Preparation of Synthetic Moderately Hard Freshwater

1. SCOPE AND APPLICATION

1.1. This method is based on and modified from EPA 821-R-02-013. It is used to prepare control diluent for freshwater toxicity tests (BSY-AS-FFS, BSY-AD-FFS, BSY-CS-FFS, BSY-CD-FFS, BSY-AS-FCD, BSY-AD-FCD, BSY-CS-FCD and BSY-CD-FCD). It is also used as culture media for *Pimephales promelas*, *Cyprinella leedsi,* and *Ceriodaphnia dubia*.

2. SUMMARY OF METHOD

2.1. Synthetic moderately hard freshwater (MHW) for fish (*P. promelas* and *C. leedsi*): $MgSO_4$, $NaHCO_3$, and KCl salts are weighed and added to a 2L Erlenmeyer flask containing deionized (DI) water. The solution is stirred until all chemicals have dissolved and then added to a 50L carboy. DI water is added to the carboy to bring the volume to about 45L. The solution is aerated overnight (~24 hours). $CaSO_4$ is weighed and added to a 2L Erlenmeyer flask containing DI water. The solution is stirred until fully dissolved and then added to the 50L carboy. DI water is added to the carboy to bring the final volume to 50L. The solution is aerated overnight (~24 hours). Aeration is removed, and the carboy is labeled. Parameters are measured, and adjustments made as needed. Record the results in LIMS.

Synthetic moderately hard freshwater (MWHCD) for *Ceriodaphnia dubia* cultures and bioassays: $MgSO_4$ and $NaHCO_3$ are weighed and added to a 2L Erlenmeyer flask containing DI water. The KCl liquid solution is measured and added to the flask with the other chemicals. The solution is stirred until all chemicals have dissolved and then added to the 20L carboy and filled with about 15L of DI water. The solution is aerated overnight (~24 hours). $CaSO_4$ is weighed and added to a 2L Erlenmeyer flask containing DI water. The sodium selenite solution and the B12 solution are measured and added to the flask with the $CaSO_4$. The solution is stirred until fully dissolved and then added to the 20L carboy. DI is added to the carboy as needed to bring the final volume to 20L. The solution is aerated overnight (~24 hours). Aeration is removed, and the carboy is labeled. Parameters are measured, and adjustments made as needed. Record the results in LIMS.

2.2. Interferences: Include but are not limited to calibration errors or improper weighing procedures; drafts; moisture; contaminated spatulas, weigh boats, glassware, carboys, or chemicals.

3. EQUIPMENT AND SUPPLIES

3.1. Carboy with lid – 50 L for fish media or 20 L for *C. dubia* media

3.2. Lab cart

3.3. 2L Erlenmeyer flasks

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3.4. Plastic weigh boats

3.5. Aeration pipette (sterile, glass)

3.6. Airline tubing– aeration is used to stabilize media and help dissolve compounds

3.7. Laboratory compressed air

3.8. Parafilm™

3.9. Magnetic stir bars (2-4 cm) and stir bar remover

3.10. Computer with access to the Laboratory Information Management System (LIMS)

3.11. Black waterproof pen

3.12. Labeling tape

3.13. 30 mL disposable plastic cup

3.14. Meters to measure physical-chemical parameters:

3.14.1. pH (Calibrate per SOP TA-06.43)

