# Using ToxCalc for Statistical Analysis of Chronic Bioassays

## 1. SCOPE AND APPLICATION

1.1. This SOP is based on procedures described in the TOXCALC Toxicity Data Analysis Software User's Guide by Tidepool Scientific Software P.O. Box 2203, McKinleyville, CA. 95519. The statistical methods are further described in Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition, EPA 821-R-02-013, and Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, EPA 821-R-02-014. Other statistical packages such as CETIS™ from Tidepool Scientific Software® and the EPA WET Spreadsheet 2012 can also be used to analyze chronic bioassay test data.

## 2. SUMMARY OF METHOD

2.1. Using TOXCALC® to analyze bioassay data involves choosing a test type, test name, endpoint, entering sample specific information, entering test concentrations (groups), entering data and performing the analysis. The TOXCALC program is activated. The test, sample, and groups are defined. Bioassay data are entered. Endpoints are selected and data are analyzed. Results are recorded on bench sheets.

2.2. It is suggested that the user concurrently read the section of the manual corresponding to the activity noted in the SOP. Also, the user should practice analyzing several sets of bioassay data for each type of bioassay that the user will be analyzing. This SOP is not intended to encompass every conceivable situation that can occur with bioassay data, but rather as an aid for the casual FDEP end-user.

2.3. It is recommended that the user review Appendix B of the ToxCalc® manual to become familiar with the TOXCALC® toolbars.

2.4. Use of this SOP constitutes acknowledgement that the user is **NOT** violating the end-user License of the TOXCALC® software. Users are expected to have read the *TOXCALC Toxicity Data Analysis Software User's Guide*.

2.5. Definitions:

2.5.1. NOEC: No Observed Effect Concentration; the exposure concentration at which there are no statistically or biologically significant differences in the frequency or severity of any effect in the exposed or control populations

2.5.2. LOEC: Lowest Observed Effect Concentration; the lowest measured effect of a toxicant or effluent that causes a statistically significant effect on a test organism during a specified exposure period

2.5.3. IC25: Inhibiting Concentration 25; it is a point estimate of the toxicant concentration that would cause a 25% reduction in a non-lethal biological measurement, such as growth or reproduction

2.5.4. LC50: Lethal Concentration 50; tells how much of a substance is needed to kill half of a group of experimental organisms in a given time


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## 3. EQUIPMENT AND SUPPLIES

3.1. Bioassay data to be analyzed

3.2. TOXCALC® program with appropriate End User License Agreement

## 4. TOXCALC METHOD FOR STATISTICAL ANALYSIS OF CHRONIC BIOASSAYS

4.1. Define Test

4.1.1. Open the *ToxCalc* program by double-clicking the *ToxCalc* icon. Select *enable macros* on the Security Warning screen and then select *yes* on the Welcome to TOXCALC screen.

4.1.2. Click on **Setup**. From the drop-down list, select the *test type* by choosing the appropriate name and then further choosing the appropriate name that pops up on the right.

4.1.2.1. DEP users will usually select ***Ceriodaphnia***, **Larval Fish**, or **Mysid** (for *Americamysis bahia* but listed as *Mysidopsis*). Larval fish species must be selected--inland silverside for *Menidia beryllina* or fathead minnow for *Pimephales promelas*.

4.1.2.1.1. For a *C. leedsi* test, select **Larval Fish, Fathead Minnow**. Although not addressed in this SOP, a code for *C. leedsi* can be created by following the instructions on pages 27-28 of the *ToxCalc* manual.

4.1.3. Click on **Endpoints**. From the drop-down list, select the *endpoint* by choosing the appropriate name. (Example: For 96-hour survival endpoint, click on **96 HR Survival**.) The data may be survival or number of young for the *C. dubia*, survival or growth by weight for *C. leedsi, P. promelas, M. beryllina*, and *A. bahia*, or cell count for *P. subcapitata (formerly S. capricornutum)*.

4.1.4. Click on the "fish" icon (first icon in second row of menu items). The *Define Test* screen will appear. Ensure that the *Title* box contains the species that you selected in Step 2. Ensure that the *Species* box is correct. For *Protocol* use the drop-down list (click on blue button) to select for acute tests, EPAAWO2-EPA/821/R-02-012; for freshwater chronic tests, EPAAWO2-EPA/821/R-02-013; for saltwater chronic tests, EPAAWO2-EPA/821/R-02-014. Click the *Done* button when finished.

