Environmental Protection Agency

Alternate Test Procedure Validation Report

for Enzymatic Reduction Method of Nitrate-N Analysis

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Table of Contents

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 wastewater, and other aqueous solutions (EPA, 1999). The enzyme Nitrate Reductase (EC 1.7.1.1, NaR) 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 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). Recombinant AtNaR2 is purified from the yeast extract to near homogeneity using immobilized metal ion affinity chromatography via the Histidine-tag built into the recombinant gene product. The purified AtNaR2 is highly stable and can be stored in a buffered solution at -80°C indefinitely. Furthermore, when the AtNaR2 is freeze-dried and stored, dry and under vacuum in an opaque package, it 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. 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 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 not directly compared to the Cadmium Reduction Method for nitrate-N (EPA Reference Method 353.2), since the automated discrete analyzers used by the participating laboratories do not run the Reference Method. However, one participating laboratory analyzed the study's sample matrices using the Cd Reduction Method running on a continuous flow analyzer and this provided a comparative data set. 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 both Drinking and Wastewater. The Study Plan was approved by Lemuel (Lem) Walker, Jr., Clean Water Act ATP Coordinator, U.S. Environmental Protection Agency, Office of Science and Technology, Engineering and Analysis Division (EAD) on 14June2013 (see Appendix A). Subsequently, the scope of the Inter-Laboratory Study was narrowed to just the Wastewater and Seawater Matrices, but the Study Plan was not altered to reflect this change. The Sample Matrices analyzed in the Inter-Laboratory Validation Study are listed in Table 1. The Enzymatic Reduction Method based on Nitrate Reductase is described according to EPA format in Appendix B, which was incorporated in the Design Plan. This Method was implemented by the Participating Laboratories which are listed in Table 2. The variety of wastewater matrices to be analyzed brings the study into compliance with the Clean Water Act (CWA).

Section 2.2 Sources of Sample Matrices and Handling of the Samples

The list of Sample Matrices is presented in Table 1. All matrices were collected on site and processed by filtration and preservation by addition of 4.5 Normal sulfuric acid to lower the pH to below 2 and cooled to <6°C, according to the Design Plan. The preservation status of the sample matrices is shown (Table 1).

The sample matrices were shipped in bulk to FACET Analytical Services and Technology, LLC, Indianapolis, IN 46217. FACET LLC filtered, acidified and aliquoted the sample matrices to certified clean bottles according to the Study Plan and EPA protocols (Appendix C). Each sample Matrix bottle was labeled with the designated matrix identification (i.e. WW-1, WW-2, etc.). 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 according to the plan developed by FACET and approved by NECi as shown in Appendix C. According to the Design Plan, once the Sample Matrices were shipped, the Participating Laboratories were to store them at 4°C and they had to complete the analysis within 30 days. The deadline for analyses completion was July 17, 2013. Certification documents for the calibrants and nitrate standards are provided in Appendix D. Each laboratory provided their own nitrite standard and the certification for the Nitrite Standard utilized by one laboratory is also included in Appendix D.

Section 2.3 Participating Laboratories and Analytical Equipment

The list of Participating Laboratories is presented in Table 2. A more detailed list of information on the Participating Laboratories is presented in Table 2 in the Study Plan (Appendix A). Laboratory 6 dropped out of the study after the samples had been shipped to them and did not complete the analysis of the samples. All the Participating Laboratories were equipped with automated discrete analyzers (DA).

Reagents for the Nitrate analysis, including the enzyme Nitrate Reductase (AtNaR2), were supplied to all laboratories by NECi. The enzyme (AtNaR2) consisted of a vacuum sealed, opaque pouch with a desiccant and a vial of freeze-dried AtNaR2 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 when stored in this form at room temperature (~25°C) is stable for up to six months. 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. As an example, the SOP for the OI Analytical DA has been added to 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 and F. The original Excel® files have been provided on a flash drive, which

accompanied the original version of this Report and have not been included with the current version of 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 previously provided data sets. An explanation table for error codes used in the KoneLab AquaKem® DA raw data files is provided in Appendix F. Supplemental Excel® File for the MDL Study by Laboratory 5 is provided in Appendix F, which is supplied by email attachment. For this section of the Report, summary tables have been prepared from the original and supplemental data in the Excel® Summary Data Sheet files Appendix E and F).

Section 3.1 Summary of Quality Control

Statistical analysis of Calibration Curves reported by each laboratory are summarized in Table 3A. In many cases, the laboratories ran more than one standard curve and the summary in Table 3 represents selected statistical data. In all cases, the correlation coefficient (r^2) = 0.999 or greater, except for Lab 7 and 10. However, both of these labs used a polynomial fit to the calibration results and found r^2 = 0.9999 and 0.9991, for labs 7 and 10, respectively. In each case, the parameters for the slope and intercept are utilized to generate an equation relating the Absorbance @ 540 nm (or 550 nm) 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. In cases where a polynomial equation is used to described the relationship of Absorbance to Nitrate-N content, the slope is replaced by constants "A" and "B", which are not listed in the table.

