INTERACTIVE EFFECTS OF MULTIPLE AGRICHEMICALS IN WETLANDS: DESIGN AND STATISTICAL OVERVIEW OF A MESOCOSM EXPERIMENT

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INTRODUCTION

Toxicology experiments often attempt to define dose-response parameters such as a no observable effect concentration (NOEC) or a concentration resulting in fifty percent mortality (LC50). In such experiments, a test organism is exposed to six or more concentrations of a single chemical. Although these types of experiments are far removed from conditions found in the field, they are thought to provide insight to dose response relationships. Under field conditions, several chemicals may be present at one time and these chemicals may interact with each other to increase (synergism) or decrease (antagonism) the individual effects of the chemicals. Organism responses in the system may directly or indirectly alter the direct toxicity of the chemicals present.

The main and interactive effects of multiple chemicals on biological response variables and the direct or indirect effects of other organisms on these response variables can be studied in mesocosms using a factorial experimental design. A four-chemical seven-level design (4^7) would require at least 2401 treatment combinations, clearly an unmanageable number. We can make the design practical by lowering the number of levels from seven to two (a low dose and a high dose). A 2^4 experiment would require only 16 treatment combinations, although some problems of resolution remain.

The disadvantage of two-level experimental designs is that they can not resolve curvilinear dose-response functions. A three-level design could resolve linear and quadratic slope components, but would require 81 treatment combinations. A more efficient design would be to use a single design point half-way between the low and high dose (center-point). The center-point would allow us to determine if there was departure from linearity (lack of fit). A center-point enhanced 2^4 design is only 17 treatment combinations. These 17 combinations would allow us to determine the main and interactive effects of four chemicals and determine if there is departure from linearity.

Another issue regarding design efficiency is replication. Replicating all design points in an experiment is costly and not necessary. For the experiment described below, the only replication used was 6 replicates of the center points. This is a 35 percent savings in experimental units over replicating all the design points (17+5 vs. 17x2). To further increase saving in experimental units, replication can be completely omitted from the design. Error can be estimated from unreplicated designs by half-normal plotting (Daniel 1959). Our goal was to determine if replication is necessary in ecotoxicology experiments set at the mesocosm scale.

Another issue we explored in this paper is how sampling of experimental units affects variability at future sampling times. Because experimental units are costly (or unreplicable) experimenters are often required to resample them. In mesocosms, sampling can cause unwanted variation. The sampling design used in this experiment allowed us to determine if sampling of mesocosms increased variation at a later sampling dates and how the time interval between resampling affected the overall variability.

The goals of this paper are to: (1) determine the frequency of response variables that respond in a non-linear fashion, (2) compare error center-point error estimates to half-normal plot error estimates, and (3) determine the amount of variability introduced by sampling the experimental units.

METHODS

A center-point enhanced 2^4 experiment was conducted at the University of Mississippi Biological Field Station. The experiment was designed to investigate the main and interactive effects of three agrichemicals (chlorpyrifos, monosodium methylarsenate (MSMA), and atrazine) and mercury on multiple wetland response variables. Atrazine, chlorpyrifos, and MSMA were added at nominal levels of 192, 51,and 219 ppb, respectively, as expected from an EEC model (U.S. EPA 1995) for wetlands downstream of agricultural fields receiving runoff two days after an agricultural application. Methyl mercury was added to the mesocosms to bring total mercury in the upper one cm of sediments to a nominal level of 0.4 mg/kg, approximately double the background concentration in the southeastern United States.

Chemicals were applied as an aqueous mixture to the 500-liter mesocosms according to a center-point enhanced 2^4 factorial design, with untreated conditions defining the low level of each chemical in the mixture. Response variables were measured 1, 2, 4, 8, 16, and 32 days after the chemicals were applied. Twenty-two mesocosms were sampled on days 1 and 8, 22 others on days 2 and 16, and the remaining 22 on days 4 and 32.

A selection of 21 response variables (Table 1), ranging from whole ecosystem responses (net primary productivity) to cellular responses (cytochrome p450 induction), were used to determine the magnitude of error produced by considering only replication at the center-points versus error estimated from a half-normal plotting. Estimating error by half-normal plotting is achieved by ranking all effects by size and converting to a normal probability scale (Miliken and Johnson 1989). The probabilities are then plotted against their respective absolute effect sizes (Figure 1). Where the slope of the effects line crosses the probability axis at 1, the respective abscissa value is the estimate of error. This value corresponds to one standard deviation. All data analysis were conducted with SAS (SAS 1987).

