**OPA Method for Measuring Submicromolar Ammonium**

Updated: July 18, 2019

References

ref: Holmes et al. 1999, Meeder et al. 2012 (incl. supplement)

**Supplies:**

Chemicals: sodium sulfite, sodium tetraborate, ethanol, opa

Lab Equipment: Fluorometer, 2 or 4 L dark bottle, repeat pippetter, quartz cuvette, something to rinse cuvette, kim wipes, gloves, sample tubes, 1 L grad cylinder, 150 ml grad cylinder, 10 ml grad cylinder, balance, stir bars, aged sample tubes.

**Fluorometer:** excitation 360 nm, emission 420 nm, low sensitivity. For concentrations above 5 uM, change emission to 500 nm. Turner makes a fluorometer specifically for this method, but you can use any fluorometer that allows you to adjust the wavelengths. You may need to adjust the sensitivity differently on different instruments.

**Reagents:**

Makes ~ 2 L.

Always use fresh MilliQ water (MQ) and very clean glassware.

1. Sodium Sulfite Solution

Add 1 g sodium sulfite (sigma S-4672) to 125 ml MQ.

Stable for ~1 month at room temperature.

2. Borate Buffer Solution

Add 80g sodium tetraborate (Sigma S-9640) to 2 L MQ.

Stir to dissolve (takes awhile).

Store at room temperature.

3. Orthophthaldialdehyde (OPA) Solution

Add 4 g OPA (Sigma P-1378) to 100 ml high grade ethanol (Carlo Erba, Fisher).

Stir to dissolve (takes awhile).

Store in the dark at room temperature.

4. Working Reagent (WR)

In a large (>2 L) brown polyethylene bottle,

Mix 2 L of 1) borate buffer solution, 2) 10 ml sodium sulfite solution, and 3) 100 ml OPA solution.

Allow WR to "age" 1 or more days before using.

Stable for 3 months when stored in the dark at room temperature.

**Standards1**

Primary Standard: 25 mM. Dissolve 0.13 g NH4Cl in 100 ml MQ.

Secondary Standard: 250 uM. 1:100 dilution of Primary.

Tertiary Standard: 2.5 uM 1:100 dilution of Secondary.

1. Choose the range of standards appropriate for your samples. For oceanic samples, 0.012 - 2 uM is appropriate.

2. Standards should be prepared in duplicate, and BF and 0 should be prepared in triplicate.

3. Put 10 ml of the appropriate sample matrix into aged tubes2, and add the volumes given in the Table below to make standards.

The sample matrix **must** match the samples. Ideally, you would choose a sample with a low ammonium concentration, prepare the standards using that sample, and then correct for the ammonium in that sample.

For **seawater** samples, use a sample with very low (<20 nM) ammonium to make standards. This can be either a deep sample (anything well below the nitracline, so 200 m or deeper) or a surface sample, if you know surface ammonium is <10 nM. This sample should have zero measurable ammonium, so you won't have to correct for background NH4 in the standards.

For **rain** samples, use MQ to make standards. NH4 may be 50-200 nM in MQ, so you may need to correct for this using the BF.

For **estuarine** samples, matrix effects may be variable between samples. I (BNW) have not run any estuarine samples using this method, so I would suggest testing the effect of the matrix by preparing multiple standard curves using different estuarine samples and comparing the slopes. Alternatively, you can use the method of standard additions on each sample (Holmes, et al, 1999).

|  |  |  |  |
| --- | --- | --- | --- |
| NH4 Concentration (uM) | Volume Tertiary Added (ul) | Volume Secondary Added (uL) | Volume Primary Added (uL) |
| BF5 | - | - | - |
| 0 | - | - | - |
| 0.012 | 50 | - | - |
| 0.025 | 100 | - | - |
| 0.049 | 200 | - | - |
| 0.119 | 500 | - | - |
| 0.250 | - | 10 | - |
| 0.500 | - | 20 | - |
| 1.24 | - | 50 | - |
| 2.48 | - | 100 | - |
| 4.90 | - | 200 | - |
| 8.45\* | - | 350 | - |
| 11.9 | - | 500 | - |
| 25.0 | - | - | 10 |
| 50.0 | - | - | 20 |
| 100 | - | - | 40 |

\* Samples/standards above 8.45 uM could not be read without dilution.