Table 3B catalogs the nitrate concentrations of the standards used in the calibration. This information is useful in comparing the results from different labs, since some labs used as many as 8 calibration concentrations, while some used as few as 5 calibration concentrations. In addition, Lab 12 used 0.10 mg N/L as the lowest calibrant, while most of the other labs using a discrete analyzer used 0.05 mg N/L (Lab 7 used 0.04 mg N/L).

Enzymatic reduction efficiencies for each laboratory are summarized in Table 4. All labs found enzymatic reduction efficiency of 95 % or greater. This establishes the effectiveness of the nitrate reductase-catalyzed enzymatic reduction of nitrate to nitrite under the conditions of the analytical method.

Initial Performance and Recovery (IPR) and Ongoing Performance and Recovery (OPR) results from all laboratories are summarized in Table 5 and 6, respectively. IPR certified standard nitrate concentrations were 2.00, 2.50, and 2.70 mg N/L (see Appendix D). IPR mean recoveries for the selected data in Table 5 were from 96 to 106%, which meets the acceptance criterion of 100 ± 10%. Other IPR analysis by the labs, which are not shown in Table 5, also fell within the acceptance criteria of 90 to 110% recovery (Appendix E - Excel® files). OPR certified standard nitrate concentrations were 2.00, 2.50, and 2.60 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 IPR and OPR results provide measures of the precision and accuracy of the Enzymatic Reduction Method of Nitrate-N Analysis. For IPR, five labs used the ERA 2.00 ± 0.02 mg N/L certified nitrate standard and obtained the mean value = 1.985 ± 0.048; RSD = 2.4391. Four labs used an 2.50 mg N/L certified nitrate standard and obtained the mean value = 2.573 ± 0.058 mg N/L; RSD = 2.2418 % and. Only one lab used the 2.70 mg N/L certified nitrate standard and found the mean value = 2.730 ± 0.026 mg N/L; RSD = 0.9463 %. Thus, there was less than a 2% difference between the values determined and the known values of the certified nitrate standards. The OPR values yielded RSD from 0.52 to 5.66 % (Table 6). Thus, the Enzymatic Reduction Method is highly precise and accurate based on these IPR and OPR results.

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 3B), 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.

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 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 Wastewater and Seawater Matrices

The Wastewater Matrices were analyzed for Nitrate-N content and spiked with 0.5 mg N/L and analyzed 6 times. These results are summarized in Table 8 and more detailed presentation of these results are presented in Table 9. Seawater was analyzed for Nitrate-N content and spiked

with 0.5 mg N/L and analyzed 6 times. These results are also summarized in Table 8 and 9. The Nitrate-N content of the matrices varied greatly and some had to be diluted before analysis. The sample matrices which were diluted to bring them within range were: WW-2, WW-5, WW-6, and WW-8. Relative Standard Deviations were less than 10%, except for three matrices. These three matrices with Relative Standard Deviations greater than 10% were ones that had Nitrate-N content less than the lowest calibrant of the Standard Curves: WW-1; WW-7; and SW-1. Nitrate-N content determined below the lowest calibrant of the Standard Curves may not be valid and would be expected to have greater error in the determination than ones falling within the range of the standard curves.

For the diluted matrices, the spiking was carried out with the diluted matrix. The acceptance criterion for spike recovery in the Wastewater and Seawater matrices was 77 to 121%. The acceptance criterion for the Relative Percent Difference (RPD) of the concentration results of the Matrix Spike (MS) and Matrix Spike Duplicate (MSD) pair was 22%.As shown in Table 8 and 9, all of the spiking results yielded data within the acceptance criteria for mean spike recovery and RPD. The mean spike recoveries varied from 85.0 to 112.4%. The RPD varied from 0.08 to 9.6%. No data from Laboratory 8 is in Table 8 or 9, since the spiking protocol specified in the Study Plan was not followed. Specifically, this laboratory did not spike samples at the specified concentration level for all pairs, resulting in data that were not comparable to those generated by the other laboratories.

Overall, none of the Wastewater Matrices, nor the Seawater Matrix, yielded a "matrix effect" on nitrate-N analysis by the Enzymatic Reduction Method, which indicates the method is not biased by the constituents of the sample matrices listed in Table 1. It is noteworthy that Nitrate-N content was successfully determined in seawater by the DA Enzymatic Reduction Method; however, the seawater matrix had such a low Nitrate-N content that the results were not valid, but the 0.5 mg N/L spikes were accurately determined. Thus, the salt content of seawater does not bias the Method even when no salt is included in the calibrants. A previous study of Nitrate-N determination in seawater by the Enzymatic Reduction Method also found that valid results were found compared to the cadmium reduction method; however, the calibrants in this study were prepared in synthetic seawater (Ringuet et al., 2011).

