Protocols for measuring ammonium in seawater

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Methods from summer 2022

# Background

Measuring ammonium using fluorometry is a relatively simple process that can be done in the field. It has benefits over other methods of analysis in that is can be done immediately after sample collection (reducing problems associated with the relatively unstable nature of ammonium that causes it to degrade relatively quickly) and also only uses one Working reagent that does not have adverse effects on the environment (i.e. can go down the drain). Care should be taken while analyzing samples using fluorometry as contamination and sources of ammonium that are introduced to samples (e.g. from exposure to atmospheric ammonium or to organic sources such as human skin) can drastically alter sample measurements. When using fluorometry the presence of ammonium in standards and variations in factors such as pH and particulates between samples and standards can also pose problems to accurate sample measurement. Therefore, the following processes should be performed with care to avoid contamination. Many of them also need to be performed in the dark since they are light sensitive. Below we present two methods; one for measuring the total ammonium in water, and one for measuring changes in ammonium over time

# Notes for washing equipment

A. Used/Dirty equipment:

Equipment that has been used for sample collection or has been exposed to sources of contaminations (such as human skin, flies, fish scales etc.) needs to be acid washed before being used for sampling, for making standards or for making reagents.

NOTE: ALWAYS wear gloves—there is no point in sterilizing if we are going to get our grubby little hands all over things ☺ (use dishwashing gloves NOT nitrile as it becomes unhappy in contact with acid)

Hot water rinse

* + Fill 1st bucket with hot water (use the kettle to boil a couple litres, add to a bucket of regular water)
  + Scrub glassware/bottles to remove large particulates if any

Acid wash

* + Wash in 2nd bucket in 5% HCL solution
  + Make solution in bucket using 31% muriatic acid and distilled water (DW). The ratio of acid to water is 1:5 (e.g. to make a 3 L solution add 500ml of acid to 2500 ml of DW)
  + Let soak for 30 min

Final DW rinse

* + Fill 3rd bucket with DW
  + Rinse all glassware by either filling and swirling or by dunking
  + Acid must be completely washed (i.e if unsure rinse, rinse again!)

Drying

* + Place glassware/bottles mouth down in drying rack on a layer of paper towel
  + Avoid breathing into bottles/putting anything that hasn’t been acid washed inside the containers!
  + Leave face down until fully dry
  + If washing caps place cap downwards so no dust settles in
  + After dry pick up glassware/bottles and immediately place caps on to reduce particles from going in!

Rinsing in the field

* Keep sample bottles closed until sample is ready to be added
* Open cap and add 50 ml of sample water
* Close cap and shake
* Pour out sample water, bottle is now ready to put sample in

# Advanced Preparation

* **Note that many of the materials that you need for this procedure require ordering from science stores (e.g. chemicals, glassfiber filters etc.) so make sure you look at the whole protocol at least 2 weeks before sample collection**

## Working Reagent

(Prepare least 24 HOURS in advance)

### Materials needed

*-1 gallon jug (fully opaque/wrapped to darken)*

*- scoopula*

*- stir stick*

*- 1 small closed bottle for OPA mixture*

*- 1 small glass beaker (lay mouth down on paper towel)*

*- 3 L DW*

*- 100 ml ethanol*

*-OPA (4g)*

*- sodium sulphite (1g)*

*- sodium tetraborate (80 g)*

*- dark room (as dark as possible)*

*- red head lamp or red light to provide some visibility*

*- small graduated cylinder*

*- large 1 L graduated cylinder*

*- microbalance scale*

1. Make sure everything has been acid washed
2. Wear nitrile gloves, labcoat and goggles
3. Prepare a dark location for mixing (i.e. a large tarp fort in the field ☺/dark room)

### Borate buffer

* + Cover a large 1 gallon nalgene in dark plastic/ ducktape
  + Add **2L DW** (get straight from the distiller)
  + Add **80 g of sodium tetraborate**
  + Using microscale measure out 80 g of sodium tetraborate
    1. Clean scoop with kimwipe and 80% acytl alcohol (let alcohol air dry off)
    2. Tare plastic weigh boat
    3. Scoop small amounts of sodium tetraborate out until you have 80 g (if you scoop too much DO NOT add it back to the bottle)
  + Put on cap and shake vigorously to mix

### Sodium Sulphite solution

* Use small glass beaker
* Add **1 g of sodium sulphite** to **125 ml DW**
* Stir with stir stick until dissolved
* Measure out 10 ml and add to borate solution (use glass pipette or 10 ml pipettor if you have one)

