Wetland Biogeochemistry Laboratory
Standard Operation Procedure

SOP #: WBL-AN-008

Determination of Nitrate + Nitrite in Extracts Using the Bran+Luebbe AutoAnalyzer 3 (NEON)

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Version 1.0

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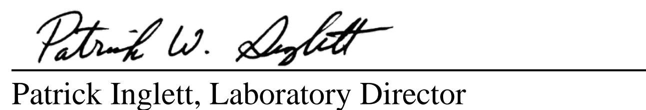

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1. APPLICABLE MATRIX

This procedure is for analysis of nitrate + nitrite in surface waters, ground waters, saline waters, porewater, and extracts.

2. SCOPE AND APPLICATION

2.1 The purpose of this SOP is to document and standardize the procedure used by laboratory personnel in the Wetland Biogeochemistry Laboratory (WBL) for the analysis of nitrate + nitrite in soil extracts. This method is specific to the needs of the National Ecological Observatory Network (NEON) project.

2.2 The WBL uses a Bran+Luebbe AutoAnalyzer 3 (AA3) with modifications to Standard Methods (SM) 4500-NO3 F for nitrate + nitrite analysis. The calibration range for nitrate + nitrite is from 0.01 to 1.0 mg N/L. Higher concentrations may be measured by using sample dilution.

2.3 This SOP is based upon a modified SM4500-NO3 F *Automated Cadmium Reduction Method* (Seal Analytical AutoAnalyzer Applications Method No. G-384-08 Rev. 6) and is applicable to KCl extracts.

2.4 Detection limit of nitrate + nitrite is **0.01** mg/L.

3. SUMMARY OF METHOD

3.1 The WBL uses an AutoAnalyzer 3, and a modified version of SM4500-NO3 F *Automated Cadmium Reduction Method* for nitrite + nitrate analysis, which uses imidazole instead of ammonium chloride.

3.2 During analysis, nitrate is reduced to nitrite by copper/cadmium metal in the form of an open tubular cadmium reactor (OTCR). The nitrite formed by cadmium reduction, plus any nitrite originally present in the sample, is determined as an azo dye at 520 nm following its diazotization with sulfanilamide and subsequent coupling with N-(1-Naphthyl)ethylenediamine dihydrochloride. These reactions occur in an acidic ($pH<2$) matrix.

3.3 Imidazole buffer reduces interferences from iron, copper, or other metals. Samples with turbidity or color that absorbs in the analytical wavelength may interfere. Turbidity or suspended matter should be removed by filtration prior to analysis to avoid blockage of the OTCR. Since nitrate is soluble filtration does not affect the analysis.

4. DEFINITIONS

See WBL-OM-003 *Acronyms and Definitions* for common laboratory terminologies.

5. INTERFERENCES

Imidazole buffer reduces interferences from iron, copper, and other metals. Samples with turbidity or color that absorbs in the analytical wavelength may also interfere.

6. SAFETY/HAZARDOUS WASTE MANAGEMENT

6.1 Read and understand the appropriate MSDS sheets on all reagents used in the analysis.

6.2 Always use caution when working with acids and/or bases.

6.3 The waste exiting the colorimeter must be collected in a satellite waste container (properly labeled as hazardous waste). When the satellite container is full, call for collection by Environmental Health & Safety.

7. APPARATUS AND EQUIPMENT

7.1 Bran+Luebbe AutoAnalyzer 3 segmented flow analysis system:
- 7.1.1 Compact Sampler
- 7.1.2 AA3 Pump
- 7.1.3 AA3 Chemistry Manifold: Heating bath temperature: 50°C
- 7.1.4 AA3 Digital Colorimeter: 10 mm flow cell / 660 nm filter
- 7.1.5 Pump Tubing / Manifold Configuration:

Sample	Blk/Blk
Imidazole Buffer	Yel/Yel
Color reagent	Org/Yel
Sample Wash	Grn/Grn
Debubbler	Orn/Orn
Air	Orn/Wht

