Production and Maintenance of *Ceriodaphnia dubia* for Chronic and Acute Toxicity Testing

1. SCOPE AND APPLICATION

1.1. This method is based on EPA 821-R-02-012 Short-term Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th Ed. October 2002 and the culturing experience of laboratory personnel. It is used to ensure that *Ceriodaphnia dubia* (*C. dubia*) bioassay test organism cultures are maintained to provide suitable organisms for bioassay testing. Tests conducted by this procedure are BSY-CS-FCD, BSY-CD-FCD, BSY-AS-FCD and BSY-AD-FCD.

2. SUMMARY OF METHOD

2.1. Cultured organisms are fed and transferred on a regular basis to 30 mL disposable plastic cups containing fresh media. The day prior to setting up bioassay tests, *C. dubia* adults are transferred to cups (one per cup) as "Adults for Neonates" (AFN). Reproduction, mortality, and health of the organisms are monitored and recorded in the relevant logbooks.

2.2. Interferences:

2.2.1. Contaminated culture vessels, contaminated culture media, and/or poor-quality food can be detrimental to the survival and reproduction of cultured *C. dubia*.

3. EQUIPMENT AND SUPPLIES

3.1. 30 mL disposable plastic cups (referred to as "cups" or "culture cups")

3.2. Disposable plastic transfer pipet

3.3. *C. dubia* culture tray(s) (120 places labeled "Adult for Neonates" or "AFN")

3.4. *C. dubia* organism collection trays

3.4.1.1. Label the first tray "*C. dubia* culture A", the second tray "*C. dubia* culture B" and the third "*C. dubia* culture C."

3.4.1.1.1. Title label goes along the front of the tray. The left rear corner contains the slot for cup #5. The right rear corner is the 30th slot and it contains the reserve cup, labeled "Res." The slots across the front of the tray from left to right are #1, #6, #11, #16, #21, and #26.

3.4.1.2. On Mondays when a C. dubia test will be initiated that week, additional trays are labeled "Adults for Neonates Culture A", "…B", and "…C"

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3.5. Clear plastic culture tray covers

3.6. 2 L beaker (2) or 20 cm Carolina dish

3.7. *Culture Records for Ceriodaphnia dubia (water flea) Adults for Neonates* logbook

3.8. Magnifying glass

3.9. Black permanent marker

3.10. Light table

4. REAGENTS AND STANDARDS

4.1. MHWCD (Moderately Hard Water for *C. dubia*), prepared per SOP TA-03.15.

4.2. YCT (Yeast, Cereal Leaves, Trout Chow), prepared per SOP TA-01.01.

4.3. *Raphidocelis subcapitata*, prepared per SOP TA-01.04.

4.4. Deionized water

5. CULTURE MAINTENANCE

5.1. Empty the DI water from the cups soaking in the plexiglass trays labeled "*C. dubia* culture". Trays A, B, and C are always in use, tray D is in use when deemed necessary or when starting a new culture. On **Mondays**, empty the DI water from the cups soaking in the plexiglass trays labeled "Adults for Neonates". If the cups were not soaked the night before, rinse new cups with DI water three times.

5.2. Pour ~20mL of MHWCD into each of the cups from Step 5.1.

5.3. Place the trays containing the *C. dubia* bioassay cultures on the light table.

5.4. On **Mondays**, count the neonates and record their number in the *Culture Records for Ceriodaphnia dubia* logbook. Also, record any dead (labeled as "X") or missing (labeled as "M") adults. Using a plastic transfer pipet, transfer one neonate to the corresponding new culture cup (1-29). Transfer 15 neonates from the old reserve cup to the new one. Replace any dead or missing adults using adults from the reserve cup. Transfer the adult from the old culture to the corresponding cup on the "Adults for Neonates" tray to produce <24-hour old neonates for use in Tuesday's test set up.

5.4.1. If a culture cup does not contain at least fifteen healthy neonates, a neonate is transferred from a cup containing fifteen or more healthy neonates. The letter "R" is noted in the culture logbook for this replacement.

5.4.2. If an adult did not produce any neonates during the week, it must be inspected by a taxonomy analysts to identify if it is a male or not. This is recorded in the *Culture Records for Ceriodaphnia dubia* logbook.

5.4.3. While transferring, do not crush the *C. dubia* by capturing them against the side or bottom of the cup. When releasing the *C. dubia* into the new cup,

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release them under the surface of the liquid by gently squeezing the transfer pipet.

5.4.4. The neonates transferred to the culture cups on Monday mature into the adults that are transferred on Wednesday and Friday. These adults are then replaced the following Monday, restarting the cycle.

