Sulfide Data Associated with the Hydroponic Wild Rice Seedling Tests

Clarifying Information for the Scientific Peer Review Panel
of the
Analysis of the Wild Rice Sulfate Standard Study: Draft for Scientific Peer Review

Minnesota Pollution Control Agency (MPCA)
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Introduction

MPCA staff received questions posed by a peer reviewer on 6/25/2014, and on 6/30/2014 MPCA staff sent initial responses to ERG for distribution to the peer review panel. The questions and initial responses are presented on pages 1 and 2 of this document. MPCA staff continued to investigate the questions posed by the reviewer, and conclusions of that investigation are presented beginning on page 3 of this document.

Initial Questions and Responses

Initial questions – Range Finder and Definitive Hydroponics Sulfide Studies

1. Are sulfate concentrations reported as SO_4 or as SO_4-S?

   Sulfate concentrations are reported as SO_4.

2. How are sulfide concentrations reported? More specifically, and as an example, in the *Rangefinder* worksheet, sulfide concentrations reported as mg/L are converted to molar concentrations (µM) by dividing by the molecular weight of sulfur (32) and then multiplying by a factor of 2.5. Why is a factor of 2.5 used?

   Sulfide concentrations were measured by two different labs: 1) by the Pastor lab in Duluth, and 2) by the Minnesota Department of Health (MDH) lab in St. Paul. The data in question were produced by the Pastor lab using a calibrated spectrophotometer that read out in mg/L. The spec had been calibrated for a one-inch cuvette, but a 1-cm cuvette was used, so the data were corrected with a factor of 2.5, where 2.54 would have been more accurate (a 1.6% error that we should correct in the future). We chose to use the Pastor data for the logistic regression because the data were produced immediately, whereas the MDH data were delayed. In addition, some of the MDH data reported values from a small subset didn't conform well to the expected concentrations from controls and the serial dilution. However, the two labs usually agreed with each other (r^2=0.97).

3. I cannot obtain the same log10 values as reported for the mean initial sulfide concentrations for any of the three studies. For example, for Model 1 from the Range Finder study (figure on page 61), the spreadsheet values for initial concentrations (averaged across the five dates) range from -0.1 to 3.1; the values shown in the model plot figure range from slightly > 0 to about 3.5.

   The data reported as mg/L in the spreadsheet were not yet corrected for the spectrophotometer path length (multiplying by 2.54) as described in question 1. Employing the correction factor will resolve the differences of the Log10 values.

4. Do negative values for initial sulfide concentrations (*e.g.,* -1 µg/L for Definitive Study 2, cells D7 and D9) indicate values below the method detection limit? If so, how were those values treated in subsequent calculations?

   The negative values are not codes for values below a method detection limit, but rather the read-out from the spectrophotometer (see answer to question number 2), for samples that were undoubtedly below the limit of quantification for that instrument. We are investigating how a method detection limit should have been developed for this procedure and will get back to you with more detailed information. Ultimately, in order to calculate a regression, a concentration has to be assigned to the control sulfide treatment, which, most likely, should have been at, or below a method detection limit.

5. If the answer to Question 4 above is indeed that the values are below the MDL, then why are other initial sulfide concentrations reported equal to zero (Range Finder and Definitive Study1 spreadsheets)?

   It appears that the Pastor Lab assigned a sulfide concentration of zero to values that should have been regarded as less than the method detection limit. We are investigating how to calculate a method detection limit for the procedure that was used.

6. Are the initial sulfide concentrations available for each nominal treatment level and replicate bottle? Only mean values for nominal treatment level are reported.

   The spreadsheet referred to in question 5 contains initial concentrations for each of the three replicates for the initial day and for each of the renewal dates. The spreadsheet does not contain any means. Please get back to us if this is not clear.

Results of MPCA Further Investigation

The peer reviewer inquiry about the sulfide concentrations initiated further review and analysis by MPCA staff, which is summarized below.

1. Background information: The Pastor lab at the University of Minnesota-Duluth (UMD) performed the hydroponic growth experiment, and quantified sulfide immediately, without storing the samples. A subset of samples were preserved and submitted to the Environmental Laboratory at the Minnesota Department of Health (MDH) in St. Paul, which also measured all the sulfide samples from the 2012-2013 field surveys.