3.3 Evaluation of the Accuracy of the Method

There were three reference standards analyzed by the participating laboratories in this study (Table 10). The first standard was SR-1 (ERA #608) and it was analyzed by 6 laboratories yielding a total of 38 analyses. The mean Nitrate-N content of SR-1 was 6.6780 ± 0.0414 mg N/L (RSD = 0.6199 %), which compared to the certified target value = 6.80 ± 0.12 mg N/L. The mean recovery of SR-1 was 98.2059 %. The two other reference standards were from the USGS (SRM-1 and SRM-2). SRM-1 was analyzed by 7 laboratories for a total 36 analyses and the

mean Nitrate-N content found was 0.4413 ± 0.0103 mg N/L (RSD = 2.3340 %). The target value was the Most Probable Value found by multiple laboratory analysis and reported on the USGS website (see Table 9 footnote for the website URL; also see Appendix D for the USGS SRM1 data) was 0.443 ± 0.011 mg N/L (RSD = 2.4831 %). The mean recovery of SRM-1 was 99.6163 %. SRM-2 was analyzed by 7 laboratories for a total 25 analyses and the mean Nitrate-N content found was 2.2868 ± 0.0691 mg N/L (RSD = 3.0217 %). The target value was the Most Probable Value found by multiple laboratory analysis and reported on the USGS website (see Table 9 footnote for the website URL; also see Appendix D for the USGS SRM2 data) was 2.300 ± 0.009 mg N/L (RSD = 0.3913 %). The mean recovery of SRM-1 was 99.4261 %.

From the results of the analysis of the 3 standard references (Table 9) and the certified nitrate standards analyzed for Quality Control (see section 3.1), it is clear that the Enzymatic Reduction Method of Nitrate-N Analysis yields highly accurate and precise results.

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

The samples matrices listed in Table 1 were also analyzed by the cadmium reduction method for Nitrate-N content (EPA Method 353.2). These results are compared to results from the Enzymatic Reduction Method in Table 10. For most of the matrices, the Enzymatic Reduction Method resulted in determination of a higher Nitrate-N content than the Cadmium Reduction Method, but two matrices were greater by CdR and two were the same for the two methods. Most of the matrices analyzed were within the range of the standard curves of the Methods (or diluted to bring them into range), but four matrices were below the lowest calibrant used to prepare the standard curves. These 4 matrices were WW-1, WW-4, WW-7, and SW-1, which are footnoted in Table 10. These matrices had RPD greater than 10%, which is not unexpected since the determined values are not really valid. One matrix with low nitrate content (WW-1) had an acceptable RPD. All the other matrices had RPD less than 10%.

It is clear from the results in Table 10, that the Enzymatic Reduction Method gave comparable results to the certified EPA reference method. In addition, extensive studies comparing the Enzymatic Reduction Method and Cadmium Reduction Method for the Nitrate-N content of natural water samples at the USGS have shown the two methods yield comparable results (Patton et al., 2002; Patton and Kryskalla, 2011; 2013). A less extensive study comparing the two methods found “no differences in variability” meaning that the standard deviation of the analysis of analyte nitrate in double-distilled water were not significantly different ($p < 0.05$) (Ringuet et al., 2011).

Section 4 Validation Conclusions

The USEPA requires for Tier 3 (nationwide) validation of an Alternate Test Procedure (ATP) Method that nine different laboratories analyze the analyte content of one sample of eight different wastewater matrices, and one sample matrix of ocean water (EPA, 1999). Thus, the ATP Method will be in compliance with the Clean Water Act (CWA) and validate the ATP Method for nationwide application as a regulatory method.

For the Enzymatic Reduction Method for Nitrate-N Analysis, the requirements of Tier 3 validation were met by analysis of eight wastewater matrices and one seawater matrix by ten laboratories (see Table 1 for list of Wastewater Sample Matrices analyzed in this study and Tables 8 and 9 for details of the analysis by 9 of the 10 laboratories listed in Table 2). The spiking studies of the Wastewater Sample Matrices (spikes of 0.5 mg N/L analyzed six times) indicated that there was little or no matrix effect on the Method by the eight Wastewater matrices (Table 8 and 9). Seawater (ocean water) was also analyzed (Table 8 and 9). No significant effect of the seawater matrix spiked with 0.5 mg N/L nitrate and analyzed 6 times, was found on the Nitrate-N content determined for seawater (Table 8 and 9).