**Samples and Laboratory Procedures**

1. Decide if sample filtration is appropriate.3 Filter if necessary.

2. To collect sample, rinse aged tube2 with sample water twice and fill to 10 ml.

3. Store in the fridge until analysis.

4. Bring all samples, standards, and WR to room temperature.

5. Turn off the lights during reagent spiking.4

6. Dilute samples that you suspect will be > 8 uM.

7. Add 2.5 ml aged WR to each sample/standard. Invert 5 times and store in the dark4. Repeat for all.

8. Put BF standard in the dark with samples WITHOUT adding WR. This will be your reagent blank.

9. Incubate samples and standards at room temperature in the dark4 2-8 hours. It is possible to read the samples up to 60 hours after spiking, but the fluorescence, and therefore the sensitivity of the method, decreases after 8 hours.

10. Turn the fluorometer on at least 30 minutes before running samples and check that the excitation and emission wavelengths and the sensitivity are set correctly.

11. Rinse the 1 cm cuvette with MQ.

12. Add 2.5 ml WR to the BF sample, transfer to the cuvette, and read immediately.

13. Use a disposable pipette to transfer each standard and sample to the fluorometer. It is not necessary to rinse the cuvette between samples unless you have just read a very high value.

14. Read one set of standards at the beginning of the run, read the samples, and then read the last set of standards. This way you can correct for instrument drift.

15. If the instrument reads 1015, this means that you have exceeded the upper limit of concentrations you can read. You should try to avoid this by diluting samples before adding WR. If you didn't do that, at this point, adjust the emission wavelength to 500 nm and re-read. You will then have to re-read standards at 500 nm to use for this sample.

15. After you are done running samples, remove the cuvette, rinse with BW, and store in the black case. Turn off fluorometer.

**Optional Filtration3**

Materials: 0.2 um membrane filter (DO NOT use a filter made of glass or containing nitrogen. DO NOT use a cartridge filter. ONLY USE a membrane filter), 47 mm swinnex filter holder with adaptor, plastic tubing, peristaltic pump (optional), 2 L container Use all plastic in the setup- NO GLASS.

1. Assemble the swinnex with tubing, adaptor, and filter. Use a zip tie to secure the tubing to the adaptor if using a pump.

2. For a new filter, pump at least 2 L sample (or full strength ASW) through the filter before collecting samples. This step is a pain, but DO NOT skimp on the rinse. You will regret it when your samples are contaminated! I use a 2 L container to collect the filtrate so I know I rinsed at least 2 L.

3. Pass the sample through the filter, rinse the tube, and directly fill the aged tube to 10 ml. Minimize sample contact with the outside of the tubing. If collecting from a Niskin bottle, you can attach the tubing right to the Niskin.

4. Before collecting the next sample, pass enough water through the tubing and filter to rinse the entire thing twice.

5. Collect the next sample.

6. Repeat steps 3-5 until the filter clogs. Then repeat step 2 and continue filtering. Filters can be re-used a lot before they clog. They can be reused for multiple days. Don't rinse with MQ. Just keep rinsing with SW.

**Calculations**

General: Use BF to correct for contamination in the standards. BF is the actual zero ammonium value. Calculate concentrations from the BF-corrected standard curve.

**Notes**

1. For both standards and samples, this recipe uses 10 ml sample. You then add reagent in a 1:4 reagent:sample ratio. So, for 10 ml you add 2.5 ml reagent. Other volumes can be used if needed, however smaller volumes should not be used for small concentrations. Smaller volumes magnify contamination.

2. All samples and standards should be prepared in "aged" tubes. Aged tubes have been stored with either a small amount of WR in them or previously reacted samples for at least 1 week (the longer the better). Over time the reagent removes all NH4 from the tube. After using a set of tubes to run samples, simply leave the unused sample+WR in the tubes and save the tubes for next time.

3. Filtration can contaminate ammonium samples substantially. Ideally, samples should be run immediately after collection without filtration. If biomass is really high, there are lots of particles, the samples must be refrigerated for more than 24 hours (for samples with low turnover rates only), or the samples must be frozen, filtration is unavoidable. In these cases, follow the Optional Filtration steps above.

4. When spiking reading samples, keep the lights off in the room. it doesn't have to be pitch dark, but try to minimize light exposure. Keep the samples in a small box or cooler and take them out only to read. While incubating, keep them very dark. I have had problems when they're stored in something like a cardboard box that doesn't keep them completely dark. I recommend putting them in a box inside something else (an cooler or drawer).

5. The background fluorescence (BF) sample is basically the blank for this method. Because the BF has not had time to react with reagent, it reflects fluorescence due to the matrix and reagent and does not reflect the ammonium contamination possibly present in the standard matrix. The BF should always be the same as or lower than the 0 standard. If it isn't, the standards may not have been incubated in absolute darkness.