Ammonium Concentration in Seawater Protocol
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References:

Holmes, R.M., et al. (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Canadian Journal of Fisheries and Aquatic Sciences* 56.10: 1801-1808.

Knap, A., A. Michaels, A. Close, H. Ducklow and A. Dickson (eds.) (1996) Chapter 8: "The Determination of nitrite, nitrate + nitrite, orthophosphate and reactive silicate in seawater using continuous flow analysis." Protocols for the Joint Global Ocean Flux Study Core Measurements.

Taylor et al. (2007) Improving the fluorometric ammonium method: matrix effects, background fluorescence, and standard additions. *Journal of The North American Benthological Society* 26(2): 167-177.

General notes

There are myriad ways to contaminate a seawater sample with NH_4^+ and generally it is best if NH_4^+ samples are drawn from the rosette first. If this is not possible (for example if gas samples need to be drawn first), duplicate or triplicate samples will improve precision. I generally prefer to wash and rinse my hands well and sample without gloves when sampling, but if someone has been helping with a lot of deck work, is new to sampling for low level nutrients, and/or is a smoker, gloves should be worn.

Make sure that no one is smoking on deck while samples are being drawn. Also be on the lookout for the use of cleaning products in the vicinity of the rosette, which can be VERY high in NH_4^+. We also learned on a recent cruise that many trace metal analysts use NH_4OH at high concentrations, which may linger long after the reagent is gone. Thus, their entire laboratory spaces should be avoided when collecting and analyzing [NH_4^+] samples.

Standard stock solutions are made up in Milli-Q/Barnstead water using the guidelines in the JGOFS seawater nutrient protocols (Knap et al. 1996) with gravimetrically-calibrated volumetric glassware. Standard salts are dried overnight prior to weighing.

For oligotrophic seawater collected in more or less the same region, I do not do the matrix effect (ME) correction suggested by Holmes et al., but instead make up standards in deep seawater from sample location (see also Taylor et al. 2007). Mesopelagic waters (> 500 m depth) have [NH_4^+] lower than most lab Milli-Q systems (thank you archaea!).

If possible, I collect water for standards from the same cast from which unknown samples are being drawn. If not, I store unfiltered mesopelagic seawater in a polycarbonate carboy at 4ºC until use. Avoid using surface water for standards, as there is usually detectable NH_4^+. Similarly, avoid near-bottom waters as they could contain NH_4^+ from sediments.

We assume that, in offshore waters, the matrix effect (ME) is identical for all samples and do not do any additional corrections beyond the deep water standard curve. This may not be a valid assumption for near-coastal/estuarine waters.

Reagents

Sodium sulfite solution
Add 1 g of sodium sulfite (e.g. Sigma S-4672) to 125 mL of Milli-Q water. The original protocol claims the resulting solution is stable for ~1 month when stored at room temperature in a glass bottle. We have found that storage times are often less than this (more like 2-3 weeks) on long cruises.

Borate buffer solution
Add 80 g of sodium tetraborate (e.g. Sigma S-9640) to 2 L of Milli-Q water. Stir or shake thoroughly to dissolve. It can take several hours for this to dissolve completely.

OPA solution
Add 4 g of OPA (e.g. Sigma P-1378) to 100 mL of ethanol. Use a high-grade, 100 proof ethanol because impurities in ethanol can autofluoresce. OPA is light sensitive, so it should be protected from light while dissolving in ethanol and stored in the dark.

Working Reagent (WR)
In a large (>2 L) brown HDPE bottle, mix 2 L of borate buffer solution, 10 mL of sodium sulfite solution, and 100 mL of OPA solution. Ideally, allow the WR to "age" for 1 day or more prior to use because its blank will decrease over time. To summarize, the final WR should contain the chemicals at the following concentrations: borate buffer (40 $g \cdot L^{-1}$, 21 mM in the WR), sodium sulfite (40 $mg \cdot L^{-1}$, 0.063 mM in the WR), and OPA in ethanol (50 $mL \cdot L^{-1}$ in the WR). The original Holmes et al. protocol states the resulting WR is stable for at least 3 months when stored in the dark at room temperature. We have found that the WR appears to lose sensitivity after about 3 weeks, which we attribute to decay of the sulfite.