3.14.2. Conductivity (Calibrate per SOP TA-06.36)

3.15. Hach kits to measure physical-chemical parameters:

3.15.1. Total Residual Chlorine (TRC) (Measure per SOP TA-06.22)

3.15.2. Alkalinity and Hardness (Measure per SOP TA-06.08)

3.15.3. Ammonia (Measure per SOP TA-06.35)

3.16. Mettler PB403-S/FACT® balance (Calibrated per SOP TA-06.38)

3.17. Magnetic stir plate

4. REAGENTS AND STANDARDS

4.1. Sodium bicarbonate ($NaHCO_3$) - Use ACS grade or equivalent

4.2. Calcium sulfate ($CaSO_4 \cdot 2H_2O$) - Use ACS grade or equivalent

4.3. Magnesium sulfate ($MgSO_4$) - Use ACS grade or equivalent

4.4. Potassium chloride (KCl) - Use ACS grade or equivalent

4.5. Potassium chloride solution 0.08 g/100 ml (for MHWCD)

4.6. Sodium Selenate working solution – prepared per SOP TA-09.08 (for MHWCD)

4.7. B12 working solution - prepared per SOP TA-09.08 (for MHWCD)

4.8. DI water

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5. PREPARATION

5.1. Cleaning the carboy

5.1.1. If carboy has been used for MHW/CD before, proceed to Step 5.1.2. For a new carboy that has not previously been used for MHW/CD, clean thoroughly with soap and water. Rinse with 10% HCl. Rinse with copious amounts of DI water. Proceed to Section 5.2.

5.1.2. If the carboy has been used for MHW/CD before, empty any remaining MHW/CD from the carboy, and rinse the carboy thoroughly with DI water. Empty all rinse water down the drain.

5.1.2.1. Scrub the carboy with soap and DI water.

5.1.2.2. Pour 20-30 mL chlorine bleach into the bottom of the carboy and add about 1L of DI water. Use the scrub brush to clean the sides and bottom of the carboy, turning carboy on its side on the floor or lab table, if necessary, to reach all areas. Wear a respirator if necessary, due to bleach fumes. Empty bleach water down the drain.

5.1.2.3. Rinse carboy several times with DI water and empty rinse water down the drain.

5.1.2.4. Add 500 mL (or enough to coat all surfaces) of 0.025N sodium thiosulfate to neutralize any remaining chlorine bleach and rotate the carboy so that it contacts all interior surfaces.

5.1.2.5. Rinse carboy with copious amounts of DI water and dispose of down the drain.

5.2. *Making the Moderately Hard Water* – prepare as needed in advance of scheduled fish bioassay tests (*P. promelas* and *C. leedsi*) and as needed to maintain *C. dubia* cultures and for *C. dubia* tests.

5.2.1. **For fish (MHW):**

5.2.1.1. Use the Mettler Toledo 403TS/00® balance per SOP TA-06.34 to weigh out the appropriate quantity of each compound listed in the table below.

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Compound	For 50 L (fish)
$NaHCO_3$	4.8 g ± 0.01 grams
$MgSO_4$	3.0 g ± 0.01 grams
KCl	0.2 g ± 0.01 grams
Day 2	
$CaSO_4$	3.0 g ± 0.01 grams

5.2.1.2. Fill a 2L flask with DI water (leave room for the addition of the chemicals).

5.2.1.3. Add $NaHCO_3$, KCl, $MgSO_4$, and a stir bar to the flask and mix on a stir plate until dissolved.

5.2.1.4. When fully mixed, pour contents of the flask into a 50L carboy.

5.2.1.5. Fill to about 45L with DI water.

5.2.1.6. Label the 50L carboy with “MHW” and “In Progress” on red tape. The water will take ~48 hours to prepare.

5.2.1.7. Connect the airline to a sterile glass pipette and then connect the airline to a laboratory compressed air outlet. Place pipette in carboy and adjust air flow to a moderate level of aeration. Place the lid over the top of the carboy and allow solution to aerate overnight (~24 hours).

5.2.1.8. After the ~24-hour aeration, add DI water to a 2L flask and add the $CaSO_4$ and a stir bar, mix on a stir plate until dissolved. This could take 1-2 hours.

5.2.1.9. Add the dissolved $CaSO_4$ solution to the 50L carboy and adjust water level with DI water as needed to reach 50L.

5.2.1.9.1. The lab has found that the Alkalinity tends to run high and needs the addition of a few extra (2-4) liters of DI water. It is good practice to check the alkalinity at this step and add water as needed to adjust properly per step 5.3.1.3.

5.2.1.10. Continue to aerate the solution for an additional ~24-hours in the same manner as the first aeration cycle.

5.2.1.11. After the second ~24-hour aeration cycle, remove the “In Progress” label and add the final label. On the label include the preparation date (date MHW was completed), the LIMS batch number (attach the LIMS sticker label), and an expiration date 14 days from the preparation date.

