Environmental Protection Agency

Alternate Test Procedure Validation

Report: Enzymatic Reduction Method of

Nitrate-N Analysis in Drinking Water

Compiled and Written by

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Report

Section 1 Introduction

This Validation Study Report supports development of Nitrate plus Nitrite Nitrogen by Nitrate Reductase (Campbell, et al., 2006), also called Enzymatic Reduction Method for Nitrate-N Analysis, for use as an USEPA Alternate Test Procedure (ATP) for Tier 3 level (nationwide) determination of nitrate plus nitrite nitrogen in drinking water (~~EPA, 1999~~). The enzyme Nitrate Reductase (EC 1.7.1.1/2, NaR; CAS #9029-27-0) catalyzes the reduction of nitrate to nitrite with the reducing power provided by the natural reductant, reduced Nicotinamide Adenine Dinucleotide (NADH), which is a thermodynamically irreversible reaction.

$$\text{Nitrate} + \text{NADH} + H^+ \rightarrow \text{Nitrite} + NAD^+ + H_2O$$

This is the basis for a "green", non-toxic method for nitrate-N analysis.

Eukaryotic NaR is a complex enzyme which contains a polypeptide chain of more than 900 amino acid residues and two metal ions (Fe^{3+} and Mo^{6+}) and three organic cofactors (Flavin Adenine Dinucleotide (FAD), Heme, and Molybdopterin) (Campbell, 1999). Since the natural NaR is of low abundance in plants, algae and fungi, recombinant DNA technology is utilized to produce the enzyme from a plant NaR gene (*Arabidopsis thaliana*) in the yeast *Pichia pastoris* which is designated AtNaR2 (Campbell et al., 2006). A second form of NaR, which is designated YNaR1 (Barbier et al., 2004), is produced from a yeast NaR gene (*Pichia angusta*, recently renamed *Ogataea angusta*, but originally named *Hansenula polymorpha*) recombinantly expressed in the yeast *Pichia pastoris.*

Recombinant AtNaR2 and YNaR1 are purified from the Pichia yeast extract to near homogeneity using immobilized metal ion affinity chromatography via the Histidine-tag built into the recombinant gene products. The purified AtNaR2 and YNaR1 are highly stable and can be stored frozen in a buffered solution at -80°C indefinitely. Furthermore, when the AtNaR2 and YNaR1 are freeze-dried and stored, dry and under vacuum in an opaque package, they can be shipped at room temperature and will remain stable for up to 6 months.

NaR-based Nitrate-N analysis is formulated as a method with a small volume, which is ideal for modern instruments such as the automated Discrete Analyzer (DA) and Flow Injection Analyzer (FIA). The method's formulation consists of a biochemical buffer to maintain pH near neutrality, the reconstituted NaR (stable for 18 hours), a precise solution of NADH, and the small volume of sample to be analyzed for Nitrate-N content. For example, in the discrete analyzer, the volume of buffered AtNaR2 is 55 µL, NADH 12 µL, and sample 5 µL (Patton and Kryskalla, 2011; 2013). Compared to EPA method 353.2, where the sample is often 20 mL, the enzymatic reduction method has obvious advantages in sample and waste handling. After the reduction of nitrate to nitrite is complete, requiring about 10 min, the nitrite is determined colorimetrically as in EPA method 353.2, which involves the sequential addition of sulfanilamide (SAN) and N (1 Naphthyl)ethylenediamine dihydrochloride (NED) and measurement at 540 ± 20 nm.

Section 2 Inter-Laboratory Validation Study

The details of the Inter-Laboratory Validation Study are presented in this section. The Nitrate Elimination Co., Inc. (NECi), Lake Linden, Michigan, coordinated the Inter-Laboratory Drinking Water Validation Study of the Enzymatic Reduction Method based on Nitrate Reductase for determination of nitrate-N and nitrate/nitrite-N. In this study the Enzymatic Reduction Method for nitrate-N is directly compared to the Cadmium Reduction Method for nitrate-N (EPA Reference Method 353.2), in each participating laboratory for each sample matrix. In addition, two published studies have compared the Enzymatic Reduction Method to the Cd Reduction Method and demonstrated the equivalence of the two methods for determining nitrate-N and nitrate/nitrite-N in aqueous samples (Patton and Kryskalla, 2011; Patton and Kryskalla, 2013).

Section 2.1 Study Objective and Design Plan

The objective of the Inter-Laboratory Study of Nitrate-N Analysis by the Enzymatic Reduction Method based on Nitrate Reductase was to demonstrate the validity of the Method according to the Design Plan presented in Appendix A. The Design Plan was developed in accordance with ~~*ATP Protocol for Organic and Inorganic Analytes* (USEPA, 1999) in order to validate the Method for Tier 3 (nationwide) status for Drinking Water; and~~ Protocol for the Evaluation of Alternate Test Procedures for Organic and Inorganic Analytes in Drinking Water, Office of Water (MS-140), EPA 815-R-15-007, February, 2015.

