Determination of Total Dissolved Nitrogen (TDN) in Fresh/Estuarine/Coastal Waters Using Alkaline Persulfate Digestion of Nitrogen to Nitrate and Measured Using Enzyme Catalyzed Reduction

1. SCOPE and APPLICATION

1.1 Potassium Persulfate is used to oxidize organic and inorganic nitrogen to NO_3 under heated alkaline conditions.

1.2 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.

1.3 A Method Detection Limit (MDL) of 0.05 mg TDN as NO_3-N/L L was determined using the Student's t value (3.14, n=7) times the standard deviation of a minimum of 7 replicates.

1.4 The Quantitation Limit for TDN as NO_3 was set at 0.15 mg TDN as NO_3-N/L.

1.5 This procedure should be used by analysts experienced in the theory and application of aqueous organic and inorganic analysis. A three month training period with an analyst experienced in the analysis of TDN in aqueous samples by cadmium reduction is required.

1.6 This method can be used for all programs that require analysis of TDN.

1.7 A portion of this procedure conforms to 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 is awaiting final approval. It is now part of the EPA Methods Update Rule 2015 and has been published to the EPA Federal Register Vol. 80 No. 33. It is recommended as an addition of approved methods at 40 CFR Part 136 and currently accepting comments.

2. SUMMARY

2.1 An exact amount of filtered samples are placed in test tubes where an exact amount of Potassium Persulfate Digestion Reagent is added. Under initially alkaline conditions and heat, nitrate is the sole nitrogen product.

2.2 The now digested samples are buffered, then 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.

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 – There are multiple analytical ranges/standard curves used for determination of TDN. See Table 1 for all analytical ranges used.

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. A **preparation batch** is composed of one to 300 environmental samples of the same matrix, meeting the above mentioned criteria and with a maximum time between the start of processing of the first and last sample in the batch to be 10 hours. An **analytical batch** is composed of prepared environmental samples (extracts, digestates, or 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 meter or other device, or the correct value for each setting of a control knob. 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, 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 which is analyzed after every 18-23 field sample analysis.

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 Instrument Detection Limit (IDL) – The minimum quantity of analyte of the concentration equivalent which gives an analyte signal equal to 3.14 times 7 replicates that make up the standard deviation of the background signal at the selected wavelength, mass, retention time absorbance line, etc.

3.24 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.

3.25 Laboratory Reagent Blank (LRB) – A blank matrix (i.e., DI 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.

3.26 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)

3.27 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. (ACS)

3.28 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.

3.29 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).

3.30 Manifold – The module whose configuration of glass connectors, fittings, mixing coils, tubing and Cadmium-Copper reduction column precisely reduces the nitrate in the sample to nitrite, followed by color production.

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

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

3.33 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.

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

3.35 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.

3.36 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)

3.37 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.

3.38 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.

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

3.40 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.

3.41 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.

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

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

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

3.45 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.

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

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

4 INTERFERENCES

4.1 Metals, highly reduced substances, and excessive amounts of nitrogen have the potential of using up potassium persulfate before all nitrogen products have been oxidized.

4.2 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.

4.3 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 should 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 Business Manager of the incident. Contact the CBL Business Manager 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 Material Safety Data Sheets (MSDS).

Chemical	Health	Flammability	Reactivity	Contact	Storage
Sodium Hydroxide	3	0	2	4	White Stripe
Potassium hydroxide	3	0	2	4	White
Potassium phosphate	2	0	3	2	Green
Sulfanilamide	0	1	1	1	Green
N-1-napthylethylenediamine dihydrochloride	2	1	1	2	Green
Nitrate Reductase (AtNaR2) from *Arabidopsis thaliana*	0	0	0	0	Green
NADH (β-Nicotinamide adenine dinucleotide reduced form disodium salt)	0	0	0	0	Green
Hydrochloric Acid	3	0	2	4	White
Potassium nitrate	2	0	3	2	Yellow
Sodium nitrite	2	0	3	2	Yellow
Chloroform	3	1	1	3	Blue
Potassium Persulfate	2	0	1	0	Yellow
Boric Acid	2	0	1	2	Green
EDTA (Ethylenediamine tetraacetic acid)	1	0	0	1	Green