### OPA

* Do this in the dark as OPA is light sensitive (in a box)
* Measure out **4 g of OPA (be very very careful!)**
* Add to **100 ml of ethanol** (measure out ethanol using graduated cylinder) in nalgene bottle
* Cap and Shake to mix!
* Pour into borate solution
* Shake the whole solution vigorously for several minutes and allow to age for 24 hours before use

## Ammonium stock solution (Prepare day before sample collection)

To calibrate the fluorometer each time it is used we need to run a standard curve of different ammonium concentrations that we can then compare our sample readings too.

In the field I will make a large stock solution of 10000 uM that I then use to make daily dilutions of 200um stock solution. Other concentrations can be used but these were the easiest to make given the materials and chemical concentrations I had, and the concentrations that are appropriate to use for a standard curve given the ammonium levels that we would expect to find in our fish samples.

### Materials needed

*-2, 1L wide mouth Nalgene bottles*

*-0.661 g Ammonium sulphate*

*-Microbalance scale*

*-filter seawater (from sample collection location)*

*- small weight boat*

*- scoopula*

*- ethanol and kimwipes for cleaning equipment*

*- graduate cylinder (100 ml)*

*- 10 ml pipettor*

### 10,000 uM solution

* Using a clean scoopula measure out 0.661 g of Ammonium sulphate
* Add 1000 ml of DW to clean Nalgene using 100 ml graduate cylinder (don’t use a larger cylinder as you want the measurements to be as accurate as possible)
* Add 0.661 g of ammonium sulphtate to Nalgene, cap tightly, and shake to dissolve
* Label bottle well and refrain from opening this solution near any samples as any contamination will skew sample readings given the strong concentration on NH4 in this bottle

### 200 uM daily solution

* Using the 10 mL pipettor, add 10 ml of the 10,000 uM stock solution to a clean wide mouth nalgene
* Add 490 ml of DW to Nalgene, cap, and shake well

Note on pipetting: Make sure that the pipette tip is fully on or it won't pull up the right amounts (which sucks as we have discovered!). When you pull up solution check to make sure it doesn't drip for a second before adding it to the sample bottle.

# Methods for measuring ammonium in seawater

# Materials needed in the field:

* 3 labelled 60 mL syringes inside one EXTRA Large ziplock bag
* Small red cooler full of crushed ice
  + Get ice from the dry lab next to the chem lab
* 6+ luer lock 0.45 um filter tips
* Notebook + pencil
* Nitrile gloves x2 pairs
* 10 x 100 mL brown Nalgene bottles (6 standard bottles, 3 sample bottles, 1 spare)
  + These bottles should either be acid washed OR still containing the last sample + OPA (the old OPA keeps them clean)
* A can-do attitude

# Field methods: Subtidal collection

* Take the XL ziplock bag of syringes down, “burp” the ziplock at the surface to get the extra air out and make the bag less floaty
* Note the depth of the RLS transect, and decide an appropriate sampling depth
* Fill inside 1 syringe, then flush it, then fill it to just past 60 mL maintaining the target depth
* Put the syringe back in the bag, seal it
* Repeat for samples 2 and 3, trying to keep all of them at the same depth (within a meter at least)
  + This makes it A) easier to remember the sampling depth
  + And B) makes it more likely that all samples have the same salinity and thus same matrix effects
* Return to the middle of your three sample locations and fill and flush the XL ziplock with water at an average of all the sample depths
* Carefully close the ziplock and commit the depths of each syringe and the bag to memory, tell someone on the boat ASAP to write them down

# Field methods: Boat processing

The goal is to get the samples filtered and iced in a reasonable amount of time, filtering the standard curve water which comes from the ziplock bag is less urgent. Wear gloves, be careful not to touch the syringe tip or filter tip to anything, don’t breathe or shed hair into the bottles

