Method for Nitrate Reductase Nitrate-Nitrogen Analysis

1. Scope and Application

1.1 This test method is applicable to the determination of nitrate plus nitrite (as nitrogen) in surface water, saltwater (saline), ground water, drinking water, wastewater, and any aqueous solution containing nitrate.

1.2 The standard range of this test method is from 0.05 to 5 mg/L of nitrate-nitrogen. Samples with greater than 5 mg N/L may be diluted with deionized water (nitrate-free) to bring them into the range of the test method. In addition, for samples with nitrate-N content lower than 0.05 mg N/L, the sample volume used in the test may be increased for a low range test method from 0.01 to 1.0 mg N/L.

1.3 Quality Control and Quality Assurance (QC/QA) procedures and criteria are delineated. However, Precision and Bias of the method will be appended at a later time when the method has been validated by the Inter-Laboratory Test Trial.

1.4 This description of the test method does not purport to address all safety concerns associated with its use. It is the responsibility of the user of this test method to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

2. Summary of Method

2.1 Reduction of nitrate in the sample is accomplished by using eukaryotic nitrate reductase (EC 1.1.7.1-3; NaR) to catalyze the conversion of nitrate to nitrite in the presence of NADH or NADPH as reductant, in a biochemical buffer with a pH near neutrality (Campbell et al., 2006).

2.2 The combined nitrite (both original nitrite and that generated by reduction of nitrate to nitrite) is reacted with sulfanilamide and subsequent coupling with N-(1-napthyl)ethylenediamine dihydrochloride to yield a pink color. The absorbance of the chromophore is measured at ~540 nm in a spectrophotometer.

2.3 Calibrants of certified nitrate standard are treated with the reduction step and colorizing reaction to yield a standard curve which is used to determine the nitrate-N content of samples, expressed as mg/L nitrate N. This step insures that NaR is functioning.

2.4 Nitrite content of the sample (expressed as mg/L nitrite-N) can be determined by omitting the reduction step and directly reacting the sample with the color dyes.

2.5 Finally, nitrate-N content can be calculated by subtracting the nitrite content (mg/L nitrite N) from the combined nitrate plus nitrite (mg/L nitrate+nitrite N), to yield the nitrate content (expressed as mg/L nitrate N).

3. Definitions

3.1 Nitrate Reductase (NaR): NADH:NaR (EC1.7.1.1 and CAS 9013-03-0) with 1 unit of enzyme activity defined as 1 µmol nitrite produced per minute at 30°C and pH 7 with NADH (refer to section 3.4) as electron donor. See Note following Section 7.6 for information on the type of NaR acceptable for this method.

3.2 Discrete Analyzer: a programmable, computer-controlled instrument that automates wet chemical analysis by using one or more robotic arms interfaced to high-precision volumetric dispensers to aspirate and dispense samples, standards, diluents and reagents.

3.3 Greiss Reaction: chemical formation of an azo-dye by diazotization of nitrite ion with sulfanilamide (CAS 63-74-1), at an acidic pH, and subsequent coupling with N-(1-naphthyl)ethylenediamine hydrochloride (CAS 1465-25-4).

3.4 NADH: β-Nicotinamide adenine dinucleotide, reduced form (CAS 53-54-9), is a coenzyme found in all living cells.

3.5 EDTA: Disodium ethylenediaminetetraacetate dihydrate (CAS 60-00-4, a heavy metal chelator.

4. Interferences

4.1 Turbid samples should be filtered prior to analysis to eliminate particulate interference.

4.2 Sample color that absorbs at wavelengths between 520 and 560 nm interferes with the absorbance measurements. When color is suspect, analyze a sample blank, omitting the N-(1-naphthyl)ethylenediamine dihydrochloride from the color reagent. Note 1: The instrumentation described in this test method may automatically correct for some turbidity and sample color.

4.3 Certain ions may cause interferences. See Table 1. However, the inclusion of EDTA in the system's phosphate buffer (section 3.6 and 7.1) overcomes most, if not all, of these heavy metal interferences.

4.4 Comprehensive studies of this method, which include analysis of interferences, have been published (Patton and Kryskalla, 2011; 2013).

5. Safety

5.1 General laboratory safe practices should be used in handling all samples and reagents in this test method.

5.2 When preparing the sulfanilamide color reagent in hydrochloric acid (section 7.4), the user should handle the concentrated hydrochloric acid with extra caution and wear heavy gloves and eye protection. The prepared sulfanilamide color reagent should also be handled with caution since it is ~3 N hydrochloric acid.

