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Standard Operating Procedure for Determination of Dissolved Inorganic Nitrate plus Nitrite (NO3+NO2) in Fresh/Estuarine/Coastal Waters Using Enzyme Catalyzed Reduction (References EPA 353.2, Standard Methods #4500-N C, 4500-NO3 F)

Document #: NASLDoc-021

Revision 2019-1
Replaces Revision 2018-1
Effective May 1, 2019
I attest that I have reviewed this standard operating procedure and agree to comply with all procedures outlined within this document.

Employee (Print)	Employee (Signature)	Date
Employee (Print)	Employee (Signature)	Date
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Employee (Print)	Employee (Signature)	Date

Revised by: _____ Date: _____

Reviewed by: _____ Date: _____

Laboratory Supervisor: _____ Date: _____

Revisions 2019

Cover Page: Updated the Nutrient Analytical Services Laboratory website.

Section 1.6: The method for Nitrate Reductase Nitrate-Nitrogen Analysis (ATP Case No. N07-0003) has been reviewed by the US EPA and has been approved. Previous versions of this SOP listed the method as awaiting final approval.

Section 2.1: Changed "concentrations found to be below the method detection limit" to "concentrations found to be below the quantitation limit" to match current lab practices.

Section 3.29: Changed MSDS to SDS (Safety Data Sheet) which shifted some of the definitions. Section 3.37 now lists SDS.

Section 5.4: Changed MSDS to SDS (Safety Data Sheet).

Section 7.12: Updated Aquakem cleaning solution; changed Clorox to bleach and 75% Clorox to 55% bleach.

Section 9.7: Updated Table 2 QCS/SRM frequency information. The QCS/SRM should be analyzed throughout the run in addition to the beginning and end.

Section 10.1: Corrected the order of working standards (lowest to highest concentration) listed under NiRMID and NiRHI.

Determination of Dissolved Inorganic Nitrate plus Nitrite (NO3+NO2) in Fresh/Estuarine/Coastal Waters Using Enzyme Catalyzed Reduction

1. SCOPE and APPLICATION

1.1 Enzyme catalyzed reduction is used to quantitatively reduce dissolved nitrate to nitrite which is then measured by colorimetric quantitative analysis of a highly colored azo dye. The method is used to analyze all ranges of salinity.

1.2 A Method Detection Limit (MDL) of 0.0057 mg NO_3+NO_2-N/L was determined using the MDL method as specified in the EPA Federal Register 821-R-16-006, titled Definition and Procedure for the Determination of the Method Detection Limit, Revision 2.The Quantitation Limit/Reporting Limit for NO_3+NO_2 was set at 0.028 mg NO_3+NO_2-N/L.

1.3 The method is suitable NO_3+NO_2 concentrations 0.028 to 5.6 mg NO_3+NO_2-N/L.

1.4 This procedure should be used by analysts experienced in the theory and application of aqueous inorganic analysis. A three month training period with an analyst experienced in the analysis of nitrate plus nitrite in aqueous samples by enzyme catalyzed reduction is required.

1.5 This method can be used for all programs that require analysis of dissolved inorganic nitrate plus nitrite.

1.6 A portion of this procedure references Standard Methods #4500-N C, 4500-NO3 F and EPA Method 353.2 (1979). Method for Nitrate Reductase Nitrate-Nitrogen Analysis (ATP Case No. N07-0003) has been reviewed by the US EPA and has been approved. It is now part of the EPA Methods Update Rule 2017 and has been published to the EPA Federal Register Vol. 82 No. 165. It is listed as an approved method in 40 CFR Part 136.

2. SUMMARY

2.1 Filtered samples are mixed with Nitrate Reductase (AtNaR2, commercially available, is a recombinantly produced form of eukaryotic Nitrate Reductase using a modified gene from the plant *Arabidopsis thaliana.* The enzyme AtNaR2 is produced in *Pichia pastoris* and purified from extracts of the yeast.) and NADH (*β*-Nicotinamide adenine dinucleotide reduced form disodium salt). The nitrite, both that which was reduced from nitrate and nitrite that was originally present, is then determined by diazotizing with sulfanilamide and coupling with N-1-napthylethylenediamine dihydrochloride to form a colored azo dye. Filtered samples with concentrations found to be below the quantitation limit are analyzed via cadmium reduction with a Technicon Bran & Luebbe AutoAnalyzer II.

3. DEFINITIONS

3.1 Acceptance Criteria – Specified limits placed on characteristics of an item, process, or service defined in a requirement document. (ASQC)

3.2 Accuracy – The degree of agreement between an observed value and an accepted reference value. Accuracy includes a combination of random error (precision) and systematic error (bias) components which are due to sampling and analytical operations; a data quality indicator. (QAMS)

3.3 Aliquot – A discrete, measured, representative portion of a sample taken for analysis. (EPA QAD Glossary)

3.4 Analytical Range – 0.028 to 5.6 mg NO_3+NO_2-N/L. The overall analytical range is comprised of three distinct yet overlapping concentration ranges. A separate calibration is performed for each range. These ranges include 0.028 to 0.28 mg NO_3+NO_2-N/L, 0.07 to 0.70 mg NO_3+NO_2-N/L and 0.56 to 5.6 mg NO_3+NO_2-N/L. Three sub-ranges are utilized so that samples can be analyzed on the most appropriate scale possible.

