Method 353.4

Determination of Nitrate and Nitrite in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

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Revision 2.0
September 1997

National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

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1.0 Scope and Application

1.1 This method provides a procedure for determining nitrate and nitrite concentrations in estuarine and coastal waters. Nitrate is reduced to nitrite by cadmium,[1-3] and the resulting nitrite determined by formation of an azo dye.[4-6]

In most estuarine and coastal waters nitrogen is thought to be a limiting nutrient. Nitrate is the final oxidation product of the nitrogen cycle in natural waters and is considered to be the only thermodynamically stable nitrogen compound in aerobic waters.[7] Nitrate in estuarine and coastal water is derived from rock weathering, sewage effluent and fertilizer run-off. The concentration of nitrate usually is high in estuarine waters and lower in surface coastal waters.

Nitrite is an intermediate product in the microbial reduction of nitrate or in the oxidation of ammonia. It may also be excreted by phytoplankton as a result of excess assimilatory reduction. Unlike nitrate, nitrite is usually present at a concentration lower than 0.01mg N/L except in high productivity waters and polluted waters in the vicinity of sewer outfalls.

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Nitrate	14797-55-8
Nitrite	14797-65-0

1.2 A statistically determined method detection limit (MDL)[8] of 0.075 µg N/L has been determined by one laboratory in seawaters of five different salinities. The method is linear to 5.0 mg N/L using a Flow Solution System (Alpkem, Wilsonville, Oregon).

1.3 Approximately 40 samples per hour can be analyzed.

1.4 This method requires experience in the use of automated gas segmented continuous flow colorimetric analyses, and familiarity with the techniques of preparation, activation and maintenance of the cadmium reduction column. A minimum of six-months training is recommended.

2.0 Summary of Method

2.1 An automated gas segmented continuous flow colorimetric method for the analysis of nitrate concentration is described. In the method, samples are passed through a copper-coated cadmium reduction column. Nitrate in the sample is reduced to nitrite in a buffer solution. The nitrite is then determined by diazotizing with sulfanilamide and coupling with N-1-naphthylethylenediamine dihydrochloride to form a color azo dye. The absorbance measured at 540 nm is linearly proportional to the concentration of nitrite + nitrate in the sample. Nitrate concentrations are obtained by subtracting nitrite values, which have been separately determined without the cadmium reduction procedure, from the nitrite + nitrate values. There is no significant salt error in this method. The small negative error caused by differences in the refractive index of seawater and reagent water is readily corrected for during data processing.

3.0 Definitions

3.1 Calibration Standard (CAL) - A solution prepared from the primary dilution standard solution or stock standard solution containing analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.2 Laboratory Fortified Blank (LFB) - An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits, and whether the laboratory is capable of making accurate and precise measurements. This is a standard prepared in reagent water that is analyzed as a sample.

3.3 Laboratory Fortified Sample Matrix (LFM) - An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.4 Laboratory Reagent Blank (LRB) - An aliquot of reagent water that is treated exactly as a sample including exposure to all labware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.5 Linear Dynamic Range (LDR) - The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.6 Method Detection Limit (MDL) - The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.[8]

3.7 Reagent Water (RW) - Type 1 reagent grade water equal to or exceeding standards established by American Society for Testing and Materials (ASTM). Reverse osmosis systems or distilling units followed by Super-Q Plus Water System that produce water with 18 megohm resistance are examples of acceptable water sources. To avoid contamination, the reagent water should be used the day of preparation.

3.8 Refractive Index (RI) - The ratio of velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as estuarine or sea water versus reagent water. The correction for this difference is referred to as the refractive index correction in this method.

3.9 Stock Standard Solution (SSS) -A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

3.10 Primary Dilution Standard Solution (PDS) - A solution prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.11 Quality Control Sample (QCS) - A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.12 SYNC Peak Solution - A colored solution used to produce a synchronization peak in the refractive index measurement. A synchronization peak is required by the data acquisition programs to initialize the peak finding parameters. The first cup in every run must always be identified as a SYNC sample. The SYNC sample is usually a high standard, but can be any sample that generates a peak at least 25% of full scale.

4.0 Interferences

4.1 Hydrogen sulfide at concentrations greater than 0.1 mg S/L can interfere with nitrite analysis by precipitating on the cadmium column .[9] Hydrogen sulfide in samples must be removed by precipitation with cadmium or copper salt.

4.2 Iron, copper and other heavy metals at concentrations larger than 1 mg/L alter the reduction efficiency of the cadmium column. The addition of EDTA will complex these metal ions.[10]

4.3 Phosphate at a concentration larger than 0.1 mg/L decreases the reduction efficiency of cadmium[11]. Dilute samples if possible or remove phosphate with ferric hydroxide[12] prior to analysis.

4.4 Particulates inducing turbidity should be removed by filtration after sample collection.

4.5 This method corrects for small refractive index interference which occurs if the calibration standard solution is not matched with samples in salinity.

5.0 Safety

5.1 Water samples collected from the estuarine and coastal environment are generally not hazardous.

However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. Laboratory personnel should obtain material safety data sheets (MSDS) for all chemicals used in this method. A lab coat, safety goggles, and gloves should be worn when handling the concentrated acid.

6.0 Equipment and Supplies

6.1 Gas Segmented Continuous Flow Autoanalyzer Consisting of:

6.1.1 Autosampler.

6.1.2 Analytical cartridge with reaction coils for nitrate analysis.

6.1.3 Open Tubular Cadmium Reactor (OTCR, Alpkem, OR) or laboratory prepared packed copper-coated cadmium reduction column (prepared according to procedures in Section 7.4 - 7.5).

6.1.4 Proportioning pump.

6.1.5 Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 540 nm interference filter (2 nm bandwidth).

6.1.6 Strip chart recorder or computer based data acquisition system.

6.1.7 Nitrogen gas (high-purity grade, 99.99%).

6.2 Glassware and Supplies

6.2.1 All labware used in the analysis must be low in residual nitrate to avoid sample or reagent contamination. Soaking with lab grade detergent, rinsing with tap water, followed by rinsing with 10% HCl (v/v) and thoroughly rinsing with reagent water is sufficient.

6.2.2 Automatic pipetters capable of delivering volumes ranging from 100 µL to 1000 µL and 1mL to 10 mL with an assortment of high quality disposable pipet tips.

6.2.3 Analytical balance, with capability to measure to 0.1 mg, for preparing standards.

6.2.4 60 mL high density polyethylene sample bottles, glass volumetric flasks and plastic sample tubes.

