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 media 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 for fish (*P. promelas* and *C. leedsi*): Chemicals are weighed and added to 2L Erlenmeyer flasks containing deionized (DI) water. The solutions are stirred until all chemicals have dissolved and then added to the carboy. DI water is added to the carboy to bring the final volume to 50L. This solution is aerated overnight (~24 hours). Aeration is removed, and the carboy is labeled. Parameters are measured, and adjustments made as needed.

2.2. Synthetic moderately hard freshwater for *Ceriodaphnia dubia* cultures and bioassays: Chemicals are weighed and added to 2L Erlenmeyer flasks containing DI water. Distilled water is added to the carboy. The solutions are stirred until all chemicals have dissolved and then added to the carboy.. DI water and/or distilled water is added to the carboy to bring the final volume to 20L. 20-mL each of 2 µg/mL selenium solution and 2 µg/mL B12 are added to the carboy. This solution is aerated overnight (~24 hours). Aeration is removed, and the carboy is labeled. Parameters are measured, and adjustments made as needed.

Note: Throughout this SOP, Synthetic moderately hard freshwater will be referred to as Moderately Hard Water (MHW). Use of this SOP constitutes acknowledgement that the section on synthetic freshwater in the above referenced EPA document (copies available in the Toxicity Lab – B246) has been read and understood.

2.3. Interferences: Include but are not limited to calibration errors or improper weighing procedures, drafts, moisture and contaminated spatulas, weigh boats, or glassware. Contamination in the chemical compound being used.

3. EQUIPMENT AND SUPPLIES

3.1. Supplies

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

3.1.2. Lab cart

3.1.3. Erlenmeyer flask, 2L (2)

3.1.4. Weigh boats

3.1.5. Aeration pipets or air stones (**MUST** be clean)

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3.1.6. Airline tubing (**MUST** be clean) – aeration is used to stabilize media and help dissolve compounds

3.1.7. Laboratory compressed air

3.1.8. Parafilm™

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

3.1.10. *Tox Record of Preparation* logbook

3.1.11. Black waterproof pen

3.1.12. Labeling tape

3.2. Equipment

3.2.1. Mettler PB403-S/FACT® balance - calibrated per SOP TA-06.31

3.2.2. Magnetic stir plate

4. REAGENTS AND STANDARDS

4.1. Reagents

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

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

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

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

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

4.1.6. Selenium working solution – prepared per SOP TA-09.08

4.1.7. B12 working solution - prepared per SOP TA-09.08

4.1.8. DI water

4.1.9. Distilled water in 1-gallon jugs purchased commercially

5. PREPARATION

5.1. Cleaning the carboy

5.1.1. If carboy has been used for MHW before, proceed to Step 5.1.2. For a new carboy that has not previously been used for MHW, 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 before, empty any remaining MHW from the carboy, and rinse the carboy thoroughly with DI water. Empty all rinse water down the floor drain. If further washing is needed (algal/fungal/slime in carboy), follow Steps 5.1.3-5.1.7. If not, proceed to Section 5.2.

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5.1.3. 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 floor drain.

5.1.4. Rinse carboy several times with DI water and empty rinse water down the floor drain. In addition to removing the bleach from the carboy this will help flush the bleach out of the floor drain.

5.1.5. Add 50 mL of 0.025N sodium thiosulfate to neutralize any remaining chlorine bleach and rotate the carboy so that it contacts all interior surfaces.

5.1.6. Rinse carboy with copious amounts of DI water and dispose of down the floor 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:

5.2.1.1. Use the Mettler PB403-S/FACT® balance per SOP TA-06.31 to weigh out the appropriate quantity of each compound listed in the table below.

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

5.2.1.2. Fill a 2L flask with DI water.

5.2.1.3. Add $CaSO_4$ and a stir bar to the flask. Place on stir plate and mix until dissolved.

5.2.1.4. Add KCl and $MgSO_4$ and mix until dissolved.

5.2.1.5. Fill a second 2L flask with DI water and $NaHCO_3$. Add stir bar, place on stir plate and mix until dissolved.

5.2.1.6. When fully mixed, pour contents of both flasks into a 50L carboy.

5.2.1.7. Fill to 50L line with DI water.

5.2.1.8. Connect the airline to the air stone and then connect the airline to a laboratory compressed air outlet. Place air stone in carboy and adjust air flow to a moderate level of aeration. Seal the carboy top opening with Parafilm™ and allow solution to aerate overnight.

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5.2.1.9. Label the MHW carboy for fish as "MHW". On the label include the date, the batch number and the initials of the preparer. Add an expiration date of 30 days from preparation date.

5.2.2. For *C. dubia*:

5.2.2.1. Use the Mettler PB403-S/FACT® balance per SOP TA-06.31 to weigh out the appropriate quantity of each compound listed in the table below.