3.5 Batch – Environmental samples, which are prepared and /or analyzed together with the same process and personnel, using the same lot(s) of reagents. An **analytical batch** is composed of prepared environmental samples (extracts, digestates, concentrates) and/or those samples not requiring preparation, which are analyzed together as a group using the same calibration curve or factor. An analytical batch can include samples originating from various environmental matrices and can exceed 20 samples. (NELAC/EPA)

3.6 Blank- A sample that has not been exposed to the analyzed sample stream in order to monitor contamination during sampling, transport, storage or analysis. The blank is subjected to the usual analytical and measurement process to establish a zero baseline or background value and is sometimes used to adjust or correct routine analytical results. (ASQC)

3.7 Calibrate- To determine, by measurement or comparison with a standard, the correct value of each scale reading on a device. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.8 Calibration – The set of operations which establish, under specified conditions, the relationship between values indicated by a measuring device. The levels of the applied calibration standard should bracket the range of planned or expected sample measurements. (NELAC)

3.9 Calibration Blank – A volume of reagent water fortified with the same matrix as the calibration standards, without the analyte added.

3.10 Calibration Curve – The graphical relationship between known values, such as concentrations, or a series of calibration standards and their analytical response. (NELAC)

3.11 Calibration Method – A defined technical procedure for performing a calibration. (NELAC)

3.12 Calibration Standard – A substance or reference material used to calibrate an instrument. (QAMS)

3.12.1 Initial Calibration Standard (STD) – A series of standard solutions used to initially establish instrument calibration responses and develop calibration curves for individual target analytes.

3.12.2 Initial Calibration Verification (ICV) – An individual standard, distinct from the Initial Calibration Standards (STD), analyzed initially, prior to any sample analysis, which verifies acceptability of the calibration curve or previously established calibration curve.

3.12.3 Continuing Calibration Verification (CCV) – An individual standard, distinct from the Initial Calibration Standards (STD), analyzed after every 18-23 field sample analyses.

3.13 Certified Reference Material – A reference material one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body. (ISO 17025)

3.14 Corrective Action – Action taken to eliminate the causes of an existing nonconformity, defect or other undesirable situation in order to prevent recurrence. (ISO 8402)

3.15 Deficiency – An unauthorized deviation from acceptable procedures or practices. (ASQC)

3.16 Demonstration of Capability – A procedure to establish the ability of the analyst to generate acceptable accuracy. (NELAC)

3.17 Detection Limit – The lowest concentration or amount of the target analyte that can be determined to be different from zero by a single measurement at a stated degree of confidence.

3.18 Duplicate Analysis – The analyses of measurements of the variable of interest performed identically on two sub samples (aliquots) of the same sample. The results from duplicate analyses are used to evaluate analytical or measurement precision but not the precision of sampling, preservation or storage internal to the laboratory. (EPA-QAD)

3.19 External Standard (ES) – A pure analyte (potassium nitrate (KNO_3)) that is measured in an experiment separate from the experiment used to measure the analyte(s) in the sample. The signal observed for a known quantity of the pure external standard is used to calibrate the instrument response for the corresponding analyte(s). The instrument response is used to calculate the concentrations of the analyte(s) in the unknown sample.

3.20 Field Duplicates (FD1 and FD2) – Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 provide a measure of the precision associated with

sample collection, preservation and storage, as well as with laboratory procedures.

3.21 Field Reagent Blank (FRB) – A aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to the sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.22 Holding time – The maximum time that samples may be held prior to analysis and still be considered valid. (40 CFR Part 136) The time elapsed from the time of sampling to the time of extraction or analysis, as appropriate.

3.23 Laboratory Duplicates (LD1 and LD2) – Two aliquots of the same sample taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.24 Laboratory Reagent Blank (LRB) – A matrix blank (i.e., reagent water) that is treated exactly as a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the instrument.

3.25 Laboratory Control Sample (LCS) – A sample matrix, free from the analytes of interest, spiked with verified known amounts of analytes from a source independent of the calibration standard or a material containing known and verified amounts of analytes. The LCS is generally used to establish intra-laboratory or analyst-specific precision and bias or to assess the performance of all or a portion of the measurement system. (NELAC)

3.26 Limit of Detection (LOD) – The lowest concentration level that can be determined by a single analysis and with a defined level of confidence to be statistically different from a blank. This is also referred to as MDL. (ACS)

3.27 Limit of Quantitation (LOQ) – The minimum levels, concentrations, or quantities of a target variable (target analyte) that can be reported with a specified degree of confidence. The LOQ is set at 3 to 10 times the LOD, depending on the degree of confidence desired. This is also referred to as the Quantitation Limit.

3.28 Linear Dynamic Range (LDR) – The absolute quantity over which the instrument response to an analyte is linear. This specification is also referred to as the Linear Calibration Range (LCR).

3.29 May – Denotes permitted action, but not required action. (NELAC)

3.30 Method Detection Limit (MDL) – The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero (Standard Methods).

3.31 Must – Denotes a requirement that must be met. (Random House College Dictionary)

3.32 Photometer – measures the absorbance of the solution in the cell in a multicell cuvette. Light passes from the lamp through the condensing lenses to the interference filter. The plane surface of the first condensing lens is coated with a material which reflects heat and infrared light. The filters are mounted on a filter wheel. There are 15 positions for filters. Each filter corresponds to a wavelength of interest. The 540 nm filter is specified by the test definition for nitrate plus nitrite. After passing through the filter the light is converted into a stream of light pulses by a chopper. Then the light is directed via a quartz fiber through a focusing lens and a slit to the beam divider. The beam divider divides the light into two parts. A specified portion is reflected to the reference detector, which monitors the light level fluctuations. The remaining major portion of the light beam goes through the liquid in the cell to the signal detector, which measures the amount of light absorbed.