* On the boat, carefully swish and empty the old working reagent and sample from a sample bottle
* Carefully open the ziplock and pull out a syringe, we will use the water in the ziplock later!
* Gently tap the syringe to get the bubbles out, screw a filter tip onto the syringe and push out the bubble this introduces
* Push 20 ml through the filter tip into dark Nalgene, shake to rinse and pour out. Push 40 ml into Nalgene, cap, place on ice in sample cooler (you will have to pour out the melted ice and scoop some out so the cooler is half full of ice
  + The 20 mL rinse is a target/approx but the **40 mL sample must be accurate**
  + Ensure the bottles get capped immediately after they have been filled with filtered water to reduce contamination
* Write down the syringe number, depth, and bottle number in the notebook
* Repeat for all 3 syringes, being careful not to spill the water in the ziplock
* For the standard bottles: Keep the last syringe used clean, and use it to pull 20 mL of water from the ziplock to rinse the syringe. Pull the plunger all the way to the end of the syringe to rinse the whole thing, then discard of the 20 ml
* Then pull 60 mL of water, tap out bubbles, screw on the filter tip, and filter 20 mL into a new bottle, rinse the bottle and discard the 20 ml, and filter the remaining 40 mL into the bottle and put on ice
* Repeat for all 6 standard curve bottles!
* If you don’t have time to filter the standard curve bottles just bring the ziplock bag of water back to BMSC and do it there. Make sure the bag is chilled on ice!

# Lab methods: Evening processing

* All of the bottles must be the same temperature! Refresh or add ice to the cooler as needed
* Step 1: calibrate all three pipettes
  + 1 mL of DI = 1 gram
  + Use the scale and DI to make sure the pipettes are accurate
* Pour out the old 200 uM NH4+ bottle in the wet lab
* Rinse the bottle with DI 5+ times, being careful to pour it down the drain and avoid contaminating the sink
* Shake any droplets of water out of the bottle once rinsed
* Add 490 mL DI to the bottle using a clean 100 mL cylinder
* Using the calibrated 10 mL pipette, add 10 mL of the concentrated NH4+ stock solution
* Using the 100 and 200 uL pipettes, add 0, 100, 300, 600, and 800 uL of 200 uM NH4+ stock to the standards-additions bottles
* Write down the bottle numbers and added volume of stock NH4+ on the fluorometry datasheet
* Turn off the lights and turn on Lamp 2
* Add 10 mL of OPA to the 5 standard-additions bottles, then spike the 3 samples as well. Leave the BF bottle unspiked. Write down the time when you spiked the samples.
* Shake bottles gently
* Leave the bottles on the counter and go home!

# Lab methods: Next morning processing

The following procedure is copied from Fiona Francis’s protocol:

Running a standard curve

* Make sure you have everything you need in the dark room, turn out the lights and avoid turning them on if any sample bottles are open as the sample will degrade very quickly with even a few seconds of light exposure
* This first step calibrates the fluorometer and creates your standard curve
* Before reading a sample shake the bottle well and keep the cap on as much as possible to avoid contamination
* Set up the fluorometer by placing the minicell adapter into the slot, closing the cap and turning on the device
* b) hit "cal" and then hit "enter" on the fluorometer
* Pipette ~ 200 ul of your zero sample into a minicell, wipe the minicell clean on the outside using a microfiber cloth (kimwipes scratch the minicells) and place the minicell into the adapter and close the lid (NOTE: to avoid bubbles place the pipette tip vertically into the minicell not on an angle and pipette out all the liquid quickly while lifting the pipette. This is a bit of an art and takes a few practices)
* hit "enter" again
* insert your standard. Use the 800 ul spike for this.
* You are aiming for a ratio of 5-35% between your blank and std
* after calibration hit the down arrow to keep the calibration and hit "diag" to get the %blank and %std
* After you have created your calibration ratio rad the rest of your standard curve. To read a sample you just hit "read" after you have placed it into the adapter
* As a rule of thumb: use a new minicell every time you read a sample that is likely a higher concentration than the previous one as it is very very hard to get all of the material out of a minicell

Running your samples

* Using the same process as for the std curve, read each sample
* All three readings must be within 3 flu units of each other. Keep running new samples in the same mini cell until you get three good readings
* After all of the samples are read, dispose of waste material down the sink with water running
* Throw out used gloves, minicells, and pipette tips into harzardous materials waste
* Keep all of the sample bottle closed and DO NOT throw out remaining contents as this will keep them sterile for the next use

# Methods for measuring excretion

## Filtered Seawater

Animals will be incubated in seawater for 30 minutes to determine rates of nitrogen excretion. This seawater needs to be pre-filtered to remove naturally occurring particles/organic material that can interfere with the fluorometry process. While a simple process it can be time consuming and also requires care to avoid adding any new material (dead skin hair) to the filtered water. I suggest wearing nitrile gloves.