7.2 Personal Computer (PC)
- 7.2.1 AACE Software Version 5.48.3

7.3 Lab supplies:
Note: All flasks listed in this SOP are volumetric.

- 7.3.1 Working standard supplies:
  10 – 50 mL volumetric flasks
  2 – 100 mL volumetric flasks

7.3.2 Reagent supplies:
2 – 250 mL amber plastic bottles
3 – 500 mL amber plastic bottles
1 – 1000 mL amber plastic bottle
1 – 1000 mL amber glass bottle
2 – 500 mL volumetric flasks
4 – 1000 mL volumetric flasks
1 – 2000 mL volumetric flask

7.3.3 Miscellaneous supplies:
Pipettes
Plastic weigh boats
Plastic disposable beakers
Spatula
Parafilm
Balance
Nalgene dropper bottle
Magnetic stir bars
Stir plate
pH meter and probe
100 mL graduated cylinder
250 mL graduated cylinder

8. REAGENTS AND STANDARDS

8.1 Chemicals
Imidazole
Hydrochloric acid (HCl), concentrated
Sulfanilamide (SAN)
Sulfuric acid (H_2SO_4), concentrated
Phosphoric acid, concentrated
Cupric sulfate
Brij-35
N-(1-Naphthyl)ethylenediamine dihydrochloride (NED)
1000 mg/L Nitrate Stock Solution (purchased/traceable)
1000 mg/L Nitrite Stock Solution (reduction efficiency, purchased/traceable)
Certified Nitrate Standard (QC, purchased/traceable)

8.2 Cupric sulfate reagent, 0.01 M (**6 months**):

Cupric sulfate	2.5 g
DDI water	to 1000 mL

Into a 1000 mL volumetric flask containing ~800 mL of DDI water, add 2.5 g of cupric sulfate. Mix well with magnetic stir bar until the cupric sulfate is dissolved and bring to volume. Store in a plastic bottle for up to 6 months.

8.3 Stock imidazole buffer (0.1 M) (**1 month**):

Imidazole	36.2 g
HCl	to pH 7.5
DDI water	to 2000 mL

Into a 2000 mL volumetric flask containing ~1600 mL of DDI water, add 32.6 g of imidazole. Mix well with magnetic stir bar until imidazole is dissolved. Suspend a pH probe into the flask and carefully add concentrated HCl dropwise until the pH has been adjusted to pH 7.5, about 2 mL of HCl (Note: Allow time between additions for the acid to be mixed into solution to obtain an accurate measurement). After using the magnetic wand to remove the stir bar and bring to volume with DDI water. Mix well and transfer a plastic bottle. Store at room temperature.

8.4 Working imidazole buffer (**daily**):

Stock imidazole buffer	750 mL
0.01 M cupric sulfate	1.8 mL
Brij-35	1 mL
DDI water	to 1000 mL

Into a 1000 mL volumetric flask, add 750 mL of the stock imidazole buffer. Add 1.8 mL of the 0.01 M cupric sulfate and 1 mL of Brij-35. Bring to volume with DDI water. Mix well by swirling. Prepare fresh daily.

8.5 Color reagent (**1 month**):

Conc. Phosphoric acid	100 mL
Sulfanilamide (SAN)	10 g
NED	0.5 g
DDI water	to 1000 mL

Into a 1000 mL volumetric flask containing ~ 500 mL of DDI water, add 100 mL of the concentrated phosphoric acid, 10 g of sulfanilamide (SAN), and 0.5 g of NED. Swirl to mix well. Add DDI water and bring to volume.

8.6 Cupric sulfate, 2% w/v (**6 months**):

Cupric sulfate	10 g
DDI water	to 500 mL

Into a 500 mL volumetric flask containing ~400 mL of DDI water, add 10 g of cupric sulfate. Mix well with a magnetic stir bar until the cupric sulfate is dissolved. Bring to volume. Store in a plastic bottle for up to 6 months. This solution is used for column (OTCR) activation only.

