Ammonium Microplate Protocol

Updated 06/09/20 – EFT

1.0 Method Summary

This method analyzes ammonium of environmental water samples or soil extracts using the salicylate-hypochlorite colorimetric reaction based on the Berthelot reaction as developed by Bower and Holm-Hansen (1980, 10.1139/f80-106). The reaction involves a three-step process where ammonium reacts with hypochlorite to form monochloramine. This reacts with salicylate, in place of phenol, in the presence of nitroprusside catalyst to produce indophenol blue. This produces a blue-green dye read at 650nm wavelength.

2.0 **Preparation**

- 2.1. Glassware
  - 2.1.1. 50mL volumetric flask
  - 2.1.2. 100mL volumetric flasks (2)
  - 2.1.3. 100mL amber bottles (3)
- 2.2. Pipettes
  - 2.2.1. 5mL adjustable pipette
  - 2.2.2. 1mL adjustable pipette
  - 2.2.3. 10µL adjustable pipette
  - 2.2.4. 100µL adjustable pipette
  - 2.2.5. 100µL multichannel pipette
- 2.3. Miscellaneous
  - 2.3.1. 2mL centrifuge tubes (6)
  - 2.3.2. 96 well microplates with cover
  - 2.3.3. Reagent reservoirs (2)
- 2.4. Reagent chemicals
  - 2.4.1. Ammonium sulfate (0.0236g)
  - 2.4.2. Sodium salicylate (22g)
  - 2.4.3. Sodium nitroprusside dehydrate (0.028g)
  - 2.4.4. Sodium citrate (10g)
  - 2.4.5. Sodium hydroxide (2.9g)
  - 2.4.6. Bleach (8.25% strength) (1mL)
  - 2.4.7. Potassium chloride (optional, 14.9g)

3.0 **Ammonium Stock and Reagents**

- 3.1. 100 mg L^{-1} NH_4^+-N stock
  - 3.1.1. Weigh 0.0236g ammonium sulfate ($(NH_4)_2SO_4$) on a weighing tray. Rinse this into a 50mL volumetric flask with ~40mL of the matrix. Dilute to 50mL with the matrix and shake to dissolve.
  - 3.1.2. For resin bag and soil extractions, the matrix is 2M potassium chloride (KCl). For other samples use DI. For KCl matrix, dissolve 14.9g KCl into 100mL of DI.
  - 3.1.3. Refrigerate 100 mg L^{-1} stock in an amber bottle for up to 1 week.

- 3.2. Salicylate reagent
  - 3.2.1. Weigh 22g sodium salicylate (HOC_6H_4COONa) and 0.028g sodium nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$). Rinse the weighing trays of each chemical into a 100mL volumetric flask using ~80mL of DI. Dilute to 100mL and shake to dissolve.
  - 3.2.2. Refrigerate for up to 3 months in an amber bottle.

- 3.3. Base solution
  - 3.3.1. Weigh 10g sodium citrate ($Na_2C_6H_5O_7$). Rinse this into a 100mL flask with ~70mL of DI.
  - 3.3.2. Weigh 2.9g sodium hydroxide (NaOH) and add to the flask. Dilute to 100mL with DI.

3.3.3. Refrigerate in an amber bottle. This is stable indefinitely, however remake yearly.

3.4. Hypochlorite reagent (**Prepare LAST**)

3.4.1. Measure 1mL of 8.25% (commercial grade) bleach (NaOCl) into a reagent reservoir.

3.4.2. Add 9mL of the base solution prepared above in two volumes of 4.5mL.

3.4.3. Wait until standards and samples are already added to the microplates to mix this reagent as it only lasts one hour. The 10mL volume is sufficient for 3 trays.

4.0 Standards

4.1. Add 1mL of stock to a 50mL flask and dilute with the matrix. This is STD6.

4.2. In six 2mL centrifuge tubes, add quantities of STD6 based on the table below to prepare 1mL quantities of STD1-6.

4.3. Standard curve takes ~30min to make and is sufficient for 6 trays. Remake standards daily.

	Amt. (µL)		**Conc (mg L^{-1})**
STD#	**STD6**	**Matrix**	
STD1	0	1000	0.0
STD2	50	950	0.1
STD3	100	900	0.2
STD4	250	750	0.5
STD5	500	500	1.0
STD6	1000	0	2.0
DL			?
QL			?

5.0 Sample Preparation

5.1. Number or label each microplate and record standard and sample names and locations for later calculations. It takes ~10min to prepare samples and ~10min to add reagent to each tray. Ensure that timing will be within the lifetime of the hypochlorite reagent.

5.2. Pipette 100µL of standards in triplicate to each microplate, using the plate layout below.

5.3. Pipette 100µL of samples in triplicate, keeping track of sample order.

5.4. Prepare the hypochlorite reagent **after** standards and samples have been added.

5.5. Add 30µL of hypochlorite reagent to each well using a pipette. Tips do not need to be replaced after every well unless the tip touches the sample in the well. Keep the pipette above the wells when adding reagent to prevent this.
5.6. Add 30µL of salicylate reagent to each well using a multichannel pipette.
5.7. Cover each microplate with seals to prevent evaporation and cross-contamination. Tap to mix.
5.8. Incubate trays 50min at lab temperatures and under a dark cover to prevent photo-degradation.
5.9. Excess reagents should be neutralized before disposal.

top left	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Std1	Std1	Std1	3	3	3	11	11	11	19	19	19
B	Std2	Std2	Std2	4	4	4	12	12	12	20	20	20
C	Std3	Std3	Std3	5	5	5	13	13	13	21	21	21
D	Std4	Std4	Std4	6	6	6	14	14	14	22	22	22
E	Std5	Std5	Std5	7	7	7	15	15	15	23	23	23
F	Std6	Std6	Std6	8	8	8	16	16	16	24	24	24
G	1	1	1	9	9	9	17	17	17	25	25	25
H	2	2	2	10	10	10	18	18	18	26	26	26

6.0 **Sample Analysis**

6.1. Start the microplate reader and connecting computer. Log in and open Gen5 v2.03.
6.2. Either start a new experiment with an existing protocol or open an existing experiment. Protocols are stored in C:\Users\Public\Documents\Protocols\Soil & Water Lab.
6.3. Optionally enter Sample IDs for each tray.
6.4. Carefully remove covers from the microplates before analysis.
6.5. Click play button to read new plate and follow instructions. The tray drive will open. Insert tray, being sure to check location of cell A1. The protocol will analyze ammonium at 650nm wavelength.
6.6. Calculate standards curves for each microplate and then convert absorbance to concentrations for each sample using the microplate_data R function.

7.0 **Method Notes**

7.1. At high ammonium concentrations or pH below 9, yellow color will form and interfere. Either dilute or further basify samples and rerun.

7.2. Sodium citrate is added to prevent metal hydroxide precipitates.