



Applied Microbiology  
&  
Biotechnology Laboratory

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## Standard Operating Procedure

### AMBL-206-E

|                     |                 |
|---------------------|-----------------|
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| <b>Prepared by:</b> | Terry E. Baxter |
| <b>Reviewed by:</b> |                 |

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## Nitrate by Cadmium Reduction and Colorimetric Determination of Combined Nitrite

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### METHOD SUMMARY

This SOP describes a procedure for determining the nitrate concentration in water by using cadmium to reduce nitrate to nitrite and then colorimetrically determining the combined nitrite concentration by sulfanilamide diazotization. Nitrate is determined after correcting for any nitrite that may be present in the sample by also measuring the sample directly for nitrite. This method is adapted from Method 4500-NO<sub>3</sub> E (Standard Methods, 2017) and Method 8039 (Hach, 2014).

### ENVIRONMENTAL HEALTH AND SAFETY

**Hazards Assessment:** The hazards assessment presented in this SOP provides a general overview of potential hazards associated with using this method but is not intended to replace the information contained in the SDS for individual chemicals. Each user of this method must thoroughly review and understand the SDS for each chemical used.

This method routinely uses small quantities of reagents in prepared packets that minimizes hazards and chemical exposure. Occasional preparation of a sock nitrate solution involves the use of a small volume of chloroform and potassium nitrate. Cadmium metal is a component of the chemical in the reagent packet used in this method and may be collected for either reuse or separate disposal as a hazardous waste. Although all chemicals are handled in small quantities that minimize exposure, the potential hazards associated with this method are as follows.

Chloroform is highly toxic and is a suspected human carcinogen (GHS Category 2) and a suspected human reproductive toxicant (GHS category 2).

**Skin Contact:** Can cause skin irritation and inflammation (GHS Category 2).

**Eye Contact:** Causes severe eye irritation (GHS Category 2) that should fully reverse in 21 days.

**Ingestion:** Acutely toxic and harmful if swallowed (GHS Category 4).

**Inhalation:** Acutely toxic if inhaled (GHS Category 3).

Potassium nitrate is an oxidizing solid that can intensify fire (GHS Category 2).

**Skin Contact:** Can cause skin irritation (GHS Category 2).

**Eye Contact:** Can cause severe eye irritation (GHS Category 2) that should fully reverse in 21 days.

**Ingestion:** Causes irritation, discomfort, nausea or vomiting if swallowed.

**Inhalation:** May cause respiratory irritation if inhaled.

NitraVer 5 Reagent (GHS Category 2).

**Skin Contact:** Can cause skin irritation and repeated or prolonged contact may cause an allergic reaction in susceptible individuals. (GHS Category 2).

**Eye Contact:** Will irritate eyes. Can cause serious eye irritation (GHS Category 2A) that should fully reversed within 21 days.

**Ingestion:** May cause gastrointestinal irritation, nausea, diarrhea or vomiting and is considered harmful if swallowed.

**Inhalation:** May cause respiratory tract irritation and can be toxic if a large amount is inhaled (GHS Category 3).

**Safety Equipment and Engineering Controls:** When preparing the stock nitrate solution, chloroform is used as a preservative and handling and adding chloroform to this solution must be done in a fume hood. An eye wash/shower station should be immediately available.

**Personal Protective Equipment (PPE):** This method requires the use of the following PPE.

Gloves (nitrile)

Safety goggles or glasses

Laboratory coat

No respiratory protection is needed when properly handling the chemicals used in this method.