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5.2.2. **For *C. dubia* (MHWCD):**

5.2.2.1. Use the Mettler Toledo 403TS/00® balance per SOP TA-06.34 to weigh out the appropriate quantity of each compound listed in the table below.

Compound	**For 20 L (*C. dubia*)**
$NaHCO_3$	1.92 g ± 0.01 grams
$MgSO_4$	1.2 g ± 0.01 grams
Day 2	
$CaSO_4$	1.2 g ± 0.01 grams

5.2.2.2. Use a sterile glass pipette to measure out the appropriate quantity of each compound listed in the table below.

Stock Solution	**For 20 L (*C. dubia*)**
KCl	100 mL
Day 2	
Sodium Selenate	20 mL
B12	20 mL

5.2.2.3. Fill a 2L flask with DI water (leave room for the addition of the chemicals).

5.2.2.4. Add $NaHCO_3$, $MgSO_4$, 100mL of KCl stock solution, and a stir bar to the flask and mix on a stir plate until dissolved.

5.2.2.4.1. The KCl stock solution is made by adding 0.08g KCl / 100mL DI water. It is customary for the lab to make up 1000mL at a time. The solution is labeled and stored in the walk-in refrigerator for up to a year.

5.2.2.5. Pour about 15L DI water into a clean 20L carboy.

5.2.2.6. When fully mixed, pour the contents of the flask into the carboy.

5.2.2.7. Label the 20L carboy with “MHWCD” and “In Progress” on red tape. The water will take ~48 hours to prepare.

5.2.2.8. Connect the airline to a sterile glass pipette and then connect the airline to a laboratory compressed air outlet. Place pipette in carboy and adjust air flow to a moderate level of aeration. Place Parafilm®

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over the top of the carboy and allow solution to aerate overnight (~24 hours).

5.2.2.9. After the ~24-hour aeration, add DI water to a 2L flask (with room) and add the $CaSO_4$, 20mL sodium selenate working solution, 20mL B12 working solution, and a stir bar, mix on a stir plate until dissolved. This could take 1-2 hours.

5.2.2.9.1. The sodium selenate and B12 working solutions are prepared per SOP TA-09.08.

5.2.2.10. Add the flask to the 20L carboy and adjust water level with DI water as needed to reach 20L.

5.2.2.10.1. The lab has found that the Alkalinity tends to run high and needs the addition of a few extra (1-2) liters of DI water. It is good practice to check the alkalinity at this step and add water as needed to adjust properly per step 5.3.1.3.

5.2.2.11. Continue to aerate the solution for an additional ~24-hours in the same manner as the first aeration cycle.

5.2.2.12. After the second ~24-hour aeration cycle, remove the "In Progress" label and add the final label. On the label include the preparation date (date MHWCD was completed), the LIMS batch number (attach the LIMS sticker label), and an expiration date 14 days from the preparation date.

5.2.2.12.1. The EPA Manual says to use a batch of MHWCD no longer than 14 days because of possible build-up of bacterial, fungal, or algal slime growth and associated problems. This lab normally uses a batch of MHWCD within 14 days, but in the absence of observable problems (cloudiness, slime, odor, etc.) the MHWCD can be recertified in LIMS for an additional 14 days to be used as culture water for C. dubia as long as the culture health remains stable. The MHWCD should not be used for tests past the 14 days.

5.2.2.13. Run parameters on the MHWCD before use (see section 5.3).

5.3. Checking Parameters

5.3.1. Prior to first use, measure the pH, conductivity, alkalinity, hardness, chlorine, and ammonia of the MHW/CD per SOPs TA-06.43, TA-06. 36, TA-06.08, TA-06.22 and TA-06.35. If values deviate more than ±10% from values listed in Steps 5.3.1.1 – 5.3.1.3 below, follow the suggested extra steps.

5.3.1.1. pH 7.4-7.8 – additional aeration may stabilize the solution and increase pH.