6.2.5 Drying oven.

6.2.6 Desiccator.

6.2.7 Membrane filters with 0.45 µm nominal pore size. Plastic syringes with syringe filters.

6.2.8 A pH meter with a glass electrode and a reference electrode. A set of standard buffer solutions for calibration of the pH meter.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Stock Sulfanilamide Solution - Dissolved 10 g of sulfanilamide ($C_6H_8N_2O_2S$, FW 172.21) in 1 L of 10% HCl.

7.1.2 Stock Nitrate Solution (100 mg-N/L) - Quantitatively transfer 0.7217 g of pre-dried (105°C for 1 hour) potassium nitrate (KNO_3, FW 101.099) to a 1000-mL glass volumetric flask containing approximate 800 mL of reagent water and dissolve the salt. Dilute the solution to the mark with reagent water. Store the stock solution in a polyethylene bottle in refrigerator at 4°C. This solution is stable for six months.

7.1.3 Stock Nitrite Solution (100 mg-N/L) - Quantitatively transfer 0.4928 g of pre-dried (105°C for 1 hour) sodium nitrite ($NaNO_2$, FW 68.99) to a 1000 mL glass volumetric flask containing approximate 800 mL of reagent water and dissolve the salt. Dilute the solution to the mark with reagent water. Store the stock solution in a polyethylene bottle in a refrigerator at 4 °C. This solution is stable for three months.

Note: High purity nitrite salts are not available. Assays given by reagent manufacturers are usually in the range of 95-97%. The impurity must be taken into account for calculation of the weight taken.

7.1.4 Low Nutrient Sea Water (LNSW) - Obtain natural low nutrient seawater from surface water of the Gulf Stream or Sargasso Sea (salinity 36 ‰, < 7 µg N/L) and filter it through 0.3 micron pore size glass fiber filters. If this is not available, commercial low nutrient sea water (< 7 µg N/L) with salinity of 35 ‰ (Ocean Scientific International, Wormley, U.K.) can be substituted.

7.2 Working Reagents

7.2.1 Brij-35 Start-up Solution - Add 2 mL of Brij-35 surfactant (ICI Americas, Inc.) to 1000 mL reagent water and mix gently.

Note: Brij-35 is a trade name for polyoxyethylene(23) lauryl ether ($C_{12}H_{25}(OCH_2CH_2)_{23}OH$, FW=1199.57, CASRN 9002-92-0).

7.2.2 Working Sulfanilamide Solution - Add 1 mL of Brij- 35 solution to 200 mL of stock sulfanilamide solution, mix gently.

Note: Adding surfactant Brij-35 to sulfanilamide solution instead of to the buffer solution is to prevent the Brij from being adsorbed on the cadmium surface, which may result in decreasing surface reactivity of the cadmium and reduce the lifetime of the cadmium column.

7.2.3 NED Solution - Dissolve 1 g of NED (N-1-naphthylethylenediamine Dihydrochloride, $C_{12}H_{14}N_2.2HCl$, FW 259.18) in 1 L of reagent water.

7.2.4 Imidazole Buffer Solution - Dissolve 13.6 g of imidazole ($C_3H_4N_2$, FW 68.08) in 4 L of reagent water. Add 2 mL of concentrated HCl. Adjust the pH to 7.8 with diluted HCl while monitoring the pH with a pH meter. Store in a refrigerator.

7.2.5 Copper Sulfate Solution (2%) - Dissolve 20 g of copper sulfate ($CuSO_4.5H_2O$, FW 249.61) in 1 L of reagent water.

7.2.6 Colored SYNC Peak Solution - Add 50 µL of red food coloring solution to 1000 mL reagent water and mix thoroughly. Further dilute this solution to obtain a peak between 25 to 100 percent full scale according to the AUFS setting used for the refractive index measurement.

7.2.7 Primary Dilution Standard Solution - Prepare a primary dilution standard solution (5 mg N/L) by dilution of 5.0 mL of stock standard solutions to 100 mL with reagent water. Prepare this solution daily.

Note: This solution should be prepared to give an appropriate intermediate concentration for further dilution to prepare the calibration solutions. Therefore the concentration of a primary dilution standard solution should be adjusted according to the concentration range of calibration solutions.

7.2.8 Calibration Standards - Prepare a series of calibration standards (CAL) by diluting suitable volumes of a primary dilution standard solution (Section 7.2.7) to 100 mL with reagent water or low nutrient seawater. Prepare these standards daily. The concentration range of calibration standards should bracket the expected concentrations of samples and not exceed two orders of magnitude. At least five calibration standards with equal increments in concentration should be used to construct the calibration curve.

If nitrate + nitrite and nitrite are analyzed simultaneously by splitting a sample into two analytical systems, a nitrate and nitrite mixed standard should be prepared. The total concentration (nitrate+nitrite) must be assigned to the concentrations of calibration standards in the nitrate+nitrite system.

When analyzing samples of varying salinities, it is recommended that the calibration standard solutions and sampler wash solution be prepared in reagent water and corrections for refractive index be made to the sample concentrations determined (Section 12.2).

7.2.9 Saline Nitrate and Nitrite Standards - If CAL solutions will not be prepared to match sample salinity, then saline nitrate and nitrite standards must be prepared in a series of salinities in order to quantify the salt error, the change in the colorimetric response of nitrate due to the change in the composition of the solution. The following dilutions of Primary Dilution Standard Solution (Section 7.2.7) to 100 mL in volumetric flasks with reagent water, are suggested:

Salinity ($‰_o$)	Volume of LNSW(mL)	Volume of PDS(mL)	Conc. mg N/L
0	0	2	.10
9	25	2	.10
18	50	2	.10
27	75	2	.10
35	98	2	.10

7.3 Open Tubular Cadmium Reactor

7.3.1 Nitrate in the samples is reduced to nitrite by either a commercial Open Tubular Cadmium Reactor (OTCR, Alpkem, OR) or a laboratory-prepared packed copper-coated cadmium reduction column.

7.3.2 If an OTCR is employed, the following procedures should be used to activate it.[10]

Prepare reagent water, 0.5N HCl solution and 2% $CuSO_4$ solution in three 50 mL beakers. Fit three 10-mL plastic syringes with unions. First flush the OTCR with 10 mL reagent water. Then flush it with 10 mL 0.5N HCl solution in 3 seconds, immediately followed by flushing with a couple of syringe volumes of reagent water. Slowly flush with $CuSO_4$ solution until a large amount of black precipitated copper come out of OTCR, then stop the flushing. Finally flush the OTCR with reagent water. Fill the OTCR with imidazole buffer for short term storage.