5.3 Potassium nitrite is toxic if swallowed, and laboratory tests suggest that it may be mutagenic or teratogenic. Gloves and safety glasses are used when handling potassium nitrite.

5.4 Potassium nitrate, unlike potassium nitrite, is not toxic to humans, unless one is chronically exposed to nitrate such as in your drinking water or food. Potassium nitrate will not explode on its own. However, it is recommended that standard laboratory safety practices such as the use of safety glasses and gloves be used when handling it.

6. Equipment and Supplies

6.1 Automated Discrete Analysis System (see section 3.2).

7. Reagents and Standards

7.1 Phosphate Buffer Solution: Dissolve 3.75 g of potassium dihydrogen phosphate (KH_2PO_4; CAS 7778-77-0), 0.01 g of disodium ethylenediaminetetraacetate dihydrate ($C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$), and 1.4 g potassium hydroxide (KOH; CAS 1310-58-3) in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. Transfer this solution to a screw-cap container and store at 4°C. This solution is stable for 6 months.

7.2 β-nicotinamide adenine dinucleotide, reduced form (NADH) stock solution (2mg/mL): dissolve 0.1 g NADH in 25 mL of reagent water contained in a 50 mL volumetric flask, dilute to the mark and mix. Transfer 1-mL aliquots to 1.5 mL snap-cap colorless polypropylene vials and store at -20°C. Stable for 1 month. Note: NADH is a hygroscopic white powder that is freely soluble in water. The solids are stable if stored dry and protected from light. Neutral solutions are colorless and stable for 1 week if stored at 4ºC, but decompose rapidly under basic or acidic conditions.

7.3 NADH working solution: thaw one 1-mL vial of NADH stock (refer to section 7.2) and dilute to 10 mL with phosphate buffer (refer to section 7.1). This reagent is stable for

about 8 hours. Prepare sufficient NADH working solution for the number of samples and standards to be analyzed. **NOTE**: NADH inhibits color formation in the Greiss reaction (refer to section 3.3). The molar concentration of NADH in the reduction medium should be about twice that of the highest calibration standard.

7.4 Sulfanilamide (SAN) Reagent (10g/L): While stirring constantly add 300 mL of concentrated hydrochloric acid (HCl, 37% w/v)) and 10 g of sulfanilamide to about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. This solution is stable for about six months when stored in a brown bottle at 20°C.

7.5 N-(1-naphthyl)ethylenediamine dihydrochloride (NED) solution (1g/L): dissolve 1 g NED in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. Transfer to a glass or amber screw-cap container. This solution is stable for 6 months at 20°C.

7.6 Nitrate Reductase (NaR): follow the manufacturer's instructions for preparing a solution of 1 unit NaR (refer to section 3.1) activity per mL of phosphate buffer (refer to section 10.1). Dilute 3 units NaR to 20 mL with phosphate buffer. Store the solution at 2-6°C, where it is stable for 8 hours. Prepare sufficient NaR for the total number of samples and standards to be analyzed. **NOTE**: For some NaR forms, high phenolic content humic substances (>2 mg dissolved organic carbon /L) have little effect on the NaR activity in the temperature range of 5-15°C, but become increasingly inhibitory in the temperature range of 20-40°C (Patton and Kryskalla, 2011; 2013) . Humic substances at the operation temperatures specified in this test method do not inhibit other forms of NaR, such as recombinant AtNaR2 (Campbell et al., 2006). If humic acids are expected to be present, the user must verify reduction efficiency of the NaR in use by analysis of Quality Control checks that approximate the sample matrix.

7.7 Nitrate Solution, Stock (1000 mg/L NO3-N): Dry potassium nitrate (KNO_3) in an oven at 105°C for 24 h. Dissolve 7.218 g in about 500 mL reagent water contained in a 1000 mL volumetric flask, dilute to the mark and mix. This solution is stable for up to 2 months with refrigeration. Alternatively, certified nitrate stock solutions are commercially available through chemical supply vendors and may be used.

7.8 Nitrate Solution, Standard (10 mg/L NO3-N): Dilute 10 mL of stock nitrate solution (7.7) to 1 L with Deionized water and store in a dark bottle. Prepare fresh as needed.