Lack of fit was determined fitting a linear model for each response variable to the 16 design points in the 2^4 model and comparing this value to the mean of the six center points. Lack of fit is indicated by departure from linear model. Lack of fit significance was tested by a F test of the sums of squares attributable to non-linearity.

We evaluated the relationship between center-point error and error estimated based on half-normal plotting by plotting the values against each other. Slopes greater or less than one indicates that the half-normal plot estimation of error over or under estimates center-point error, respectively.

To determine the amount of variance introduced into the mesocosms caused by sampling and how this variation would be dependent on time since last sampling, we compared overall variability (root mean square error) relative to the mean response for each sampling date with the variability (relative to the mean) on the next resampling date. Fish response variables were not considered in the analysis as most fish were removed from the mesocosms by day 16.

RESULTS

Analysis of lack of fit for the overall model (days 1-32) found no lack of fit for any of the response variables. A few response variables had significant lack of fit on a given sample date. Overall, lack of fit was rarely observed.

Error estimated from replicated center-points or obtained from half-normal plots were essentially identical for the whole model (Figure 2) for each of the 21 response variables examined. On a date by date basis, there was a near perfect match between the two error estimates; r^2 ranged from 0.93 to 0.98. The only apparent pattern was that half-normal plot error estimates of nitrate nitrogen were consistently lower than their corresponding center point values on all sample days. The distribution of the nitrate response, being highly skewed to non-detectable responses, may have been the cause of this pattern.

Variability due to resampling of mesocosms was not apparent between day 1 and day 8 (Figure 3). This is seen by most responses falling close to the 1:1 line. The exception to this pattern was that both sediment percent organic matter and sediment percent solids were more variable on day 1 than on day 8. Variability was higher for most responses on day 2 than when the respective mesocosms were resampled on day 16 (Figure 4). The comparison variability between days 4 and 32 show a wide scattering of the responses above and below the 1:1 line (Figure 5).

DISCUSSION

Lack of fit was not important to all the end-points measured in this experiment. Using one center-point ($1/17^{th}$ of our financial resources) can not be considered too wasteful to determine this result. Clearly, had we used a 3-level design (81 units) trying to find non-linearity that was not there would have been wasteful. The reason for lack of fit not being important probably came from the choice of high level dose for the chemicals. The dose range chosen was probably in the linear portion of an underlying dose-response function. If high level factorial experiments are attempted, the dose levels must be spaced so that there is non-linearity in the response.

The comparison of error generated from center-point replication vs. error estimated from the half-normal plots clearly indicated that replication of factorial experiments in wetland mesocosms is not required. The error estimates generated by half-normal plotting and the six center points were almost identical in all cases. There is no inherent reason why we should believe one estimate to be more robust than the other. Because the center-points were not necessary for error estimation, this experiment could have saved an additional 22 percent (5/22) of its financial resources, or directed them towards the analysis of other end points.

The pattern of variability seen between successive repeated sampling of the same mesocosms can best be interpreted by considering when the chemicals were expected to have their largest effects. Most of the responses were expected to occur within the first few days of the experiment while the chemical concentrations were still high in the mesocosms. That is why variability is unchanged between days 1 and 8 (Figure 3). This result also suggests that our sampling did not affect variability at the later sampling dates. The largest difference in variability is seen in the contrast between day 2 and 16 mesocosms (Figure 4). By day 16, most response variables have stopped responding to the chemicals because of their lower concentrations in the water column and have lower variabilities compared to day 2.