8.7 Sample wash:

Brij-35	0.5 mL
DDI water	to 1000 mL

Into a 1000 mL volumetric flask containing ~750 mL of DDI water, add 0.5 mL of Brij-35. Mix well and bring to volume.

9. STANDARDS

Note: Nitrate standards must be prepared in DDI due to known issues with nitrate contamination of KCl.

9.1 Nitrate 10.0 mg N/L stock standard (**monthly**):

Into a 100 mL volumetric flask containing ~80 mL of DDI, add 1 mL of 1000 mg/L nitrate standard. Bring to volume and mix well.

9.2 Calibration standards (concentrations in mg N/L):

Blank, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 (or higher).

Using the 10 mg/L nitrate stock standard and DDI water, prepare the calibration standards according to the following table:

Standard (mg/L)	Vol. of 10 mg/L Stock	Final Volume
blank	-	100 mL
0.01	0.1 mL	100 mL
0.02	0.2 mL	100 mL
0.05	0.5 mL	100 mL
0.1	1.0 mL	100 mL
0.2	2.0 mL	100 mL
0.4	4.0 mL	100 mL
0.6	6.0 mL	100 mL
0.8	8.0 mL	100 mL
1.0	10.0 mL	100 mL

9.3 Reduction Efficiency Check solutions (1.0 mg N/L as nitrite)

Prepare a working reduction check solution by adding 0.1 mL of the 1000 mg/L nitrite stock solution (8.1) into a 100 mL flask containing ~80 mL of DDI. Bring to

volume and mix well. This solution should be run after a 1.0 mg/L nitrate standard to compare responses and calculate the efficiency of the OTCR to reduce nitrate to nitrite.

9.4 Quality Control Check Standard:

A certified nitrate QC standard, purchased from a secondary source (e.g., ERA), is used as a stock solution to prepare a working QC solution. Prepare and analyze this solution to validate the calibration curve. These solutions must be prepared according to the manufacturer's instructions using appropriate clean flasks and DDI.

10. SAMPLE HANDLING AND PRESERVATION

10.1 Samples for nitrate + nitrite determination must be stored at -20°C. Samples should be analyzed within 48 hours of thawing and can be stored at 4°C for up to 21 days thawed after the initial analysis if reruns are required.

10.2 Samples that are stored at -20°C must be allowed to come to room temperature before processing.

11. QUALITY CONTROL

The following quality control checks are used with each analytical set.

Type	**Frequency**
Instrument Calibration	7-10 Standards, daily or failure of CCCS.
Method Reagent Blank	1st analysis of blank immediately after standard curve.
Continuing Calibration Blank (CCB)	Continued analysis of blank: 1 per 10 samples if analytical set <100, or 1 per 20 samples if analytical set >100.
Practical Quantification Limit (PQL)	At least 1 per analytical set <100, or at least 2 per analytical set >100, at a concentration of 3-5 times the MDL.
Continuing Calibration Check Standard (CCCS)	Continued analysis of 0.8 ppm std. 1 per 10 samples if analytical set <100, or 1 per 20 samples if analytical set >100.
Quality Control Check Standards (QC)	Analyzed at the beginning of each analytical set to verify the standard curve. One QC is also analyzed 1 per 20 samples.
Reduction Efficiency Check	Analyzed once after the highest nitrate standard during the gain check to determine if cadmium reduction (OTCR) is effectively reducing nitrate to nitrite.
Repeat (Rep)	At least 1 per analytical set and 1 per 20 samples analyzed.

Spike (spike added prior to sample analysis) (Sp)	At least 1 per analytical set and 1 per 20 samples analyzed.

12. CALIBRATION AND STANDARDIZATION

12.1 At the beginning of each analytical run, a blank and the following working standards are analyzed to calibrate the instrument: 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, and 1.0 mg N/L. Quality control and calibration checks are also used to determine the accuracy of calibration (Section 9.4 and 11).

12.2 Only Class A volumetric flasks and properly calibrated pipettes are used for preparing calibration and check standards. Intermediate stocks and standards are prepared using a purchased, traceable certified standard solution. Quality control (secondary source) samples are prepared using certified solutions from commercial vendors.

