Using EPA Whole Effluent Toxicity (WET) Statistics for Acute and Chronic Bioassay Tests

1. SCOPE AND APPLICATION

1.1. This method is based on the EPA's WET Analysis Spreadsheet v2.3.

1.2. The spreadsheet is used to analyze valid WET test data to obtain acute and chronic test endpoints (e.g. survival, reproduction/growth) identified in EPA's WET test methods under the NPDES program. EPA's WET Analysis Spreadsheet calculates WET test endpoints recommended in EPA's Technical Support Document (TSD) (e.g. LC50, NOEC, LOEC, IC25) as well as EPA's Test of Significant Toxicity (TST; EPA 833-R-10-003, June 2010). The spreadsheet can also be used for reasonable potential analysis, permit compliance determination, and permit limit development.

2. SUMMARY OF METHOD

2.1. Information about the test is entered on the "Start" tab in the spreadsheet. On the "Input" tab, data endpoints are entered (growth, reproduction, or survival). Results are calculated by the program and put in the "Output" tab. Statistics are saved to the "Statistics" tab. A "Generated Report" is created and saved as a PDF. All statistics (LC50, NOEC, LOEC, PMSD, IC25, and Control % CV) are recorded in a Bioassay Bench Sheet logbook. A second analyst checks that all the endpoint counts were entered correctly as well as performs the calculations again to verify the final stats were generated and recorded correctly.

2.2. Interferences:

2.2.1. Non-valid test data entered, or data entered incorrectly.

2.2.2. Excel files not saved properly.

3. EQUIPMENT AND SUPPLIES

3.1. Computer with access to the internet, "ToxCommon" folder, and Excel

3.1.1. The EPA WET Analysis Spreadsheet is located at https://www.epa.gov/npdes/whole-effluent-toxicity-wet-npdes-spreadsheet. A new spreadsheet should be obtained from EPA's website for each new test (EPA updates the file periodically).

3.4. Bioassay Bench logbooks and SRT logbooks.

4. METHOD

4.1. All Test Statistics (*P. promelas*, *M. beryllina*, *A. bahia* and *C. dubia*)

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

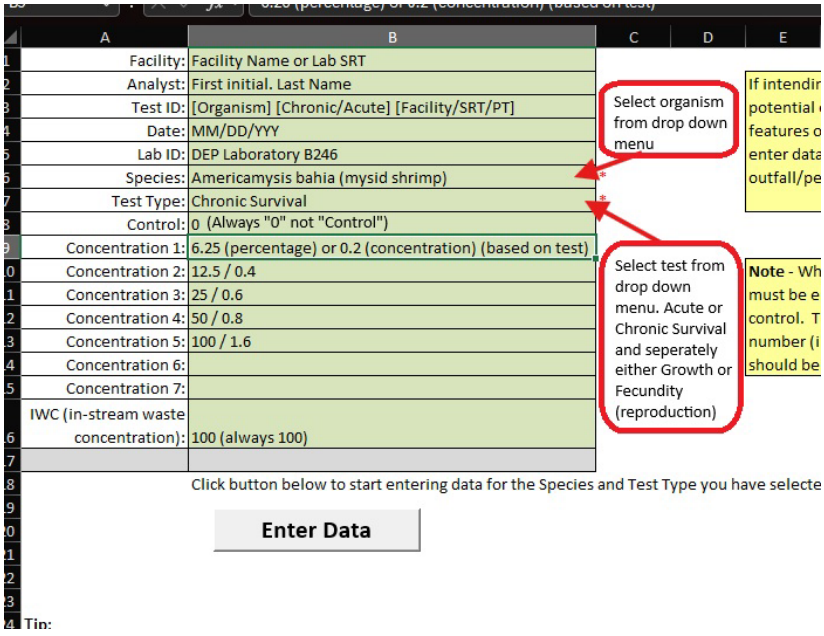
4.1.1. Go to https://www.epa.gov/npdes/whole-effluent-toxicity-wet-npdes-spreadsheet and open "Whole Effluent Toxicity (WET) Analysis Spreadsheet (xlsm)".