ACKNOWLEDGEMENTS

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Table 1. Listing of all variables used in the analyses

Variable code	Description
TPKEN	Water column total phosphorus (ug/L).
FLUOROM	Fluorometry (florometric units)
CHLA	Chlorophyll a (ug/L).
CHLB	Chlorophyll b (ug/L).
RESPSED	Glucose mineralization in sediment (ug/L/hr.) .
BRESPWAT	Glucose mineralization in sediment (ug/L/hr.).
PERSOL	Pecent solids in sediment.
PERORG	Percent organic matter in sediment.
AMMASN	Ammonia nitrogen concentration.
PLANTCPF	Chlorpyrifos concentration in Juncus spp. tissue (nG/G).
PLANTATR	Atrazine concentration in Juncus spp. tissue (nG/G).
NETPPR	Net primary production (mg O_2 /L/day).
GROSSPPR	Gross primary production (mg O_2 /L/day).
TURBID	Water column turbidity (NTU).
TAA	Total ascorbic acid in *Juncus spp.* stems.
AA	Ascorbic acid in *Juncus spp* stems.
DAAPER1	Ratio of dehydroxy ascorbic acid to ascorbic acid.
SI	Super oxide production in fish cells.
ACETYCO	Acetycholine esterase activity.
CYTP450	Cytochrome p450 activity in liver cells.
LIVERIND	Ratio of fish liver mass to fish body mass.
CONDITION	Fish condition (length cubed / weight).
AODCWAT	Acridince orange count of bacteria in water (counts per ml).
AODCSED	Acridince orange count of bacteria in sediment. (count per ml).

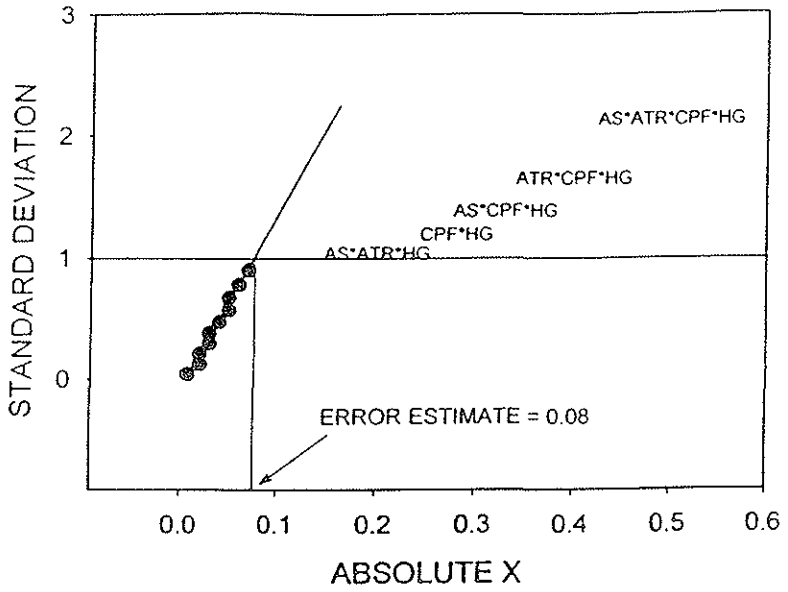

Figure 1. Example of a half-normal plot. Non-significant effects fall along the straight. Significant effects fall off the line. Where the straight line crosses 1 on the y-axis, the corresponding x-axis value is the error estimate.