7.9 Nitrite Solution, Stock (1000mg/L NO2-N): Place about 7 g of potassium nitrite (KNO_2) in a tared 125-mL beaker and dry for about 24 h to a constant weight in a desiccator containing a suitable desiccant. Adjust the weight of the dry potassium nitrite to 6.072 g. Add 50 mL of water to the beaker, stir until dissolved, and transfer quantitatively to a 1000-mL volumetric flask. Dilute to the mark with deionized water and store in a sterilized bottle under refrigeration. Prepare fresh as needed. Alternatively, certified nitrite stock solutions are commercially available through chemical supply vendors and may be used. **NOTE**: Potassium nitrite is easily oxidized; use only dry, free flowing white, or yellowish white crystalline powder of this reagent.

7.10 Nitrite Solution, Standard (10 mg/L NO2-N): Dilute 10 mL of stock nitrite solution (7.9) to 1 L with water. This solution is unstable; prepare fresh as needed.

8. Sample Collection, Preservation, and Storage

8.1 Sample preservation and holding time requirements for drinking water samples are as follows:

8.1.1 For nitrate: Chill the sample to ≤4°C and analyze within 48 hours. If the sample is chlorinated, the holding time for an unacidified sample kept at 4°C is extended to 14 days.

8.1.2 For nitrite: Do not add acid. Chill the sample to ≤4°C and analyze within 48 hours of collection.

8.1.3 For nitrate-nitrite: Acidify to pH<2 with H_2SO_4 at the time of collection, and analyze within 28 days of collection.

8.2 Sample preservation and holding time requirements for wastewater samples or other samples to be used for compliance monitoring under the Clean Water Act.

8.2.1 For nitrate: Chill the sample to ≤6 C and analyze within 48 hours of collection.

8.2.2 For nitrite: Chill the sample to ≤6 C and analyze within 48 hours of collection.

8.2.3 For nitrate-nitrite: Acidify to pH <2 with H2SO4 at the time of collection, and analyze within 28 days of collection.

8.3. In all cases, samples should be analyzed as soon as possible after collection.

9. Quality Control (QC)

9.1 **Requirement for QC Analysis:** To be certain that analytical values obtained using this test method are valid and accurate within the confidence limits of the test, the following QC procedures must be followed when analyzing nitrite-nitrate in water.

9.2 **Initial Demonstration of Performance (IDP):** IDP is used to characterize instrument performance and laboratory performance prior to performing analyses by this test method and periodically as specified below. The laboratory must complete the IDP tests and demonstrate compliance with the acceptance standards before beginning to analyze samples. **NOTE:** For the discrete analyzer (DA), this type of QC evaluation is done in every analytical run.

9.2.1 **Linear Calibration Range (LCR):** LCR must be determined initially and verified every six months or whenever a significant change in the instrument response is expected or observed. The initial demonstration of linearity must employ a number of standards sufficient to insure that the results are linear. Verification of linearity must use a minimum of a blank and three standards. If any verification data exceeds the nominal value of the standard by ±10%, linearity must be reestablished. If any portion of the linearity test range is found to be nonlinear, sufficient standards must be used to clearly define the linear range. **NOTE:** For the DA which is automated and programed to self-calibrate at the beginning of each analytical run, this section is a virtually meaningless. In fact, the DA system calculates the best regression equation fit to the results of the analysis of each standard versus the nominal concentration of nitrate in the standard calibrants. The best fit is not always linear and the program often uses a polynomial equation for calibration and to calculate the nitrate content (mg N/L) of unknown samples as well as QC standards. Thus, there seems to be little value in determining the LCR for a DA method.

9.2.2 **Quality Control Sample (QCS):** QCS is a standard of known concentration from an external source and different from calibration standards. QCS must be analyzed initially and quarterly or as required to meet QC requirements. If the QCS analysis result is not within ±10% of the known concentration, the test has failed. The source of the problem must be identified and corrected before continuing with IDP. **NOTE:** Again for the DA, a 2^{nd} source independent reference standard (SSC – Section 9.3) is included in every analytical run and must pass the acceptance limit of ±10% of the known values of the certified nitrate standard before the run can be continued. This is equivalent to the QCS, but done with every analytical run on the DA.

9.2.3 **Reduction Efficiency (RE):** Analyze a nitrite standard (Section 7.6) for determining the Reduction Efficiency. The nitrite standard will contain 2.5 mg NO_2^- N/L and the absorbance at 540 nm will be compared to the Second-source calibrant (2^{nd}RS) absorbance at 540 nm:

RE = [(2nd Source A-540)/(Nitrite A-540) X 100].