4.1.1.1. Click "Enable Editing" and save excel file in the appropriate location [year, type of test (facility, SRT, or PT)] within the "EPA WET Spreadsheet">"!EPA Stats SRT and Facility" folder.

4.1.1.1.1. Follow the naming convention in the folder. Generally, it's "Facility Organism Test Type Date Started". SRT or PT tests are named "Organism Test Type Date Started". Chronics are additionally labeled with "Survival" or "Growth/Reproduction".

4.1.1.2. In the "Start" tab, fill out the following fields: Facility, Analyst (first initial, full last name), Test ID (ex. C. dubia Chronic SRT or A. bahia Acute Facility), Date (start date, must use MM/DD/YYYY, Lab ID (DEP Laboratory B246), Species (drop down menu, C. leedsi is not an option, select "Cyprinodon veriegatus (sheepshead minnow)"), Test Type (drop down menu), and Dilutions (ex. 0, 6.25, 12.5, 25, 50, 100. When entering concentrations, they must be entered from low to high starting with the Control. The control value needs to be a number ("0")). Enter "100" in the IWC field.

Figure 1 – *Data entry*



4.1.1.3. If the "Enter Data" button on the "Start" tab is not working, you need to enable macros. To do this, select File>Options>Trust Center>Trust Center Settings>Macro Settings. Make sure the

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

"Enable VBA macros" is selected. Select "OK", and "OK" again to clear all windows. Exit the program and re-open to use.

4.1.1.3.1. To use the WET Spreadsheet, macros must be turned on. To view a completed spreadsheet without adding/altering data, turn the macros off.

4.1.2. Under the "Input1" (sublethal) or "Input2" (survival) tab, enter the data endpoints for each concentration replicate. Press the "Calculate Results" button and the "Output" tab should appear.

Figure 2 *(Sublethal [growth] data entry example)*

Non-percent Endpoints (Growth, Reproduction, and Germ-tube Length)

Replicate	0.00	0.13	0.25	0.50	0.75	1.00
1	0.518	0.423	0.513	0.51	0.359	0.122
2	0.536	0.433	0.581	0.531	0.323	0.061
3	0.565	0.496	0.562	0.51	0.44	0.108
4	0.559	0.533	0.564	0.54	0.436	0.09
5						
6						

Figure 3 *(Survival data entry example; Top: Original Number of Organisms; Bottom: Surviving Organisms*)

Percent Endpoints (Survival, Fertilization, Larval Development, and Germination)

	Replicate	0.00	6.25	12.50	25.00	50.00	100.00
Number of Organisms Exposed or Counted per Replicate	1	5	5	5	5	5	5
	2	5	5	5	5	5	5
	3	5	5	5	5	5	5
	4	5	5	5	5	5	5
	5	5	5	5	5	5	5
	6	5	5	5	5	5	5
	7	5	5	5	5	5	5
	8	5	5	5	5	5	5
	9						
	10						
	11						
	12						
	13						
	14						
	15						
	16						
	17						
	18						
	19						
	20						

	Replicate	0.00	6.25	12.50	25.00	50.00	100.00
Number of Organisms Surviving or Responding	1	5	5	5	5	4	3
	2	5	5	5	5	3	2
	3	5	5	5	5	1	2
	4	5	5	5	5	5	5
	5	5	5	5	5	5	2
	6	5	5	5	5	4	4
	7	5	5	5	5	5	3
	8	5	5	5	5	3	1
	9						
	10						
	11						

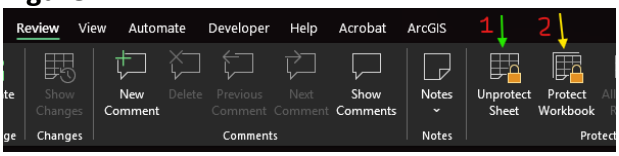
The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

4.1.2.1. When entering endpoints in for a chronic test, the survival endpoint results must be done first. See section 4.2 for mor information.