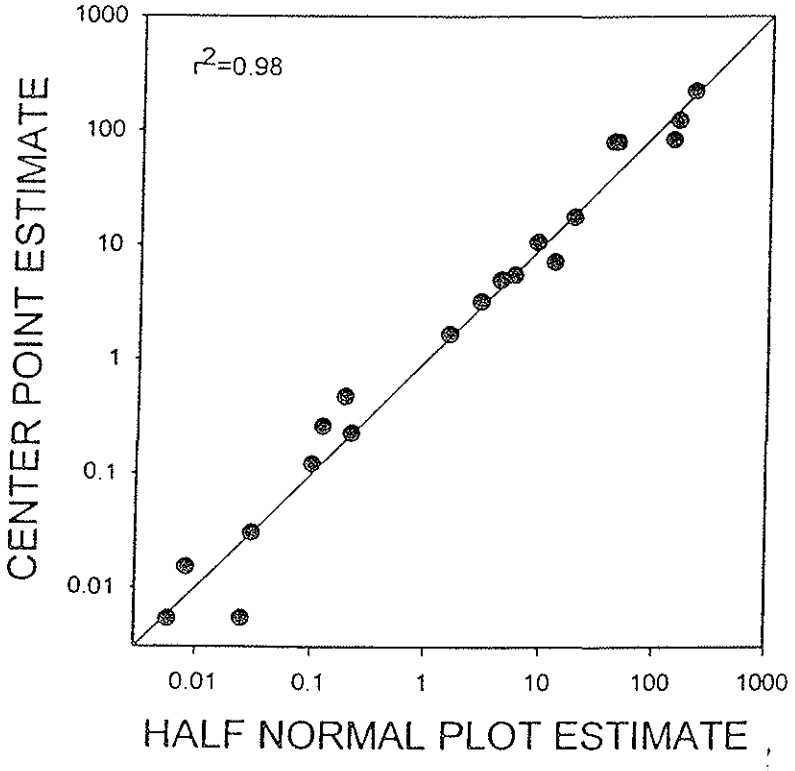

Figure 2. Comparison of error estimated from center-points vs. error generated from half-normal plots for full model.

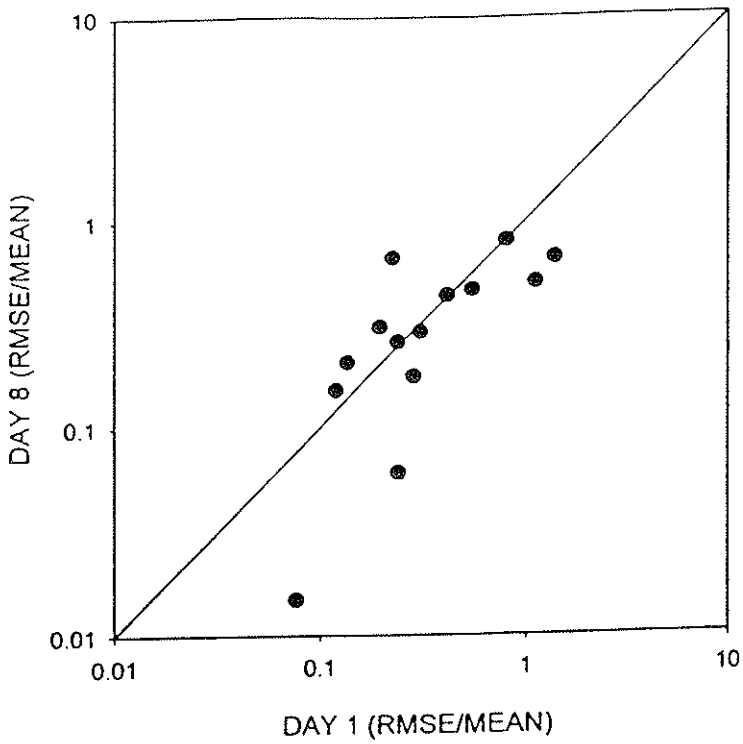

Figure 3. Comparison of standardized error (root mean square error (RMSE) / mean) for sample day 1 and day 8.

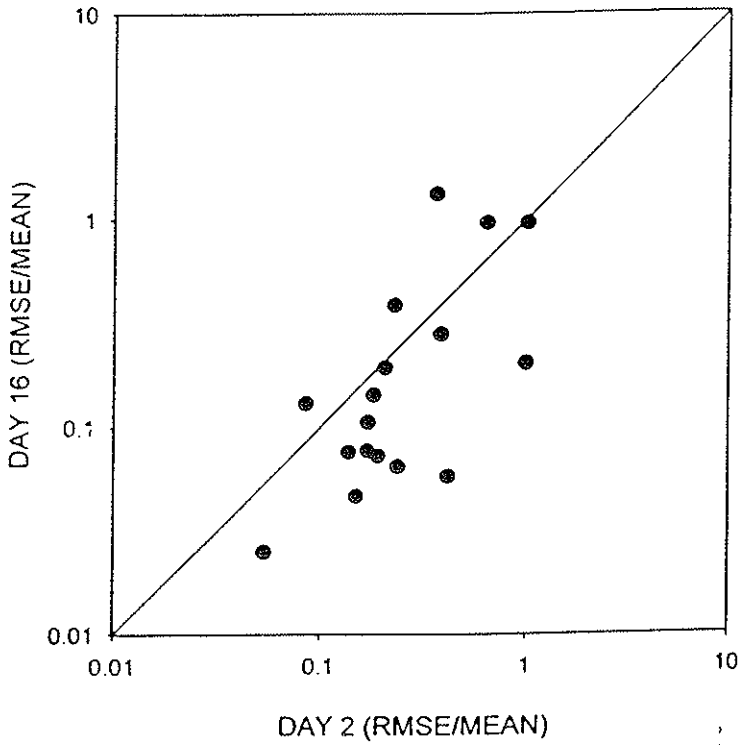

Figure 4. Comparison of standardized error (root mean square error (RMSE) / mean) for sample day 2 and day 16.