13. SAMPLE ANALYSIS

13.1 Switch on the surge protector. This turns on power to all parts of the AA3 except the PC.

13.2 Start up the PC, open AACE, select NO2+NO3 analysis and begin charting.

13.3 Place the reagent lines into the dedicated plastic bottle for DDI water with Brij-35, engage the platen and begin pumping. During this time you can check for leaks, flow problems, or problems with the bubble pattern that are independent of the OTCR.

13.4 After flow has been established with DDI water and Brij-35, place the appropriate pump tubes into the respective reagents. Connect the Nitrogen gas bag to the Orn/Wht pump tube used for segmentation immediately before the point where the OTCR would be connected and open the valve. Wait ~10 minutes and check the baseline. If the baseline is ok then stop the pump while leaving the platen engaged and remove the jumper made from tubing and place OTCR into the flow path, connecting the OTCR to the mixing coil closest to the pump first. (Note: When removing the OTCR from the system it is important to remove the end that is farthest from the pump first. By doing so, you avoid a possible siphon through the OTCR which helps avoid the admission of air into the OTCR.). Once reagents have been pumping through the system and a good bubble pattern has been established you can monitor the baseline for stability.

13.5 Now it is necessary to load several aliquots of the 1.0 mg/L NO3-N high standard onto the autosampler so the Gain can be set. With a 1.0 mg/L high standard passing through the photometer, use the Gain in AACE to set the peak height at ~90% of full scale. Ideally, you will be able to set the standard calibration by the first cup or two of high standard so the other cups will give you an opportunity to check the stability of the high standard response.

13.6 A 1.0 mg/L NO2 standard should be the last cup run when setting the standard calibration. This standard should be prepared in DDI water without acidification, as the addition of acid can oxidize NO2 to NO3. This standard is run as a reduction check. The response of this standard can be compared to the 1.0 mg/L NO_3-N to check the efficiency of the OTCR reducing NO3 to NO2. For reduction efficiency below 90% the column needs to be reactivated.

13.6.1 Proper performance of the OTCR is a critical component of this analysis. When the coil has poor reduction efficiency there will be a low recovery of nitrate as nitrite. If the coil is overactive, it is possible that nitrite will be further reduced.

13.6.2 In the case of poor reduction efficiency, the OTCR should be reactivated. This is accomplished by diluting the Cupric sulfate (2% w/v solution) 1:1 with stock imidazole buffer in a plastic disposable beaker. Disconnect one end of the OTCR from the coupling used to store the coil and place that end in the buffered copper solution. Using a syringe, draw this solution through the OTCR and let the solution stand in the coil for ~10 minutes. Draw stock imidazole buffer through the OTCR to remove the buffered copper solution from the OTCR. Note: It is important that air is not introduced into the OTCR during handling. After an OTCR has been reactivated it should be conditioned similar to the process used for an overactive column. (See 13.6.3). The OTCR need not be reactivated every day before beginning analysis but only when poor reduction efficiency is observed.

13.6.3 In the case of an overactive OTCR it is necessary to condition the coil by aspirating a 1.0 mg/L NO3-N standard continuously for ~10 minutes (or longer if it appears that the response shows that there is significant drift). Another way to condition the OTCR is to run multiple cups of the 10.0 mg/L NO_3-N with the autosampler.

13.6.4 After conditioning an overactive coil or a newly reactivated coil, run several cups of 1.0 mg/L NO_3-N, check the gain setting, run one 1.0 mg/L NO2 standard and recalculate the efficiency. Note: When running the NO2 standard check for tailing of the NO2 peak. If the NO2 peak shows tailing it is advisable to clean out the system. Disconnect the OTCR from the system and put the jumper in the OTCR's place. Fill the OTCR with stock imidazole buffer and put the coupling in place to avoid the introduction of air. Put the color reagent line in DDI water. Introduce 1N NaOH through the imidazole buffer line and the sample probe after the OTCR has been removed from the system. Flush with 1 N NaOH for ~15 minutes, followed by DDI water for five minutes, 5 N H2SO4 for ~15 minutes, and finally with DDI water for an additional 15 minutes. Rerun the 1.0 mg/L NO_3-N cups and 1.0 mg/L NO2 cup and recalculate the reduction efficiency.