The Study Plan was approved, 7July2015, by Dr. Steven Wendelken, Safe Drinking Water Act ATP Coordinator, U.S. Environmental Protection Agency, (Steven C. Wendelken, PhD, U.S. EPA, OGWDW/SRMD/TSC, 26 W. Martin Luther King Dr., Cincinnati, OH 45219).

The Sample Matrices analyzed in the Inter-Laboratory Drinking Water Validation Study are listed in Table 1. The Enzymatic Reduction Method based on Nitrate Reductase is described according to EPA format in Appendices B and C, which were incorporated in the Design Plan. This Method was implemented by the Participating Laboratories which are listed in Table 2, and directly compared to EPA Method 353.2.

The variety of drinking water matrices analyzed, along with direct comparison to the EPA Reference Method, validates the Nitrate-N Analysis by the Enzymatic Reduction Method based on Nitrate Reductase for compliance monitoring under National Primary Drinking Water Regulations (NPDWRs).

Section 2.2 Sources of Sample Matrices and Handling of the Samples

The list of Sample Matrices is presented in Table 1. The sample matrices were shipped in certified clean bottles according to the Study Plan and EPA protocols. Each sample Matrix bottle was labeled with the designated matrix identification (i.e. DW-2, and DW-3). Certified Nitrate Standards and Calibrants were purchased from ERA in individual, labeled bottles such that there was no handling of these until they reached the participating Laboratories. The packaged and sealed Sample Matrices and Nitrate Standards were shipped on "blue ice" to the Participating Laboratories by NECi (See Table 3). According to the Design Plan, once the Sample Matrices were shipped, the Participating Laboratories were to store them at 4°C until they completed the analysis. Certification documents are provided in Appendix D.

Section 2.3 Participating Laboratories and Analytical Equipment

The list of Participating Laboratories is presented in Table 2. Laboratory 4 dropped out of the study after the samples had been shipped to them and did not complete the analysis of the samples. Two Participating Laboratories (Labs 1 and 2) were equipped with automated discrete analyzers (DA). One Participating Laboratory (Lab 3) used a Lachat Flow Injection Analyzer with off-line nitrate reduction as described in Appendix C. Each Participating Laboratory also analyzed the sample matrices using an automated Cadmium Reduction Analyzer running EPA Method 353.2.

Reagents for the Nitrate analysis, including the enzyme Nitrate Reductase (AtNaR2 or YNaR1), were supplied to all laboratories by NECi. The enzyme (AtNaR2 or YNaR1) consisted of a vacuum sealed, opaque pouch with a desiccant and a vial of freeze-dried AtNaR2 or YNaR1 containing one unit of enzyme activity, where the unit of enzyme activity is defined as the amount of enzyme catalyzing the NADH-driven reduction of 1 µmol of nitrate to nitrite per min at 30°C and pH 7.5. AtNaR2 and YNaR1 when stored in this form at room temperature (~25°C) are stable for up to one year. Each laboratory reconstituted the enzyme in phosphate buffer, pH 7.5, at the time of analysis according to instructions provided with the enzyme packet. For each type of DA being used in the study, a specific set of Standard Operating Procedures (SOP) has been developed which condenses the Method in Appendix B.

Section 3 Validation Study Results and Discussion

The results from the Inter-Laboratory Validation Study are contained in the Excel® files in Appendix E. The original Excel® files have been provided on a flash drive, which accompany this Report. Included within the Summary Excel® file are the "bench sheets" where possible and, in some cases, as PDF or other files in the directory for each laboratory in the provided data sets. An explanation table for error codes used in the KoneLab AquaKem® DA raw data files is provided in Appendix E. For this section of the Report, summary tables have been prepared from the original Excel® Summary Data Sheet files (Appendix E).

Section 3.1 Summary of Quality Control

While summary data are present in this report for Quality Control Samples, it is to be understood that all analytical analysis done in this study were for data sets that were in compliance with QC acceptance standards. This stipulation applies for both the Enzymatic Reduction Method for Nitrate Analysis using Nitrate Reductase and Cadmium Reduction Method for Nitrate Analysis (Reference Method EPA 353.2).

Statistical analysis of Calibration Curves reported by each laboratory are summarized in Table 4. In many cases, the laboratories ran more than one standard curve and the summary in Table 4 represents selected statistical data. In all cases, the correlation coefficient (r^2) = 0.999 or greater. In each case, the regression fit parameters are utilized to generate an equation relating the Absorbance @ 540 nm (or 550 nm or Area in volts/sec) to the Nitrate-N content of the unknown sample, such that the concentration of Nitrate-N (mg N/L) can be calculated from the Absorbance or area of the absorbance peak in the case of FIA.