**Analysis-derived Wastes and Disposal:**

| Waste Generated   | Hazardous<br>( Y / N) | Disposal   |
|---|-----------------------|--|
| This procedure generates small volumes (10 mL) of an expended sample containing several small cadmium metal granules from the reagent used in the test. | Y                     | The expended sample is first filtered through a glass fiber filter to remove the cadmium and then may be disposed in a sink. The cadmium is removed from the filter and accumulated in a separate container. |

**METHOD DESCRIPTION****1.0 Introduction and Applicability**

Nitrate is a form of nitrogen that is readily available for uptake and use by aquatic plants and phytoplankton that, when present in excessive amounts, can significantly increase plant and phytoplankton productivity resulting in accelerated eutrophication and degraded water quality. Measurement of nitrate is important for assessing the quality of surface waters and for monitoring and control of biological processes treating municipal and industrial wastewaters. Fertilized agricultural soils will not only release nitrate to surface waters, but will also contribute to nitrate to groundwater where their presence can become a serious health concern (infant methemoglobinemia) if the groundwater is used as a drinking water source.

This method is used to reduce nitrate to nitrite with a cadmium catalyst and then measures the nitrite resulting from this reduction along with any nitrite that was already present in the sample. Measuring nitrite in the sample without the cadmium reduction step is required to correct the nitrate results for any pre-existing nitrite. This method is applicable for measuring nitrate in natural surface water and groundwater, agricultural runoff water, municipal wastewater, industrial wastewater and seawater.

**2.0 Apparatus**

- a. Membrane filter, 47 mm diameter, 0.45  $\mu\text{m}$  pore size
- b. Glass-fiber filter, with a 47 mm diameter, nominal pore size  $\leq 2.0 \mu\text{m}$  and  $\geq 1.0 \mu\text{m}$
- c. Filtration funnel assembly for a 47 mm size diameter filter.
- d. Vacuum suction flask, 1000 mL capacity.
- e. Glass test tube, 50-mL, to collect filtered sample. If possible, have one filtrate collection tube for each sample filtered.
- f. 50-mL, 20-mL, 10 mL and 5-mL volumetric pipets, Class A
- g. 1000-mL and 100 mL volumetric flasks, Class A

- h. 10 mL sample cells, matching pair of 1-cm square cuvettes, (Hach 2495402). Inspect sample cells before use to verify that there are no abrasions, scratches or other marks on the glass walls. Replace the matching pair if either cell is damaged.
- i. DR 3900 or DR 2010 spectrophotometer (Hach), or an equivalent spectrophotometer capable of being set at a single wavelength of 500 nm to 543 nm.
- j. Rubber stoppers for 10-mL sample cells (size 1)
- k. Glass or ceramic Buchner funnel with a waste sample collection flask.

### 3.0 Reagents

- a. Stock nitrate solution: Dry potassium nitrate ( $\text{KNO}_3$ ) for 24 hours at a temperature of  $103 - 105^\circ\text{C}$ . Dissolve  $721.8 \text{ mg} \pm 0.5 \text{ mg}$  dried  $\text{KNO}_3$  in reagent water and dilute to 1000 mL ( $1.00 \text{ mL} = 100 \mu\text{g NO}_3^- \text{ as N}$ ). Transfer to a 1-L reagent bottle, preserve with 2 mL chloroform ( $\text{CHCl}_3$ ). This solution is stable for 6 months.
- b. Intermediate nitrate solution. Dilute 100 mL of stock nitrate solution to 1000 mL with reagent water ( $1.00 \text{ mL} = 10.0 \mu\text{g NO}_3^- \text{ as N}$ ). Transfer to a 1-L reagent bottle, preserve with 2 mL chloroform ( $\text{CHCl}_3$ ). This solution is stable for 6 months.
- c. NitraVer 5 Nitrate reagent packet for 10-mL sample (Hach, 2106169)
- d. Reagent water, distilled water.

### 4.0 Filtration of Samples

- a. Remove sample turbidity or color by filtering through a  $0.45 \mu\text{m}$  membrane filter. Pre-filter samples through a pre-washed (see 4.0 c) glass fiber filter if filtration time is greater than 10 minutes.
- b. Wash filters before use to remove trace amounts of nitrite and nitrate. Prepare only the number of filters (membrane and glass fiber) that you plan to use.
  - 1. Presoak filters overnight in 2 L of reagent water.
  - 2. Decant reagent water and soak again for another 1 hour in 2 L of reagent water.
  - 3. Decant reagent water, remove filters and store them in a smaller holding container (a glass petri dish works just fine). Add fresh reagent water.
- c. Carefully remove a filter from the holding container, place it on the filtration apparatus support screen and draw a slight vacuum to remove all excess water from the filter to near dryness. Secure the filtration funnel over the filter and turn off the vacuum.