### Materials needed

* 10L and 20 L Nalgene for filtered seawater
* two large water jugs (20 L each)
* gast vacuum pump
* tygon tubing
* vac guard filter
* manifold
* whatman glassfiber filters (0.7 um) (at least 10)
* tweezers

### Filtering process

* Make sure that the Nalgenes are clean. Rinse several times using distilled water and always store with a cap on. Wear nitrile gloves for this process.
* Assemble the vacuum pump and tubing as shown in picture below and replace the Nalgene cap with the nippled cap that can attach to the tubing.
* Disconnect the manifold from the Palen lab pump and attach to tubing running to Nalgene cap.
* Make sure all of the stop cocks are closed on the manifold
* Wearing gloves, use tweezers to place a glass fiber filter into two of the manifold filter cups
* Pour seawater into both cups until full.
* Close vacuum valve on pump and turn on. Pressure should rise to 1.3-1.5 PSI. Open valve on pump to keep pressure constant. Water should start running through the filters and into the nalgenes
* Continue filling cups and try not to let them completely drain before adding more water (completely draining the cups may rip the filter papers from suction)
* Filter at least 30 L
* If the processes becomes very slow (the filters will clog over time) drain the cup and change the filter paper )
* After filtering water, turn off pump and vent by turning the valve all the way open. Detach manifold and reattach to Palen lab pump. Put normal caps on nalgenes.

# Sample Collection Protocols

## Sample Collection in the Field

Collected animals will be incubated in ziplocs of filtered seawater for 30 minutes. Water samples will be collected from each Ziploc pre and post incubation to test for NH4 concentrations. Therefore at least two sample bottles are required for each animal. Empty ziplocs with filtered water and no animals will also be test to make sure no change in NH4 occurs over the incubation period from contamination from the bags or other activity. 7 sample of filtered water will also be collected and spiked with known concentrations to run a standard curve to compare our samples two. A new standard curve needs to be run each sample collection day since water conditions will vary over time. Sample collection and subsequent sample analysis is time consuming so I suggest collecting no more than 15 animals per day since this will add up to at least 43 samples (15animals\*2 + 3controls\*2 + 7 standard curve concentrations).

The collection process is quite time sensitive since animal incubations will all start and end around the same time so running fewer animals a day will reduce the chance of sample contamination due to haste. For example, if it takes you three minutes to take a pre sample and start an animal incubating, by the time to have started 10 animals, 30 minutes will be over and it will be time to start processing the post samples fromt the first animals. If it takes you longer than three minutes you should processes fewer animals in one go. The more batches you do the longer your day will be (an extra hour makes a difference at 11 PM ☺ )

### Materials needed

* 2 watches
* 45 x 100 ml nalgenes (darkened) and acid washed
* nitrile gloves
* 3 x 60 ml syringes
* 3 ziplocs for placing syringes in between uses
* 20 x luer lock 0.45 um filter tips (fill in type)
* 30 x clean large numbered ziplocs
* large cooler for holding animals
* large cooler for holding ziplocs
* collection bag
* small cooler for collected samples
* ice (for small cooler)
* scale (for weighing animals)
* ruler
* large graduated cylinder
* small ziplocs

### General Collection technique

* Collect animals and place in a cooler on the dock.
* WEAR GLOVES! Any contamination at this point could really mess up the samples!
* Pour a reasonable amount of filtered water into a new, labelled Ziploc bag. Write the sample number and Ziploc number on data sheet.
* Use two people for this: Take a dark Nalgene and write the bottle number on the sample sheet.
* One person should open the Ziploc while the other person take a 60 ml syringe and pulls up ~20 ml to flush and rinse syringe
* Pull up 60 ml, put on a luer lock tip, close ziploc
* Push 20 ml through tip into dark Nalgene, shake to rinse and pour out. Push 40 ml into Nalgene, cap, place on ice in sample cooler
* Put syringe and tip into a clean Ziploc and set aside
* Place animal into Ziploc (wearing gloves and trying not to touch the inside of the bag with anything but the animal!), write down time on sample sheet
* Repeat with remaining bags and animals of set, try to also run a control bag (same process just don’t put an animal in), luer lock tip should be changed when it gets hard to push through
* After 30 minutes, open first sample bag, collect post sample like pre
* Take out animal, keep bag with remaining water (set aside to measure volume of after all animals are processed)
* Hold animal over a new labelled Ziploc to drain for 1 minute
* In addition to sample collection make sure that you fill at least 7 additional bottles with 40 ml of filtered seawater (I take it from one of the control bags) to use to make your standard curve back in the lab

## Sample Processing in the Lab

### Material needed

* OPA reagent mixture
* 10 ml glass pipette
* 1 ml pipettor and tips
* Dark room
* Nitrile gloves
* Waste beaker
* 200 um stock solution
* Tray large enough to hold all samples so they can be easily moved around and kept in order
* Waste bucket for OPA
* Fan to keep cool in the dark
* Comfortable shoes
* Water bottle and music (trust me it get’s hot and boring other wise!!)