7.4 Packed Cadmium Reduction Column

The following procedures are used for preparation of a packed cadmium reduction column.[13]

7.4.1 File a cadmium stick to obtain freshly prepared cadmium filings.

7.4.2 Sieve the filings and retain the fraction between 25 and 60 mesh size (0.25-0.71 mm).

7.4.3 Wash filings two times with 10% HCl followed with reagent water.

7.4.4 Decant the reagent water and add 50 mL of 2% $CuSO_4$ solution. While swirling, brown flakes of colloidal copper will appear and the blue color of the solution will fade. Decant the faded solution and add fresh $CuSO_4$ solution and swirl. Repeat this procedure until the blue color does not fade.

7.4.5 Wash the filings with reagent water until all the blue color is gone and the supernatant is free of fine particles. Keep the filings submersed under reagent water and avoid exposure of the cadmium filings to air.

7.4.6 The column can be prepared in a plastic or aglass tube of 2 mm ID. Plug one end of column with glass wool. Fill the column with water and transfer Cd filings in suspension using a 10 mL pipette tip connected to one end of column. While gently tapping the tube and pipette tip let Cd filings pack tightly and uniformly in the column without trapping air bubbles.

7.4.7 Insert another glass wool plug at the top of the column. If a U- shape tube is used, the pipette tip is connected to the other end and the procedure repeated. Connect both ends of the column using a plastic tube filled with buffer solution to form a closed loop.

7.4.8 If an OTCR or a packed cadmium column has not been used for several days, it should be reactivated prior to sample analysis.

7.5 Stabilization of OTCR and Packed Cadmium Reduction Columns

7.5.1 Pump the buffer and other reagent solutions through the manifold and obtain a stable baseline.

7.5.2 Pump 0.7 mg-N/L nitrite standard solution continuously through the sample line and record the steady state signal.

7.5.3 Stop the pump and install an OTCR or a packed column on the manifold. Ensure no air bubbles have been introduced into the manifold during the installation. Resume the pumping and confirm a stable baseline.

7.5.4 Pump 0.7 mg-N/L nitrate solution continuously through the sample line and record the signal. The signal will increase slowly and reach steady state in about 10-15 minutes. This steady state signal should be close to the signal obtained from the same concentration of a nitrite solution without the OTCR or packed cadmium column on line.

7.5.5 The reduction efficiency of an OTCR or a packed cadmium column can be determined by measuring the absorbance of a nitrate standard solution followed by a nitrite standard solution of the same concentration. Reduction efficiency is calculated as follows:

$$\text{Reduction Efficiency} = \frac{\text{Absorbance of Nitrate}}{\text{Absorbance of Nitrite}}$$

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection - Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Go-Flo or equivalent) that are attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either

electronically or via a mechanical messenger when the bottles have reached the desired depth.

8.1.2 In a submersible pump system, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for sample processing.

8.1.3 For collecting surface samples, an acid - cleaned plastic bucket or a large plastic bottle can be used as a convenient sampler. Wash the sampler three times with sample water before collecting samples.

8.1.4 Turbid samples should be filtered as soon as possible after collection.

8.1.5 60-mL high density polyethylene bottles are used for sample storage. Sample bottles should be rinsed 3 times with about 20 mL of sample, shaking with the cap in place after each rinse. Pour the rinse water into the cap to dissolve and rinse away salt crusts trapped in the threads of the cap. Finally, fill the sample bottle about 3/4 full, and screw the cap on firmly.

8.2 Sample Preservation - After collection and filtration, samples should be analyzed as soon as possible. If samples will be analyzed within 3 hours then keep refrigerated in tightly sealed, high density polyethylene bottles in the dark at 4°C until analysis can be performed.

8.3 Sample Storage - Natural samples usually contain low concentrations of nitrite (< 14 g N/L) and no preservation techniques are satisfactory.[14] Samples must be analyzed within 3 hours of collection to obtain reliable nitrite concentrations.[15]

Samples containing high concentrations of ammonia or nitrite may change in nitrate concentration during storage due to microbial oxidation of ammonia and nitrite to nitrate. These samples should be analyzed as soon as possible.

Natural samples containing low concentrations of nitrite and ammonia (< 10% of the nitrate concentration) can be preserved for nitrate analysis by freezing. A maximum holding time for preserved estuarine and coastal water samples for nitrate analysis is one month.[16]

The results of preservation of natural samples are shown in Tables 1 and 2 for nitrate and nitrite, respectively.

9.0 Quality Control

9.1 Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance by determining the MDL and LDR and laboratory performance by analyzing quality control samples prior to analysis of samples using this method.

9.2.2 A method detection limit (MDL) should be established for the method analytes using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots of water which have been processed through the entire analytical method. Perform all calculations defined in the method and report concentration in appropriate units. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where, S = the standard deviation of the replicate analyses

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined every six months or whenever a significant change in background or instrument response occurs or a new matrix is encountered.

9.2.3 The LDR should be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (Absorbance Units Full Scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to that of samples, therefore a correction factor for salt error, or refractive index, will not be necessary. Normalize

responses by multiplying the response by the Absorbance Units Full Scale output range setting. Perform the linear regression of normalized response vs. concentration and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response, R, of a standard no longer yields a calculated concentration C_C, that is within 100 ± 10% of known concentration, C, where C_C = (R-b)/m. That concentration defines the upper limit of the LDR for the instrument. Should samples be encountered that have a concentration that is ≥ 90% of the upper limit of LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) - A laboratory should analyze at least one LRB with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Laboratory Fortified Blank (LFB) - A laboratory should analyze at least one LFB with each set of samples. The LFB must be at a concentration that is within the daily calibration range. The LFB data are used to calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90 -110%, the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

Upper Control Limit = x + 3S
Lower Control Limit = x - 3S

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery - Laboratory Fortified Sample Matrix (LFM)

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one sample per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater than the MDL.

9.4.2 Calculate percent recovery of analyte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

where,

R = percent recovery

C_S = measured fortified sample concentration (background + addition in mg N/L)

C = sample background concentration (mg N/L)

S = concentration in mg N/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be the matrix related and the sample data should be flagged.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared fresh daily for system calibration. The calibration concentrations should bracket the concentrations of samples and the range should not be over two orders of magnitude.

10.2 A calibration curve should be constructed for each sample set by analyzing a series of calibration standard solutions. A sample set should contain no more than 60 samples. For a large number of samples make several sample sets with individual calibration curves.