- d. Remove the vacuum apparatus from the vacuum flask and carefully place the 50-mL tube in the flask. Replace the vacuum apparatus so that the filtrate tube on the bottom of the filtration apparatus is inside the test tube.
- e. Turn on the vacuum just enough to filter the sample without causing sample to be splash out of the filtrate collection tube. Collect approximately 40 mL of filtered sample.
- f. Remove the vacuum apparatus from the vacuum flask and carefully remove the 50-mL tube from the flask. Analyze as soon as possible or cover the tube with a wax film or transfer this sample to a clean 60-mL plastic sample container and secure the cap.
- g. Before filtering the next sample, thoroughly rinse the vacuum apparatus and the 50-mL filtrate collection tube with reagent water. Remove excess water from the vacuum apparatus by placing it on the flask and apply a vacuum until dry. Remove excess water from the collection tube by inverting and shaking the water out, and then leave the tube inverted (in a tube rack) to finish drying.

## 5.0 Procedure for Analysis of Nitrate

### 5.1 Select the Instrument Mode

The instruments used to measure nitrate can be set to perform this measurement in either a stored program mode or a manual mode. The manual mode is preferred over the stored program mode.

- a. Turn on the DR3900 or DR 2010 spectrophotometer allowing it to initialize and warm up according to manufacturer's instructions. The instrument is ready to use after initialization has completed.
- b. Once the instrument is ready, select to measure nitrate either by using a stored program, or by using a manual mode.
- c. Stored Program Mode Measurements. The stored program mode uses a preprogrammed calibration that is recalled from memory by entering the appropriate stored program code.
  1. DR 3900. On the main menu screen, select Stored Programs to display the alphabetical list of stored programs and program code numbers. Scroll through the list to find **N Nitrate HR PP** and touch the screen to highlight this method and press START to run the program.

Alternately, after selecting Stored Programs from the main menu screen, you may press the "Select by Number" option from the bottom, enter program code 355 and then press OK. Press START to run the program.

2. DR 2010. Enter program code 355 (press keys 3-5-5-Enter) and dial the wavelength setting to 500 nm.
3. When using the stored program mode the default instrument readout is typically set to display units of mg/L for the  $\text{NO}_3^-$  - N chemical form. This is the chemical form desired.
- d. Manual Measurement Mode (preferred). The manual mode is used to obtain sample absorbance readings that are then compared to a concentration-absorbance calibration curve to determine the concentration of the unknown sample.
  1. DR 3900. On the main menu screen select Single Wavelength to display the options menu for single wavelength mode. Check that the reading option displayed is set to absorbance (Abs), otherwise press Abs/%Trans./Ext to switch between % transmittance, concentration and absorbance until Abs is displayed. Press the wavelength icon ( $\lambda$ ) and, using the numeric keypad, enter 500. NOTE: You can also display absorbance readings when using the stored program mode by pressing the Abs, % Trans, Conc Option key from the Options menu until "Abs" is displayed.
  2. DR 2010. Press the SHIFT key and then the ABS (8) key, and then dial the wavelength setting to 500 nm. NOTE: Pressing the SHIFT and ABS keys will also display the absorbance reading when running a stored program.