4.1.3. Press the “Save to Statistics” button and the “Statistics” tab will become available. In the “Statistics” tab, all the statistical data for the test will be generated (some can be found on the Output tab as well).

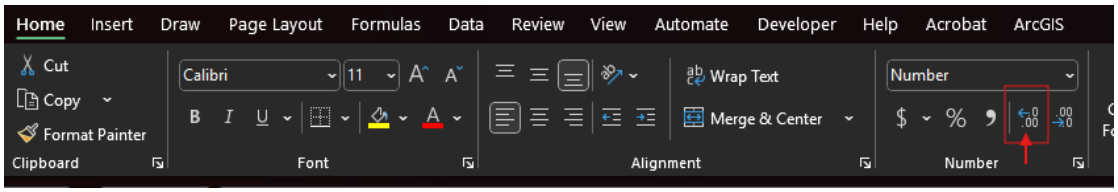
4.1.3.1. Unprotect the sheet (Review > Unprotect sheet) (Green arrow in image below) and expand out the number of decimals shown for either the IC/EC25 or LC50 column (whichever has results, depending on the test type).

Figure 4



4.1.3.1.1. To expand out the decimal places shown on a number, go to Home > Number and adjust the decimal places.

Figure 5



4.1.3.2. Transcribe the appropriate results based on the bioassay logbook. This could include NOEC, LOEC, IC(/EC)25, LC50 Lower 95% C. I., Upper 95% C. I., PMSD, and Control CV.

Figure 6

IWC	NOEC	LOEC	IC/EC25	LC50	Lower 95% C.I.	Upper 95% C.I.	TST
100	0.5	0.75	0.6398	NA	0.61	0.69	NA

Figure 7

MSD	PMSD	Control Mean (Transformed)	Control Mean	Control Stdev	Control CV	TAC-Control Mean Response Upper Bound
NA	NA	1.37	0.98	8.1%	0.059423	NA

4.1.3.3. Protect the sheet again by selecting Review > Protect Workbook and entering the password (see yellow arrow in Figure 4).

4.1.4. Go back to the “Output” tab and click the “Generate Report” button. A new tab named “OutputReport” will open. Save the “OutputReport” tab as a PDF in the “Generated Reports” folder within the “EPA WET Spreadsheet” folder.

4.1.4.1. Before saving the Generate Report as a PDF, expand out the necessary results. The IC25 or LC50 and the PMSD should have four

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

significant figures. Ensure other values such as the NOEC and LOEC are expanded out to show their true value (0.5 will round to 1, expand out to show 0.5).

4.2. Chronic Survival Statistics (*P. promelas*, *M. beryllina*, *A. bahia*, *C. dubia*)

4.2.1. For chronic tests, survival data must be analyzed first. If there is a survival effect for a given concentration (highlighted yellow with a "Y" in the statistical result column on the Output tab), then those concentrations are not entered for the sublethal endpoint to get the NOEC and the LOEC values (not necessary for SRT tests since NOEC and LOEC isn't used for control charts). The data is included to get the IC/EC25 value.

Figure 8

Statistical Data	Conc.	Mean (Transformed)	Mean	Stdev	CV	Dunnett test
Statistics are based on the transformed data used for endpoint calculations	0	1.371273527	0.975	0.08148517	0.059	
	0.125	1.412016112	1	0	0.000	NS
	0.25	1.412016112	1	0	0.000	NS
	0.5	1.371273527	0.975	0.08148517	0.059	NS
Data are normally distributed.	0.75	0.862007509	0.575	0.098313807	0.114	Y
	1	0.357224818	0.125	0.070948527	0.199	Y

4.2.1.1. On the "Instructions" tab of the WET spreadsheet, the instructions for "Analyzing Dual Biological Test Endpoints" contains a typo. Where it says "… highlighted yellow with a "N" …" the "N" should say "Y". EPA has been made aware of this typo and plans to fix it when they are able to. There are emails to confirm this saved in the "EPA WET Spreadsheet" folder under "ToxCommon".