3.33 Precision – The degree to which a set of observations or measurements of the same property, obtained under similar conditions, conform to themselves; a data quality indicator. Precision is usually expressed as standard deviation, variance or range, in either absolute or relative terms. (NELAC)

3.34 Preservation – Refrigeration, freezing, and/or reagents added at the time of sample collection (or later) to maintain the chemical and or biological integrity of the sample.

3.35 Quality Control Sample (QCS) – A sample of analyte of known and certified concentration. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials. Also referred to as CRM.

3.36 Run Cycle – Typically a day of operation – the entire analytical sequence from sampling the first standard to the last sample of the day.

3.37 Safety Data Sheets (SDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.38 Sample Segment – Bar-coded metal tray that holds up to fourteen four milliliter auto analyzer vials containing samples or standards. The user identifies each vial in the operating software.

3.39 Sample Segment Holder – An automated temperature controlled carousel that contains up to six sample segments. This carousel spins in clockwise or counterclockwise manner to move the sample

segments into position for analysis. This carousel format allows for continuous processing.

3.40 Sensitivity – The capability of a test method or instrument to discriminate between measurement responses representing different levels (concentrations) of a variable of interest.

3.41 Shall – Denotes a requirement that is mandatory whenever the criterion for conformance with the specification requires that there be no deviation. (ANSI)

3.42 Should – Denotes a guideline or recommendation whenever noncompliance with the specification is permissible. (ANSI)

3.43 Standard Reference Material (SRM) – Material which has been certified for specific analytes by a variety of analytical techniques and/or by numerous laboratories using similar analytical techniques. These may consist of pure chemicals, buffers, or compositional standards. The materials are used as an indication of the accuracy of a specific analytical technique. Also referred to as CRM.

3.44 Test Definition – A photometric test consisting of a user defined testing sequence, reagent additions, calibration standards, incubations and absorption results.

3.45 Test Flow – Functions to define the parameter for reagent and sample dispensing, dilution, incubation and measurement.

4 INTERFERENCES

4.1 Suspended matter in the sample will scatter light as it passes through the cuvette to the detector. High blank responses will result. The identified sample will be reanalyzed.

4.2 Blemishes in the cuvette, as result of the manufacturing process, will result in high blank responses. The identified sample will be reanalyzed.

5 SAFETY

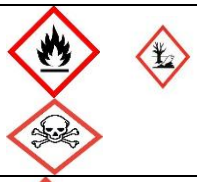
5.1 Safety precautions must be taken when handling reagents, samples and equipment in the laboratory. Protective clothing including lab coats, safety glasses and enclosed shoes must be worn. In certain situations, it will be necessary to also use gloves and/or a face shield. If solutions come in contact with eyes, flush with water continuously for 15 minutes. If solutions come in contact with skin, wash thoroughly with soap and water. Contact Solomons Rescue Squad (911) if emergency treatment is needed and also inform the CBL Associate Director of Administration and Facilities Maintenance of the incident. Contact the CBL Associate Director of Administration and Facilities Maintenance if additional treatment is required.

5.2 The toxicity or carcinogenicity of each reagent used in this procedure may not have been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known hazardous materials and procedures.

5.3 Do not wear jewelry when troubleshooting electrical components. Even low voltage points are dangerous and can injure if allowed to short circuit.

5.4 The following hazard classifications are listed for the chemicals used in this procedure. Detailed information is provided on Safety Data Sheets (SDS).

Table 1

Chemical	Health Hazard	Fire Hazard	Instability Hazard	Specific Hazard	
Nitrate Reductase (AtNaR2) from *Arabidopsis thaliana*	0	0	0		
NADH (*β*-Nicotinamide adenine dinucleotide reduced form disodium salt)	0	0	0		
Potassium hydroxide	3	0	2	4	
Sulfanilamide	1	1	0		
N-1-napthylethylenediamine dihydrochloride	1	0	0		
Hydrochloric Acid	3	0	2	ACID, COR	
Potassium nitrate	1	0	0	OXY	
Sodium nitrite	2	0	1		
Potassium phosphate	2	0	0		
EDTA (Ethylenediamine tetraacetic acid	1	0	0		

On a scale of 0 to 4 the substance is rated on four hazard categories: health, flammability, reactivity, and contact. (0 is non-hazardous and 4 is extremely hazardous)
HAZARD RATING

Health Hazard - Blue: 4 – deadly, 3 – extreme danger, 2 – hazardous, 1 – slightly hazardous, 0 – normal material

Fire Hazard - Red: Flash Points: 4 – below 73° F, 3 – below 100° F, 2 – below 200° F,

1 – above 200° F, 0 – will not burn

Instability Hazard - Yellow: 4 – may detonate, 3 – Shock and heat may detonate, 2 – violent chemical change, 1 – unstable is heated, 0 - stable

Specific Hazard - White: Acid = ACID, Alkali = ALK, Corrosive = COR, Oxidizer = OXY

6 EQUIPMENT AND SUPPLIES

6.1 Aqaukem 250 multi-wavelength automated discrete photometric analyzer. Aquakem 250 control software operates on a computer running Microsoft Windows NT, XP, or 7 operating system.