5.4.5. If *C. dubia* tests are not being initiated, transfer the adults to a glass Carolina dish instead. Label the dish "Adults for Neonates", the date, and the MHWCD batch #.

5.5. On **Wednesdays** and **Fridays**, count neonates similar to Mondays.

5.6. Feed the newly transferred *C. dubia* (refer to SOP TA-02.00). Food can be added to the culture cups after the MHWCD is poured, and prior to the transfers. Place the trays in Incubator #4 (maintained at ~25 ºC).

5.7. Empty the remainder of the neonates and adults from the old culture cups into the culture room sink.

5.8. Rinse the inside of each empty cup with a strong stream of DI water, then fill the emptied cups with DI water and allow to soak until the next transfer.

5.8.1. If a cup has a film build-up that the DI water cannot dislodge, discard the cup and replace it with a new one.

5.9. Record daily feeding in the *Feeding Log and Culture Record for C. dubia* logbook. (Feedings on Culture transfer days are also recorded in the *Culture Records for Ceriodaphnia dubia* logbook).

6. MASS CULTURE PREPARATION

6.1. Label a clean 2 L beaker for each tray as "Mass Culture" along with the tray letter. Write the current date on the label along with "*C. dubia*".

6.2. After the AFN have been transferred on Mondays, empty remaining *C. dubia* into the beakers labeled "Mass Culture".

6.3. Add MHWCD to the new "Mass Culture" beakers to bring the volume to approximately 1.8 liters.

6.4. Take the old "Mass Culture" beakers to the sink and empty them. Rinse with water and place the dirty beakers on the dirty glassware cart.

6.5. Place the new "Mass Culture" beakers in Incubator #1.

6.6. Feed cultures per SOP TA-02.00.

6.7. Record feeding times, media used, and weekly parameters in the *Feeding Log and Culture Records for Ceriodaphnia dubia* logbook.

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6.7.1. Parameters for the “Mass Culture” beakers are typically done on Fridays unless the lab will be closed. DO, pH, temperature, and conductivity are measured and recorded.

7. BIOASSAY CULTURE PREPRATION

7.1. On the day prior to test initiation, set up the *C. dubia* culture tray labeled “Adults for Neonates” with adults that have produced ≥ 1 brood from the *C. dubia* Culture trays (A, B, C, and sometimes D). Feed per SOP TA-02.00.

7.1.1. For chronic toxicity tests, neonates < 24 hours old and born within an eight-hour period are required. For acute tests, neonates < 24 hours old are needed.

7.2. After setting up the “Adults for Neonates” trays, check cups containing adults periodically (generally every one to two hours) for new neonates. When this occurs, record the cup ID (i.e. C14) and number of neonates present.

7.2.1. To collect *C. dubia* for chronic tests the day prior to initiation, conduct hourly counts and record the number of neonates.

7.2.1.1. If there are less than 8 neonates in a cup, use a black permanent marker to mark the rim of the cup.

7.2.1.2. If there are ≥ 8 neonates in a cup, mark the side of the cup with the cup ID, number of neonates, and time of count (ex. A19(11) 10:00)

7.2.1.3. Transfer the adult from the cups having ≥ 8 neonates to new cups containing media and food, then return to appropriate AFN slot. Place the cup containing the neonates on the organism collection tray for use in testing.

7.2.1.4. Eight to twelve cups containing ≥ 8 neonates are needed for a chronic definitive test. Conduct hourly counts until sufficient organisms are collected.

7.2.1.4.1. Keep in mind that neonates need to be ≤ 24 hours old and within 8 hours of each other. Time born is assumed to be the prior count time, since it could have been born any time between that count time and the time they were actually counted.

7.2.2. To collect *C. dubia* for acute tests, on the day of initiation all AFN cups will be counted and the number of neonates will be recorded. Once all cups have been counted, the top four to five from each tray will be set aside and mixed for use in the acute test(s).

7.2.2.1. A minimum of 120 neonates are needed for an acute definitive test, so the number of neonates in all of the cups chosen should exceed this amount.

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7.2.2.2. Cups with ≥ 8 neonates pulled during the hourly counts can be used for the acute test as long as they are ≤ 24 hours old at test initiation.