## 5.2 Prepare the Sample for Measurement

- a. When using the DR 2010 a 10-mL cell riser must be inserted into the cell compartment. Check if a cell riser is already in the cell compartment and if it is not, insert one.
- b. If sample concentration is known to be greater than the upper limit of the calibration curve, dilute the sample with reagent water to bring the diluted sample concentration to a value within the calibration range. Use the dilution factors in the following table as guidance when diluting samples.

| Expected Final<br>Concentration Range,<br>$\text{NO}_3^-$ - N mg/L | Dilution Factor | Diluted Concentration<br>Range, mg/L |
|--|-----------------|--------------------------------------|
| 32 to 60   | 1/2             | 16 to 30                             |
| 62 to 150  | 1/5             | 12.4 to 30                           |

- c. Quantitatively transfer 10.0 mL of the same sample into each of two optically matched 10-mL sample cells. One of these sample cells will be the sample blank used to zero the instrument.
- d. Add the entire contents of the NitraVer 5 nitrate reagent packet to one of the sample cells – this is the measured sample. Start the mixing timer and immediately place the rubber stopper in the sample cell and mix vigorously for 1 minute.
  1. DR 3900. On the Toolbar display, located on the right side of the screen, press Timer to select and activate the timer function. If using a Stored Program, the mixing timer is pre-programmed. Press OK to begin the 1-minute mixing period. If using the Single Wavelength (manual mode) the numeric keypad is displayed. Enter 1-0-0 and press OK to begin the 1-minute mixing period. The timer function display will show the time remaining. A sound will be emitted when the mixing period is finished.
  2. DR 2010. When using a Stored Program mode, the timer is pre-programmed. Press the SHIFT key and then the Timer (5) key. This begins the 1-minute mixing period and the display will show the time remaining. If the stored program does not have a pre-programmed time or if using Manual measurement mode, press the SHIFT key and then the TIMER key to display “Timer=MM:SS” and then press 1-0-0. Press the ENTER key to begin the 1-minute mixing period. Three series of four beeps will sound when the reaction period is finished.

At the end of the mixing period, undissolved reagent and small cadmium granules will settle to the bottom of the sample cell. This is normal and will not interfere with the measurement as long as the undissolved reagent and granules remain settled.

- e. Immediately after the mixing period has finished (the timer beeps have finished), start the second instrument timer, which will provide a 5-minute reaction time.
  1. DR 3900. On the Toolbar display, located on the right side of the screen, press Timer to select and activate the timer function. If using a Stored Program, the reaction timer is also pre-programmed. Press OK to begin the 5-minute reaction period. If using the Single Wavelength (manual mode) the numeric keypad is displayed. Enter 5-0-0 and press OK to begin the 5-minute reaction period. The timer function display will show the time remaining. A sound will be emitted when the reaction period is finished.
  2. DR 2010. When using a Stored Program mode, the reaction timer is pre-programmed. Press the SHIFT key and then the Timer (5) key. This begins the 5-minute reaction period and the display will show the time remaining. If the stored program does not have a pre-

programmed time or if using Manual measurement mode, press the SHIFT key and then the TIMER key to display "Timer=MM:SS" and then press 5-0-0. Press the ENTER key to begin the 5-minute reaction period. Three series of four beeps will sound when the reaction period is finished.

- f. During the 5-minute reaction time, use a soft cloth to clean the outside glass of both the blank and the sample cells.

### 5.3 Conduct the Measurement

- a. When the reaction period has completed (the instrument will emit a sound or series of beeps), the instrument must be zeroed before the sample measurement reading can be obtained.
- b. Insert the blank sample cell into the cell compartment and close the cover. Zero the instrument.
  1. DR 3900. The 10-mL mark on the sample cell must face towards the front of the instrument. Press Zero. This also activates the Read key. Repeat if the instrument does not display 0.00 mg/L or 0 absorbance.
  2. DR 2010. The 10-mL mark on the sample cell must face towards the left of the instrument. Press the Zero key. Repeat if the instrument does not display 0.00 mg/L or 0 absorbance.

Open the cover and remove the blank sample cell.