6.2 Freezer, capable of maintaining -20 ± 5° C.

6.3 Lab ware – All reusable lab ware (glass, Teflon, plastic, etc) should be sufficiently clean for the task objectives. This laboratory cleans all lab ware related to this method with a 10% HCl (v/v) acid rinse.

7 REAGENTS AND STANDARDS

7.1 Purity of Water – Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. Freshly prepared water should be used for making the standards intended for calibration. The detection limits of this method will be limited by the purity of the water and reagents used to make the standards.

7.2 Purity of Reagents – Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without compromising the accuracy of the determination.

7.3 Ethylenediamine tetraacetic acid (EDTA, 25 mM) 9.3 g

In a 1 L volumetric flask add approximately 800 mL reagent water. Dissolve 9.3 g ultrapure EDTA in reagent water and bring to volume. Write name of preparer, preparation date, reagent manufacturer, manufacturer's lot number in the Analytical Reagent log book. Store the flask at room temperature out of direct sunlight. The reagent is stable for one year.

7.4 Phosphate Buffer-

Potassium di-hydrogen phosphate (KH_2PO_4)	1.88 g
Potassium hydroxide (KOH)	0.7 g
EDTA (0.25 M)	5.0 mL

In a 500mL volumetric flask dissolve 1.88 g KH_2PO_4, 0.7g KOH and 5.0 mL EDTA (0.25M) in approximately 400 mL reagent water. Bring flask to volume. Store the flask at room temperature. Write name of preparer, preparation date, reagent manufacturers, manufacturers' lot numbers in the Analytical Reagent log book. The reagent is stable for six months.

7.5 Nitrate Reductase (AtNaR2)-

Nitrate reductase from *Arabidopsis Thaliana*	3.0 unit vial
Phosphate Buffer	20 mL

Transfer 1mL phosphate buffer to the 3.0 unit vial of AtNaR2 to affect dissolution. Shake several times over a thirty minute period. Transfer this to the 20mL reagent bottle quantitatively with four 1 ml aliquots of the phosphate buffer. Add 15mL of phosphate buffer to the reagent bottle. Shake bottle to complete the reagent preparation. This is enough reagent for approximately 300 analyses. Write name of preparer, preparation date, reagent manufacturers, manufacturers' lot numbers in the Analytical Reagent log book. This reagent is stable for eight hours in the refrigerated reagent compartment of the instrument.

7.6 NADH-

(β-Nicotinamide adenine dinucleotide reduced form disodium salt)	2.4 g vial
Phosphate Buffer	11 mL

Carefully transfer NADH crystals from vial to 20 mL reagent bottle. Place 1 mL phosphate buffer in vial and shake thoroughly. Transfer to reagent bottle. Add 10 mL phosphate buffer to the reagent bottle. Shake to complete reagent preparation. This is enough reagent for approximately 300 analyses. Write name of preparer, preparation date, reagent manufacturers, manufacturers' lot numbers in the Analytical Reagent log book. This reagent is stable for eight hours in the refrigerated reagent compartment of the instrument.

7.7 Sulfaniamide-

Sulfanilamide	5 g
Hydrochloric Acid (concentrated)	150 mL

Add 250 mL reagent water to a 500 mL volumetric flask. Carefully add 150 mL concentrated hydrochloric acid to the flask. Then add 5 g sulfanilamide to the flask. Bring the flask to volume with reagent water. Once dissolution is complete transfer reagent to a brown poly-bottle and store in the refrigerator. Write name of preparer, preparation date, reagent manufacturers, manufacturers' lot numbers in the Analytical Reagent log book. This reagent is stable for six months.

7.8 N-1-napthylethylenediamine dihydrochloride –

N-1-napthylethylenediamine dihydrochloride	0.5 g

Place 0.5 g N-1-napthylethylenediamine dihydrochloride in a 500mL volumetric flask. Bring flask to volume with reagent water. Once dissolution is complete transfer reagent to a brown poly-bottle and store in refrigerator. Write name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Reagent log book. This reagent is stable for six months.

7.9 Nitrate Stock Standard, 5000 µM –

Potassium nitrate (KNO_3), primary standard grade, dried at 45ºC	0.253 g

In a 500mL volumetric flask, dissolve 0.253 g of potassium nitrate in approximately 400 mL reagent water. Bring flask to volume with reagent water (1 mL contains 5 µmoles N). Write name of preparer, preparation date, standard manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh every 6 months or when <20% remains in bottle.

7.10 Stock Nitrite Standard –
Sodium nitrite ($NaNO_2$), primary standard grade, dried at 45ºC
0.1725 g
In a 500mL volumetric flask, dissolve 0.1725 g of sodium nitrite in approximately 400 mL of reagent water. Dilute to volume with reagent water (1 mL contains 5 µmoles N). Add 1 mL of chloroform as a preservative. Write name of preparer, preparation date, standard manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh every 6 months or when <20% remains in bottle.