4.1.4.1. Example: If *Ceriodaphnia* was selected in **Setup** and **96 HR Survival** was selected under **Endpoints**, the title will have *CD-Ceriodaphnia Survival and Reproduction test*.

4.1.4.2. If you want to add a test name or species, refer to the TOXCALC® manual, pp. 16-20, *Adding New Test Types-Adding New Test Names*. Example: If you want the *Title* box to have *Ceriodaphnia Survival*, follow the manual instructions to add this *Test Type* and *Test Name*.


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4.1.4.3. The *Protocol* box will initially reference an EPA ’91 method. Change to protocol referenced by current method manual.

4.2. Define Sample

4.2.1. Open the **Define Sample** box using one of the following methods:

4.2.1.1. Click on **Setup**, then choose *Sample* from the drop-down list.

4.2.1.2. Click on the *Define Sample* icon (second row, third icon from the left).

4.2.2. In the *Define Sample* box, type an alphanumeric ID in the *Test ID* edit box (mandatory entry-limit of 10 characters).

4.2.2.1. Optional: In the *Ref Tox ID* box, enter the Ref Tox ID corresponding to the Test ID of the appropriate reference toxicant text. (Limited to 10 characters.)

4.2.3. Select the appropriate *Sample ID, Sample Type,* and *Laboratory ID* from edit box(es) drop-down list(s).

4.2.3.1. If the codes you need are not listed, choose the *Codes* button to add a code. Refer to TOXCALC® manual pp. 16-20 and 27-28.

4.2.4. Enter Test Start, Test End, and Sample Dates & Times (optional). Use “mm/dd/yy hh:mm,” 24-hour format.

4.2.5. Select concentration unit from the *Conc. Units* edit box drop-down list. Or, type in any unit you need. Unless the test is an SRT, the unit will usually be %.

4.2.6. In the *Comments* edit box, enter any comments you want (maximum of 70 characters).

4.2.7. When finished, click the *Done* button to exit the **Define Sample** box.

4.3. Define Groups (Test Concentrations)

4.3.1. Open the *Assign Groups* dialogue box using one of the following methods:

4.3.1.1. Click on **Setup**, then choose *groups* from the drop-down list.

4.3.1.2. Click on the *Assign Groups Method 1* icon (second row, fourth icon from the left).

4.3.2. Select the *Test Data Sheet* from the *Place Groups On:* edit box. If the Test Data Sheet is not showing in the edit box, click the blue button and select.

4.3.3. Under *Control Assignments* select a control, usually D-control (diluent control). If there is a brine (DEP usually uses sea-salt control) or reagent control, select it from the first drop-down edit box and select the diluent control from the second edit drop down box.

4.3.3.1. For instructions on how to assign numerical concentrations to controls, refer to page 24 of the TOXCALC® manual.


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4.3.4. In the *Group Assignments* edit boxes, enter each concentration (low to high). Use the Tab key to move through the boxes. **MUST** be numbers only, no letters. (Example: 6.25, 12.5, 25, 50, 100. **NOT** 6.25A, 12.5B, etc.)

4.3.4.1. This program generally works with 3 to 10 groups, unless there are four replicates per group; then, results can be generated with 2 groups (screening test).

4.3.5. If there are equal replicates for each concentration and control, go to the *Equal Reps* edit box and click the 'up' arrow to select the number of replicates; then, click on the *Equal Reps* button. If the replicates are unequal, type in the number of replicates (use tab key to move between edit boxes).

4.3.6. When finished, click on the *Done* button.

4.3.7. Look at the Test Data Sheet and ensure that the groups (concentrations) are correct. Correct errors by selecting the error, deleting the error, and entering the correct data. If there are a number of errors (incorrect data entered into the *Assign Group* dialogue box), return to the *Assign Group* dialogue box and re-enter the correct data.

4.4. Enter Data (Test Data)

4.4.1. Activate the Test Data worksheet by clicking on the **Test Data** tab at the bottom of the file (**Test Data Sheet** that appears when *Assign Groups* is completed.

4.4.2. Enter data as appropriate for the test type.

4.4.2.1. Acute and chronic tests: Survival data

4.4.2.2. Chronic larval fish and chronic mysid tests: Total weight, tare weight, and weight count. The weight count should be the same as the start count for each replicate. Enter weigh as mg to two decimal places (xx.xx)

**Note:** If a concentration for growth (larval fish and mysids) has no surviving organisms, enter the same weight for the tare and the total for each replicate.