Accuracy of the Method was shown to be very high by analysis of 3 reference standards (Table 10) and the precision of the Quality Control results (Tables 5 and 6). The MDL evaluation of the equipment used in the study demonstrated that the Method has a detection limit of less than 0.050 mg N/L on the DA 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 eight wastewater and seawater matrices and three reference standards by the Enzymatic Reduction Method and the certified Cadmium Reduction Method (EPA Method 353.2) indicated that very similar results were obtained (Table 11). 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 Wastewater and Seawater 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 3, 4, 5, 6, and 7) 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 Wastewater

in the field. For Discrete Analyzers running the Method, the MDL ranged from 0.008 to 0.046 mg N/L (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.

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

Campbell, Wilbur H. (1999) Nitrate Reductase Structure, Function and Regulation: Bridging the Gap between Biochemistry and Physiology, Annual Review of Plant Physiology and Plant Molecular Biology 50:277-303.

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

Patton CJ, AE Fischer, WH Campbell & ER Campbell (2002) Corn leaf nitrate reductase: A nontoxic alternative to cadmium for photometric nitrate determinations in water samples by air-segmented continuous-flow analysis. Environmental Science and Technology, 36: 729-35.

Patton, C.J., and Kryskalla, J.R., 2011, Colorimetric determination of nitrate plus nitrite in water by enzymatic reduction, automated discrete analyzer methods: U.S. Geological Survey Techniques and Methods, book 5, chap. B8, 34 p. (Available on line at http://pubs.usgs.gov/tm/05b08/)

Patton, C.J., and Kryskalla, J.R., 2013, Analytical properties of some commercially available nitrate reductase enzymes evaluated as replacements for cadmium in automated, semi-automated, and manual colorimetric methods for determination of nitrate plus nitrite in water, USGS Scientific Investigations Report 2013-5033 (http://pubs.usgs.gov/sir/2013/5033/).

Ringuet, R., L. Sassano, and Z. L. Johnson (2011) A Suite of microplate reader-based colorimetric methods to quantify ammonium, orthophosphate, and silicate concentrations for aquatic nutrient monitoring. Journal of Environmental Monitoring, 13: 370-376.

Glossary:

DA = Automated Discrete Analyzer

IPR = Initial Performance and Recovery

OPR = Ongoing Performance and Recovery

MDL = Method Detection Limit

MS = Matrix Spike

MSD = Matrix Spike Duplicate

QA = Quality Assurance

QC = Quality Control

Table 1. List of Sample Matrices for Analysis

Sample Number	Sample Type	Matrix Identifier	Filtered	Acidified
Waste Water Samples				
1	Denver area treatment plant Influent wastewater	WW-1	Yes	Yes
2	Denver area treatment plant Wastewater effluent #1	WW-2	Yes	Yes
3	Denver area treatment plant Wastewater effluent #2	WW-3	Yes	Yes
4	Michigan paper mill waste stream effluent	WW-4	Yes	Yes
5	Denver area metal finisher waste stream effluent	WW-5	Yes	Yes
6	Denver area Commercial laundry waste stream effluent	WW-6	Yes	Yes
7	Environmental Resources Associates #507 Hardness WasteWatR reference material	WW-7	Yes	Yes
8	Michigan Confined Animal Feeding Operation (CAFO) effluent from tiled field	WW-8	Yes	Yes
Other Sample				
9	Low-nutrient seawater (collected offshore Hawaii)	SW-1	Yes	No
Reference Standards				
	ERA # 608 Reference Standard	SR-1	Yes	Yes
	USGS PE N-116 (low nutrient-fortified river water)	SRM-1	Yes	No
	USGS PE N-115 (high nutrient-fortified river water)	SRM-2	Yes	No

Table 2 Participant Laboratories

Lab Number	Lab Name	Contact	Analytical Method
2	USGS/NWQL	Charles Patton	Discrete Analyzer #1
3	USGS/NWQL	Charles Patton	Discrete Analyzer #2
4	OI Analytical	William Lipps	Discrete Analyzer
5	ThermoFisher	Stephen White	Discrete Analyzer #1
6*	ThermoFisher	Stephen White	Discrete Analyzer #2
7	ThermoFisher	Stephen White	Discrete Analyzer #3
8	Univ. of Maryland/Solomons	Jerry Frank	Discrete Analyzer
9	Klamath Tribes	Kris Fischer	Discrete Analyzer
10	Geochemical Testing	Tim Boergstresser	Discrete Analyzer
11	Unity/Westco Scientific	Bill Georgian	Discrete Analyzer
12	Astoria-Pacific	Winston Pavitt, CEO	Discrete Analyzer

*Laboratory 6 dropped out of the study.
Laboratory 1 employed a different analytical procedure than that used by the other laboratories (Lab Numbers 2-12). Therefore, those data have not been included as part of this final study report.