7.11 Secondary Nitrite Standard –
Stock Nitrite Standard 0.70 mL
In a 100 mL volumetric flask, dilute 0.70 mL of Stock Nitrite Standard to volume with reagent water to yield a concentration of 35 µM NO_2 –N/L (0.49 mg N/L). Write name of preparer, preparation date, standard manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh every 4 months.

7.12 Aquakem Cleaning Solution –
Bleach 55.0 mL
In a 100 mL volumetric flask, dilute 55.0 mL of bleach to volume with 45mL reagent water to yield a concentration of 55% bleach. Write name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Reagent log book. Reagent is stable for six months.
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8 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Water collected for NO_3+NO_2 should be filtered through a Whatman GF/F glass fiber filter (nominal pore size 0.7 µm), or equivalent.

8.2 Water collected for NO_3+NO_2 should be frozen at ≤-20° C. The AutoAnalyzer vial container (sample cups) should be clean and sample rinsed.

8.3 Frozen NO_3+NO_2 samples may be stored up to 28 days. It has been shown that frozen QCS samples up to a year old still fall well within the control limits.

8.4 NO_3+NO_2 samples may be refrigerated at 4° C for no longer than one day.

9 QUALITY CONTROL

9.1 The laboratory is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory instrument blanks and calibration standard material, analyzed as samples, as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of data generated.

9.2 Initial Demonstration of Capability

9.2.1 The initial demonstration of capability (iDOC) – is used to characterize instrument performance (MDLs) and laboratory performance (analysis of QC samples) prior to the analyses conducted by this procedure.

9.2.2 Linear Dynamic Range – LDR (Linear Calibration Range) should be established for NO_3+NO_2 using appropriate eight point calibration curve.

9.2.3 Quality Control Sample (QCS/SRM) – When using this procedure, a quality control sample is required to be analyzed at the beginning and end of the run, to verify data quality and acceptable instrument performance. If the determined concentrations are not within ± 3s of the certified values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with analyses.

9.2.4 Method Detection Limits (MDLs) – Initial MDLs should be established for NO_3+NO_2 using a spiked water sample, typically two to 10 times the estimated MDL and no more than ten times higher than the estimated MDL. Process a minimum of seven spiked samples and seven blank samples through all steps of the method. The samples used for the MDL must be prepared and analyzed in at least three batches on three separate calendar dates. If multiple instruments are used for this analysis, MDLs must include data/calculations from all instruments.

9.2.4.1 Make all computations as specified in the analytical method and express the final results in the method-specified reporting units.

9.2.4.2 Calculate the sample standard deviation (S) of the replicate spiked sample measurements and the sample standard deviation of the replicate method blank measurements from all instruments to which the MDL will be applied.

9.2.4.3 Compute the MDLs (the MDL based on spiked samples) as follows:

$$MDL_S = t_{(n-1,\ 1-\alpha=0.99)} S_S$$

where:
MDLs = the method detection limit based on spiked samples
$t_{(n-1,\ 1-\alpha\ =\ 0.99)}$ = the Student's t-value appropriate for a single-tailed 99th percentile
t statistic and a standard deviation estimate with n-1 degrees of freedom.
S_S = sample standard deviation of the replicate spiked sample analyses.

9.2.4.4 Compute the MDL_b (the MDL based on method blanks) as follows:

If none of the method blanks give numerical results for an individual analyte, the MDLb does not apply. A numerical result includes both positive and negative results, including results below the current MDL, but not results of "ND" (not detected) commonly observed when a peak is not present in chromatographic analysis.

If some (but not all) of the method blanks for an individual analyte give numerical results, set the MDLb equal to the highest method blank result. If more than 100 method blanks are available, set MDLb to the level that is no less than the 99^{th} percentile of the method blank results. For "n" method blanks where $n \geq 100$, sort the method blanks in rank order. The (n * 0.99) ranked method blank result (round to the nearest whole number) is the MDL_b. For example, to find MDL_b from a set of 164 method blanks where the highest ranked method blank results are ... 1.5, 1.7, 1.9, 5.0, and 10, then 164 x 0.99 = 162.36 which rounds to the 162nd method blank result.

Therefore, MDL_b is 1.9 for n =164 (10 is the 164th result, 5.0 is the 163rd result, and 1.9 is the 162nd result). Alternatively, you may use spreadsheet algorithms to calculate the 99th percentile to interpolate between the ranks more precisely.

If all of the method blanks for an individual analyte give numerical results, then
calculate the MDLb as:

$$MDL_b = \bar{X} + t(n-1, 1-\alpha=0.99)S_b$$

where:
MDL_b = the MDL based on method blanks
$\bar{X}$ = mean of the method blank results (use zero in place of the mean if the mean is negative)

$t_{(n-1, 1-\alpha = 0.99)}$ = the Student's t-value appropriate for the single-tailed 99th percentile
t statistic and a standard deviation estimate with n-1 degrees of freedom.
S_b = sample standard deviation of the replicate method blank sample analyses.

9.2.4.5 The verified MDL is the greater of the MDLs or MDLb. If the verified MDL is within 0.5 to 2.0 times the existing MDL, and fewer than 3% of the method blank results (for the individual analyte) have numerical results above the existing MDL, then the existing MDL may optionally be left unchanged. Otherwise, adjust the MDL to the new verification MDL. (The range of 0.5 to 2.0 approximates the 95th percentile confidence interval for the Initial MDL determination with six degrees of freedom.)