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5.3.1.2. Hardness 80-100 mg/L (± 10%) as $CaCO_3$ – Adding DI water will decrease the hardness or adding more of the four compounds listed in the table below in the suggested amounts will increase the hardness. (The quantities given in the table will increase the hardness by 10-13 mg/L as $CaCO_3$)

Compounds	Amount
$NaHCO_3$	12.0 ± 0.1 mg
$MgSO_4$	7.5 ± 0.1 mg
KCl	0.5 ± 0.1 mg
$CaSO_4 \cdot 2H_2O$	7.5 ± 0.1 mg

5.3.1.3. Alkalinity 57-64 (± 10%) as $CaCO_3$ – Adding DI water will decrease the alkalinity or adding more of the four compounds listed above in Step 5.3.1.2 in the suggested amounts will increase alkalinity. (The quantity listed will increase the alkalinity by 10-13 mg/L as $CaCO_3$)

Note: It may be necessary to empty the "out of parameter" MHW/CD and start again if you are having difficulty adjusting MHW/CD after initial preparation.

5.3.1.4. Conductivity, chlorine, and ammonia have no recommended EPA ranges. These parameters are measured for the lab's reference. Typical MHW/CD readings are as follows: conductivity ~270-300 µmhos, chlorine <0.07 mg/L, and ammonia <0.2 mg/L.

5.3.1.5. Record batch information in LIMS/Laboratory/Technical/Laboratory Logbook. Record the results in the appropriate LIMS logbooks per SOP TA-06.42.

6. DATA ARCHIVAL

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

6.2. 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.

6.2.1. The corrected entry is written as near as possible to the error or in the comment box on the appropriate bench sheet.

6.2.2. If the correction requires an explanation, write a comment and reference the error with a letter. Initial and date the explanation. Do not attempt to put all information (correction and comments) into a small space.

6.2.3. 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.).

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6.3. FDEP Laboratory Information Management System (LIMS).

7. QUALITY CONTROL

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

8. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

8.2. Refer to Laboratory Quality Assurance Manual for sample disposal.

8.3. Refer to the DEP Health and Safety Manual and Hazardous Waste Management Plan and MSDS Information Sheets.

9. REFERENCES

9.1. EPA-821-R-02-013, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

9.2. DEP Health and Safety Manual

9.3. DEP Laboratory Quality Assurance Manual

9.4. MDSD Information Sheets

9.5. DEP SOP TA-06.08, Measuring Total Alkalinity and Hardness

9.6. DEP SOP TA-06.22, Measuring Total Residual Chlorine Using the Hach® Pocket Colorimeter™ II Test Kits

9.7. DEP SOP TA-06.34, Calibration and Use of the Mettler Toledo 403TS/00 Balance

9.8. DEP SOP TA-06.35, Measuring Total Ammonia using the Hach® Ammonia Nitrogen Pocket Colorimeter™ II Test Kit

9.9. DEP SOP TA-06.36, Calibration and Use of the Fisherbrand™ Traceable™ Bench Conductivity Meter for Conductivity and Salinity

9.10. DEP SOP TA-06.38, Calibration and Use of Mettler Toledo PB403-S/FACT Balance

9.11. DEP SOP TA-06.42, LIMS Entry for Laboratory Parameters

9.12. DEP SOP TA-06.43, Calibration and Use of Fisherbrand™ accumet™ AB315 pH/mV Meter

9.13. DEP SOP TA-09.08, Preparation of B12 and Sodium Selenate for use in 20% DMW, MHWCD, and *Raphidocelis subcapitata* for *Ceriodaphnia dubia* Food

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APPENDIX OF CHANGES

05/06/2008 Added Scope and Application, Summary of the Method and Appendix of Changes

04/13/2009 Added References section.

01/24/2012 Revised (major) the SOP to include preparation of SMHW for *Ceriodaphnia dubia*.

12/20/2013 Added “DATA ARCHIVAL” and “QUALITY CONTROL” sections and moved “SAFETY/HAZARDOUS WASTE MANAGEMENT” section.

02/10/2015 Formatted for 508 compliance.

03/04/2026 Changed expiration period from 30 days to 14 days with the allowance for recertification of MHWCD for an addition 14 days for use as culture water only.

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