Table 3 Calibration of Enzymatic Reduction Method Summary

A. Statistics for the Calibration Curve for Each Laboratory

	NaR Method	Slope	A-540 nm	Correlation Coefficient
		A-540 per mg N/L	Intercept	r^2
Lab 2	DA	0.1392	0.0027	0.9999
Lab 3	DA	0.1468	0.0019	0.9999
Lab 4	DA	0.0954	0.0026	0.9999
Lab 5	DA	0.1159	-0.0006	0.9994
Lab 7	DA	0.1420	-0.0048	0.9963 (0.9999)[i]
Lab 8	DA	0.1361	0.0148	0.9994
Lab 9	DA	0.1352	-0.0015	0.9997
Lab 10	DA	0.1328	0.0014	0.9980 (0.9991) [i]
Lab 11*	DA	0.0643	0.0027	0.9986
Lab 12	DA	0.0530	0.0084	0.9999

*Lab 11 used Absorbance @ 550 nm; Polynomial Fit.

B. Calibration Nitrate Concentrations (mg N/L) used.

Lab 2	Lab 3	Lab 4	Lab 5	Lab 7	Lab 8	Lab 9	Lab 10	Lab 10	Lab 12
0.05	0.05	0.05	0.05	0.04	0.05	0.05	0.05	0.05	0.10
0.25	0.25	0.50	0.25	0.50	0.25	0.50	0.25	0.10	0.50
0.50	0.50	1.00	0.50	0.75	0.5	0.75	0.50	0.25	1.00
0.75	0.75	2.50	0.75	1.00	0.75	2.50	0.75	0.50	2.50
1.25	1.25	5.00	1.25	1.25	1.25	5.00	1.25	1.00	5.00
2.50	2.50		2.50	2.50	2.5		2.50	2.50	
3.75	3.75		3.75	3.75	3.75		3.75	5.00	
5.00	5.00		5.00	5.00	5		5.00		

Table 4 Enzymatic Reduction Efficiency Summary

Acceptance Standard is 90% or greater reduction efficiency.

2nd Source = Nitrate Standard in mg N/L and Nitrite Standard = mg N/L

			A-540 nm	mg N/L	Reduction Efficiency
Lab 2	2nd Source	2.50	0.35217	2.51	**102.1%**
	Nitrite Standard	2.50	0.34507	2.46	
Lab 3	2nd Source	2.50	0.36664	2.48	**101.3%**
	Nitrite Standard	2.50	0.36190	2.45	
Lab 4	2nd Source	2.50	0.2456	2.548	**107.2%**
	Nitrite Standard	2.50	0.2292	2.376	
Lab 5	2nd Source	2.50	0.295	2.59	**116.6%**
	Nitrite Standard	2.50	0.252	2.22	
Lab 7	2nd Source	2.50	0.33422	2.3576	**94.9%**
	Nitrite Standard	2.50	0.3514	2.4833	
Lab 8	2nd Source	2.50	0.37078	2.557	**103.8%**
	Nitrite Standard	2.50	0.35867	2.464	
Lab 9	2nd Source	2.50		2.506	**103.7%**
	Nitrite Standard	2.50		2.417	
Lab 10	2nd Source	2.50	0.358	2.4872	**102.3%**
	Nitrite Standard	2.50	0.350	2.4310	
Lab 11*	2nd Source	3.04	0.2127	3.00	**98.6%**
	Nitrite Standard	3.04	0.2151	3.04	
Lab 12	2nd Source	2.50	0.146	2.598	**105.0%**
	Nitrite Standard	2.50	0.140	2.475	

*Lab 11 used Absorbance @ 550 nm

Table 5 Initial Performance and Recovery (IPR) Summary

Lab	# Analyses	Spike Conc. (mg/L)	Mean Recovery (%)	RSD (%)	Minimum Recovery (%)	Maximum Recovery (%)
2	4	2.5	100.67	0.71	99.67	101.30
3	4	2.5	101.82	0.54	101.29	102.40
4	4	2.0	96.16	2.23	93.50	98.70
5	4	2.5	106.38	0.73	105.82	107.52
7	4	2.0	101.79	0.78	100.63	102.37
8	4	2.5	102.76	0.92	101.77	103.93
9	4	2.0	100.50	1.13	98.97	101.66
10	4	2.0	98.45	0.66	97.77	99.21
11	4	2.7	101.11	0.95	100.17	102.43
12	4	2.0	99.34	2.45	95.85	101.50

Table 6 Ongoing Performance and Recovery (OPR) Summary

RSD = Relative Standard Deviation

Lab	OPR mg N/L	# Analyses*	Mean Recovery %	RSD %	Minimum Recovery (%)	Maximum Recovery (%)
2	2.50	13	97.50	2.74	94.67	101.23
3	2.50	16	96.38	1.48	94.36	99.74
4	2.50	11	101.04	2.12	98.00	105.00
5**	2.50	4	104.49	0.68	103.48	105.48
5**	2.00	9	96.30	5.66	89.74	107.41
7	2.00	31	102.89	1.06	100.54	104.58
8	2.50	22	99.75	0.52	98.43	102.41
9	2.50	39	103.26	1.61	99.76	106.43
10	2.00	27	102.95	1.11	100.54	104.58
11	2.60	13	101.34	1.69	98.17	104.42
12	2.00	11	99.60	5.49	92.65	108.45

*Some Labs did more OPR than are summarized here for reasons of repeated analyses.