Enzymatic and cadmium reduction efficiencies for each laboratory are summarized in Table 5 by showing one example for each lab. All labs found enzymatic and cadmium reduction efficiency of 93% or greater, which meets the acceptance criterion of 90% or greater reduction efficiency. This establishes the effectiveness of the nitrate reductase-catalyzed enzymatic reduction of nitrate to nitrite under the conditions of the analytical method, as well as the cadmium reduction efficiency under the conditions of the analytical method. In Lab 1, reduction efficiencies were about the same for both methods; while Lab 2 found greater reduction efficiency with the NaR reduction method and Lab 3 greater reduction efficiency for cadmium reduction method.

Initial Performance and Recovery (IPR) and Ongoing Performance and Recovery (OPR) results from all laboratories are summarized in Table 6. IPR certified standard nitrate concentration was 2.01 mg N/L (see Appendix D). IPR individual and mean recoveries for the selected data in

Table 6 were from 95 to 105% for NaR Reduction Analysis, which meets the acceptance criterion of 100 ± 10%. IPR individual and mean recoveries for the selected data in Table 6 were from 93 to 99% for Cadmium Reduction Analysis, which meets the acceptance criterion of 100 ± 10%. IPR recoveries were greater for NaR Reduction Analysis than Cadmium Reduction Analysis in all three labs.

OPR certified standard nitrate concentrations were 2.01, 2.50, and 12.00 mg N/L (see Appendix D). OPR mean recoveries for the selected data in Table 6 were from 96 to 104% which meets the acceptance criterion of 100 ± 10%. All other OPR recoveries, which are not shown in Table 6, were within the acceptable range of 90 to 110% recovery(Appendix E - Excel® files).

The final Quality Control evaluation is the determination of Minimum Detection Limit (MDL) for each laboratory's equipment; these results are summarized in Table 7. All the MDL values are below the level of the lowest calibrant for these analyzers (Table 4), which indicates that the calibration curve for these analyzers is completely valid with respect to detecting nitrate-N at the lowest level of the calibration. Lab 1 had the highest MDL values found in this study for both the Nitrate Reductase Method on the AquaKem Discrete Analyzer, 0.0097 mg N/L, and the Cadmium Reduction Method, 0.03436 mg N/L (Table 7). Lab 2 found almost identical MDL values for the two methods: Nitrate Reductase Method, 0.006507 mg N/L; and Cadmium Reduction Method, 0.006694 mg N/L. Lab 3 running the FIA system found a higher MDL for the Nitrate Reductase Method, 0.009429, than the Cadmium Reduction Method, 0.006507 mg N/L. Since the MDL is dependent on the analytical equipment used with the Methods, the MDL values from the different labs cannot be compared directly.

Three published studies evaluated the MDL for Enzymatic Reduction Method for Nitrate-N Analysis (Patton et al., 2002; Patton and Kryskalla, 2011; Patton and Kryskalla, 2013). When the Method was run on an Air-segmented Continuous Flow Analyzer (Patton et al., 2002), the MDL was reported to be 0.006 mg N/L. When the Method was run on a Discrete Analyzer (Patton and Kryskalla, 2011), the MDL was reported to be 0.02 mg N/L. When the MDL of the reference method, EPA Method 353.2, was determined on an Air-segmented Continuous Flow Analyzer (Patton et al., 2002; Patton and Kryskalla, 2013), it was found to be 0.003 mg Nitrate-N/L. The lower MDL for the reference method is apparently due to differences in the analyzer equipment: Air-segmented Continuous Flow Analyzer uses the same cuvette for analyzing all samples and blanks; and the Discrete Analyzer uses a different cuvette for every sample and blank. Indeed, the Enzymatic Reduction Method for Nitrate-N Analysis, has a lower MDL for the Air-segmented Continuous Flow Analyzer than the Discrete Analyzer. Although the Discrete Analyzer uses a correction for background absorbance, it apparently does not correct for all the differences between the cuvettes (Patton and Kryskalla, 2011).

Section 3.2 Nitrate-N Content and Spike Analysis of Drinking Water Matrices

Each laboratory participating in the Study analyzed local tap water (DW-1) and the tap water spiked with 2 to 4 mg/L free chlorine (DW-1-Cl) using both Methods for Nitrate-N Analysis. In all cases the tap water was from a regulated municipal drinking water supply: Lab 1, Chicago City Water; Lab 2, New Haven, Connecticut, City Water; and Lab 3, Loveland, CO, City Water.