7.2.3. The organism batch number is the date (mm/dd/yy) that the tests will be initiated.

7.3. Maintain the AFN culture until Thursday of the test (Day 2).

7.4. Record batch information in the *Culture Records for Ceriodaphnia dubia Adults for Neonates* logbook.

8. CULTURE CRASH PROTOCOL

8.1. In the event of a culture crash, a new tray may be reestablished via two methods.

8.1.1. If there is at least one healthy tray you may use *C. dubia* in its mass culture beaker to set up a new tray.

8.1.2. If none of the trays seem healthy, or the lab wants to bring in fresher cultures, an order must be placed for new *C. dubia.* Refer to SOP TA-04.20, steps 6.1-6.14.

9. DATA ARCHIVAL

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

9.2. Record the culture set up, transfers, batch numbers, and number of neonates in the *Culture Records for Ceriodaphnia dubia Adults for Neonates* logbook. Record transfers in the *Culture Records for Ceriodaphnia dubia* logbook. Record culture maintenance and feedings in the *Feeding Log and Culture Records for Ceriodaphnia dubia* logbook.

10. QUALITY CONTROL

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

10.2. Autoclaved glassware, clean-rinsed cups, suitable media, and food is used to minimize the potential for contamination of the cultures.

10.3. Culture records are maintained and checked for performance. Appropriate staff are notified in the event of culture problems.

10.4. New disposable plastic cups are soaked with DI water overnight or rinsed three times with DI water before use.

10.5. Mass cultures are replaced weekly, typically on Monday.

10.6. *C. dubia* cultures are typically transferred every Monday, Wednesday, and Friday.

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10.7. Every Monday and Friday, *C. dubia* culture disposable plastic cups are replaced with fresh disposable plastic cups.

10.8. The method recommends tests and test cultures be maintained at 25±1°C (A. bahia tests are recommended to be maintained at 26±1°C). The method requires test temperatures not deviate more than 3° during the test period (max-min).

10.8.1. Because of the imprecise nature and thermal cycling of laboratory incubators, the Tox lab strives to maintain tests and cultures at 25±1°C but follow the 3° requirement as a test acceptability criterion. The incubators are set to alert if the temperature exceeds the range by a degree on either side for an hour. Temperature fluctuations below this threshold are minor enough to not impact the test/culture water temperatures significantly.

10.8.2. To ensure accuracy of test and culture temperatures within the incubators, the Tox lab has set correction factors to the continuous monitoring temperature probes within the incubators to ensure the temperature they are recording is as close to the actual test/culture water temperature as possible. The correction factors were established by taking an average of the test/culture temperatures on the shelf the monitor is on with a NIST-traceable thermometer and correcting against the temperature the continuous monitoring probe was recording.

10.8.3. In the case that a test exceeds the three-degree test acceptability criterion, a comment must be added to the data as follows:

10.8.3.1. Test temperature >3°, no toxicity seen: “Temperature of test solutions deviated from method specifications.”

10.8.3.2. Incubator temperature >3°, no toxicity seen: “Continuous temperature monitoring in surrogate test vessels deviated from method specifications.”

10.8.3.3. Test temperature >3°, with toxicity seen: [“J” qualify] “Estimated value. Deviation of test solution temperatures from method specifications may be contributing to toxicity.”

11. SAFETY/HAZARDOUS WASTE MANAGEMENT

11.1. Proper laboratory protection, including lab hood or ventilation system, lab coat, gloves and safety glasses, is required when working with chemicals and unprocessed samples.

11.2. Excess/unneeded organisms are humanely euthanized, and waste is disposed per FDEP Laboratory Quality Assurance Manual.

11.3. Refer to the FDEP Health and Safety Manual and Hazardous Waste Management.

11.4. Read MSDS information sheets for reagent handling and disposal.

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12. REFERENCES

12.1. EPA-821-R-02-012

12.2. DEP Laboratory Quality Assurance Manual

12.3. DEP Health and Safety Manual

12.4. MSDS Information Sheets

12.5. DEP SOP TA-01.01, *YCT (Yeast, Cereal Leaves, Flake-Fish Food, or Trout Chow) Preparation*

12.6. DEP SOP TA-01.04, *Raphidocelis subcapitata culture for Ceriodaphnia dubia Food*

12.7. DEP SOP TA-03.15, *Preparation of Synthetic Moderately Hard Freshwater*

12.8. DEP SOP TA-02.00, *Feeding Test-Organism Cultures*

12.9. DEP SOP TA-04.20, *Receiving and maintaining Purchased Test Organisms*

APPENDIX OF CHANGES

06/06/2013 Combined and Reformatted TA-05.01-3.14 and TA-05.02-1.13. SOP TA-05.01 was removed from the LIMS tracking system. The new SOP was named TA-05.02-2.0.

02/11/2015 Formatted for 508 compliance.

03/28/2025 Combined SOPs TA-05.02 and TA-04.01.

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