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)
STORAGE
Red – Flammability Hazard: Store in a flammable liquid storage area.
Blue – Health Hazard: Store in a secure poison area.
Yellow – Reactivity Hazard: Keep separate from flammable and combustible materials.
White – Contact Hazard: Store in a corrosion-proof area.
Green – Use general chemical storage (On older labels, this category was orange).
Striped – Incompatible materials of the same color class have striped labels. These products should not be stored adjacent to substances with the same color label. Proper storage must be individually determined.

6 EQUIPMENT AND SUPPLIES

6.1 Aquakem 250 multi-wavelength automated discrete photometric analyzer. Aquakem 250 control software operates on a computer running Microsoft Windows NT or XP 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. Test tubes used in this analysis are predigested and rinsed with copious amounts of deionized water.
6.4 Pressure Cooker with pressure regulator and pressure gauge.
6.5 Hot plate with variable heat settings.

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 deionized water. Dissolve 9.3 g ultrapure EDTA in deionized 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 (25 mM)	5.0 mL

In a 500mL volumetric flask dissolve 1.88 g KH_2PO_4, 0.7g KOH and 5.0 mL EDTA (25mM) in approximately 400 mL deionized water. Bring flask to 500 mL 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	10 g
Hydrochloric Acid (concentrated)	300 mL

Add 500 mL deionized water to a 1 L volumetric flask. Carefully add 300 mL concentrated hydrochloric acid to the flask. Then add 10 g sulfanilamide to the flask. Bring the flask to volume with deionized 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	1.0 g

Place 1.0 g N-1-napthylethylenediamine dihydrochloride in a 1 L volumetric flask. Bring flask to volume with deionized 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's 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	0.5055 g
Deionized water	up to 1000 mL

In a 1000 mL volumetric flask, dissolve 0.5055 g of potassium nitrate in ~800 mL of deionized water. Dilute to 1000 mL with deionized water (1 mL contains 5 µmoles N). Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh every 4 months or when < 20% remains in bottle.

7.10 Working Nitrate Standard for TDN – See Table 1 for all working Nitrate Standards for TDN.
Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh for every digestion batch.

7.11 Glutamic Acid Stock Standard, –

Glutamic Acid dried at 45°C	0.3705 g
Deionized water	up to 500 mL
Chloroform ($CHCl_3$)	0.5 mL

In a 500 mL volumetric flask, dissolve 0.3705 g of glutamic acid in about 400 mL of deionized water and dilute to 500 mL with deionized water. Add 0.5 mL of chloroform as a preservative. Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Standard log book.

7.12 Working Glutamic Acid Standard for TDN – See Table 1 for all working Glutamic Acid Standards for TDN.
Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Standard log book. Make fresh for every digestion batch.

7.13 Potassium Persulfate Digestion Reagent –

Sodium Hydroxide (NaOH)	3 g
Potassium Persulfate ($K_2S_2O_8$), Low N	20.1 g
Deionized water	up to 1000 mL

In a 1000 mL volumetric flask, dissolve 3g of sodium hydroxide and 20.1 g of potassium persulfate in ~800mL of deionized water. Dilute to 1000 mL with deionized water. Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Reagent log book. Make fresh daily. See Table 1 for Targeted Watershed Samples (TWS).

7.14 Borate Buffer Solution –

Boric Acid (H_3BO_3)	61.8 g
Sodium Hydroxide (NaOH)	8 g
Deionized water	up to 1000 mL

In a 1000 mL volumetric flask, dissolve 61.8 g of boric acid in ~ 300mL deionized water. Add 8g of sodium hydroxide and dilute to 1000mL with deionized water. Write the name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Reagent log book. Make fresh every 4 months.