10.3 Analyze the calibration standards, in duplicate, before actual samples.

10.4 The calibration curve containing five or more data points should have a correlation coefficient, r, of 0.995 or better.

10.5 Place a high CAL solution followed by two blank cups to quantify the carry-over of the system. The difference in peak heights between two blank cups is due to the carry over from the high CAL solution. The carry-over coefficient, k, is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}}$$

where,

P_{high} = the peak height of the high nitrate standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample.

The carry over coefficient, k, for a system should be measured in seven replicates to obtain a statistically significant number. k should be remeasured with any change in manifold plumbing or upon replacement of pump tubung.

The carry over correction (CO) on a given peak i is proportional to the peak height of the preceding sample, P_{i-1}.

$$CO = (k)(P_{i-1})$$

To correct a given peak height reading, P_i, subtract the carry over correction,[17,18]

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is corrected peak height. The correction for carry over should be applied to all the peak heights throughout a run. The carry over coefficient should be less than 5% in this method.

10.6 Place a high standard nitrate solution followed by a nitrite standard solution of same concentration at the beginning and end of each sample run to check for change in reduction efficiency of OTCR or a packed cadmium column. The decline of reduction efficiency during a run should be less than 5%.

10.7 Place a high standard solution at the end of each sample run (60 samples) to check for sensitivity drift. Apply sensitivity drift correction to all the samples. The sensitivity drift during a run should be less than 5%.

Note: Sensitivity drift correction is available in most data acquisition software supplied with autoanalyzers. It is assumed that the sensitivity drift is linear with time. An interpolated drift correction factor is calculated for each sample according to the sample position during a run. Multiply the sample peak height by the corresponding sensitivity drift correction factor to obtain the corrected peak height for each sample.

11.0 Procedure

11.1 If samples are frozen, thaw the samples at room temperature. If samples are stored in a refrigerator, remove samples and equilibrate to room temperature. Mix samples thoroughly prior to analysis.

11.2 Turn on the continuous flow analyzer and data acquisition components and warm up at least 30 minutes.

11.3 Set up the cartridge according to the type of cadmium reductor used for nitrate + nitrite analysis (configuration for OTCR shown in Figure 1 and packed cadmium column in Figure 2). Configuration for analysis of nitrite alone is shown in Figure 3.

Note: When a gas segmented flow stream passes through the OTCR, particles derived from the OTCR were found to increase baseline noise and to cause

interference at low level analysis. Packed cadmium columns are, therefore, preferred for nitrate analysis at low concentrations.

11.4 Set spectrophotometer wavelength at 540 nm.

11.5 Set the Absorbance Unit Full Scale (AUFS) range on the spectrophotometer at an appropriate setting according to the highest concentration of nitrate in the samples. The appropriate setting for this method is 0.2 AUFS for 0.7 mg N/L.

11.6 Prepare all reagents and standards.

11.7 Begin pumping the Brij-35 start-up solution (Section 7.2.1) through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has been stabilized, reset the baseline.

NOTE: To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the sample line with reagent water, 1 N HCl solution, reagent water, 1 N NaOH solution for a few minutes each at the end of the daily analysis. Make sure to rinse the system well with reagent water after pumping NaOH solution to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulate. Filter the reagents and samples if necessary.

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flowcell can often be eliminated by simply attaching syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohol was found to be effective in removing air bubbles from the flow cell.

For samples of varying salinities, it is suggested that the reagent water used for the sampler wash solution and for preparing calibration standards and procedures in Sections 12.2 and 12.3 be employed.

11.8 Check the reduction efficiency of the OTCR or packed cadmium column following the procedure in Section 7.5.5. If the reduction efficiency is less than 90% follow the procedure in Section 7.5 for activation and stabilization. Ensure reduction efficiencies reach at least 90% before analysis of samples.[19]

11.9 A good sampling rate is approximately 40 samples per hour for 60 second sample times and 30 second wash times.

11.10 Use cleaned sample cups or tubes (follow the procedures outlined in Section 6.2.2). Place CAL solutions and saline standards (optional) in sampler. Complete filling the sampler tray with samples, laboratory reagent blanks, laboratory fortified blanks, laboratory fortified sample matrices, and QC samples. Place a blank after every ten samples.

11.11 Commence analysis.

12.0 Data Analysis and Calculations

12.1 Concentrations of nitrate in samples are calculated from the linear regression, obtained from the standard curve in which the concentrations of the calibration standards are entered as the independent variable, and their corresponding peak heights are the dependent variable.

12.2 Refractive Index Correction for Estuarine and Coastal Samples

12.2.1 If reagent water is used as the wash solution and to prepare the calibration standard solutions, the operator has to quantify the refractive index correction due to the difference in salinity between sample and standard solutions. The following procedures are used to measure the relationship between sample salinity and refractive index for ***a particular detector***.

12.2.2 First, analyze a set of nitrate or nitrite standards in reagent water with color reagent using reagent water as the wash and obtain a linear regression of peak height versus concentration.

Note: The change in absorbance due to refractive index is small, therefore low concentration standards should be used to bracket the expected absorbances due to refractive index.

12.2.3 Second, replace reagent water wash solution with Low Nutrient Seawater wash solution.

Note: In nitrate and nitrite analysis absorbance of the reagent water is higher than that of the LNSW. When using reagent water as a wash solution, the change in refractive index causes the absorbance of seawater to become negative. To measure the absorbance due to refractive index change in different salinity samples, Low Nutrient Seawater must be used as a wash solution to bring the baseline down.

12.2.4 Replace NED solution (Section 7.2.4) with reagent water. All other reagents remain the same. Replace the synchronization sample with the colored SYNC peak solution (Section 7.2.6).

12.2.5 Prepare a set of different salinity samples with LNSW. Commence analysis and obtain peak heights for different salinity samples. The peak heights for the refractive index correction must be obtained at the same AUFS range setting and on the same spectrophotometer as the corresponding standards (Section 12.2.2).

12.2.6 Using Low Nutrient Seawater as the wash water, a maximum absorbance will be observed for reagent water. No change in refractive index will be observed in the seawater sample. Assuming the absolute absorbance for reagent water (relative to the seawater baseline) is equal to the absorbance for seawater (relative to reagent water baseline), subtract the absorbances of samples of various salinities from that of reagent water. The results are the apparent absorbance due to the change in refractive index between samples of various salinities relative to the reagent water baseline.