### Standard curve bottles

* Create a standard curve with at least 7 concentrations with at least one zero. These concentrations might vary depending on the measurements you expect from your animal collections as you want your standard curve to encompass the full range of sample concentrations you expect to get
* Once you have determined your curve concentrations, add the appropriate amount of 200 um stock solution to each of your prepared sample bottles (see field collection above)

### Adding reagent and bottle incubation

* Bring all of your samples into the dark room
* Add 10 ml of OPA solution to each sample bottle as well as to each bottle in the standard curve using the 10 ml pipettor
* Shake each bottle gently
* Let sit for at least 4 hours

### Running a standard curve

* Make sure you have everything you need in the dark room, turn out the lights and avoid turning them on if any sample bottles are open as the sample will degrade very quickly with even a few seconds of light exposure
* This first step calibrates the fluorometer and creates your standard curve
* Before reading a sample shake the bottle well and keep the cap on as much as possible to avoid contamination
* Set up the fluorometer by placing the minicell adapter into the slot, closing the cap and turning on the device
* b) hit "cal" and then hit "enter" on the fluorometer
* Pipette ~ 200 ul of your zero sample into a minicell, wipe the minicell clean on the outside using a microfiber cloth (kimwipes scratch the minicells) and place the minicell into the adapter and close the lid (NOTE: to avoid bubbles place the pipette tip vertically into the minicell not on an angle and pipette out all the liquid quickly while lifting the pipette. This is a bit of an art and takes a few practices)
* hit "enter" again  
  - to the read a different zero without completing the calibration hit "esc" and then repeat steps b) and c) (you will want to do this until you find the lowest reading zero measurement
* once you find a zero you are happy with, keep the calibration and then insert your standard. I usually use my 700 ul spike for this.
* You are aiming for a ratio of 5-35% between your blank and std
* after calibration hit the down arrow to keep the calibration and hit "diag" to get the %blank and %std
* After you have created your calibration ratio rad the rest of your standard curve. To read a sample you just hit "read" after you have placed it into the adapter
* As a rule of thumb: use a new minicell every time you read a sample that is likely a higher concentration than the previous one as it is very very hard to get all of the material out of a minicell

### Running your samples

* Using the same process as for the std curve, read each sample starting with the pre-measurements followed by the samples themselves
* You don’t have to follow this pattern but I like reading all of the pres first as they are likely going to all be low in NH4 compared to the samples which reduces the risk that you will contaminate things
* After all of the samples are read, dispose of waste material down the sink with water running
* Throw out used gloves, minicells, and pipette tips into harzardous materials waste
* Keep all of the sample bottle closed and DO NOT throw out remaining contents as this will keep them sterile for the next use

#### Dilutions

* It is very likely that you will have samples that are too highly concentrated for the fluorometer to read (it took a very long time to trouble shoot this but this is part of the reason we had to use minicells)
* If this happens you will get a too high warning on the fluorometer and will need to dilute the samples
* To do this, pipette 1 ml of a low pre sample into a cuvette along with 250 ul of the sample. This will create a 1/4 dilution which in most cases is low enough to read with the animals we are using
* Mix this well using the pipette and then try reading this in the flurometer
* Record the dilution you are using

# Additional things

## Matrix effects and Background Fluorescence

Standards-additions method

* Accounts for ME by making standards with sample water
* Protocol 1 is used to estimate the ME-corrected concentration of a single unknown, protocol 2 is for multiple unknowns with similar ME can be estimated
* BF corrected fluorescence of 3 to 5 samples with known amounts of NH4 added is measured and regressed against their nominal spike concentrations and the ME-corrected concentrations of the sample is estimated by extrapolating the curve to the x-axis
* Regress BF corrected fluorescence of standard additions against their nominal concentrations using linear regression