7.15 Aquakem Cleaning Solution –

Clorox	75.0 mL

In a 100 mL volumetric flask, dilute 75.0 mL of Clorox to volume with deionized water to yield a concentration of 75% Clorox. Recent (2012) trends in commercially available Clorox, have necessitated altering this formula to 55.0 mL Clorox in 100 mL flask. Write name of preparer, preparation date, reagent manufacturer, manufacturer lot number in the Analytical Reagent log book. Reagent is stable for six months.

8 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Water collected for TDN should be filtered through a Whatman GF/F glass fiber filter (nominal pore size 0.7 μm), or equivalent.
8.2 Prior to initial use, capped 30 mL test tubes must be digested with Digestion Reagent, then rinsed thoroughly with deionized water.
8.3 A prescribed amount (typically 10mL) of sample should be added to each sample rinsed, capped 30mL test tube.
8.4 Water collected for TDN should be frozen at -20° C.
8.5 Frozen TDN samples may be stored longer than 28 days. It has been shown that frozen QCS samples up to a year old still fall well within the control limits.
8.6 Digested TDN samples may be stored up to three months.
8.7 TDN 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 (TDN) – 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 Quality Control Sample (QCS/SRM) – When using this procedure, a quality control sample is required to be analyzed during the run, to verify data quality and acceptable instrument performance. If the determined concentrations are not within ± 10% 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.

9.2.3 Method Detection Limits (MDLs) – MDLs should be established for TDN using a low level ambient water sample, typically three to five times higher than the estimated MDL. To determine the MDL values, analyze seven replicate aliquots of water. Perform all calculations defined in the procedure (Section 13) and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = S t_{(n-1,1-\alpha=0.99)}$$

Where,

S = Standard deviation of the replicate analyses.
n=number of replicates
$t_{(n-1,1-\alpha=0.99)}$ = Student's *t* value for the 99% confidence level with n-1 degrees of freedom (*t*=3.14 for 7 replicates.)

MDLs should be determined annually, whenever there is a significant change in instrumental response, change of operator, or a new matrix is encountered.

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 Nanopure water treated the same as the samples. An amount of analyte above the MDL (TDN) 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 ± 3σ 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 sample 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 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. Set up an accuracy chart by using percent recovery since the concentration of the QCS/SRM varies. Enter QCS/SRM results on the chart each time the sample is analyzed

9.3.5 Continuing Calibration Verification (CCV) – Following every 18-23 samples, two CCV are analyzed to assess instrument performance. The CCVs are made from the different material than the calibration standards (KNO_3), and are to be within TV ± 3σ. 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. Specific CCV's can be found in Table 1.

9.4 Assessing Analyte Recovery - % Recovery

9.4.1 Analyte recovery is assessed through percent recoveries of laboratory spikes. Analyte recovery is also assessed through the percent recovery of an organic standard that was digested with each batch of samples.

9.4.2 Percent Recovery for each spiked sample should fall within 80-120%. Where:
%SR = (Actual/Expected) x 100

9.5 Assessing Analyte Precision – Relative Percent Difference (RPD)

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 (LRB) and CCV are tracked daily in the raw data file, copied to Reagent Blank (LRB) and CCV Control Charts.

10 CALIBRATION AND STANDARDIZATION

10.1 Calibration – Daily calibration must be performed before sample analysis may begin. Multiple point calibrations (See Table 1) are used with the Aquakem 250. ASTM Type I water is used as the “zero point” in the calibration.

10.2 Working TDN Standards – See Table 1 for all working TDN Standards.

10.3 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 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 0.990.

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 deionized water.

11.3 Begin daily bench sheet documentation. Remove nitrate reductase and NADH vials from freezer.

11.4 Once water reservoir is full, "perform washes" – complete five wash cycles and then initiate "start-up" at main menu.