The results from Lab 1 for determination of the Nitrate-N Content and Spiking Studies are presented in Table 8. For the NaR Reduction Method, the Nitrate Content of the "as is" tap water (DW-1) and chlorinated tap water (DW-1-Cl), were virtually the same, 0.3237 ± 0.003698 and 0.3185 ± 0.01625 Nitrate-N mg/L, respectively, for the mean of 7 replicates and the standard deviation (Table 8, first page). Both DW-1 and DW-1-Cl were spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the NaR Reduction Method (Table 8, first page). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. There was virtually no difference between the results of spiking studies for DW-1 and DW-1-Cl, which indicates that chlorination of the tap water did not cause a matrix effect in the NaR Reduction Method for these samples.

For Lab 1 applying the Cd Reduction Method, the Nitrate Content of the "as is" tap water (DW-1) and chlorinated tap water (DW-1-Cl), were virtually the same, 0.2259 ± 0.008633 and 0.2186 ± 0.007162 Nitrate-N mg/L, respectively, for the mean of 7 replicates and the standard deviation (Table 8, second page). However, these Nitrate-N contents were lower than found by the NaR Reduction Method. Both DW-1 and DW-1-Cl were spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the Cd Reduction Method (Table 8, second page). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. There was virtually no difference between the results of spiking studies for DW-1 and DW-1-Cl, which indicates that chlorination of the tap water did not cause a matrix effect in the Cd Reduction Method for these samples.

Similar results were found by Lab 2 and Lab 3 for analysis of their tap water with and without chlorination. These results are not summarized in a table in this report, but may be found in the data included in Appendix E in the Summary Data Sheets. Labs 2 and 3, also found that there was no matrix effect for either the NaR Reduction Method or Cd Reduction Method for chlorination of their local tap water.

For the DW-2 High TDS Drinking Water Matrix, Lab 3 results for determination of the Nitrate-

N Content and Spiking Studies are presented in Table 9. For the NaR Reduction Method, the Nitrate Content of the DW-2 was, 0.2628 ± 0.001390 Nitrate-N mg/L, respectively, for the mean of 7 replicates and the standard deviation (Table 9, left column). DW-2 was spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the NaR Reduction Method (Table 9, left column). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. Analysis of the Nitrate Content of DW-2 with the Cd Reduction Method yielded 0.2606 ± 0.0045 Nitrate-N mg/L (Table 9, right column). DW-2 was spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the Cd Reduction Method (Table 9, right column). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. Thus, it is clear that the two methods produce very similar results for DW-2 and Drinking Water with High Total Dissolved Solids does not produce a matrix effect in either method.

For the DW-3 High TOC Drinking Water Matrix, Lab 2 results for determination of the Nitrate-N Content and Spiking Studies are presented in Table 10 For the NaR Reduction Method, the Nitrate Content of the DW-3 was undetectable, -0.020617 ± 0.001497 Nitrate-N mg/L, respectively, for the mean of 7 replicates and the standard deviation (Table 10, left column). DW-3 was spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the NaR Reduction Method (Table 10, left column). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. Analysis of the Nitrate Content of DW-3 with the Cd Reduction Method yielded 0.002450 ± 0.002696 Nitrate-N mg/L (Table 10, right column). DW-2 was spiked in duplicate according to the Study Plan with 3 levels of Nitrate-N mg/L, 0.5, 1.0, and 2.5, for analysis with the Cd Reduction Method (Table 10, right column). All the spike recoveries and recovery percentages were within acceptable limits of 85 to 115% and the Relative Percent Difference between the MS and MSD were also acceptable as less than 20%. Thus, it is clear that the two methods produce very similar results for DW-2 and Drinking Water with High Total Organic Carbon does not produce a matrix effect in either method.

3.3 Evaluation of the Accuracy of the Method

There was one reference standard analyzed by all the Participating Laboratories in this study (Table 11). The standard was SR-1 (ERA #698) with certified target value = 5.14 ± 0.094 mg N/L. Since this level of Nitrate-N content is greater than the highest calibrant for most the Participating Laboratories for either method, the matrix was diluted prior to analysis. Lab 1 running the Cd Reduction Method had a calibration range going up to 20 mg Nitrate-N/L and did

not need to dilute the sample prior to analysis with this method. Each Lab found the mean Nitrate-N content SR-1 to be within 10% of the ERA certified value using both methods (Table 11). Clearly, both methods are equally accurate for determining Nitrate-N content.