2. The original plan was to perform statistical analyses of the hydroponic data using the MDH analytical data, to eliminate the possibility of differences in results between the two laboratories.

3. The statistical analyses by MPCA staff were performed with the UMD analytical data in early 2014 because the MDH data were not yet available. The UMD data were produced using a calibrated spectrophotometer that read out in mg/L. The spectrophotometer had been calibrated for a 1-inch cuvette, but a 1-cm cuvette was used, so a correction factor of 2.54 cm/inch needed to be employed to adjust the data. The data received by the MPCA had been adjusted with a factor of 2.5, rather than 2.54, so before proceeding with any statistical analyses MPCA staff had further adjusted the data (a 1.6% correction). The MPCA statistical analyses presented in the document for peer review used data that had been adjusted using a factor of 2.54, and therefore does not need any further adjustment.

4. However, the data in the UMD report to the MPCA and in associated spreadsheets available to the public and the peer reviewers were not adjusted for the 2.54 factor, and the adjustment factor of 2.5 was not explained in the spreadsheets. As a result of an inquiry by a peer reviewer, MPCA staff investigated the UMD analytical data and found:

a) The sulfide data in the report to the MPCA should be adjusted upwards by a factor of 1.6%, and
b) Sulfide data from the control hydroponic samples (which had not had any sulfide added and were undoubtedly below the method detection limit) had been used in the logistic regressions performed by MPCA staff and presented in the MPCA draft Analysis for scientific peer review.

5. For the analytical method used at UMD, Dr. Nathan Johnson (UMD) had determined a method detection limit (22.1 µg sulfide/L) and a reporting limit (60.6 µg sulfide/L); these values can be used in the statistical analysis involving the control samples, which had no sulfide added and are expected to be essentially zero. All of the values used in the MPCA analysis from the controls were below the method detection limit of 22.1 µg sulfide/L. The lowest treatment concentration where sulfide was added was a target concentration of 3 micromolar, or about 100 µg sulfide/L.

5. As a result of Conclusion 4 above, MPCA determined:

a) The logistical regressions should be recalculated to investigate the sensitivity of the results to a range of assumed sulfide concentrations for the control, which were below the limits of quantification (see Conclusion 6, below, for that analysis), and
b) The results from the recalculations in (a) should be compared to the results of logistical regressions performed with the sulfide data produced by the MDH laboratory (see Conclusions 7 and 8, below, for the comparison of MDH results to UMD results).

6. The logistic regressions for the three juvenile seedling sulfide experiments have been re-calculated with a range of assumed concentrations for the control. In the following table it can be seen that estimates of EC20 and EC50 are not sensitive to the particular value assumed for the sulfide concentration of the control treatment, as long as the values are at or below the detection limit of the analytical method for sulfide. All of the analyses for the control treatments were below the detection limit.

Table 1. Sensitivity of EC20 and EC50 values to assumed sulfide concentrations for controls below the detection limit.

Test	Effect Concentration µg Sulfide/L	Sulfide concentration assumed for control treatment			
		Reported values (used in draft Analysis for peer review)	Low Value 1.1 µg Sulfide/L	Half the Detection Limit 11.05 µg Sulfide/L	Detection Limit 22.1 µg Sulfide/L
Range Finder	EC20	239	239	240	242
Definitive 1		210	210	210	211
Definitive 2		322	322	322	322
Range Finder	EC50	459	459	459	460
Definitive 1		326	326	327	327
Definitive 2		365	365	365	365

7. An additional way to assess the reliability of the EC20 and EC50 estimates based on the UMD sulfide analytical data is to compare results using UMD data to results from MDH data. The first step is to compare analytical results from the two laboratories. For samples above the reporting limits that were split between the UMD and MDH labs (all of the initial samples from the 3 seedling experiments), the data are similar, with an r^2 of 0.99, a slope near one (0.997), and a intercept near zero (-2.2) (Figure 1; $p < 0.0001$). A parametric paired T-test showed no difference between the two laboratories ($p= 0.70$). A non-parametric Wilcoxon test also showed no difference between the laboratories ($p= 0.46$). There is no statistically significant difference between the data produced by the two labs. (Note: The r^2 of 0.97 reported in the initial response to the peer reviewer inquiry, see response to Q #2 on Page 1, included data produced below the reporting limits for both labs).