For acceptable results, RE must be 90% or greater. If the RE is unacceptable, the source(s) of the problem must be determined and the DA re-calibrated.
NOTE: The likely source of a problem with the RE is - either the NaR is bad and needs to be replaced, or the NADH has expired and needs to be replaced.

9.2.4 **Method Detection Limit (MDL):** The MDL must be established for the analyte using reagent water (blank) fortified at a concentration near the detection limit of the instrument. Perform the MDL study according to "40 CFR Part 136 Appendix B". Samples should be prepared using reagent water fortified with nitrate at a concentration between 1 to 5 times the expected MDL. First, calibrate the instrument (see section 9.3). MDL is determined by analyses of seven replicates of fortified reagent water (spiked) and processed through the entire analytical method. Calculate the replicates analyte concentration in system units using the instrument calibration (see Section 10). Calculate MDL as follows:

$$MDL = (t) \times (S)$$

Where,

t = Student's t value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom (t = 3.143 for seven replicates) and

S = standard deviation of the seven replicate analyses.

If the concentration of the sample (spike) used to determine the MDL is greater than 5X the calculated MDL repeat the MDL determination at a lower concentration for the sample (spike) studied. MDL should be determined every six months, when a new operator begins work, or whenever there is a significant change in the background or instrument response.

9.3 **Calibration and Calibration Verification:** Prior to the analysis of samples, calibrate the instrument using at least four working standards containing concentrations of nitrate in water that bracket the expected sample concentration. Before each analysis, verify the calibration with a mid-range second source calibrant (SSC) from an external source and different from calibration standards. The result should fall within ± 10 % of the known concentration. If the SSC is not within ± 10%, determine the problem, and recalibrate the instrument.

9.4 **Initial Performance and Recovery (IPR):** If a laboratory has not performed this test method before, or if there has been a major change in the measurement system, for example, new analyst, new instrument, and so forth, a precision and bias study must be performed to demonstrate laboratory capability. Analyze four replicates of a standard solution prepared from a Certified Independent Reference Material (CRM) containing a mid-range concentration of nitrite-nitrate in water. The matrix and chemistry of the solution should be equivalent to the solution used in the collaborative study. Each replicate must be taken through the complete analytical test method including any sample preservation and pretreatment steps. Calculate the mean and standard deviation of the four values and compare to the acceptable ranges provided by the manufacturer of the CRM. Do not use this test method to analyze samples unless the IPR recoveries are within the accepted limits.

9.5 **Ongoing Performance and Recovery Sample (OPR):** To ensure that the test method is in control, analyze a OPR containing a mid-range concentration of nitrite-nitrate in water with each batch or 10 samples. If large numbers of samples are analyzed in the batch, analyze the OPR after every 10 samples. The OPR must be taken through all of the steps of the analytical method including sample preservation and pretreatment. The result obtained for the OPR shall fall within ± 10% of the known concentration. If the result is not within these limits, analysis of samples is halted until the problem is corrected, and either all the samples in the batch must be reanalyzed, or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

9.6 **Method Blank:** Analyze a reagent water test blank with each batch or 10 samples. The concentration of nitrite-nitrate in water found in the blank should be less than the MDL. If the concentration of nitrite-nitrate in water is found above this level, analysis of samples is halted until the contamination is eliminated, and a blank shows no contamination at or above this level, or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

9.7 **Matrix Spike (MS) and Matrix Spike Duplicate (MSD):** To check for interferences in the specific matrix being tested, perform MS and MSD on at least one sample from each batch by spiking an aliquot of the sample with a known concentration of nitrite-nitrate in water and taking it through the analytical method. The spike concentration plus the background concentration of nitrite-nitrate in water must not exceed the high calibration standard. The spike must produce a concentration in the spiked sample that is 2 to 5 times the nitrite-nitrate in water concentration in the unspiked sample, or 10 to 50 times the detection limit of the test method, whichever is greater.

Calculate the percent recovery of the spike (P) using the following formula:

$$P = 100 \times \frac{[A \times (V_s + V) - (B \times V_s)]}{C \times V}$$

In this equation, A = nitrite+nitrate concentration (mg/L) in spiked samples, B = nitrite+nitrate concentration (mg/L) in unspiked samples, C = nitrite+nitrate concentration (mg/L) in the spiking solution, V_s = sample volume (mL) used, and V = added spiking solution volume (mL).