Supplies

50 mL polypropylene centrifuge tubes (Falcon tubes) in a rack – at least one per sample depth and two per standard concentration. Pre-mark tubes at the 40mL mark for ease of filling.
Bottle top pipettor, set to 10 mL
Micro pipettors for adding NH_4^+ standard spikes
WR, enough for 10 mL per sample plus standards
50 mM NH_4Cl solution (1º stock, stored frozen; see Knap et al. 1996 reference for

general considerations on the preparation of nutrient standards)
50 µM NH_4Cl solution (2º stock, made fresh, stored at 4ºC, and used within one week)
Fluorometer with appropriate filter set (we use a Turner AquaFluor hand-held unit)
Cuvettes (our fluorometer uses 2 mL plastic cuvettes)
Waste collection container for spent reagent

Procedure

Pre-cruise/pre-sampling

1. Pre-soak sample tubes with WR: add 20-30 mL of WR reagent, shake vigorously and allow to sit overnight. After soaking, WR can be dumped back into WR stock or discarded. Pre-conditioned tubes can and should be reused from cruise to cruise – rinse with Milli-Q water between cruises and do not allow WR to dry in sample tubes. New Falcon tubes (especially generic brand tubes) have a very high NH_4^+ blank. The blank in the tubes continues to drop with time and there is no reason to use new tubes . . .we have been using the same tubes at sea for ~6 years.

2. Make secondary NH_4Cl stock (50 µM): dilute 50 mM NH_4Cl primary stock 1000X by diluting 100 µL of 50 mM NH_4Cl to 100 mL Milli-Q water in a volumetric flask, or make gravimetrically.

Sample collection

1. Rinse sample tubes vigorously 3x with sample water from each sample depth, making sure to rinse cap and threads well, and then fill to the 40 mL mark. Do not use tubing to fill sample tubes.

2. Rinse and fill duplicate tubes with water for standards (see General Notes above) and one background fluorescence (BF) sample to 40 mL.

3. Samples should be analyzed immediately, if possible, or stored at 4ºC in the dark for no more than 2-3 hours.

Laboratory procedure

1. Prepare standards by adding 50 µM secondary ammonium stock solution to 40 mL standard matrix (deep mesopelagic water) in duplicate. We suggest additions of 0, 25, 50, 125, and 250 µL 50 µM NH_4Cl for oligotrophic seawater.

2. Add 10 mL WR to all samples and standards, remembering <u>not</u> to add WR to the BF sample. If using a pump style bottle-top pipettor to dispense WR, discard the first aliquot that is dispensed before adding to samples.

3. After incubating for at least 2–3 h, read samples and standards on the fluorometer.

Begin by measuring the BF sample. Add 10 mL of WR to the BF sample and read immediately on the fluorometer. Again, if using a pump style bottle-top pipettor to dispense WR, discard the first aliquot that is dispensed before adding to samples. Pour the sample directly from the reaction container into a cuvette (rinse the cuvette three times with sample) and immediately read on the fluorometer.

4. After the BF sample is read, proceed with the standards and samples. Pour the sample directly from the reaction container into a cuvette and immediately read on the fluorometer. We use a single cuvette for the entire run and triple rinse the cuvette with sample prior to reading. Keep samples in the dark between readings. We have found high precision with our fluorometer and only make a single fluorometer reading of each sample and standard. We read one standard curve at the beginning of measurements and one at the end to bracket the reaction period of the samples.

5. Even though the original protocol says the reagent is stable for only 8-10 h, we have regularly read samples the next day (12 h) and still found standards linear within the range of most marine samples.

Data analysis

Use the volume of the standard additions to calculate the actual concentration in each standard as in Holmes et al. 1999. For the standard additions suggested here, the final concentrations in each standard are:

Volume of 2º standard addition (µL)	Volume of seawater dilution matrix (mL)	Final [NH4+] (µM)
0	40	0
25	40	0.031
50	40	0.062
125	40	0.156
250	40	0.311

Subtract the BF reading from all samples and standards and use the standard curve to calculate the concentration of the unknowns. We use 'Protocol I' in Taylor et al. 2009.

We calculate our detection limit as three times the precision (standard deviation) of the lowest standard.

Revision history

v. 2.0: written for SPOT project, aes and sml
v. 2.1: clarifications and standard curve concentrations added for submission to BCO-

DMO, 12 Oct 2018 aes
v. 2.2: clarifications added for fluorometer reading procedure and other additional clarifications, 11 Jul 2019 aes
v. 2.3: added note about NH_4OH contamination, 21 Sept 2020