12.2.7 For each sample of varying salinity, calculate the apparent nitrate or nitrite concentrations due to refractive index from its peak height corrected to reagent water baseline (Section 12.2.5) and the regression equation of nitrate or nitrite standards obtained with color reagent being pumped through the system (12.2.2). Salinity is entered as the independent variable and the apparent nitrate or nitrite concentration due to refractive index is entered as the dependent variable. The resulting regression allows the operator to calculate apparent nitrate or nitrite concentration due to refractive index when sample salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.8 An example of typical results follows:

Salinity ($‰_o$)	Apparent concentration (µg N/L) Nitrate	Nitrite
0.0	0.000	0.000
3.8	0.026	0.015
9.2	0.096	0.040
13.8	0.142	0.055
18.1	0.190	0.086
26.8	0.297	0.153
36.3	0.370	0.187

Note: You must calculate the refractive index correction for your particular detector. Moreover, the refractive index must be redetermined whenever a significant change in the design of flowcell or a new matrix is encountered.

12.2.9 An example of typical linear equations is:

Apparent nitrate (µg N/L) = 0.01047S

Apparent nitrite (µg N/L) = 0.00513S

where S is sample salinity. The apparent nitrate and nitrite concentration due to refractive index so obtained should be added to samples of corresponding salinity when reagent water is used as wash solution and standard matrix.

If nitrate and nitrite concentrations are greater than 100 and 50 µg N/L respectively, the correction for refractive index is negligible and this procedure can be optional.

12.3 Correction for Salt Error in Estuarine and Coastal Samples

12.3.1 When calculating concentrations of samples of varying salinities from standards and the wash solution prepared in reagent water, it is common to first correct for refractive index errors, and then correct for any change in color development due to the differences in composition between samples and standards (so called salt error).

12.3.2 Plot the salinity of the saline standards (Section 7.2.9) as the independent variable, and the apparent concentration of analyte (mg N/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for all saline standards. The resulting regression equation allows the operator to correct the

concentrations of samples of known salinity for the color enhancement due to matrix effect, e.g., salt error. Following are typical results for the nitrate and nitrite systems:

Salinity (‰)	Apparent concentration (µg N/L) Nitrate	Nitrite
0.0	569.64	558.15
3.8	570.50	565.50
9.2	572.74	563.00
13.8	568.96	564.94
18.1	566.44	563.00
26.8	558.74	559.06
36.3	559.86	554.67

12.3.3 As shown in above results, salinity has no systematic effect on the nitrate and nitrite signal and therefore salt error correction is not recommended.

12.4 Results of sample analyses should be reported in mg N/L or in µg N/L.

mg N/L = ppm (parts per million)
µg N/L = ppb (part per billion)

13.0 Method Performance

13.1 Single Laboratory Validation

13.1.1 Method Detection Limit- A method detection limit (MDL) of 0.075 µg N/L has been determined by one laboratory from LNSW of five different salinities fortified at a nitrate concentration of 0.28 µg N/L.

Salinity (‰)	SD (µg N/L)	Recovery (%)	MDL (µg N/L)
36.5	0.0234	103.5	0.0734
36.5	0.0298	98.9	0.0935
36.5	0.0148	110.3	0.0464
36.5	0.0261	103.6	0.0819
27.5	0.0203	105.4	0.0638
27.5	0.0321	102.3	0.1009
27.5	0.0314	103.8	0.0986
27.5	0.0335	100.1	0.1052
18.6	0.0167	105.8	0.0523
18.6	0.0170	101.6	0.0534
18.6	0.0229	106.4	0.0720
18.6	0.0229	104.5	0.0719
9.4	0.0222	105.3	0.0698
9.4	0.0229	106.4	0.0720
9.4	0.0197	91.5	0.0620
0.0	0.0260	103.9	0.0817
0.0	0.0306	106.9	0.0961
0.0	0.0160	111.0	0.0501
0.0	0.0248	109.5	0.0780

13.1.2 Single analyst precision - A single laboratory analyzed three samples collected from the Miami River and Biscayne Bay, Florida. Seven replicates of each sample were processed and analyzed with salinity ranging from 0.019 to 32.623‰. The results were as follows:

Sample	Salinity (‰)	Concentration (µg N/L)	RSD (%)
		Nitrate	
1	32.623	48.22	2.59
2	13.263	206.41	1.07
3	0.019	276.38	1.99
		Nitrite	
1	32.623	5.21	1.62
2	13.263	31.03	0.58
3	0.019	54.07	0.49

13.1.3 Laboratory fortified sample matrix - Laboratory fortified sample matrices were processed in three different salinities ranging from 0.019 to 32.623 and ambient nitrate concentrations from 48.22 to 276.38 µg N/L. Seven replicates of each sample were analyzed and the results were as follows:

Salinity (‰)	Concentration ambient (µg N/L)	Concentration fortified (µg N/L)	RSD (%)	Recovery (%)
32.623	48.22	139.94	1.50	106.4
13.263	206.41	139.94	1.25	102.6
0.019	276.38	139.94	1.19	102.3

13.2 Multi-Laboratory Validation

Multi-laboratory data is unavailable at this time.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The USEPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society, Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

1. Morris, A. W. and Riley, J.P., 1963. Determination of nitrate in sea water. Anal. Chim. Acta. 29:272-279.

2. Brewer P. G. and J. P. Riley 1965. The automatic determination of nitrate in seawater. Deep-Sea Res., 12:765-772.

3. Wood, E.O., Armstrong, F.A.J., and Richards, F.A., 1967. Determination of nitrate in seawater by cadmium-copper reduction to nitrite. J. Mar. Biol. Assn. U.K., 47:23-31.

4. Bendschneider, K. and R. J. Robinson, 1952. A new spectrophotometric method for the determination of nitrite in sea water. J. Marine Res., 11:87-96.

5. Fox, J.B. 1979. Kinetics and mechanisms of the Griess reaction. Analytical Chem. 51:1493-1502.

6. Norwitz, G., P.N. Keliher,, 1984. Spectrophotometric determination of nitrite with composite reagents containing sulphanilamide, sulphanilic acid or 4- nitroaniline as the diozotisable aromatic amine and N-(1-naphthyl)ethylenediamine as the coupling agent. Analyst, 109:1281-1286.

7. Spencer, C.P. 1975, The micronutrient elements. In *Chemical Oceanography* (Riley, J. P. and G. Skirrow, Eds.), Academic Press, London and New York, 2nd Ed. Vol 2, Chapter 11.

8. 40 CFR, 136 Appendix B. Definition and Procedure for the Determination of Method Detection Limit. Revision 1.11.