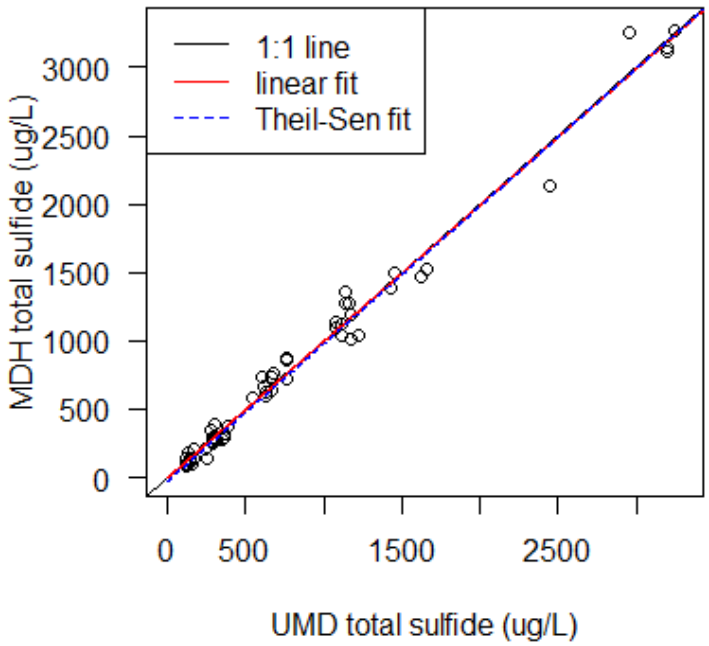


Figure 1. Comparison of sulfide data produced by the UMD lab and the MDH lab from the fourth vessel of each treatment in the hydroponic seedling experiments. $r^2= 0.99$, n=59; the linear regression equation is: MDH = 0.997 UMD – 2.2. The Theil-Sen fit refers to a non-parametric regression method, which confirms the similarity of the sulfide analyses performed by the two laboratories.

8. A second step in assessing the reliability of the EC20 and EC50 estimates based on the UMD sulfide analytical data would be to estimate EC20 and EC50 using the MDH data. There is a critical difference, however, between the two data sets: The UMD analytical data were produced from the three actual replicate incubation vessels for each sulfide treatment level, whereas the MDH data for each treatment level consists of just one analysis from a fourth vessel that was prepared at the same time and in the same manner as the treatment vessels, but was not actually used for the incubation (the data comparison in Figure 1 are samples from the fourth vessel). The purpose of the fourth vessel was to provide samples for analysis for both the UMD and MDH labs; plants were never introduced into the vessel. MDH analytical data could not be obtained from each of the three replicate 700 mL incubation vessels that did contain plants because the MDH lab required a sample volume of 40 to 60 mL, and removing that volume for sampling purposes would have left an unacceptably large headspace in the incubation vessel (as samples were withdrawn, nitrogen gas replaced the volume removed). In contrast, the UMD lab was able to quantify sulfide with equivalent accuracy using a 2.5 mL sample volume.

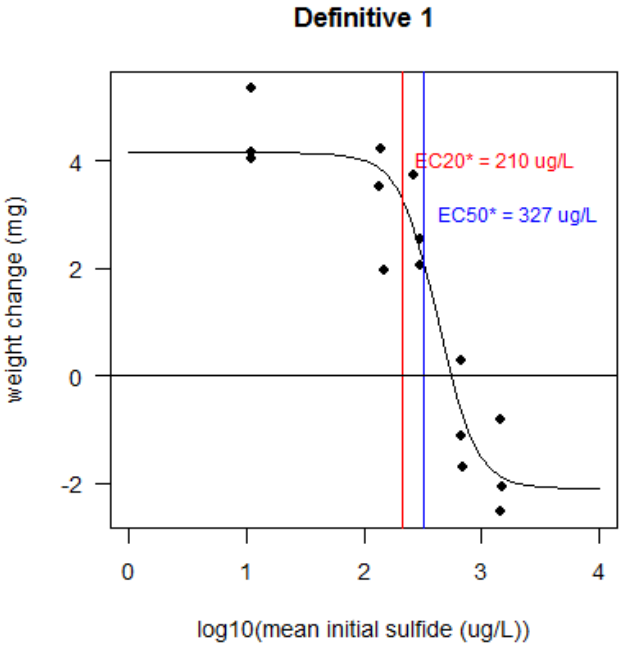
When MPCA staff used the MDH-produced sulfide data to run the R routines to model the logistic regressions, the models for the Rangefinder and Definitive-1 experiments were comparable to those produced from the UMD data (Table 2). However, the R routine crashed when modeling the Definitive-2 experiment when using the MDH data, which may be a result of two factors:

