Protocol: **Santoro lab urea concentration in seawater**

Version 1.1
8/16/2016

Pre-run preparation

1. At least 2.5 hours before you intend to start reactions turn on the water bath in the fume hood to 80 °C. Make sure bath has enough water to cover 13 mL of a 15 mL Falcon tube

2. Make Reagents #1 and #2. Make enough of each reagent to account for all 3 of the standard curves you will do (18 tubes), plus all samples which are typically run in duplicate or triplicate. The amounts shown here will allow you to run ~125 total reactions.

    a. Reagent #1 (500 mL); combine in a **fume hood**, **<u>on ice</u>**, and in 1 L HDPE bottle
        i. 300 mL MilliQ H_2O
        ii. 150 mL H_2SO_4
        iii. 50 mL H_3PO_4
        iv. Allow to cool on ice to room temperature
        v. 50 mg $FeCl_3$- $6H_2O$
        vi. Shake to dissolve $FeCl_3$- $6H_2O$

    b. Reagent #2 (100 mL); combine in a **fume hood** and in 1 L HDPE bottle
        i. 100 mL MilliQ H_2O
        ii. 2.0 g Diacetylmonoxime (2,3-Butanedione-monoxime)
        iii. 0.02 g Thiosemicarbizide
        iv. Shake to dissolve

3. Create Standard curve **in triplicate,** in 15 mL Falcon tubes

    a. Warm primary standard to room temperature
    b. Dilute primary standard 100x in a 100 mL volumetric flask, this secondary standard is good for 1 day and then should be re-made.
    c. Always rinse beakers with small amounts of the primary or secondary standard before using them
    d. Standard Curve Additions: Always use good pipetting technique

Desired Concentration (µM N)	Addition of Secondary Standard (µL)	MilliQ in Falcon Tube (mL)	Actual Concentration (µM N)
Matrix Blank	0	8	0
0.10	8	8	0.0999
0.25	20	8	0.2494
0.50	40	8	0.4975
1.0	80	8	0.9901
2.0	160	8	1.9608

1 [Urea]

e. 2 curves are done at the beginning of a run and 1 is done at the end
f. If using new reagents, the 1st two standard curves should always be completed before reagent is added to samples.

4. Reduce all seawater samples to 8 mL with a serological pipette, discard the excess

Reacting standards and samples

1. Add 3.0 mL of Reagent #1 to each Falcon tube
    a. Do addition in a fume hood
    b. Use the repeat pipette with a 50 mL tip

2. Add 800 µL of Reagent # 2 to each Falcon tube
    a. Do addition in a fume hood
    b. Use the repeat pipette with a 5 mL tip

3. Cap and vortex on high for 2 seconds

4. Place entire tube rack in the 80 °C water bath and start a **40-minute** timer immediately

5. Before removing tubes from the 80 °C water bath turn of all room lights and block most sunlight

6. **Immediately** transfer tube rack to a slushy ice bath

7. Intermittently feel the 15 mL Falcon tubes **until they reach room temperature**. Normally this takes less than 5 minutes but the amount of time depends on the temperature of the ice bath. See the note at the end of this protocol for more information about this step, it leads to the most common problems with this method.

8. Remove the Falcon tubes from the ice bath and place them at room temperature in the dark

9. Read each tube on the spec immediately, however the compound evolved should be relatively stable in the dark for 2 hours after the reaction has cooled

Reading standards and samples

1. Power up UV/Vis spectrophotometer (ours is Thermo Evolution 60S) and select “Basic ATC” from the start screen

2. Set wavelength to 525 nm

3. Install 10 cm (14 mL) cuvette in the spec
    a. Install holder platform in the chamber
    b. Match sharpie line on cuvette to holder so as to center the cuvette in the light path

4. Blank Spec using MilliQ H_2O from a squirt bottle

5. Carefully vacuum out cuvette with vacuum pump before reading the first sample

6. Pour standard or sample into cuvette and record the absorbance once reading is relatively stable (See Note below on what to do if absorbance will not stabilize)

7. Carefully vacuum out cuvette with vacuum pump between samples

Preparing the Primary Standard (5 mM urea, 10 mM urea N)
Urea, H_2NCONH_2 MW=60.6

1. Dry urea overnight at ~50 °C
2. In a 500 mL Volumetric flask dissolve 0.15 g of urea and bring volume up to 500 mL with a squirt bottle
3. Keep this stock solution at 4 °C when not using it

Note on Step 6 of "Reacting standards and samples":
The most common problems with this method come from the cooling step immediately following the reaction for 40 minutes at 80 C. Two different problems can occur during this step; the reaction can fail to completely stop, or the tubes can get too cold. It is important for the ice bath to be very cold. It is most important that the tubes cool rapidly and completely. Overcooling the tubes should be secondary concern. If tubes are not completely cooled when read you will observe *an absorbance that continues to rise in the cuvette*, slowly and steadily. If tubes are over cooled, then pouring overcooled reaction into the cuvette can cause condensation giving *an artificially high absorbance initially that will slowly fall*. These are the most common issues with this method. Overcooling the reaction is a much preferable mistake to not completely stopping the reaction. Overcooling can be remedied by placing cold tubes in a room temperature water bath, in the dark, for 5-10 minutes then reading.

Citations:
Price and Harrison, 1987, *Mar. Biol.* 94:305-317
L. Goeyens et al., 1998, *Estuarine, Coastal, and Shelf Science.* 47, 415-418
Bronk Lab "Urea analysis: Monoxime Procedure"
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4 [Urea]