9.2.4.6 MDLs should be determined annually, whenever there is a significant change in instrumental response or a significant change in instrument configuration. Data for annual MDL calculation and verification is analyzed at least quarterly, throughout the year.

9.3 Assessing Laboratory Performance

9.3.1 Laboratory Reagent Blank (LRB) – The laboratory must analyze at least one LRB with each batch of samples. The LRB consists of reagent water treated the same as the samples. An amount of analyte above the MDL found in LRB indicates possible reagent or laboratory environment contamination. LRB data are used to assess and correct contamination from the laboratory environment.

9.3.2 Quality Control Sample (QCS)/ Standard Reference Material (SRM) – When using this procedure, a quality control sample is required to be analyzed at the beginning of the run and end of the run, to verify data quality and acceptable instrument performance. If the determined concentrations are not within ± 3*s* of the certified values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with the analyses. The results of these QCS/SRM samples shall be used to determine batch acceptance.

9.3.3 The QCS are obtained from a source external to the laboratory and different from the source of calibration standards.

9.3.4 Control Charts – The Accuracy Control Chart for QCS/SRM samples and reagent blanks is constructed from the average and standard deviation of the 20 most recent QCS/SRM measurements. The accuracy chart includes upper and lower warning levels (WL=±2*s*) and upper and lower control levels (CL=±3*s*). These values are derived from stated values of the QCS/SRM. The standard deviation (*s*) is specified relative to statistical confidence levels of 95% for WLs and 99% for CLs. Enter QCS/SRM results on the chart each time the sample is analyzed

9.3.5 Calibration Verification, Initial and Continuing (ICV/CCV) – Immediately following calibration (ICV) and following every 18-23 samples (CCV), one CCV of 10 µM NO_3 (0.14 mg N/L) NiRMID, 35 µM NO_3 (0.49 mg N/L) NiRHI , 200 µM NO_3 (2.8 mg N/L) NiRXHI is analyzed to assess instrument performance. The CCVs are made from the same material as calibration standards (KNO_3), and are to be within TV ± 3*s*. Failure to meet the criteria requires correcting the problem, including reanalysis of any affected samples. If not enough sample exists, the data must be qualified if reported.

9.3.6 Reduction Efficiency Verification (REV) – The REVs are made from $NaNO_2$, 35 µM NO_2 (0.49 mg N/L) and are to be within the expected value ± 3*s* of the equivalent CCV, 35 µM NO_3 (0.49 mg N/L). Failure to meet the criteria requires correcting the problem.

9.4 Assessing Analyte Recovery – Percent Recovery

9.4.1 Analyte recovery is assessed through percent recoveries of laboratory spikes of samples.

9.4.2 Percent Recovery = (Actual/Expected) x 100

9.5 Assessing Analyte Precision – Relative Percent Difference

9.5.1 Analyte replication is assessed through duplicate analyses of samples – Relative Percent Difference.

9.5.2 RPD = (Laboratory Duplicate Result 1 – Laboratory Duplicate Result 2)/[(Laboratory Duplicate Result 1 + Laboratory Duplicate Result 2)/2] X 100

9.6 Corrective Actions for Out of Control Data

9.6.1 Control limit – If one measurement exceeds Accuracy Control Chart CL, repeat the analysis immediately. If the repeat measurement is within the CL, continue analyses; if it exceeds the CL, discontinue analyses and correct the problem.

9.6.2 Warning limit – If two out of three successive points exceed Accuracy Control Chart WL, analyze another sample. If the next point is within WL, continue analyses; if the next point exceeds the WL, evaluate potential bias and correct the problem.

9.6.3 Trending – If seven successive Accuracy Control Chart measurements are on the same side of the central line, discontinue analyses and correct the problem.

9.6.4 When external QCS samples are out of control, correct the problem. Reanalyze the samples analyzed between the last in-control measurement and the out-of-control one.

9.6.5 When external CCV samples are out of control, correct the problem. Reanalyze the samples analyzed between the last in-control measurement and the out-of-control one.

9.7 General Operation - To assure optimal operation and analytical results, the Reagent Blank and CCV are tracked daily in the raw data file, copied to Reagent Blank and CCV Control Charts.

Table 2

QC Indicator	Acceptance/ Action Limits	Action	Frequency (Batch)
Correlation Coefficient	≥ 0.995	If <0.995, evaluate data points of the calibration curve. If any data point is outside established limits, reject as outlier.	1 per batch if acceptable.
Quality Control Sample (QCS)/ Certified Reference Material (CRM)	± 10%	If QCS value is outside ± 10% of the target value reject the run, correct the problem and rerun samples.	Beginning and end of run as well as scattered throughout the run.
Initial Calibration Verification (ICV)	± 10%	Recalibrate if outside acceptance limits.	Beginning of run following standard curve.
Continuing Calibration Verification (CCV)	± 10%	If outside 10%, correct the problem. Rerun all samples following the last in-control CCV.	After every 18-23 samples.
Method Blank/Laboratory Reagent Blank (LRB)	≤ Method Quantitation Limit	If the LRB exceeds the quantitation limit, results are suspect. Rerun the LRB. If the concentration still exceeds the quantitation limit, reject or qualify the data, or raise the quantitation limit.	Following the ICV and after every 18-23 samples following the CCV.
Laboratory Fortified Sample Matrix Spike	± 10%	If the recovery of any analyte falls outside the designated acceptance limits and the	1/10 (spike OR duplicate)