11.5 Gather reagents from refrigerator during start-up and assess reagents. Remake anything that has exceeded the time over which it is considered stable. Nitrate reductase and NADH reagents are made fresh for every analytical run.

11.6 Once startup is complete, check that the instrument water blank of water from the reservoir has performed within acceptance limits. If any of the instrument functions 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.

11.7 Load reagents into reagent carousel and place into refrigerated reagent compartment.

11.8 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.

11.9 Select the methods to be calibrated. Three different methods may be calibrated – TDN LOW, TDN XHigh and TDN TWS are the method names to be selected in the software.

11.10 Begin calibration – 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 the 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.

11.11 Organize and sub-sample into cups the samples, reagent blanks, check standards and all quality control samples while instrument performs calibrations.

11.12 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.

11.13 Once the calibration curve is accepted, samples are loaded into the segments and analyzed. CCV (Continuing Calibration Verification) samples 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 analytical precision and analyte recovery, respectively. The total number of duplicates and spikes performed will be equal or greater to ten percent of the total number of samples in the analytical batch.

11.14 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 calibration range it was run within, the samples are automatically diluted by the instrument and reanalyzed.

11.15 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 July 1, 2015 would be named 070115. The file is converted to Microsoft Excel for data work up. Remaining samples are discarded.

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

11.17 Aquakem Cleaning Solution is inserted into the instrument and shut down procedures are initiated. Daily files are cleared from the instrument software, the software is exited and the instrument is shut down. The computer is shut down.

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

12 PROCEDURE – SAMPLE DIGESTION

12.1 TDN/TDP samples are digested simultaneously in the same ampule. In our procedures, this ampule is a 30 mL screw cap test tube. (See Table 1 for all TWS samples.)

12.2 Prepare working standards, QCS, and CCV in labeled 100 mL volumetric flasks:

12.2.1 Select concentration range for both TDN/TDP that best fits the sample batch from Table 1.

12.2.2 Fill 100 mL volumetric flasks with 80 mL deionized water.

12.2.3 Add appropriate amount of KNO_3 stock standard and KH_2PO_4 secondary standard solution to each labeled working standard volumetric flask from Table 1.

12.2.4 Add appropriate amount of glutamic acid and glycerophosphate working standard solutions to each labeled CCV and % recovery volumetric flask from Table 1.

12.2.5 Bring up to 100 mL volume with deionized water.

12.2.6 Mix each 100 mL labeled volumetric flask thoroughly

12.3 Sub-sample working standards into 30mL screw cap test tubes:

12.3.1 Prepare 2, 30mL labeled test tubes for each working standard concentration.

12.3.2 Sample rinse each test tube with the appropriate working standard.

12.3.3 Add exactly 10mL of each working standard to each test tube.

12.3.4 Prepare 2 labeled test tubes with exactly 10 mL deionized water for "0" in the calibration curve.

12.3.5 Set aside 2 empty labeled test tubes to be digested with the batch with digestion reagent only.

12.3.6 Prepare 2, 30mL labeled test tubes for glutamic/glycerophosphate for % recovery and CCV by adding exactly 10mL to each test tube.

12.3.7 Thaw a Quality Control Sample (CRM) stored in freezer and sub-sample exactly 10mL into a labeled 30mL test tube to be used for QCS.

12.4 Prepare Digestion Reagent by dissolving 20.1 g Potassium persulfate and 3 g Sodium hydroxide in a 1000 mL volumetric flask:

12.4.2 Rinse volumetric flask with deionized water.
12.4.3 Add 20.1 g Potassium Persulfate directly to the volumetric flask.
12.4.4 Add deionized water until the meniscus is slightly below full volume.
12.4.5 Add 3 g Sodium Hydroxide to the persulfate and water solution, cap immediately and mix thoroughly.
12.4.6 Bring to volume with deionized water.
12.4.7 Make fresh daily.
12.4.8 Digestion Reagent has a shelf life of about 4 hours.