9. Timmer-ten Hoor, A., 1974. Sulfide interaction on colorimetric nitrite determination. Marine Chemistry, 2:149-151.

10. Alpkem Corporation. 1990. RFA Methodology: Nitrate+Nitrite Nitrogen. Method A303-S170. Alpkem Corporation, Clackamas, Oregon.

11. Olson, R.J. 1980. Phosphate interference in the cadmium reduction analysis of nitrate. Limnol. Oceanogr., 25(4)758-760.

12. Alvarez-Salgado, X.A., F.Fraga and F.F.Perez. 1992, Determination of nutrient salt by automatic methods both in seawater and brackish water: the phosphate blank. Marine Chemistry, 39:311-319.

13. Grasshoff , K. 1983, Determination of Nitrate, In *Methods of Seawater Analysis* (Grasshoff, K., M. Ehrhardt and K. Kremling, Eds) Weinheim, Verlag Chemie, Germany. pp143-150.

14. Takenaka, N., A.Ueda and Y. Maeda 1992, Acceleration of the rate of nitrite oxidation by freezing in aqueous solution. Nature, Vol. 358, p736-738.

15. Grasshoff , K. 1983, Determination of Nitrite, In *Methods of Seawater Analysis* (Grasshoff, K., M. Ehrhardt and K. Kremling, Eds) Weinheim, Verlag Chemie, Germany. pp139-142.

16. MacDonald, R.W. and F.A. McLaughlin. 1982. The effect of Storage by freezing on dissolved inorganic phosphate, nitrate, and reactive silicate for samples from coastal and estuarine waters. Water Research, 16:95-104.

17. Angelova, S, and H.W.Holy. 1983. Optimal speed as a function of system performance for continuous flow analyzers. Analytica Chimica Acta, 145:51-58.

18. Zhang, J.-Z. 1997. Distinction and quantification of carry-over and sample interaction in gas segmented continuous flow analysis . Journal of Automataic Chemistry, 19(6):205-212.

19. Garside, C. 1993. Nitrate reductor efficiency as an error source in seawater analysis. Marine Chemistry 44: 25-30.

17.0 Tables, Diagrams, Flowcharts, and Validation Data

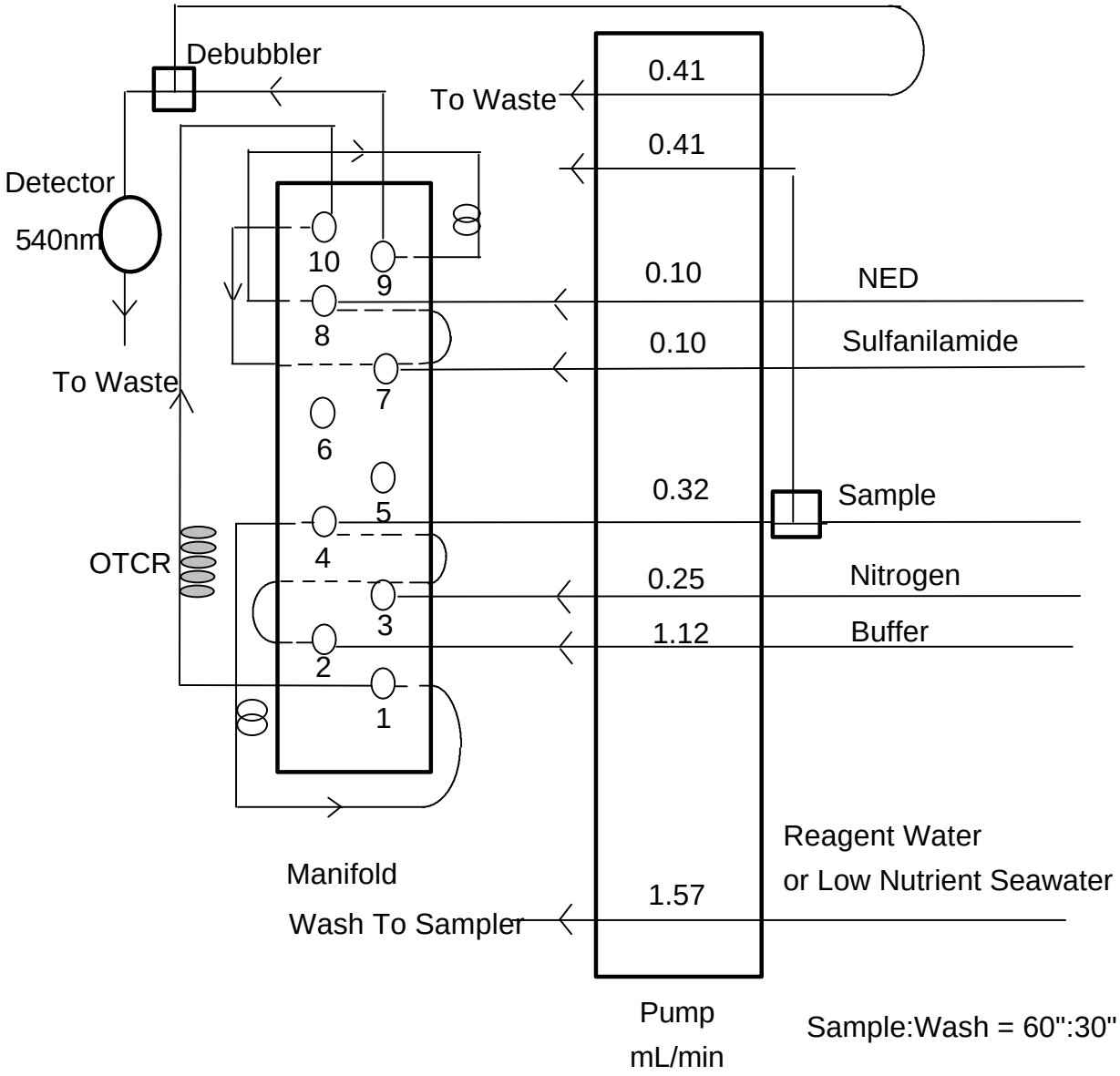

Figure 1. Manifold configuration for nitrate + nitrite analysis using an Open Tubular Cadmium Reactor.