The percent recovery of the spike shall fall within ±10%, based on the nitrite-nitrate in water concentration. If the percent recovery is not within these limits, a matrix interference may be present in the sample selected for spiking. Under these circumstances, one of the following remedies must be employed: the matrix interference must be removed, all samples in the batch must be analyzed by a test method not affected by the matrix interference, or the results must be qualified with an indication that they do not fall within the performance criteria of the test method.

9.8 **Independent Reference Material (IRM):** In order to verify the quantitative value produced by the test method, analyze an Independent Reference Material (IRM) submitted as a regular sample (if practical) to the laboratory at least once per quarter. The concentration of the IRM should be in the concentration mid-range for the method chosen. The value obtained must fall within the control limits established by the laboratory.

10. Calibration and Standardization

10.1 Using the standard nitrate solution (section 7.8) prepare calibration standards by using the automated calibration function of the discrete analyzer (section 3.2). Table 2 specifies suggested calibrants. **NOTE**: Most discrete analyzers generate calibration standards and calibration curves automatically. Follow the manufacturer's instructions for calibrating with individual calibration standards, if an automatic calibration function is not available.

10.2 Prepare at least one calibration standard from the standard nitrite solution (section 7.10) at the same concentration as one of the nitrate standards to verify the efficiency of the reduction. Verify that reduction efficiency is greater than 90% with each batch of enzyme. See Table 3 for an example of Reduction Efficiency Analysis.

10.3 Run the calibration analysis as described in section 11 for sample analysis.

10.4 Prepare a standard curve by plotting the absorbance of each processed calibration standard against its known concentrations. **NOTE**: Most discrete analyzers generate calibration curves automatically.

11. Procedure

11.1 Removal of Color Interferences: If there is a possibility that the color of the sample might absorb in the photometric range from 540 ± 20 nm, determine the background absorbance. **NOTE**: Many discrete analyzers automatically compensate for background absorbance and turbidity on each sample. Follow the manufacturer's instructions.

11.2 Prepare a method in the discrete analyzer software following these specifications:

- 11.2.1 Dispense 55 µL of NaR (section 7.6) and 5 µL of sample. Mix.
  NOTE: Larger volumes of samples and reagents may be used while maintaining the same ratio (See Table 4).
- 11.2.2 Add 12 µL of NADH (section 7.3). Mix and measure the background absorbance.
- 11.2.3 Incubate 600 seconds at 37ºC.
- 11.2.4 Add 25 µL of SAN reagent (section 7.4). Mix and incubate 120 seconds at 37ºC.
- 11.2.5 Add 25 µL of NED reagent (section 7.5). Mix and incubate 120 seconds at 37ºC.
- 11.2.6 Measure absorbance at 540 nm, using an optional background subtraction at 700 nm. If automatic background subtraction is not available, prepare a reagent blank (with SAN only added) for background subtraction at 540 nm.

11.3 When determining nitrite alone, replace NaR reagent (section 7.6) with Phosphate Buffer (section 7.1).

12. Data Analysis and Calculations

12.1 Determine the concentration of nitrate or nitrite nitrogen in the samples in mg N/L using the computer based data handler provided with the automated discrete analyzer software. **NOTE**: The discrete analyzer will automatically calculate the net absorbance by subtracting the background absorbance from the measured absorbance of the color developed sample. Use the net absorbance to determine the concentration of nitrogen in the sample.

12.2 Where separate values are required for nitrite-nitrogen and nitrate-nitrogen, calculate the nitrate-nitrogen by subtracting the nitrite-nitrogen from the total nitrate-nitrite nitrogen content.

12.3 Report the nitrogen content in mg N/L as:

Nitrite-Nitrogen (NO_2-N), mg N/L;
Nitrate-Nitrogen (NO_3-N), mg N/L; and
Combined Nitrate-Nitrite Nitrogen (NO_3, NO_2-N), mg N/L.

13. Method Performance

13.1 A comparison of results by this method and cadmium reduction method (EPA 353.2) is shown in Table 5A and Table 5B. Also Figure 1 presents a graphic showing the comparison of the EPA Method 353.2 to NaR Nitrate Method for two enzyme forms using environmental water samples.