12.5 When ready to digest, thaw frozen samples at room temperature.
12.6 Rinse dispensing vessel with deionized water and sample rinse with digestion reagent.
12.7 Add thoroughly mixed digestion reagent.
12.8 Adjust and check dispensing vessel for desired dispensing volume.
12.9 Add desired amount of digestion reagent (Typically 5mL), cap tube, shake for mixing and add test tube to pressure cooker.
12.10 Add desired amount of digestion reagent (5mL) to the standards at the beginning, middle and end of the sequence of loading the samples.
12.11 When all samples and standards have received digestion reagent and have been loaded into the pressure cooker, place pressure cooker on hot plate, add deionized water until tubes are 75% immersed, wet the gasket on the lid with a few drops of water and place lid on the pressure cooker.
12.12 Turn the hot plate on maximum temperature and have the pressure cooker come up to full steam. (This takes about 1 hour.)
12.13 When full steam is achieved, place the pressure regulator on the steam vent. Maintain heat for the cooker containing samples and standards at 3-4 psi for 1 hour.
12.14 Turn off pressure cooker and unplug the hot plate when finished. Keep the lid on the pressure cooker.
12.15 After samples have cooled, usually the next day, remove the pressure cooker lid, add 1 mL Borate Buffer to each tube, cap, and shake.
12.16 Sample batch is now ready to analyze and is stable for 3 months.

13 DATA ANALYSIS AND CALCULATIONS

13.1 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 July 1, 2015 would be named 070115. The file is converted to Microsoft Excel and then to Lotus 123 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.

14 METHOD PERFORMANCE

14.1 On 32 separate dates from May 2009 through February 2010, 32 replicate analyses of SPEX® Corporation QC 6-51 NUT 2 were performed by TDN Enzyme Catalized Reduction. This produced a mean value of 0.55 mg TDN as NO_3-N/L, SD 0.03, Relative Percent Difference of 4.4% from the expected value of 0.525 ± 10%. This is a mean recovery of 105%.

15 REFERENCES

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15.4 Patton, et al. (2002). Corn leaf nitrate reductase – a nontoxic alternative to cadmium for photometric nitrate determinations in water samples by air-segmented continuous-flow analysis, Environ. Sci Tech. 2002, 36, 729-735. http://www.nitrate.com/pattonetal2002.pdf

15.5 http://www.nitrate.com/nar-nam1.htm

Range	umoles NO3/L	mg N/L	ml 1 NO3 std/100ml	Potassium Persulfate	Spike Conc.	Glutamic/Glycerophosphate for % Recovery	CCV
	0	0	DI H2O				
Low	10	0.14	0.2	20.1 g/L and 3g/L NaOH	200 umole NO3 12 umole PO4	1 ml Glutamic	1.0 mL Glutamic
10 ml sample	25	0.35	0.5				
5 ml persulfate	35	0.49	0.7				
	50	0.70	1.0				
	75	1.05	1.5				
XHigh	0	0	DI H2O		400 umole NO3 12 umole PO4	3 ml Glutamic	3.0 mL Glutamic
10 ml sample	25	0.35	0.5	20.1 g/L and 3g/L NaOH			
5 ml persulfate	50	0.70	0.7				
	75	1.05	1.5				
	100	1.4	2.0				
	150	2.1	3.0				
	200	2.8	4.0				
	400	5.6	8.0				
TWS TDN	0	0	DI H2O				
5 ml sample	150	2.1	3.0	13.4 g/2000 mL and 2 g NaOH 6.7 g/L and 1g NaOH	2.5 ml of 400 umole NO3 & 12 umole PO4 Added to 2.5 ml sample prior to digestion	4 ml Glutamic	4 ml Glutamic
15 ml persulfate	300	4.2	6.0				
	400	5.6	8.0				
	500	7.0	10.0				

Table 1. Methods and Standards Used for TDN Enzyme Catalyzed Nitrate

April 14, 2015