4.4.3. When data entry is finished, a second person should verify the entered data; or, if another person is not available, click on **Data** and click the *Double Entry* command. Re-enter the data (a pop-up notification will appear if the data differ upon re-entry). Press [Return] to move to the next lower cell; use the mouse or the arrow keys to move between columns. When finished, press [Esc] to exit the *Double Entry* mode.

4.4.3.1. If there is an entry error, select the cell containing the incorrect data and enter the correct data. Press [Return].

4.5. Analyze Data

4.5.1. It is strongly recommended that the user review Appendices A, C, and D of the ToxCalc® user manual.


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4.5.2. ToxCalc® analyzes data by hypothesis based tests to yield NOEC(s) and LOEC(s) and by point estimate tests to determine a level of effect (IC25, LC50).

4.6. Hypothesis Calculation

4.6.1. Open *Hypothesis Testing* dialogue box using one of the following methods:

4.6.1.1. Click on **Data** then *analyze*

4.6.1.2. Click on the *Analyze Data* icon (tenth icon from the left on toolbar)

4.6.2. If not showing, select *EPA methods* in the *Method* edit box.

4.6.3. In the *Tails* edit box, select either *one-tailed* or *two-tailed*.

4.6.3.1. **One-tailed** if the data is expected to show either a positive or negative effect.

4.6.3.2. **Two-tailed** if the effect is unknown.

4.6.4. Click in the *Auto* checkbox to select automatic data transformation. TOXCALC® automatically selects *Arcsin Square Root* for proportional data (ex: survival) and no transform for quantitative data (ex: weight).

4.6.5. Click **Calculate** to analyze data.

4.6.6. Review the results in the *Results* box. Click the *Summary Report* tab on the **TOXCALC® Data** sheet.

4.6.7. Click the **Save Data** button to save the data and the summary report. Refer to the TOXCALC® manual Chapter 6 Page 34 Item#12 for saving options.

4.7. Point Estimate Calculation (EC50, LC50, ICxx determinations)

4.7.1. Click on the *Point Estimate* button in the *Hypothesis Testing* dialogue box.

4.7.2. Select the desired point estimate method: Maximum Likelihood-Probit, Trimmed Spearman-Karber, or Linear Interpolation (ICp).

4.7.2.1. If the *Auto* box is checked, the *Maximum Likelihood-Probit* method will be automatically selected for proportional data and the *Linear Interpolation (ICp)* method will be selected for quantitative data.

4.7.3. Click on the **Calculate** button.

4.7.4. Review the results in the *Results* box. View the summary by clicking on the *Summary Report* tab on the **TOXCALC® Data** sheet.

4.7.5. Click the **Save Data** button to save the data and the summary report. Refer to the TOXCALC® manual Chapter 6 Page 34 Item #12 for saving options.

## 5. DATA ARCHIVAL

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

5.2. Data is saved in the TOXCALC® computer program. Results are recorded on the appropriate bench sheets. They are then entered into the Laboratory Information


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Management System (LIMS) using **Results Editor** (LIMS/Laboratory/Technical/Results Editor).

5.3. To correct raw data entries, place a single line through the incorrect or illegible entry. Write the corrected entry near the error, date and initial the correction. Do not overwrite entries.

5.3.1. The corrected entry is written as near its appropriate space as possible or else in the comment box on the appropriate bench sheet.

5.3.2. If the correction requires an explanation, write a comment and reference the error. Initial and date the explanation.

5.4. Use sequential alphabetical annotation to identify errors and corresponding comments (use A for first error and use A for corresponding explanatory comment; use B for second error, etc.).

## 6. QUALITY CONTROL

6.1. Data entry is verified either by a second person or by the *Double Entry* command in the TOXCALC® program.

6.2. Refer to the Bureau Quality Assurance Manual for corrective action and handling out-of-control data.

## 7. REFERENCES

7.1. EPA 821-R-02-013 Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition, October 2002.

7.2. EPA 821-R-02-014 Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd Ed. October 2002.

7.3. TOXCALC Toxicity Data Analysis Software User's Guide: http://www.toxicitylab.mobi/content/toxicity_testing/wet_glossary.php

7.4. DEP Laboratory Quality Assurance Manual http://www.dep.state.fl.us/labs/library/lab_sops.htm

## APPENDIX OF CHANGES

08/12/2013 Reformatted SOP.

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