13.2 The performance of this method was validated by an Inter-Laboratory Study (See Plan for Inter-Laboratory Test Trial) and preliminary results of that study are appended here. Comparison of this Method to EPA Method 353.2 are summarized in Table 6. These data are plotted in a comparison in Figure 2. The regression line fit for Drinking Water matrices shown in Fig. 2A, indicates that the NaR Reduction Method results were ~95% of Cd Reduction Method for the 4 Drinking Water matrices. The linear regression fit for Waste Water matrices shown in Fig. 2B, indicates that the NaR Reduction Method results were ~98% of Cd Reduction Method for 7 Waste Water matrices (WW-5 was omitted from graphic). When the results for the WW-5 matrix are included, the NaR Reduction Method results were 100% of Cd Reduction Method for 8 Waste Water matrices. Basically, the two methods are equivalent. More detailed analysis of the results of the Inter-Laboratory Validation Study of the Nitrate Reductase Reduction Method will be published separately.

14. Pollution Prevention

All reagents and standards should be prepared in volumes consistent with laboratory use to minimize the generation of waste.

15. Waste Management

All waste should be disposed of in a manner consistent with local regulations. **NOTE**: This method uses very small volumes of samples and reagents and, therefore, less waste is generated than with most analysis methods.

16. References

Campbell, Wilbur H., P Song, GG Barbier (2006) Nitrate Reductase for Nitrate Analysis in Water. Environmental Chemistry Letters, 4: 69-73.

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Patton, C.J., and Kryskalla, J.R., 2013, Analytical properties of some commercially available nitrate reductase enzymes evaluated as replacements for cadmium in automated, semiautomated, and manual colorimetric methods for determination of nitrate plus nitrite in water: U.S. Geological Survey Scientific Investigations Report 2013–5033, 36 p., http://pubs.usgs.gov/sir/2013/5033/.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1993. Methods for Chemical Analysis of Water and Wastes. Method 353.2. Revision 2.0. U.S. Environmental Protection Agency, Washington, DC

17. Tables, Diagrams, Forms, Flowcharts, and Validation Data

Table 1 Determination of Nitrate in the Presence of Potential Interferences

Species	Concentration Added (mg/L)	Unspiked Sample Result (mg N/L)	Spiked Sample Result (mg N/L)	Spike Added (mg N/L)	Percent Recovery
Cl^-	500	0.02	0.23	0.200	105
		0.17	2.54	2.50	95
F^-	500	0.01	0.22	0.200	105
Br^-	500	<0.01	0.21	0.200	100
		0.15	2.65	2.50	100
SO_4^{2-}	500	<0.01	0.21	0.200	105
		0.14	2.53	2.50	96
Fe^{3+}	500	0.17	2.60	2.50	97
	1.0	<0.01	0.21	0.200	105
		0.168	2.59	2.50	96
Zn^{2+}	1.0	<0.01	0.22	0.200	110
		0.14	2.64	2.50	100
Al^{3+}	1.0	<0.01	0.21	0.200	105
		0.14	2.53	2.50	96
BrO_3^-	1.0	<0.01	0.22	0.200	110
		0.17	2.64	2.50	99
ClO_2^-	1.0	0.01	0.22	0.200	110
		0.14	2.54	2.50	96
ClO_3^-	1.0	0.23	2.45	2.50	89

TABLE 2 Example Concentrations of Calibration Standards

NO_3^--N or NO_2-N, mg/L	**mL of 10 mg N/L Standard per 100 mL final volume**
0.01	0.1
0.05	0.5
0.1	1.0
0.5	5.0
1.0	10
2.0	20
3.0	30
5.0	50

Table 3. Determination of Reduction Efficiency

NO_2-N/L	NO_3-N/L	Reduction Efficiency
5.19	4.89	94%
5.18	4.99	96%
5.24	4.91	94%

Table 4. Scaled sample and reagent volumes for implementation at larger assay volumes.

Volume (µL)	x1	x2	x3	x4	x5
Sample	5	10	15	20	25
AtNaR2	55	110	165	220	275
NADH	12	24	36	48	60
SAN	25	50	75	100	125
NED	25	50	75	100	125
Total Assay	122	244	366	488	610

Table 5A.
Comparison of Nitrate Analysis by Cadmium Reduction (EPA Method 353.2) and the Nitrate Reductase (NaR) Method

The Cadmium Reduction analysis of Nitrate-N plus Nitrite-N in samples was carried out using an automated continuous flow analyzer system which meets the specifications defined in EPA Method 353.2 Revision 2.0 (EPA, 1993). The analysis was done in complete compliance with EPA Method 353.2 by OI Analytical.