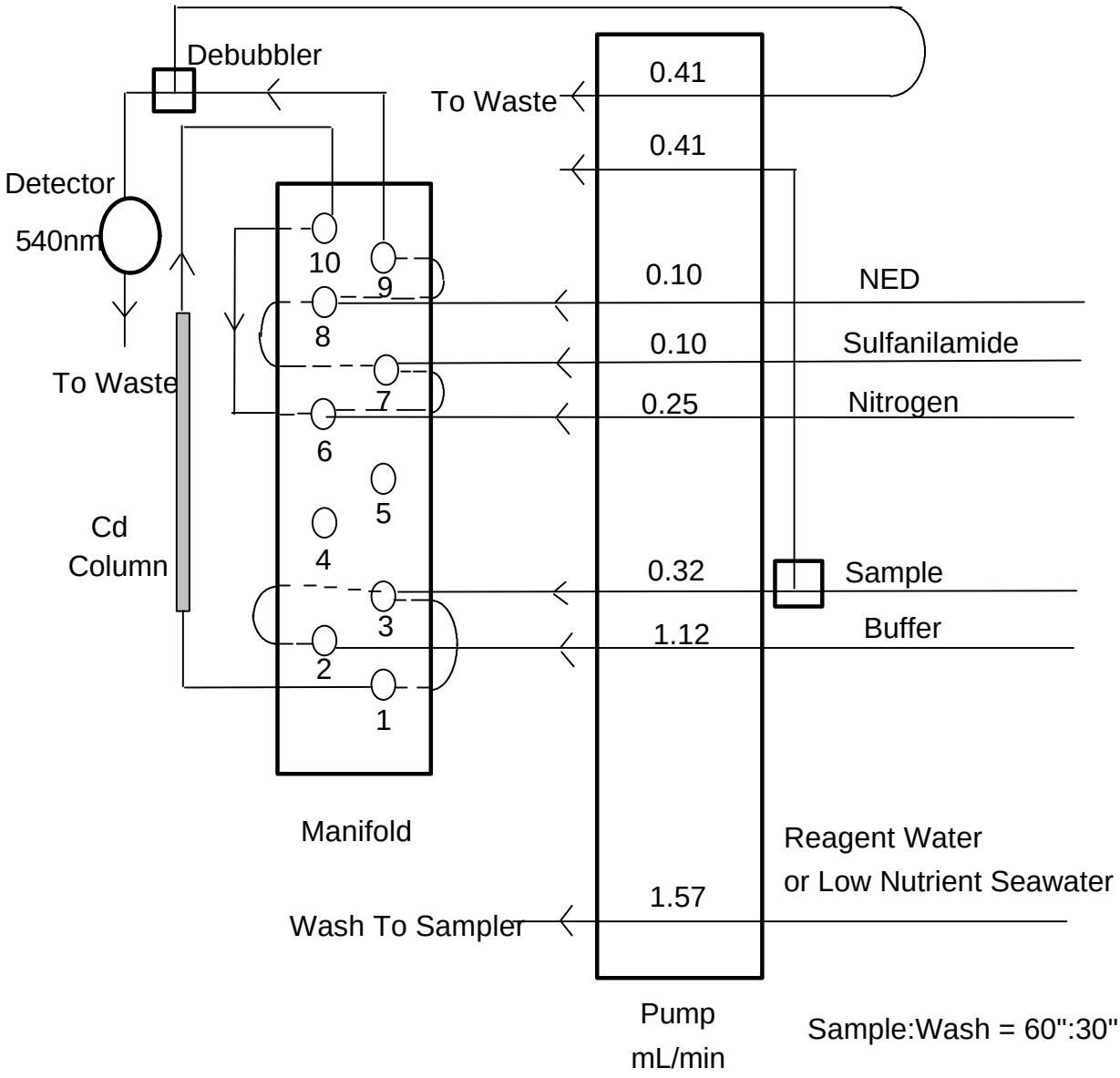

Figure 2. Manifold configuration for nitrate + nitrite analysis using a homemade packed copper-coated cadmium reduction column.

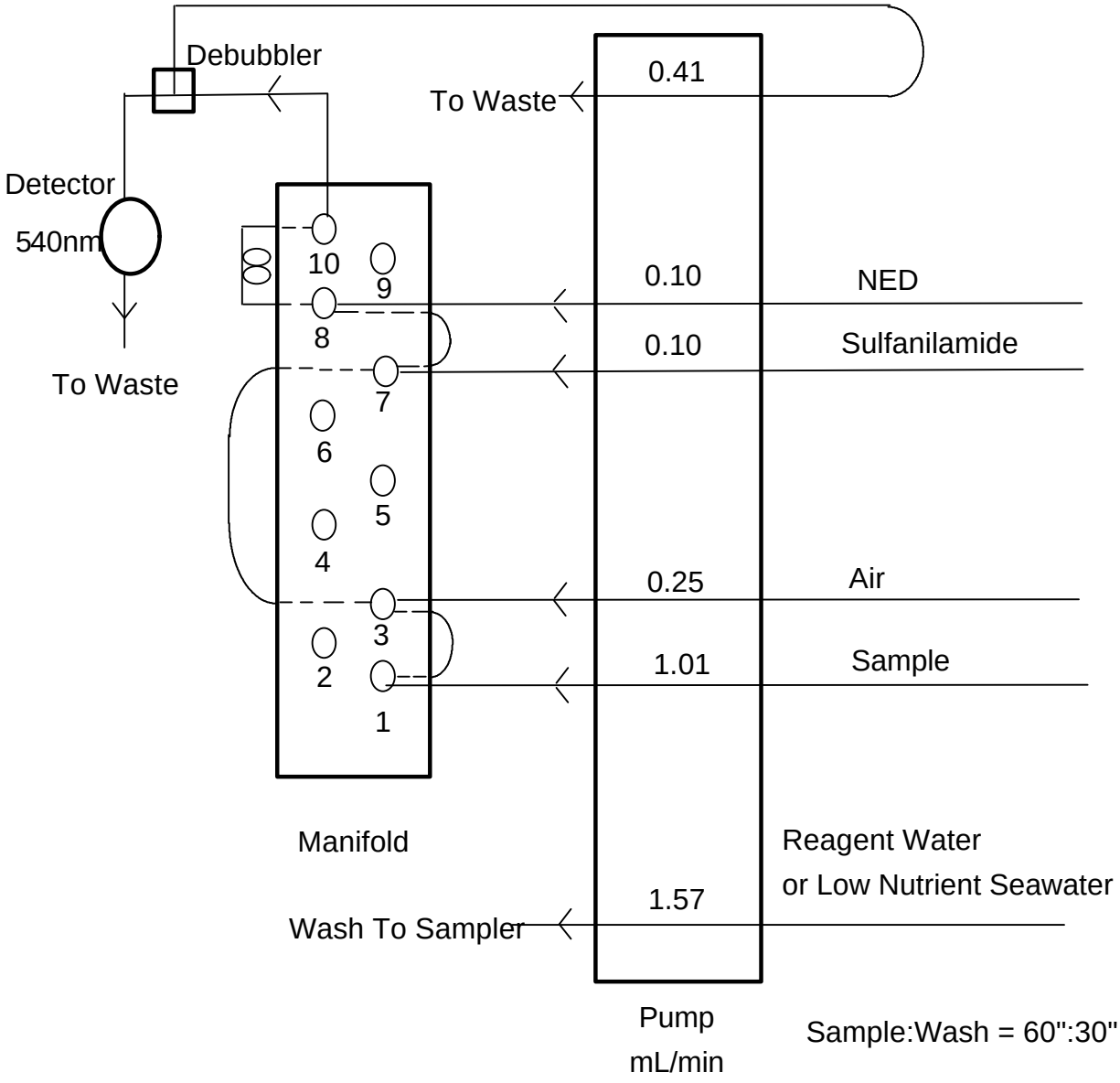

Figure 3. Manifold configuration for nitrite analysis.

