rice & Maness (March, 2004, 208-215). The term "bioassay" is both misdefined and misapplied, and the wide scattering of the data suggest that the method may be inaccurate. The definition that a bioassay must use a "naive" species is incorrect. Many bioassays use species that are normally exposed to the chemical being assayed. There are several phytohormone bioassays that have been used in teaching including the lettuce hypocotyl elongation bioassay for gibberellins (Reiss, 1994), the oat coleoptile curvature test for auxins, the dandelion leaf disk chlorophyll retention bioassay for gibberellins, and the soybean callus bioassay for cytokinins (Witham et al., 1971). As with many biology terms, the definition of bioassay has been corrupted by some over the years. Dictionaries and textbooks still usually define it similar to the following: *appraisal of the biological activity of a substance by testing its effect on an organism and comparing the result with some agreed standard.* (http://www.thefreedictionary.com/bioassay)

The brine shrimp exercise lacked the standard curve required for a bioassay. An accurate description of the exercise would be an LC50 determination or simply a dose-response curve. A bioassay is very different. A bioassay quantifies the amount of a chemical, such as auxin, by using a living organism or living tissue as a substitute for an analytical technique or analytical instrument, such as a high performance liquid chromatograph.

Brine shrimp can be used in true bioassays. Michael et al (1956) used brine shrimp to quantify pesticides. They also described a simple technique to easily separate hatched shrimp from eggs using phototaxis. They evaluated results by determining how long it took for the brine shrimp to drop from the top of the solution.

The statistics were incomplete, as there were no correlation coefficients for the fitted curves. The curves seem to fit poorly. The wide data scatter in the three graphs indicates a potential problem with the method. However, there were too many sources of variation to determine if the method itself was the cause of the variability. Each data point represented a different leaf so there could have been substantial variation in toxin content from leaf to leaf. Differences in technique from student to student was another variable in Figures 1 and 3. Possible types of student variation include differences in how finely students cut the leaves before extraction with alcohol, and inconsistency among students in determining whether the brine shrimp were dead or alive.

Figure 1 has several points with zero or very low percent survival at low concentrations, yet several higher concentrations gave 100% survival. The authors did not address this strange result but one possible explanation is a wide variation in the number of brine shrimp per ml. If there are just enough leaf toxins to kill all shrimp at 50 shrimp/ml, there may not be enough to kill any shrimp at 75 shrimp/ml.

To determine the accuracy of the method itself, one person should do five or more tests

with a single leaf extract and a single concentration that gives about 50% survival. A standard deviation of those data would provide an indication of the variability due to the method alone.

In addition to curve fitting, a more informative statistical approach would be to have five or more replicates for each leaf concentration. For the Figure 1 experiment, concentrations of 10, 20, 30, 40 and 50 mg/ml could be used. The standard error for each concentration could be included on the graph so the students could see how the standard error quantifies the variability in the data.

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Response

David Hershey is correct that there are other uses of the word "bioassay" than the use that we employed in our study (Rice & Maness, 2004). Specifically, a bioassay need not be performed on naïve organisms. We did not intend our article to present a comprehensive definition of all possible bioassay studies, such as the ones Hershey mentions.

The most important reason for using an assay on naïve organisms is that we do not know which compound/s may be responsible for toxicity. If we did know which compounds had biological activity, it would make more sense to directly measure those compounds; for example, the tannin assay to which we referred in the article. Because we do not know which compound/s caused the toxicity, it was not possible to make the standard curve that Hershey insists is necessary for a bioassay. We used the term "bioassay" in the more general sense in which it was used in a research article by Almedia-Cortez, Shipley & Aranson (1999) and as the term "assay" was used in the teaching article by Opler, Mizell, Robert, Cervantes-Cervantes, Kincaid & Kennelly (2002). We believe that the distinction between "bioassay," using the definition Hershey has presented, and "dose-response curve," as he has described our project, will not be of major importance to instructors who wish to use this technique in the classroom.

Instructors will wish to use the level of statistical sophistication that is appropriate for their classroom activities. We did not present the statistical analyses (although we performed them), as most classes taught by readers of this journal would not be prepared for these analyses. The results in Figure 1 were presented to illustrate a particular sampling problem. Figures 2 and 3 show clear distinctions between the two oak species, and between box elder and birch, respectively. An analysis of variance indicated that both the effects of concentration and the differences between the two species were significant at $p<0.0001$. If the method was invalid, such a distinction would not have emerged. There is indeed a wide scatter of variability, but this did not affect the overall conclusion. Each data point represented a separate leaf, as we were examining the variability of leaf toxicity in the field; the very thing Hershey cites as a problem is, in fact, what we intended to sample. Hershey is correct that variability in student technique contributes to the variability in these graphs; however, we wanted to show that the technique was robust against such student differences, which every instructor will encounter. We were not attempting to determine the accuracy of a toxicity estimate for any one particular leaf.We believe that only advanced classes would want to perform multiple assays on extracts from single leaves, and then perform the nested analysis of variance that would then be necessary. We believe the statistical analyses presented in the earlier study published in *The American Biology Teacher* (Opler et al., 2002) are also sufficient for our study and we did not need to repeat their instructions.

Therefore, while an instructor may choose to employ the refinements Hershey has suggested for our method, his suggestions do not demonstrate inaccuracy in the method that we published.

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