3.4 Comparison of the Enzymatic Reduction Method to the Cadmium Reduction Method

Every Drinking Water Sample Matrix analyzed in this Validation Study listed in Table 1 was analyzed by both the Nitrate Reductase Reduction and Cadmium Reduction Method, the EPA Reference Method (EPA Method 353.2). In general, no significant difference was found between the results obtained by the Nitrate Reductase Method and the Cadmium Reduction Method in Nitrate-N content in mg/L within the statistical limits of the methods in any of the Drinking Water Matrices. Perhaps, the greatest difference between the two methods was found for analysis of Nitrate-N content of DW-3 by Lab 2 (Table 10). Here it was found that the Sample Matrix had undetectable Nitrate-N content by the NaR Reduction Method, but that it had 3 ppb Nitrate-N by the Cd Reduction Method; however, this level of Nitrate-N is below the MDL of the method reported in Table 7. Lab 1 also analyzed DW-3 by both methods and found 36 ppb by the NaR Reduction Method, and an undetectable level by Cd Reduction Method. Clearly, the two methods gave similar results for this Drinking Water Matrix at the limit of detection of Nitrate-N in water.

With regard to Matrix Spike analysis, DW-1, DW-1-Cl, DW-2, DW-3 (see Table 1) were all analyzed by the Matrix Spike protocol using both the NaR Reduction Method and the Cd Reduction Method (Table 8, 9, and 10). No differences were found in the results of these studies. The results showed that none of these matrices produced a Matrix Effect on the results of either Method by demonstration of completely acceptable Spike Recoveries and acceptable comparison between the pairs of each Spike MS and MSD.

Section 4 Validation Conclusions

The USEPA requires for Tier 3 (nationwide) validation of an Alternate Test Procedure (ATP) Method that three different laboratories analyze the analyte content of one sample of three different finished drinking water matrices (EPA, 1999). Thus, the ATP Method will be in compliance with the Safe Drinking Water Act (CWA) and validate the ATP Method for compliance monitoring under National Primary Drinking Water Regulations (NPDWRs).

For the Enzymatic Reduction Method for Nitrate-N Analysis, the requirements of Tier 3 validation were met by analysis of three different finished drinking water matrices (see Table 1 for list of Drinking Water Sample Matrices analyzed in this study and Tables 8, 9, and 10 for

details of the analysis by 3 of the 4 laboratories listed in Table 2). The spiking studies of the Drinking Water Sample Matrices (spikes of 0.5, 1.0 and 2.5 mg Nitrate-N/L analyzed in duplicate) indicated that there was little or no matrix effect on the Method by the three drinking water matrices (Table 8, 9, and 10); including chlorination of tap water.

Accuracy of the Method was shown to be very high by analysis of the standard reference SR-1 (Table 11) and the precision of the Quality Control results (Tables 6). The MDL evaluation of the equipment used in the study demonstrated that the Method has a detection limit of 0.050 mg N/L or less on the DA and FIA analyzers used in the study (Table 7). Few interfering substances, if any, were discovered in the present study. Previous analysis of interferences with specific compounds showed that there was little interference with the Method (Patton and Kryskalla, 2011; Patton and Kryskalla, 2013). See also data on interferences in The Method description in Appendix B.

Comparison of the analysis of the three drinking water matrices and the reference standard by the Enzymatic Reduction Method and the certified Cadmium Reduction Method (EPA Method 353.2) indicated that very similar results were obtained (Table 8, 9, and 10). Previous studies have also found the two methods yielded similar results (Patton et al., 2002; Patton and Kryskalla, 2011; 2013; Ringuet et al., 2011).

In summary, the Enzymatic Reduction Method for Nitrate-N Analysis has been validated by a robust Inter-Laboratory Study of Drinking Water Sample Matrices. All laboratories analyzing the Sample Matrices met all Quality Control criteria for valid analyses prior to analyzing the samples. The Calibration Curve, Nitrate Reduction Efficiency, and IPR/OPR recoveries (Tables 4, 5, and 6) were within the acceptable range before analyzing the unknown Sample Matrices. Analysis of certified Nitrate Standards indicated that the Method is highly accurate and capable of providing definitive analysis of Nitrate-N content of Drinking Water in the field. For Discrete Analyzers running the Method, the MDL ranged from 0.0066 to 0.0097 mg N/L; while the Flow Injection Analyzer had an MDL = 0.0094 (Table 7). Thus, the requirements of the ~~Tier 3 level~~ Alternate Test Procedure Protocol have been met for validation of the Enzymatic Reduction Method for Nitrate-N Analysis by the Inter-Laboratory Validation Study reported herein. The Method is ready for compliance monitoring under National Primary Drinking Water Regulations (NPDWRs).