** Lab 4 initially used OPR = 2.50 mg N/L and then switched to OPR = 2.00 mg N/L.

Table 7 Method Detection Limit (MDL) Summary

Abbreviations: DA = Discrete Analyzer; NA = Not Analyzed.

Lab	Method	MDL	Replicates	Spike	Spike/MDL
		mg N/L		mg N/L	Ratio
2	DA	0.0079	8	0.040	5.068
3	DA	0.0148	8 (7)*	0.040	2.701
4	DA	0.0130	7	0.050	3.832
5	DA	0.0055	7	0.025	4.522
7	DA	0.0226	7	0.050	2.215
8	DA	0.0310	7	0.050	1.615
9	DA	0.0260	7	0.075	2.881
10	DA	NA			
11	DA	0.0060	7	0.045	7.541
12	DA	0.0463	7	0.100	2.160

*One replicate discarded due to issue with blank

Table 8 Nitrate-N Content and Spike Analysis of Waste Water and Seawater Matrices

Abbreviations: RSD = Relative Standard Deviation; RPD = Relative Percent Difference

Laboratory	Matrix	Unspiked Sample Summary					Spiked Sample Summary		
		Nitrate-N (mg N/L)	Standard Deviation (mg N/L)	RSD (%)	Dilution	Final Nitrate-N (mg N/L)	Mean Spike 1 % Recovery (%RPD)	Mean Spike 2 % Recovery (%RPD)	Mean Spike 3 % Recovery (%RPD)
2	WW-6	2.4732	0.0249	1.0080	2X	4.9464	100.1677 (1.0138)	103.2587 (0.7319)	99.9327 (0.6115)
3	WW-8	2.9017	0.01752	0.6037	5X	14.5083	112.3524 (0.8002)	108.9310 (0.8933)	111.1024 (0.3202)
4	WW-7	0.0070	0.00872	124.5429	None	0.0070	96.1024 (4.7179)	91.9024 (4.5016)	90.3024 (0.6543)
5	WW-5	2.7849	0.07318	2.6276	100X	278.4896	98.4712 (2.4315)	98.9524 (1.0010)	94.3448 (0.5755)
9	WW-4	0.0400	0.0010	2.5000	None	0.0400	107.0000 (2.4348)	105.6000 (3.5211)	102.3000 (1.2693)
10	WW-3	0.2251	0.0114	5.0473	None	0.2251	103.0767 (0.3376)	103.4267 (2.2096)	100.1567 (1.3639)
11	WW-2	4.0667	0.0139	0.3410	2X	8.1333	99.3867 (0.1534)	98.6767 (0.0768)	85.0667 (1.6296)
12	WW-1	0.0403	0.0307	76.0286	None	0.0403	95.9400 (1.5385)	95.9400 (9.6154)	92.1400 (1.5968)
7	SW-7	0.0094	0.0139	148.4678	None	0.0094	100.4180 (2.4635)	101.3640 (1.9760)	104.6390 (1.1698)

Table 9 Detailed Data Supporting Table 8

Data in this table are summarized from each Laboratory's Summary Excel File in Appendix F.
Standard Curve Data are in Table 3. Spiking was done at 0.5 mg N/L