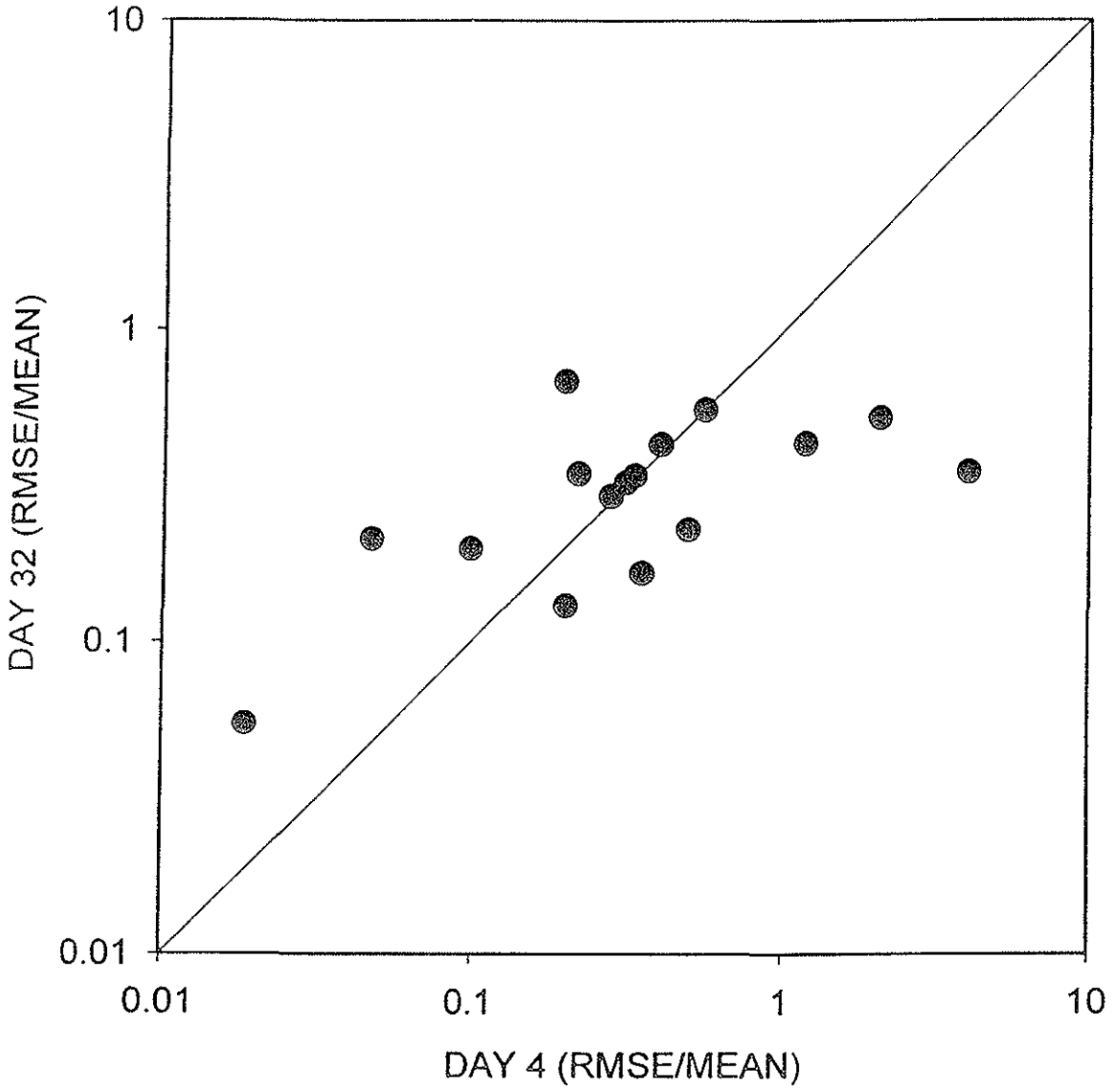

Figure 5. Comparison of standardized error (root mean square error (RMSE) / mean) for sample day 4 and day 32.