Section 5 References and Glossary

~~*Protocol for EPA Approval of Alternate Test Procedures for Organic and Inorganic Analytes in Wastewater and Drinking Water*, USEPA, 1999.~~
~~http://water.epa.gov/scitech/methods/cwa/atp/upload/2007_02_06_methods_atp_EPA821B98003.pdf~~

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Glossary:

DA = Automated Discrete Analyzer

ERA = http://www.eraqc.com/

FIA = Flow Injection Analyzer

IPR = Initial Performance and Recovery

OPR = Ongoing Performance and Recovery

MDL = Method Detection Limit (http://www.gpo.gov/fdsys/pkg/CFR-2012-title40-vol24/pdf/CFR-2012-title40-vol24-part136- appB.pdf)

MS = Matrix Spike

MSD = Matrix Spike Duplicate

QA = Quality Assurance

QC = Quality Control

SDWA= Safe Drinking Water Act

TDS = Total Dissolved Solids

TOC = Total Organic Carbon

Table 1.
Drinking Water Matrices for Analysis

Each Sample Matrix (except DW-1) will be provided to Participating Laboratories as "Ready to Analyze" (samples will be filtered with 0.45 µm filter and preserved by acidifying) and in sufficient volume to permit multiple analysis. We will supply each lab with Chlorine Standard Solution, 25-30 mg/L as Cl_2, 2 mL PourRite Ampules (NIST)

Sample Matrix	Identifier	Number Replicates	Spikes*	Laboratories
Finished drinking water: from cold water tap of participating laboratories on day of analysis	DW-1	7	3 MS & MSD At 0.5 mg N/L At 1.0 mg N/L At 2.5 mg N/L	All
Local Tap Water with 2-4 mg/L free chlorine added	DW-1 + Cl_2	7	3 MS & MSD At 0.5 mg N/L At 1.0 mg N/L At 2.5 mg N/L	All
Drinking water with high TDS (~ 500 ppm)	DW-2	7	3 MS & MSD At 0.5 mg N/L At 1.0 mg N/L At 2.5 mg N/L	Lab 1 and 4
Drinking water with high TOC (~2 ppm)	DW-3	7	3 MS & MSD At 0.5 mg N/L At 1.0 mg N/L At 2.5 mg N/L	Lab 2 and 3
ERA #698 Standard Reference	SR-1**	7	NONE	All

*Each MS and MSD sample will be analyzed once. See Appendix D for method of preparing spikes and the layout of a set of analysis of typical DW-1 and its spikes.

SR-1 is Reference Standard of known Nitrate-N mg/L concentration (not acidified) and **must be analyzed by all Participating Laboratories. **SR-1 will require dilution with reagent water.**

Table 1, NOTES:

A. Preparation of Chlorinated Tap Water:

Provided Chlorine Standard Solution, 25-30 mg/L as Cl_2, 2 mL PourRite Ampules (NIST)

1. In clean tube, pipet 9.0 mL of tap water.
2. Open ampule of Chlorine Standard Solution.
3. Pipet 1.0 mL of Chlorine Standard Solution into tube with tap water.
4. Mix to prepare chlorinated tap water with 2.5 to 3.0 mg/L free chlorine.
5. Analyze the chlorinated tap water as DW-1+CL with 7 replicates by both methods.
6. Also analyzed chlorinated tap water as DW-1+CL spiked at 0.5, 1.0 and 2.5 mg N/L in duplicate (MS and MSD). One analysis each for MS and MSD.

B. Non-Acidified DW-2 and DW-3:

1. By request, Laboratory 4 Sample Matrices DW-2 and DW-3 will be filtered but not acidified.

C. Material Holding:

1. Sample Matrices, Standard Calibrants, and QC Samples **will be held at 4°C until analyzed**.
2. The list of materials to be shipped to each laboratory are enumerated in Table 3.
3. The materials will be shipped on "blue ice" (i.e. ~4°C) via overnight shipping.

D. Analysis Window:

1. Each Participating Laboratory will have thirty days (30 days) to complete analysis from the day they receive the materials.

Table 2.
Participating Laboratories

Lab #	Lab Name	Contact	Analytical Equipment
1	USEPA Chicago Region 5 Lab	Francis Awanya, Chemist, Group Leader	Discrete Analyzer And Cd-Reduction Analyzer
2	Unity/Westco Scientific	Bill Georgian Method Development	Discrete Analyzer And Cd-Reduction Analyzer
3	Lachat Instruments	Lynn Egan	Flow Injection Analyzer – Both NaR and CdR Methods
4	Univ. of Maryland/Solomons	Jerry Frank	Discrete Analyzer And Cd-Reduction Analyzer

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~~**Lab 4**~~
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Table 3

List of Materials Shipped to Four (4) Participating Labs

And

Two (2) Complete Sets Retained by NECi

Certification Documents for Items are in Appendix D

Item	Source	Preparation	Volume (mL)	Date Shipped
DW-2	NECi	NECi	50	8Jul2015
DW-3	ERA	NECi	50	
SR-1	ERA	NECi	50	
Chlorine Standard Solution	Hach	Sealed Ampule	2.0	
2.01 mg Nitrate-N/L	ERA	NECi	50	
2.50 mg Nitrate-N/L	ERA	NECi	50	
15.0 mg Nitrate-N/L	ERA	NECi	50	
50.0 mg Nitrate-N/L	ERA	NECi	50	
1000 mg/L Nitrite-N	ERA	NECi	10	