13.7 With the gain set, a stable baseline and the demonstration of acceptable reduction efficiency, the system is ready for analysis.

13.8 During the analysis, log the order that standards and samples are loaded onto the tray using the tray map and update the tray protocol in AACE. Check the linear regression in AACE once the standards are run. Then calculate the QC standard recovery using the QC template. If the correlation coefficient and the recovery of the QC standard fall within acceptance criteria, then the run should be allowed to progress. Check the recoveries of the QC, Continuing Calibration Standard, the matrix spike and the precision of the analytical replicate every 20 samples to make sure the OTCR continues to perform during the analysis.

13.9 At the end of the run after the final baseline has been marked by AACE, remove the OTCR from the system by stopping the pump with the platen engaged and removing the connection that is farthest from the pump first. Remove the other connection and replace the jumper on the instrument. Draw Stock Imidazole Buffer through the OTCR using a disposable beaker and a 60 ml plastic syringe. Install the coupler to the ends of the OTCR for storage. Place all three reagent lines in the bottle dedicated for DDI water with Brij-35, start the pump and allow to flush. Also, place wash lines in bottle for DDI only if the wash water was acidified. Flush system for ~20-30 minutes. Disengage platen, turn off surge protector for instrument. Review your chart and proofread your tray protocol. Once you are satisfied that everything is correct, export your run as a .slk file. Then open Windows Explorer and locate your file under C:/AACE/Data and copy both the .slk and .tmp files on a flash drive to edit in Excel.

14. CALCULATIONS AND DATA REPORTING

14.1 After completion of the analysis, select Retrieve/View Chart to check that AACE has marked all the peaks correctly. Correct if necessary and save the corrections.

14.2 Select Set Up/Analysis and proofread your tray protocol to make sure all samples are correctly named and assigned to the proper cup positions on the sampler tray. It is best to check the Tray Protocol for typographical errors before the run is completed so the corrections may be made in AACE. If an error is noticed in the Tray Protocol after the run is completed, the error is noted on the tray map and the correction is made in Excel after the run is downloaded from AACE.

14.3 To reformat the data from the Bran+Luebbe report format to a form that can be downloaded directly into Excel, select File/Export/ASCII file. Press Export, Ok, and Ok.

14.4 Open Windows Explorer/AACE/Data and copy the .slk and .tmp files on to a disk. In Excel this file can be opened to edit the file. Copy and paste the edited file into an Excel report template and do the final data summary. Note: When closing the .slk file after editing, copying and pasting, say No to Save Changes. This preserves the original, unedited .slk file on the disk.

14.5 Submit the final data and QC summary to the QA officer for approval.

15. DATA ARCHIVAL

Individual runs are archived on the PC. Archival of all files generated by AACE is done by saving on an external hard drive. Note: When performing file maintenance on the PC, delete analytical run data from AACE, not Windows Explorer.

16. METHOD PERFORMANCE

This method was validated through a single laboratory study and an inter-laboratory method validation study (SM4500-NO3 F modified). Historical performance based on recoveries from 2011 is as follows:

Precision – 2.3% (UCL: 14%)
Accuracy – 96% (LCL-UCL: 73-119%)
Spike recovery – 98% (LCL-UCL: 76-120%)