Table 1 . Percentage recovery of nitrate from natural water samples preserved by freezing and refrigeration.

Method[A]	Sample[B]	Salinity	Time (Day)								
			0	7	14	21	28	35	46	62	92
25C, P	river	0.019	100	192.5	279	287.3	267.5	262.4	300.7	228.1	260.8
	estuary	13.263	100	108.5	106.2	124	103.9	139.3	258.9	188.5	229.1
	coast	32.623	100	102	128.8	153.8	93.3	89	44.2	72.4	84.9
25C, G	river	0.019	100	257	294.9	316.4	298.2	225.4	135.4	77.6	66.9
	estuary	13.263	100	108.8	108.5	122.5	90.6	79.2	81.5	56.2	128.2
	coast	32.623	100	98	135.2	150.9	98.5	84.3	36.9	56.1	66.6
4C,P	river	0.019	100	105	90	111.6	100.7	82.7	112.2	97.3	104.7
	estuary	13.263	100	104.5	90.4	107.1	102.6	95.9	109	82.4	101.4
	coast	32.623	100	127.6	65.7	149.1	82.3	93.3	43.3	73.5	89.2
4C,G	river	0.019	100	158.2	88.1	108.4	99.4				
	estuary	13.263	100	103.1	84.5	107.4	95.9				
	coast	32.623	100	100.9	54.4	123	68.9				
4C,P,	river+	0.019	100	105.5	99.2	106.1	96.2	91	114.8	98.4	96.9
	estuary+	13.263	100	110.2	116.4	104.8	102.9	93	110.9	85	99.7
	coast+	32.623	100	112.7	112.7	103.8	93.3	90.6	102.4	75.4	98.6
4C,G,	river+	0.019	100			105.7	98.3	101	114.5		
	estuary+	13.263	100			100.1	98	93.3	109.1		
	coast+	32.623	100			104.4	93.6	90.2	99.5		
Fr,P	river	0.019	100	100.5	100.4	103.9	95.8	88.6		85.7	95.9
	estuary	13.263	100	114.1	115.5	105.6	97.9	104.6	98.8	72.8	87.6
	coast	32.623	100	130.5	100.9	128.2	92.7	98.5	42.2	50.9	87.5
Fr,P,	river+	0.019	100	101.9	103.2	103.1	95.4	91.2	82.5	87.4	90.2
	estuary+	13.263	100	102	106.7	102.4	97.4	95	78.5	78	94.7
	coast+	32.623	100	103.2	111.1	101.3	91.5	92.1	104.7	69.6	92.3

Table 2 . Percentage recovery of nitrite from natural water samples preserved by freezing and refrigeration

Method[A]	Sample[B]	Salinity	Time(day)								
			0	7	14	21	28	35	46	62	92
25C, P	river	0.019	100	220	0.3	0	0	0	0	0	0
	estuary	13.263	100	110.6	456.8	920.2	957.8	661.5	58.7	0	0
	coast	32.623	100	104.1	92.2	74.1	89.5	74.1	94.6	72.2	0
25C, G	river	0.019	100	182.8	0.3	0	0	0	0	0	0
	estuary	13.263	100	108.5	519.1	1026.3	1079.1	867.5	843.1	705.7	209.2
	coast	32.623	100	100	87.8	73.8	89.5	73.5	95.9	85.7	66.5
4C,P	river	0.019	100	104.2	88.2	31.8	93.9	0	65	84.1	0
	estuary	13.263	100	102.8	101.8	38.9	0	91	17.8	8.5	0
	coast	32.623	100	68.4	65.7	33.2	70.5	50.5	0	0	0
4C,G	river	0.019	100	104.9	97.8	99.8	96.7				
	estuary	13.263	100	104.4	98.8	100.6	91				
	coast	32.623	100	94.3	87	71.1	97.6				
4C,P	river+	0.019	100	47.6	98.9	98.5	97.2	67.8	0	2.2	75.0
	estuary+	13.263	100	95.4	21.1	0	0	0	2.7	0	0
	coast+	32.263	100	0	0	0	0	0	0	0	0
4C,G	river+	0.019	100			97.9	95.8	84.6	85.9		
	estuary+	13.263	100			100.6	91.6	94.1	100		
	coast+	32.623	100			69.5	97.6	65.9	87.6		
Fr,P	river	0.019	100	70.6	86.2	98	77.3	68.1		74.9	77.3
	estuary	13.263	100	1.3	0.7	0	0	0	96	13.3	57.3
	coast	32.623	100	78.6	4.9	0	0	0	8.6	80	27.8
Fr,P	river+	0.019	100	97	87.2	95.4	75.9	75.9	63.1	75.2	69.2
	estuary+	13.263	100	103.5	98.6	95.9	52	90.5	74.2	0	77.6
	coast+	32.623	100	99.7	95.9	56.5	92.2	67	100.5	80	65.9

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next page

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[A] Methods of preservation:
25C,P and G: Store the samples in high density polyethylene carboys (P) or glass bottles (G) at room temperature (~25°C).
4C, P and G: Store samples in high density polyethylene bottles (P) or glass bottles (G) in a refrigerator (4°C) in the dark.
Fr,P and Fr,P: Freeze the samples in high density polyethylene bottles (P) and store at -20°C in a freezer in the dark.
Glass and high density polyethylene bottles were used to study the effect of type of sample bottles on the recovery of nitrite and nitrate from refrigeration.

[B] For salinity and concentration of nitrate in river, estuary and coast samples see section 13.1.2.
Sample river+, estuary+ and coast+ are the fortified river, estuary and coast samples, respectively, at nitrate concentrations 139.94 µg N/L.