Table 4

Calibrants and Standard Calibration Curve Equation for Participating Labs

	Lab 1		Lab 2		Lab 3	
Calibration	**NaR-R**	**Cd-R**	**NaR-R**	**Cd-R**	**NaR-R**	**Cd-R**
Reagent Blank	0.00	0.00	0.00	0.00	0.00	0.000
Calibrant 1	0.05	0.10	0.05	0.05	0.05	0.050
Calibrant 2	0.25	0.25	0.10	0.10	0.10	0.125
Calibrant 3	0.50	0.50	0.25	0.25	0.50	0.250
Calibrant 4	0.75	1.00	0.50	0.50	1.00	0.500
Calibrant 5	1.25	5.00	1.25	1.25	2.50	1.000
Calibrant 6	2.50	10.00	2.50	2.50	5.00	2.500
Calibrant 7	3.75	20.00	5.00	5.00		5.000
Calibrant 8	5.00					
Linear Regression	**Nitrate (mg N/L) = (Absorbance Sample - Intercept) / Slope**					
Slope	0.118					
Intercept	0.022					
R^2	0.99989					
Polynomial Regression	**Nitrate (mg N/L) = (A x Conc x Conc) + (B x Conc) + C or = (A x A-550 x A-550) + (B x A-550) + C**					
Constant A		-0.029	-0.1988	0.4224	0.13991	0.18204
Constant B		1.262	15.0219	5.1881	4.0092	11.7730
Constant C		0.018	-0.5308	-0.1879	0.071083	0.020945
R^2		0.99996	1.0000	1.0000	0.99999	1.0000

Table 5

Enzymatic and Cadmium Reduction Efficiency

Evaluated using 2.50 Nitrate-N mg/L and 2.50 Nitrite-N mg/L

	Lab 1		Lab 2		Lab 3	
	NaR-R	Cd-R	NaR-R	Cd-R	NaR-R	Cd-R
Nitrate Absorbance 540 nm, 550 nm, Or Area/sec	0.318	0.495	0.2080	0.4915	9.162	28.501
Nitrite Absorbance 540 nm, 550 nm, Or Area/sec	0.341	0.527	0.1963	0.5082	8.997	27.381
Catalytic Reduction Efficiency (NO_3/NO_2) Percent	93.2551%	93.9279%	105.9603%	96.7139%	101.8339%	104.0904%

Table 6

Initial Performance and Recovery (IPR)

and

Ongoing Performance and Recovery (OPR)

	Lab 1		Lab 2*		Lab 3	
	NaR-R	**Cd-R**	**NaR-R**	**Cd-R**	**NaR-R**	**Cd-R**
IPR Nitrate-N mg/L	**2.01**	**2.01**	**2.01**	**2.01**	**2.01**	**2.01**
IPR-1	2.0740	1.9128	2.0126	1.9411	2.0999	1.9787
IPR-2	2.0671	1.8777	1.9992	1.9561	2.1037	1.9791
IPR-3	2.0581	1.9454	1.9962	1.9052	1.9156	1.9765
IPR-4	2.0826	1.9619	1.9932	1.9113	2.0050	1.9701
Mean IPR	**2.0705**	**1.9245**	**2.0003**	**1.9284**	**2.0311**	**1.9761**
Mean IPR Recovery (%)	**103.0075**	**95.7438**	**99.5174**	**95.9415**	**101.0473**	**98.3134**
OPR Nitrate-N mg/L	**2.01**	**12.00**	**2.50**	**2.50**	**2.50**	**2.50**
Number OPR	**7**	**8**	**8**	**7**	**7**	**6**
Mean OPR	2.1388	11.8393	2.4726	2.5542	2.4740	2.5373
Standard Deviation of Mean	0.03685	0.208999	0.06118	0.116486	0.015445	0.011914
Relative Standard Deviation (%)	1.7233	1.7653	2.4744	4.5606	0.6243	0.4696
Mean Recovery (%)	**106.4065**	**98.6610**	**98.9035**	**102.1674**	**98.9600**	**101.4913**