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

5.2.2.2. Fill a 2L flask with DI water (can use distilled water).

5.2.2.3. Add $CaSO_4$ and a stir bar to the flask. Place on stir plate and mix until dissolved.

5.2.2.4. Add $MgSO_4$ and mix until dissolved.

5.2.2.5. Add 100mL of 0.08g KCl/100mL stock solution. Prepared by adding 0.8 g KCl to 1 L of DI or distilled water. Labeled and stored in walk-in refrigerator.

5.2.2.6. Fill a second 2L flask with DI water (can use distilled water) and add $NaHCO_3$. Add stir bar, place on stir plate and mix until dissolved.

5.2.2.7. Pour three 1-gallon jugs of distilled water into a 20L carboy.

5.2.2.8. When fully mixed, pour contents of both flasks into carboy.

5.2.2.9. Add 20mL of selenium working solution (prepared per SOP TA-09.08).

5.2.2.10. Add 20mL of B12 working solution

5.2.2.11. Fill to 20L line with DI water (can use distilled water).

5.2.2.12. Connect the airline to the air stone and then connect the airline to a laboratory compressed air outlet. Place air stone in carboy and adjust air flow to a moderate level of aeration. Seal the carboy top opening with Parafilm™ and allow solution to aerate overnight.

5.2.2.13. Label the MHW carboy for *C. dubia* as "MHWCD." On the label include the date, the batch number and the initials of the preparer. Add an expiration date of 30 days from preparation date.

Note: EPA Manual suggests using a batch 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 MHW

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within 14 days, but in the absence of observable problems (cloudiness, slime, odor, etc.) it can be used up to 30 days.

5.3. Checking Parameters

5.3.1. Prior to first use, measure the pH, alkalinity and hardness of the MHW per SOPs TA-06.23 or TA-06.24, TA-06.08.. If values deviate more than ±10% from values listed in Steps 5.3.1.1-5.3.19.3, follow the suggested extra steps.

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

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

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

5.3.1.3. Alkalinity 57-67 (± 10%) as Ca CaCO3 – 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 CaCO3.

Note: Although wasteful, it may be expedient to empty the "out of parameter" MHW and start again taking precautions to ensure that the correct quantities of reagents are used if you are having difficulty adjusting MHW after initial preparation.

5.3.1.4. Record batch information in the *Tox Record of Preparation* logbook.

6. DATA ARCHIVAL

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

6.2. Record information in the *Tox Record of Preparation* logbook.

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

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

6.3.2. If the correction requires an explanation, write a comment and reference the error.

6.3.3. Initial and date the explanation.

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6.3.4. 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.).

7. QUALITY CONTROL

7.1. Refer to Laboratory QA 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 Manual for sample disposal.

8.3. Refer to the Laboratory Health and Safety Manual and Hazardous Waste Management

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. Laboratory Health and Safety Plan https://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents.

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

9.4. Environment Canada Report EPS 1/RM/21: Biological Test Method: Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia.*

9.5. DEP SOP TA-60.31, Calibration and Use of Mettler PB403-S/FACT Balance

9.6. DEP SOP TA-09.08, Preparation of Selenium Solution for use in 20% DMW

9.7. DEP SOP TA-06.23, Calibration and Use of Accumet Model AR10 pH Meter

9.8. DEP SOP TA-06.24, Calibration and Use of Mettler Toledo SevenMulti for Measuring pH

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

APPENDIX OF CHANGES

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

05/12/2008 Inserted comment The EPA manual suggests using a batch of dilution water no longer than 14 days because of the possible build-up of bacterial, fungal, algal slime growth and associated problems. Dilution water is usually used within 14-days in this laboratory; however, in the absence of observable problems (growth) a batch of dilution water can be used up to 30 days for Step 7 under **Day 2**--- Allows diluent to be used up to 30 days absent observable problems.

04/13/2009 Added References section. Changed SOP references in Day 2 Comments from TA-06.16 to TA-06.15, TA-06.03 to TA-06.23 and TA-09.09 to TA-09.08.

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04/07/2010	Deleted "It can be used, by adjusting the quantity of additives, to prepare synthetic water having a different hardness." from Scope and Application. Deleted "Vinyl gloves can be substituted for individuals allergic to latex." from comment of Safety Section. Added "CAUTION Individuals allergic to latex should not use latex gloves. A suitable substitute should be found and used." to Safety Comment. Updated reference to Biology Section Quality Manual.
03/17/2011	Updated references to Biology Section Quality Manual, now Bureau Quality Manual. Changed Mettler 440 Balance to PB403-S/FACT. Changed SOP TA-06.15 to TA-06.31.
01/24/2012	Updated references to Biology Section Quality Manual. Revised (major) the SOP to include preparation of SMHW for *Ceriodaphnia dubia*.
01/08/2013	Reformatted.
12/20/2013	Added "DATA ARCHIVAL" and "QUALITY CONTROL" sections and moved "SAFETY/HAZARDOUS WASTE MANAGEMENT" section.
11/20/2014	Clarified and reduced verbiage describing process.
02/10/2015	Formatted for 508 compliance.
11/04/2015	Updated links.
01/08/2016	Corrected referenced numbers for parameters. Updated references.
11/30/2017	Updated references. Included use of B12.

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