17. DATA ASSESSMENT, ACCEPTANCE CRITERIA AND CORRECTIVE ACTIONS FOR QC MEASURES

Type	Purpose	Acceptance Criteria	Corrective Action
Instrument Calibration	Standardize the instrument.	**Linear regression $R^2 > 0.995$** All standards must be within 10% of their true value	Reanalyze standards. If same response is obtained, re-optimize instrument and restart analysis. If same response is obtained, prepare new standards and restart analysis.
Method Reagent Blank	Determine if there is any bias or contamination contributed by the method itself. When blanks are found to be positive, the impact on the samples is determined; sample results maybe qualified if concentrations are <5x the blank value.	**Value <MDL or <0.02 mg/L, whichever is higher**	Prepare new blank and restart analysis. If same response is obtained, determine cause of contamination (reagents, calibration standards, environment, equipment failure, etc.) and eliminate the source of contamination.
Continuing Calibration Blank (CCB)	Determine stability of calibration over the course of the run.	**Mean of observed values for an analytical set are <MDL or <0.02 mg/L, whichever is higher**	Recalibrate and reanalyze the affected portion of the run.
Practical Quantification Limit (PQL)	Determine stability of calibration on the low end of the curve; determine ability to consistently achieve the detection limit.	**Mean of observed values for an analytical set within Accuracy 90-110%**	Reevaluate system, recalibrate and reanalyze the affected portion of the run.
Continuing Calibration Check Standard (CCCS)	Determine stability of calibration over the course of the run.	**Mean of observed values for an analytical set within Accuracy 95-105%**	Recalibrate & reanalyze the affected portion of the run.

Quality Control Check Standards (QC)	Determine if calibration is accurate.	**Accuracy 95-105%**	Reanalyze or re-prepare QC check standard. If same response is obtained, prepare new primary and calibration standards. If that fails, check against an alternate QC source and stock solution. Obtain approval from QA officer or staff. Discard unacceptable QC once confirmed and document findings on QC result log.
Reduction Efficiency Check	Determine if cadmium reduction (OTCR) is effectively reducing nitrate to nitrite.	**Reduction Efficiency 90-110%**	Below 90% the column needs to be reactivated. For reduction efficiency above 110% the column needs to be conditioned.
Repeat (Rep)	Assess analytical precision.	**Precision (RSD) < 20%**	Determine and eliminate cause of problem (baseline drift, carryover, etc). Reanalyze all affected samples.
Spike (spike added prior to sample analysis) (Sp)	Determine if there is matrix interference.	**Recovery 85-115%**	Check pipette, remake spike and reanalyze. Meantime spike a DDI or Blank Matrix. If acceptable, reanalyze affected samples. If not acceptable, but the recovery of DDI or blank matrix is acceptable, then it is likely sample matrix interference. Make proper notation on the analytical report.

Data for actual samples that do not meet the above criteria are qualified as such, or the nature of non-conformance is reported to the customer, project manager, or principal investigator.

18. REFERENCES

18.1 Alpkem RFA Method #A303-S170.

18.2 Standard Methods for the Examination of Water and Wastewater, 18th Edition, 1992, 4500NO3 F Automated Cadmium Reduction Method. American Public Health Association, Washington, D.C. (pg 4-91).

18.3 F. Nydahl, Talanta 23, p. 349-357. (1976).

18.4 Methods for Chemical Analysis of Water and Wastes, August 1993, EPA-600/R-93/100, Methods 353.2 Determination of Nitrate-Nitrite Nitrogen by Automated Colorimetry.

18.5 Patton, C.J., Doctoral Dissertation, Michigan State University, 1982, p. 87-121.

18.6 Fox, J.B., Anal. Chem., 51, p. 1493. (1979).

18.7 G. Norwitz and P.N. Keliher, Analyst, 109, p. 1281. (1984).

18.8 AA3 Operators Manual.

18.9 Seal Analytical. Autoanalyzer Applications, Method No. G-384-08 Rev. 6., Nitrate and Nitrite in Water and Seawater (with Cd coil), pp. 10.

19. REVISIONS

Version 1.0 - 2018:

This revision updates reagent and standard preparation instructions provided in the WBL-AN-008 (2013). Documentation changes were made to the imidazole buffer concentration (section 8.3, 8.4) and use of conc. phosphoric acid for color reagent (section 8.5). Color reagent preparation section is added (section 8.5). These changes followed the Seal Analytical AutoAnalyzer Applications Method No. G-384-08 Rev. 6 for nitrate and nitrite determination in water and seawater.