*Lab 2 Cd-R - 1 OPR greater than 110%

Table 7

Minimum Detection Limit

Abbreviations: DA, Discrete Analyzer; FIA, Flow Injection Analyzer

	Lab 1		Lab 2		Lab 3	
	NaR-R	Cd-R	NaR-R	Cd-R	NaR-R	Cd-R
Analytical Equipment	**DA**	**DA**	**DA**	**DA**	**FIA**	**FIA**
Spike mg Nitrate-N/L	**0.050**	**0.050**	**0.025**	**0.020**	**0.010**	**0.025**
MDL mg Nitrate-N/L	**0.0097**	**0.03436**	**0.006507**	**0.006694**	**0.009429**	**0.006507**
Ratio Spike/MDL	5.1453	1.4553	3.8422	2.9877	1.0606	3.8422

Table 8

See attached PDF

Table 9

DW-2 High TDS Drinking Water Matrix Nitrate-N Content and Spike Analysis

Abbreviation: RPD = Relative Percent Difference

Lab 3 NaR Reduction Method DW-2

Nitrate-N mg/L	Mean Nitrate-N mg/L	Standard Deviation mg N/L	Relative Standard Deviation
0.2907	0.2959	0.004749	1.6048%
0.2973			
0.3023			
0.2985			
0.2939			
0.2992			
0.2893			

0.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
1.944	0.7900	0.4941	98.8226	0.0486
1.9437	0.7898	0.4939	98.7746	

1.0 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
2.8302	1.2489	0.9530	95.3003	0.3875
2.8375	1.2526	0.9567	95.6703	

2.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
5.8573	2.8417	2.5458	101.8321	0.4251
5.8371	2.8309	2.5350	101.4001	

Lab 3 Cd Reduction Method DW-2

Nitrate-N mg/L	Mean Nitrate-N mg/L	Standard Deviation mg N/L	Relative Standard Deviation
0.2659	0.2677	0.001071	0.4000%
0.2684			
0.2678			
0.2679			
0.2680			
0.2693			
0.2669			

0.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
15.418	0.7536	0.4858	97.1697	0.4125
15.386	0.7516	0.4838	96.7697	

1.0 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
24.932	1.2425	0.9748	97.4759	0.2050
24.970	1.2445	0.9768	97.6759	

2.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
53.061	2.7602	2.4925	99.6983	0.5512
52.407	2.7465	2.4788	99.1503	

Table 10

DW-3 High TOC Drinking Water Matrix Nitrate-N Content and Spike Analysis

Abbreviation: RPD = Relative Percent Difference

Lab 2 NaR Reduction Method DW-3

Nitrate-N mg/L	Mean Nitrate-N mg/L	Standard Deviation mg N/L	Relative Standard Deviation
-0.0213	-0.020617	0.001497	7.2622%
-0.0228			
-0.0199			
-0.0199			
-0.0185			
-0.0213			
-0.0143			

0.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.0507	0.4212	0.4418	88.3633	5.5884
0.0525	0.4466	0.4672	93.4433	

1.0 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.0870	0.9320	0.9526	95.2617	1.0341
0.0863	0.9222	0.9428	94.2817	

2.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.1878	2.3364	2.3570	94.2807	0.7068
0.1866	2.3198	2.3404	93.6167	

Lab 2 Cd Reduction Method DW-3

Nitrate-N mg/L	Mean Nitrate-N mg/L	Standard Deviation mg N/L	Relative Standard Deviation
0.0044	0.002450	0.002696	110.0301%
0.0018			
0.0057			
-0.0021			
0.0031			
0.0018			
0.0025			

0.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.1106	0.5147	0.5123	102.4500	3.0723
0.1082	0.4992	0.4968	99.3500	

1.0 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.1923	1.0417	1.0393	103.9250	1.5373
0.1948	1.0578	1.0554	105.5350	

2.5 mg N/L Spike				
A-540	mg N/L	Spike mg N/L	Recovery %	RPD %
0.4405	2.6422	2.6398	105.5900	3.2766
0.4273	2.5571	2.5547	102.1860	

Table 11

Standard Reference - SR-1

Seven Replicates Analyzed

ERA 698 Certified Value SR-1 = 5.14 ± 1.83% mg Nitrate-N/L

	Dilution	Diluted Mean mg Nitrate-N/L	Standard Deviation mg Nitrate-N/L	Relative Standard Deviation %	Final mg Nitrate-N/L	Compared to ERA
Lab 1 - NaRR	Auto-diluted 1:10	5.3989	0.03375	0.6251	5.3989	105.0195%
Lab 1 - CdR	NONE	5.0937	0.07998	1.5701	5.0937	99.0992%
Lab 2 - NaRR	Auto-diluted 1:4	4.9795	0.07552	1.5166	4.9795	96.8783%
Lab 2 - CdR	1:4	1.0128	0.02132	2.1052	5.0640	98.5214%
Lab 3 - NaRR	1:2	2.5629	0.005100	0.1990	5.1258	99.7218%
Lab 3 - CdR	1:2	2.5902	0.01816	0.7011	5.1800	100.7782%