The NaR Method was carried out on the OI Analytical Discrete Analyzer using AtNaR2 with a protocol modified from the Method described herein to adapt it to this equipment, which basically involves doubling of sample and all reagent volumes.

Sample #	Analysis by Cd Reduction (mg NO_3+NO_2-N/L)	Analysis by NaR Reduction (mg NO_3+NO_2-N/L)	Analysis by NaR Reduction (mg NO_3+NO_2-N/L) Non-Preserved
1	0.96	0.94	0.88
2	0.04	0.05	0.06
3	0.32	0.24	0.55
4	0.68	0.68	0.58
5	10.1	11.6	Lost
6	0.75	0.79	0.77
7*	2.5	3.11	2.88

*Sample #7 contains sulfide. Sulfide reacts with cadmium forming cadmium sulfide decreasing the efficiency of the reduction

Table 5B. Comparison of Cadmium Reduction and Nitrate Reductase Reduction Methods.

Data from Table 5 Comparison of Third Party Check Determination for Cadmium Reduction Method by Continuous Flow Analysis (CFA) and Nitrate Reductase (YNaR1) Reduction Method by CFA (Patton and Kryskalla, 2013), and Table 12 Third Party Check Determination for Nitrate Reductase (AtNaR2) Reduction Method by Discrete Analyzer (DA). (Patton and Kryskalla, 2011)

Target Concentration (mg N/L)	Sample Identification	Number of Analysis	Mean Concentration Found ± Standard Deviation (mg N/L) And (Relative Standard Deviation)		
			Cadmium Reduction by CFA	NaR Reduction	
				CFA with YNaR1	DA with AtNaR2
0.20	092 L	63	0.19 ± 0.01 (3.53 %)	0.19 ± 0.01 (3.67 %)	
0.50	TPC-L	143			0.49 ± .02 (3.3 %)
2.00	092 M	62	2.00 ± 0.05 (2.26 %)	2.00 ± 0.05 (2.69 %)	
2.00	TPC-M	143			1.99 ± 0.06 (3.1 %)
4.00	092 H	63-65*	4.07 ± 0.07 (1.61 %)	4.02 ± 0.11 (2.63 %)	
4.00	TPC-H	143			4.03 ± 0.11 (2.7 %)

*For 092 H sample, 65 analyses were done for Cadmium Reduction and 63 analyses for NaR Reduction.

Table 6. Comparison of Cadmium Reduction Method to Nitrate Reductase Method.

Cadmium Reduction Method (EPA 353.2) was carried out by Continuous Flow Analyzer (CFA) and the Nitrate Reductase (NaR) Method (this document) was done by Discrete Analyzer (DA) in a multiple laboratory study involving 10 different laboratories. The samples matrices are identified in the Study Plan and consisted of 4 Drinking Water matrices (DW), 8 Waste Water matrices (WW), Seawater (SW), and 3 Standard Reference Materials (SRM) from the US Geological Survey. Abbreviations: CdR = Cadmium Reduction Method; NaRR = Nitrate Reductase Reduction Method; RPD = Relative Percent Difference; and RSD = Relative Standard Deviation.

Matrix	Cd Reduction	NaR Reduction	RPD
Method	CFA (mg N/L)	DA (mg N/L)	CdR - NaRR
		Mean ± RSD	%
DW2	0.83	0.77 ± 2.3%	7.50
DW3	1.21	1.22 ± 1.8%	-0.82
DW4	7.02	6.70 ± 2.5%	4.66
DW5	0.49	0.44 ± 4.5%	10.75
WW1	0.03	0.03 ± 67%	0.00
WW2	8.27	7.7 ± 3.9%	7.14
WW3	0.26	0.23 ±4.3%	12.24
WW4	0.03	0.06 ± 100%	-66.67
WW5	272.6	273 ± 4.0%	-0.15
WW6	4.8	4.9 ± 4.1%	-2.06
WW7	0.06	0.02 ± 50%	100.00
WW8	14.1	13.9 ± 3.6%	1.43
SW1	0.03	0.02 ± 50%	0.00
SRM-1	0.48	0.44 ± 4.5%	8.70
SRM2a	2.36	2.30 ± 2.6%	2.58
SRM2b	ND	0.23 ± 8.7%	X

Figure 1. Snapshot of Figure 12 from Patton and Kryskalla, 2011.
Graphic illustrates the equivalency of Cadmium Reduction and NaR Reduction Methods with real world samples.