4.2.2. Under the "Input2" tab enter the number of organisms exposed or counted per replicate into the first data table. Enter the data endpoint (number of organisms surviving per replicate) into the second data table. Press the "Calculate Results" button and the "Output" tab should appear.

4.3. Chronic Growth Statistics (*P. promelas*, *M. beryllina*, and *A. bahia*)

4.3.1. In the "EPA WET Spreadsheet" folder, go to the "Organism Final Weights" folder. Open a blank template (fish species or *A. bahia*). Enter pan weight (g) and pan plus organism weight (g) from the *Chronic Weights* logbook to the excel template. Transcribe pan weight (g), pan plus organism weight (g), organism weight (g), mean organism weight (mg), and mean concentration weight (mg) in the bioassay bench sheets.

4.3.2. Under the "Input1" tab enter the data endpoint for growth (the organism weights in milligrams) for each concentration's replicate. Press the "Calculate Results" button and the "Output" tab should appear.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

4.4. Chronic Reproduction Statistics (*C. dubia*)

4.4.1. Open the “Chronic C. dubia Counts” folder within the “EPA WET Spreadsheet”. Open the “C. dubia Calculations Sheet” excel template. Click “File” and Save As with the name of the test facility or SRT and date. Save the file to the current year folder in the “Chronic C. dubia Counts” folder.

4.4.2. Fill in Analyst initials, Test ID, and Date at the top. Fill in the number of neonates for each replicate per day on the data table. Enter the amount of surviving original organisms for each day in the column titled “Surviving Original Organisms” for each concentration.

4.4.2.1. When there is a split brood, type in “s” and then the two-digit number (Ex. S04, S10) and that number will be added to the previous day’s total (line above it). When there is a fourth brood, type in “f” and the two-digit number and the number will not be added to the total. Reproduction statistics only take the first three broods for calculations.

4.4.3. When all the numbers have been filled in, have another analyst verify that all the numbers have been transcribed correctly from the bioassay bench sheets into the excel sheet. Have the analyst who verifies add their initial at the top of the spreadsheet.

4.4.4. Open “Whole Effluent Toxicity (WET) Analysis Spreadsheet (xlsm)”.

4.4.5. Under the “Input1” tab enter the data endpoints (number of total neonates in first three broods) for the replicates of each concentration. Press the “Calculate Results” button and the “Output” tab should appear.

4.5. Acute Statistics (*P. promelas*, *M. beryllina*, *C. leedsi*, *C. dubia* and *A. bahia*)

4.5.1. Survival is the only endpoint for acute tests.

4.5.1.1. When using C. leedsi as the test species, select “Cyprinodon variegatus (sheepshead minnow)”. The EPA spreadsheet does not include C. leedsi.

4.5.2. Under the “Input2” tab enter the number of organisms counted per replicate into the first data table. Enter the data endpoints (number of organisms surviving per replicate on termination day) into the second data table. Press the “Calculate Results” button and the “Output” tab should appear.

5. DATA ARCHIVAL

5.1. Use a black, waterproof pen for all bench sheets and logbooks.

5.2. Record statistics in appropriate spaces in bench sheets and SRT logbooks.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

5.3. Save EPA WET Statistics Spreadsheets in the appropriate folders under the "EPA WET Spreadsheet" folder in "ToxCommon".

6. QUALITY CONTROL

6.1. Refer to DEP Laboratory Quality Assurance Manual for corrective action and handling out-of-control data.

6.2. Have another analyst verify that the statistical analysis was run correctly, and that the data was entered into the excel spreadsheets from the logbooks correctly.

7. REFERENCES

7.1. EPA's Technical Support Document (TSD)

7.2. EPA's Test of Significant Toxicity (TST; EPA 833-R-10-003, June 2010)

7.3. Whole Effluent Toxicity (WET) NPDES Spreadsheet

7.4. FDEP Laboratory Quality Assurance Manual

Appendix of Changes

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.