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 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 50L carboy. DI water is added to the carboy to bring the volume to about 48L. The solution is aerated overnight (~24 hours). The final chemical 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 is added to the carboy as needed 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.

2.2. Synthetic moderately hard freshwater for *Ceriodaphnia dubia* cultures and bioassays: Chemicals are weighed and added to 2L Erlenmeyer flasks containing DI water. 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. Record the results in LIMS.

> **Note:** Throughout this SOP, Synthetic moderately hard freshwater for fish will be referred to as MHW. Synthetic moderately hard freshwater for *Ceriodaphnia dubia* will be referred to as MHWCD. Use of this SOP constitutes acknowledgement that the section on synthetic freshwater in the above referenced EPA document (copies available in the Toxicology Lab – B246) has been read and understood.

2.3. 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 pipets or air stones (MUST be clean)

3.6. Airline tubing (MUST be clean) – 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

4.9. Distilled water in 1-gallon jugs purchased commercially

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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 floor drain.

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 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.

5.1.6. 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 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 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
$CaSO_4$	3.0 g ± 0.01 grams

5.2.1.2. Fill a 2L flask with DI water.

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

5.2.1.4. At the same time, fill a second 2L flask with DI water and add the $NaHCO_3$ and a stir bar, mix on a stir plate until dissolved.

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

5.2.1.6. Fill to about 48L with DI water.

5.2.1.7. Label the 50L carboy with "MHW" on light blue tape. On the label include the date, the LIMS batch number (including the LIMS sticker label), and an expiration date 30 days from the preparation date.

5.2.1.7.1. Include a label that says, "In Progress" since the solution will take 48-hours to prepare.

5.2.1.8. Connect the airline to a clean 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. Place the lid over the top of the carboy and allow solution to aerate overnight (~24 hours).

5.2.1.9. 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.

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

5.2.1.10.1. 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.

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

5.2.1.12. After the second 24-hour aeration cycle, remove the "In Progress" label and run parameters on the MHW before use (see section 5.3).

5.2.2. **For *C. dubia*:**

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*)
$CaSO_4$	1.2 g ± 0.01 grams
$MgSO_4$	1.2 g ± 0.01 grams

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$NaHCO_3$	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 $MgSO_4$ and a stir bar to the flask. Place on stir plate and mix until dissolved.

5.2.2.4. 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.

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

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

5.2.2.7. To the carboy, add 100mL of 0.08g KCl/100mL stock solution which is prepared by adding 0.8 g KCl to 1 L of DI or distilled water. It is labeled and stored in the walk-in refrigerator.

5.2.2.8. Add 20mL of sodium selenate working solution (prepared per SOP TA-09.08).

5.2.2.9. Add 20mL of B12 working solution (prepared per SOP TA-09.08).

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

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.

5.2.2.11. Label the MHW for *C. dubia* carboy as "MHWCD" on light blue tape. On the label include the date, the LIMS batch number (including the LIMS sticker label), and an expiration date 30 days from the preparation date.

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 (~24 hours).

5.2.2.13. After the 24-hour aeration period and before use, run parameters on the MHWCD (see section 5.3).

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 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

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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.

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

5.3.1.4. Conductivity, chlorine, and ammonia have no limitations according to the EPA. These parameters are measured for the lab's personal reference. Typically, MHW(CD) readings are as follows: conductivity= ~270-300 µmhos, chlorine= <0.03 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.

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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.).

6.3. FDEP Laboratory Information Management System (LIMS).

7. QUALITY CONTROL

7.1. Refer to DEP Laboratory Quality 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. Health and Safety Manual

9.3. Quality Manual

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

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

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

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

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

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

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

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

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

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.

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.

01/08/2016 Corrected referenced numbers for parameters. Updated references.

11/30/2017 Updated references. Included use of B12.

11/16/2018 Updated SOPs. Updated current balance. Changed font from Times New Roman to Calibri.

10/31/2019 Updated SOPs and links.

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11/10/2020	Replaced Tox Record of Preparation logbook with LIMS. Updated equipment and supplies section. Updated data archival section with universal language. Updated Health and Safety Plan link. Fixed pH, conductivity, ammonia, chlorine and balance SOP mentions. Updated QC section with universal language.
10/29/2021	Updated for universal language and changed font in one section to be the same as the rest.
11/07/2022	Updated pH meter in references and SOP for using the pH meter.
11/02/2023	Updated links. Checked grammar and punctuation.
11/12/2024	Updated section 5.2 to reflect the EPA method for making MHW. Other corrections throughout to reflect the changes made in section 5.2. Edited for grammar, continuity, and clarity. Updated references.

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