		QCS is in control, the recovery problem is judged matrix induced. Repeat the LFM and if the sample results are again outside the acceptable recovery range, the sample should be reported with a "matrix induced bias" qualifier.	
Laboratory Duplicate	± 10%	If the RPD fails to meet the acceptance limits, the samples should be reanalyzed. If the RPD again fails to meet the acceptance limits, the sample must be reported with a qualifier identifying the sample analysis result as not having acceptable RPD for duplicate analysis.	1/10 (spike OR duplicate)

10 CALIBRATION AND STANDARDIZATION

10.1 Calibration – Daily calibration must be performed before sample analysis may begin. Eight point calibrations are used with each of the three sub-calibrations that cover the analytical range. Five working nitrate standards are used to produce the calibrators for each set of three calibration curves. The instrument performs serial dilutions of working standards to produce the eight calibrators defined for each curve. The following outlines the preparation of the working standards and the following table describes the subsequent serial dilutions the instrument performs to make each standard for each of the three calibration curves.

NO23 Working Standards:

NiRMID

Working Standard	0.28 mg N/L	(0.4 mL stock to 100 mL)
Working Standard	0.7 mg N/L	(1.0 mL stock to 100 mL)

NiRHI

Working Standard	0.7 mg N/L	(1.0 mL stock to 100 mL)
Working Standard	2.8 mg N/L	(4 mL stock to 100 mL)

NiRXHI

Working Standard	5.6 mg N/L	(8.0 mL stock to 100 mL)
Working Standard	22.4 mg N/L	(32 mL stock to 100 mL)

NO23 Calibrators:

	Working Standard mg/L N	Dilution Factor	Concentration mg/L N
NiRMID	0.28	9+1	0.028
	0.28	7+1	0.035
	0.28	5+1	0.04667
	0.28	3+1	0.07
	0.28	1+1	0.140
	0.7	3+1	0.175
	0.7	2+1	0.233
	0.28	0+1	0.280
NiRHI	0.7	9+1	0.070
	0.7	5+1	0.11667
	0.7	4+1	0.140
	0.7	2+1	0.233
	0.7	1+1	0.350
	2.8	5+1	0.46667
	2.8	4+1	0.560
	0.7	0+1	0.700
NiRXHI	5.6	9+1	0.560
	5.6	5+1	0.933
	5.6	4+1	1.120
	5.6	2+1	1.86667
	5.6	1+1	2.800
	22.4	5+1	3.733
	22.4	4+1	4.480
	5.6	0+1	5.600

10.2 The instrument software prepares a standard curve for each set of calibrators. This is viewed by displaying a second order calibration. The second order graph plotting measured absorbance against standard concentration is presented for review and approval. If acceptance criteria are not met the entire curve can be reanalyzed or individual standards can be reanalyzed. One standard value (original or reanalyzed) for each and every standard is incorporated in the curve. The coefficient of determination (Pearson's r value) for the calibration curve as well as the calculated concentration of each calibrator is reviewed. The calculated value of each calibrator must be within ten percent of the expected value. The coefficient of determination (Pearson's r value) for the calibration curve must be greater than 0.995.

11 PROCEDURE – DAILY OPERATIONS QUALITY CONTROL

11.1 Turn on computer. Computer will automatically initiate Konelab software. Once software is running, turn on instrument and allow connection between instrument and computer to complete.

11.2 Discard any water remaining in the water reservoir from the previous analytical run. Fill the water reservoir with fresh reagent water.

11.3 Remove from freezer samples to be analyzed. Allow samples to begin thawing. Begin daily bench sheet documentation. Remove nitrate reductase and NADH vials from freezer.

11.4 Place cuvette waste box into cuvette waste sliding drawer.

11.5 Once water reservoir is full, use instrument software to click More, Instrument Actions, and Perform Water Wash.– complete at least five perform water wash cycles.

11.6 After performing water washes, clean the dispensing needle by performing test washes. Click More, Instrument Actions, More, Adjustment Program. Once in the Adjustment Program click, 4-Dispensing Unit, 1-Dispenser, 8-Test Wash. Perform 8 to 10 Test Washing. When complete, press "Q" to quit until you are able to bring up the Main Page.

11.7 Perform Start Up operations by clicking Start Up at the bottom of the Main Page.

11.8 Gather working standards and reagents from refrigerator during startup. Assess standards and reagents. Remake anything that has exceeded the time over which it is considered stable. Nitrate reductase and NADH reagents are to be made fresh for every analytical run.

11.9 Once startup is complete, check the instrument water blank by clicking More, Instrument Actions, More, Check Water Blank. If any of the instrument blanks are outside their predefined and software controlled limits, the user will be notified on the main menu page. User takes corrective action to return instrument functions to controlled limits.

11.10 Load reagents into reagent carousel and place into refrigerated reagent compartment. Reagent positions can be found by clicking reagents at the top of the main page.

11.11 Load working standards into a sample segment, identify the standards in their positions from the drop down menus at the individual segment positions, and load into instrument. (Click Samples from the top of the Main Page, then click desired segment number.)

11.12 Select the methods to be calibrated by clicking Calibr./QC Selection on the bottom of the main page.. Click NiRMID, NiRHI and NiRXHI as the three methods to be calibrated, then click Calibrate at the bottom of the page. The three methods will now show as pending. Return to the main page..