			A-540	Nitrate-N	Nitrate-N	Spike 1			Spike 2			Spike 3		
Matrix ID	**Lab**	**Dilution Factor**	**Matrix Replicates**	**Measured (mg-N/L)**	**Final (mg-N/L)**	**Spike A-540**	**Spiked (mg-N/L)**	**Spike Recovery %**	**Spike A-540**	**Spiked (mg-N/L)**	**Spike Recovery %**	**Spike A-540**	**Spiked (mg-N/L)**	**Spike Recovery %**
WW-1	**12**	1												
WW-1 MS			0.012											
WW-1 MSD			0.009	**0.0403**	**0.0403**	0.037	0.524	96.7400	0.038	0.545	100.9400	0.036	0.505	92.9400
			0.012			0.036	0.516	95.1400	0.035	0.495	90.9400	0.035	0.497	91.3400
WW-1			**Mean Spike Recovery (%)**			**95.9400**			**95.9400**			**92.1400**		
			Relative Percent Difference (%)			**1.5385**			**9.6154**			**1.5968**		
	Lab		A-550											
WW-2	**11**	2	0.2635											
WW-2 MS			0.2630	**4.0667**	**8.1333**	0.2962	4.5671	100.0867	0.2958	4.5618	99.0267	0.2937	4.5286	92.3867
WW-2 MSD			0.2646			0.2957	4.5601	98.6867	0.2956	4.5583	98.3267	0.2889	4.4554	77.7467
WW-2			**Mean Spike Recovery (%)**			**99.3867**			**98.6767**			**85.0667**		
			Relative Percent Difference (%)			**0.1534**			**0.0768**			**1.6296**		
	Lab		A-540											
WW-3	**10**	1	0.041											
WW-3 MS			0.039	**0.2251**	**0.2251**	0.116	0.7417	103.3267	0.115	0.734	101.7867	0.114	0.7308	101.1467
WW-3 MSD			0.038			0.115	0.7392	102.8267	0.117	0.7504	105.0667	0.113	0.7209	99.1667
WW-3			**Mean Spike Recovery (%)**			**103.0767**			**103.4267**			**100.1567**		
			Relative Percent Difference (%)			**0.3376**			**2.2096**			**1.3639**		
	Lab		A-540											
WW-4	**9**	1	0.0053											
WW-4 MS			0.0055	**0.0400**	**0.0400**	0.0787	0.582	108.4000	0.0781	0.578	107.6000	0.0750	0.555	103.0000
WW-4 MSD			0.0054			0.0768	0.568	105.6000	0.0754	0.558	103.6000	0.0741	0.548	101.6000
WW-4			**Mean Spike Recovery (%)**			**107.0000**			**105.6000**			**102.3000**		
			Relative Percent Difference (%)			**2.4348**			**3.5211**			**1.2693**		
	Lab		A-540											
WW-5	**5**	100	0.2992											
WW-5 MS			0.3463	**2.7849**	**278.4896**	0.3752	3.2374	90.5024	0.3820	3.2961	102.2354	0.3764	3.2472	92.4706
WW-5 MSD			0.3228			0.3845	3.3171	106.4400	0.3782	3.2632	95.6694	0.3785	3.2660	96.2190
WW-5			**Mean Spike Recovery (%)**			**98.4712**			**98.9524**			**94.3448**		
			Relative Percent Difference (%)			**2.4315**			**1.0010**			**0.5755**		
	Lab		A-540											
WW-6	**2**	2	0.35647											
WW-6 MS			0.35077	**2.4732**	**4.9463**	0.42141	2.9589	97.1527	0.42419	2.9785	101.0707	0.42467	2.9819	101.7507
WW-6 MSD			0.34998			0.42569	2.9891	103.1827	0.42730	3.0004	105.4467	0.42209	2.9637	98.1147
WW-6			**Mean Spike Recovery (%)**			**100.1677**			**103.2587**			**99.9327**		
			Relative Percent Difference (%)			**1.0138**			**0.7319**			**0.6115**		
	Lab		A-540											
WW-7	**4**	1	-0.0005											
WW-7 MS			0.0008	**0.0070**	**0.0070**	0.0454	0.4760	93.8024	0.0455	0.4770	94.0024	0.0439	0.4600	90.6024
WW-7 MSD			0.0017			0.0476	0.4990	98.4024	0.0435	0.4560	89.8024	0.0436	0.4570	90.0024
WW-7			**Mean Spike Recovery (%)**			**96.1024**			**91.9024**			**90.3024**		
			Relative Percent Difference (%)			**4.7179**			**4.5016**			**0.6543**		
	Lab		A-540											
WW-8	**3**	5	0.40495											
WW-8 MS			0.40953	**2.9017**	**14.5083**	0.4873	3.4773	115.1238	0.4808	3.4309	105.8524	0.48528	3.4627	112.2095
WW-8 MSD			0.40572			0.4834	3.4496	109.5810	0.4851	3.4617	112.0095	0.48373	3.4516	109.9952
WW-8			**Mean Spike Recovery (%)**			**112.3524**			**108.9310**			**111.1024**		
			Relative Percent Difference (%)			**0.8002**			**0.8933**			**0.3202**		
	Lab		A-540											
SW-1	**7**	1	-0.00121											
SW-1 MS			-0.00448	**0.0094**	**0.0094**	0.0735	0.5178	101.6780	0.0726	0.5111	100.3440	0.0761	0.5357	105.2620
SW-1 MSD			-0.00476			0.0717	0.5052	99.1580	0.0740	0.5213	102.3840	0.0752	0.5295	104.0160
SW-1			**Mean Spike Recovery (%)**			**100.4180**			**101.3640**			**104.6390**		
			Relative Percent Difference (%)			**2.4635**			**1.9760**			**1.1698**		

Table 10 Standard Reference Materials Summary

Labs 5 and 8 did not analyze these matrices.