1) In this third experiment the sulfide concentration range was compressed, and
2) There was less variability in the sulfide concentrations being modeled, since each treatment level was characterized by just one value, whereas there were three different values characterized by the UMD data (for example, see Figure 2).

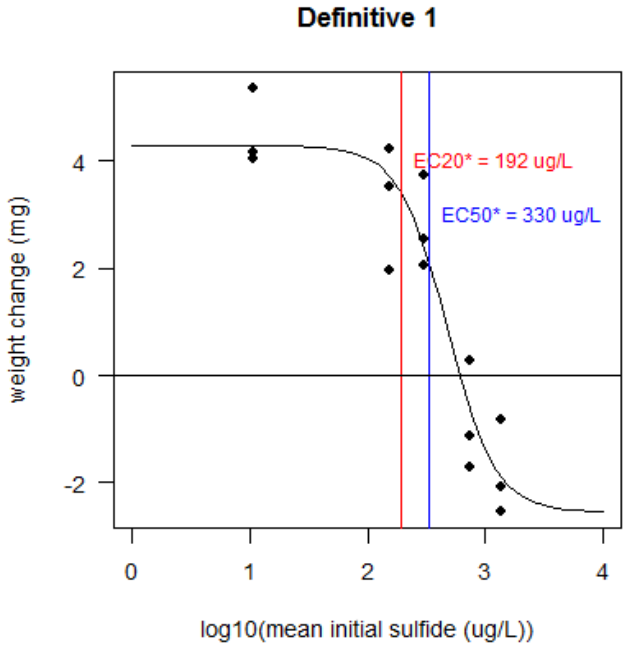
So, there was relatively little MDH data to define the middle part of the logistic curve. Fitting a 4-parameter model to data with no x-axis variation within treatment replicates, where the treatment range was compressed, was not possible for the R routine that was employed. The richer UMD data set provided a greater amount of information about the middle part of the curve to run the model.

Table 2. EC20 and EC50 estimates calculated from UMD sulfide data compared to estimates from MDH data. "*" indicates that the R routine could not be successfully run with the MDH data set.

Test	Effect Concentration µg Sulfide/L	Data Source for Sulfide Treatments	
		UMD Lab	MDH lab
Range Finder	EC20	239	168
Definitive 1		210	192
Definitive 2		322	*
Range Finder	EC50	459	391
Definitive 1		326	330
Definitive 2		365	*



A. Based on UMD sulfide data.



B. Based on MDH sulfide data.

Figure 2. Comparison between logistic regressions conducted with sulfide data produced by the UMD lab (A) and the MDH lab (B). Note that there is slight variation in the three sulfide values for a given treatment in (A), whereas the three sulfide values in (B) are all precisely the same because the only value available was from the fourth vessel.

9. Based on this further review and analysis, MPCA staff conclude the following:
   a) The UMD sulfide data used by MPCA staff had been properly adjusted to account for the path length of the spectrophotometer prior to statistical analysis,
   b) The UMD and MDH analytical laboratories produce sulfide data that are statistically indistinguishable, and
   c) It is preferable to estimate EC20 and EC50 sulfide values using the UMD data, as is presented in the Draft Analysis for Scientific Review, because the UMD samples were taken from each of the triplicate exposure vessels, whereas the MDH samples were taken from a fourth vessel that was used just for characterization of the treatment concentrations. This procedure also resulted in there being only one average MDH sulfide value to characterize all three replicates, whereas UMD data were produced for each of the three replicates.