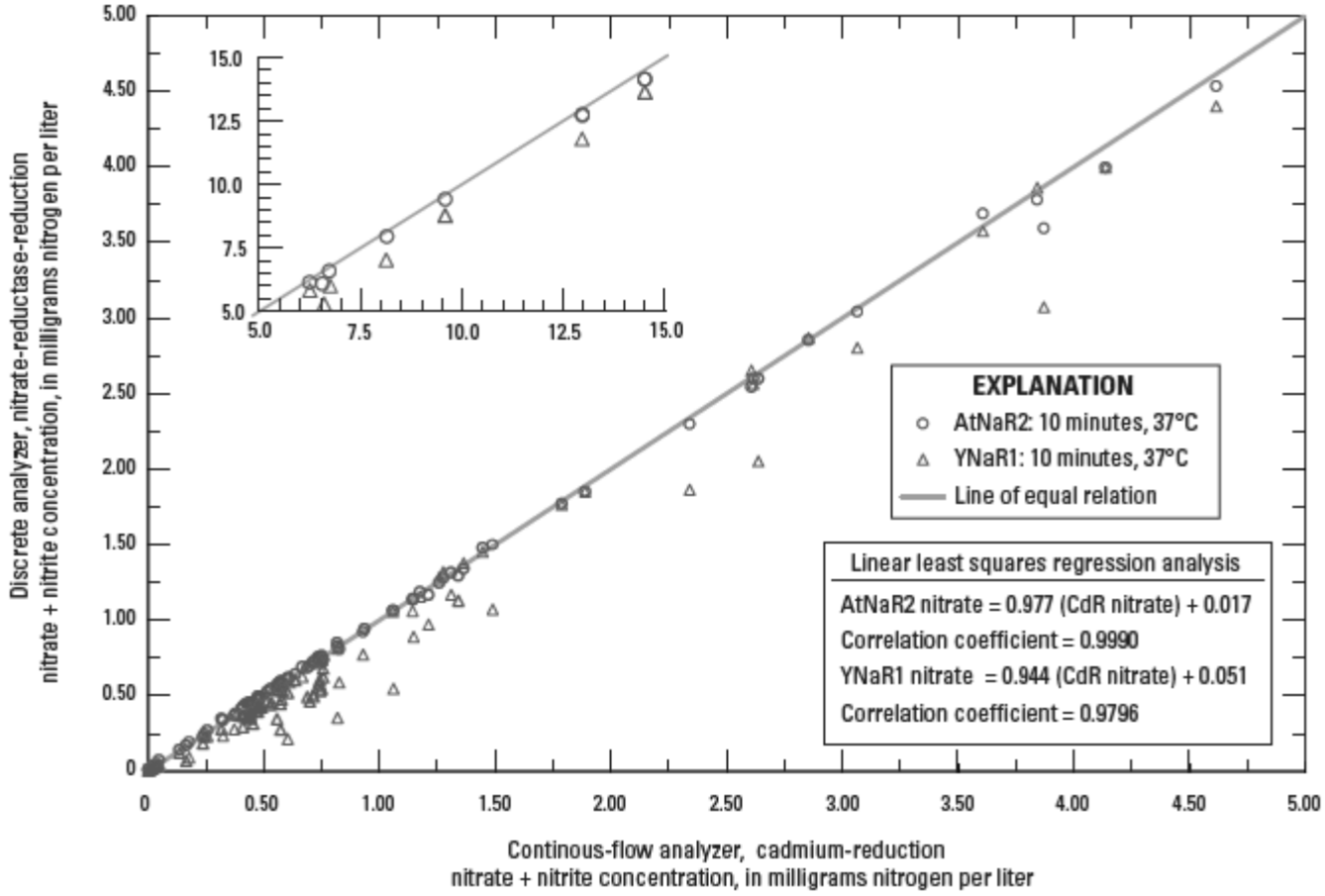

Figure 12. Comparison of nitrate + nitrite concentrations for 115 environmental water samples analyzed by automated continuous-flow, cadmium-reduction (CdR) method and automated discrete analyzer (DA) enzymatic-reduction method with NAD(P)H:YNaR1 nitrate reductase and NADH:AtNaR2 nitrate reductase. DA determinations were consecutive on November 9, 2005.

Figure 2. Comparison of Cadmium Reduction Method and Nitrate Reductase Method Results using Table 6 data.