Sample Matrix ERA #698 - Finished drinking water (Target value from ERA)

Sample	Target	Lab	# Analyses	Concentration Result (mg N/L)			Recovery (%)			RSD
	mg N/L			Mean	Min.	Max	Mean	Min.	Max	%
ERA #698	6.80 ±0.12 (1.82%)	1	7	6.7809	6.7316	6.8038	99.72	98.99	100.06	0.38
		2	7	6.6154	6.5942	6.6497	97.28.	96.97	97.79	0.31
		3	7	6.5852	6.4661	6.6443	96.84	95.09	97.71	0.94
		4	0							
		6	7	6.3445	6.2929	6.4189	93.30	62.54	94.39	0.66
		7	7	6.8857	6.8000	6.9700	101.26	100.00	102.50	1.00
		9	3	6.8565	6.8295	6.8885	100.83	100.43	101.30	0.43
		10	0							
		N = 38		Mean = 6.6780 ± 0.62%			Mean Recovery = 98.20%			

Sample Matrix SRM-1 - USGS PE Sample N-116

(Target Most Probable Value from USGS*)

Sample	Target	Lab	# Analyses	Concentration Result (mg N/L)			Recovery (%)			RSD
	mg N/L			Mean	Min.	Max	Mean	Min.	Max	%
SRM-1	0.443 ±0.011 (2.44%)	1	3	0.4477	0.4476	0.4477	100.60	100.59	100.60	0.01
		2	3	0.4605	0.4581	0.4626	103.48	102.95	103.96	0.49
		3	7	0.4190	0.3520	0.4420	94.16	79.10	99.33	7.28
		4	3	0.4046	0.4023	0.4064	90.92	90.41	91.32	0.51
		6	7	0.4304	0.4269	0.4335	96.72	95.93	97.42	0.56
		7	10	0.4579	0.4430	0.4710	102.90	99.56	105.26	2.86
		9	0							
		10	3	0.4690	0.4410	0.4950	105.39	99.10	105.26	7.65
		N = 36		Mean = 0.4413 ± 2.34%			Mean Recovery = 99.62%			

Table 10 Continued

Sample Matrix SRM-2 - USGS PE Sample N-115

(Target Most Probable Value from USGS*)

Sample	Target	Lab	# Analyses	Concentration Result (mg N/L)			Recovery (%)			RSD
	mg N/L			Mean	Min.	Max	Mean	Min.	Max	%
SRM-2	2.300 ±0.009 (0.38%)	1	3	2.2653	2.2209	2.3022	99.36	97.41	100.97	1.82
		2	3	2.3215	2.3019	2.3407	101.82	100.96	102.66	0.84
		3	3	2.2647	2.2590	2.2680	99.33	99.08	99.47	0.22
		4	3	2.1676	2.0896	2.2353	95.07	91.65	98.04	3.39
		6	7	2.3783	2.3497	2.4028	104.31	103.06	105.39	0.78
		7	3	2.3267	2.2700	2.4000	102.05	99.56	105.26	2.86
		9	0							
		10	3	2.2833	2.1210	2.4680	100.15	93.03	108.25	7.65
		N = 25		**Mean = 2.2868 ± 3.02%**			**Mean Recovery = 99.43%**			

*Data from US Geological Survey (see link below and Appendix D of the Final Report)
(http://bqs.usgs.gov/srs_study/reports/round_details.php?PHPSESSID=vifh972qads22dirctsvevi841)

For SRM-1, which is USGS N-116,
25 analysis were reported by colorimetric methods with the following results (units = mg N/L):
MPV = 0.443; Fps = 0.016 (Fps = StDev/1.4826) therefore, StDev = 0.011 (2.44%)

For SRM-2, which is USGS N-115,
25 analysis were reported by colorimetric methods with the following results:
MPV = 0.230; Fps = 0.013; StDev = 0.009 (0.38%)

MPV = Most Probable Value; Fps = F-pseudo-sigma or MAD = Median Absolute Deviation

Table 11 Comparison of Nitrate-N Content of Sample Matrices and Standard References Determined by Discrete Analyzer Enzymatic Reduction Method (NaR) and Cadmium Reduction Air-Segmented Continuous Flow Analyzer Method (CdR)

Abbreviations: RPD = Relative Percent Difference for NaR-CdR (+ = NaR higher; - = CdR higher)

Note: NaR and CdR Method were done by Laboratory 2 on 19Jul2013 and values are mean of 3 replicates.

Sample Matrix	NaR	CdR	RPD
	mg N/L	mg N/L	%
WW-1*	0.03	0.03	0.0000
WW-2	7.8	7.6	+2.5974
WW-3	0.23	0.26	-12.7656
WW-4*	0.04	0.03	+28.5714
WW-5	270.8	272.6	-0.6625
WW-6	4.8	4.8	0.0000
WW-7*	0.05	0.06	-18.1818
WW-8	13.77	14.1	-2.3681
SW-1*	0.027	0.030	-10.5263
SR-1	6.80	7.02	-3.1838
SRM-1	0.45	0.48	-6.4516
SRM-2	2.28	2.36	-3.4188

*These values at or below the lowest calibrant of the method standard curve.