11.13 Start instrument and calibration by clicking Page Up on the keyboard. This may also be a green button on the keyboard – See test flow below for stepwise instrument functions for the analysis of standards and samples.

Test Flow – Method of Analysis, Stepwise

- 55 µL NiR AtNaR to cuvette
- 5 µL sample to cuvette with mixing
- 15 µL NiR NADH to cuvette with mixing
- Incubation, 600 seconds, 37ºC
- 25 µL sulfanilamide (SAN) reagent to cuvette with mixing
- Incubation, 120 seconds, 37ºC
- 25 µL N-1-Napthylethylenediamine dihydrochloride (NED) reagent to cuvette with mixing
- Incubation, 120 seconds, 37ºC
- End point absorbance measurement, 540 nm
- Side-wavelength measurement, 700 nm
- Software processes absorbance value, side wave length value and uses calibration curve to calculate analyte concentration (mg/L N as NO2)
- User is notified if any measured values used to calculate final concentration are outside preset limits. If so, analyst has options to accept results, rerun the sample or rerun the sample diluted to a user or software specified factor.
- User is notified of each side wave length value. Side wave length >0.005 absorbance units indicates a scratched cuvette or turbid sample. If the side wave length value exceeds 0.005 absorbance units, the analyst specifies that the sample is reanalyzed. If the side wave length of the reanalyzed sample is <0.005 absorbance units, the reanalyzed result is accepted. If the same concentration and side wave length >0.005 absorbance units is again obtained, the results are accepted.

11.14 Organize samples, reagent blanks, check standards and all quality control samples while instrument performs calibrations.

11.15 As calibration curves are produced by the instrument, review them for acceptability. The instrument software prepares a standard curve for each set of calibrators. A graph plotting measured absorbance against standard concentration is presented for review and approval. If acceptance criteria are not met, either the entire curve shall be reanalyzed or individual standards shall be reanalyzed, depending on the violation. One standard value (original or reanalyzed) for each and every calibrator is incorporated in the curve.

11.16 Once calibration curves are accepted, samples are loaded into the sample segments and loaded into the instrument for analysis. After the Reagent Blank, the first samples analyzed should be ICV (initial calibration verification) samples. There should be one sample for each calibration curve, of a concentration close to the middle of each range. The following are the recommended ICV samples for each curve: 0.14 mg N/L NiRMID, 0.49 mg N/L NiRHI and 2.8 mg N/L NiRXHI. Secondary Nitrite Standard (REV) (0.49 mg N/L) should be analyzed and compared with 0.49 mg N/L ICV to determine reduction efficiency.

11.17 Samples are loaded into the segments and analyzed. CCV (Continuing Calibration Verification) samples (one for each of the three calibration ranges) follow every 18-23 samples. Standard Reference Material (SRM) samples, as well as Laboratory Reagent Blanks (LRB) are scattered throughout the analytical batch. Throughout the analytical batch, samples are chosen as laboratory

duplicates and laboratory spikes to assess analyte precision and analyte recovery, respectively. The total number of duplicates and spikes performed will be equal to or greater than ten percent of the total number of samples in the analytical batch.

11.18 As sample analysis is complete, results must be reviewed and accepted manually. If results fall outside acceptance limits, the sample should be reanalyzed. If sample result exceeds the highest standard of the highest calibration range, the samples can be automatically diluted by the instrument and reanalyzed. If the result is such that it will fall within a higher calibration range, it should be reanalyzed in the higher range. If the result is such that it will fall within a lower calibration range, it should be reanalyzed within the lower range. If the result falls below the lowest standard of the lowest calibration range, the result should be discarded and the sample should be analyzed via cadmium reduction method.

11.19 Upon completion of all analysis, results are saved to a daily report file. Click Report on the bottom of the main page, More, Results To File, and select one row per result. The file is then named by the run date. The daily report file for analytical batch of January 1, 2015 would be named 010115. The file is converted to Microsoft Excel for data work up. Remaining samples are discarded.

11.20 All reagents are removed from the reagent chamber and returned to the refrigerator. Reagents that have exceeded their stability period are discarded.

11.21 Click on Stand By on the bottom of the main page and insert Aquakem Cleaning Solution into the instrument. This initiates shut down procedures. Daily files are cleared from the instrument software by clicking More, Management, Clear Daily Files. The software is exited and the instrument is turned off. The computer is turned off.

11.22 The waste is flushed down the drain with copious amounts of tap water. The waste cuvette box is moved to the fume hood and covered. The incubator cover plate is removed. The incubator is wiped clean. The cover is cleaned and returned to its original position.

12 DATA ANALYSIS AND CALCULATIONS

12.1 Upon completion of all analysis, results are saved to a daily report file. The file is named by the run date. The daily report file for analytical batch of January 1, 2015 would be named 010115. The file is converted to Microsoft Excel for data work up. The instrument software has calculated final sample concentration from the designated standard curve, correcting each concentration for associated side wave length and also for any user or instrument specified dilution. Dilution by the instrument is noted by software as analysis ensues and, also, documented in the Excel data report file. The analyst examines each row of data. Results are eliminated that are outside the limits of the calibration range, or have an unrepeated side wave length measurement greater than 0.005 absorbance units.

13 REFERENCES

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