- c. Remove the rubber stopper from the sample cell, and then insert the sample cell into the cell compartment with the 10-mL mark on the sample cell facing the same direction as the blank sample cell (see 6.3b above).
  1. DR 3900. The display will show the results or press the Read icon to refresh the display.
  2. DR 2010. The display will show the results or press the Read key to refresh the display.

Open the cover and remove the sample cell.

- d. Immediately after finishing with each pair of blank and sample cell measurements, pour the contents of both sample cells onto a glass fiber filter in the Buchner funnel and waste sample collection flask to separate the cadmium granules from the waste sample. Rinse the sample cells as necessary to remove all granules. Tap water is acceptable. The collected waste sample and rinse water can be disposed in the sink.
- e. Continue to rinse the sample cells and the rubber stopper with reagent water. **NEVER allow sample or water to remain in a sample cell.** Place the sample cells upside down on a clean adsorbing pad to dry. To avoid scratching the glass walls, **NEVER place the sample cells on the**



**drying rack or with other glassware on the drain shelf or in the Contrad solution bath.** To avoid etching the sample cell matching numbers from the glass, **NEVER use the Contrad solution bath to clean the cells.**

## 6.0 Calibration Curve

- a. Preparation and use of an external calibration curve is preferred and recommended to ensure greater control of the measurement process. However, the pre-programmed calibration provided with a stored program is acceptable as long as it has been verified using a known calibration check sample.
- b. Prepare calibration standard solutions as follows.

| To obtain a calibration solution having this concentration of $\text{NO}_3^-$ as N | Dilute this volume of stock or intermediate nitrate solutions to 100 mL. |
|--|--|
| 30 mg/L  | 30.0 mL <sup>a</sup>   |
| 25 mg/L  | 25.0 mL <sup>a</sup>   |
| 20 mg/L  | 20.0 mL <sup>a</sup>   |
| 15 mg/L  | 15.0 mL <sup>a</sup>   |
| 10 mg/L  | 10.0 mL <sup>a</sup>   |
| 5.0 mg/L   | 50.0 mL <sup>b</sup>   |
| 1.0 mg/L   | 10.0 mL <sup>b</sup>   |
| 0.03 mg/L  | c  |
| Blank  | d  |

a Using stock nitrate solution (1.00 mL = 100  $\mu\text{g}$ ).

b Using intermediate nitrate solution (1.00 mL = 10.0  $\mu\text{g}$ ).

c Prepare the 0.03 mg/L standard by diluting 3 mL of the 1.0 mg/L standard to a total volume of 100 mL.

d A calibration blank, consisting of reagent water and reagents and also known as a reagent water blank, is included.

- c. Allow prepared calibration standards to equilibrate to the same temperature as the samples, equilibrating both to the temperature of the room.
- d. Using reagent water as the blank, conduct the measurement of each calibration standard in the manual mode as described above in 5.1.d or 5.2.
- e. The most recent calibration curve data for both the DR 2010 and DR 3900 are available in an Excel spreadsheet file that may be downloaded from <https://www.cefn.s.nau.edu/~teb/amb/amb SOPs.html>. When

updating the calibration data, enter the standard concentration values, if different than provided, in the “Concentration” column, and enter the absorbance readings for each concentration in the “Abs” column. The Excel file automatically calculates the slope, intercept and r-squared values from a linear regression analysis of the calibration data. The resulting calibration curve and a basic calculator that uses the linear calibration curve equation for calculating a sample’s concentration are also provided in this file.

## 7.0 Calculation and Reporting

- a. Direct Readout. When using the direct readout mode, the nitrate-nitrogen concentration is taken directly from the display and no calculation is necessary. In order to correct for any nitrite that may have been in the sample prior to the nitrate reduction step, a separate aliquot of sample must also be measured directly for nitrite without having gone through the cadmium reduction step (SOP 205D). The corrected nitrate-nitrogen concentration is calculated as follows:

$$\text{NO}_3^- \text{-N, mg/L} = \left( \frac{R}{D} \right) - C$$

where  $R$  = reading,  $\text{NO}_3^- \text{-N, mg/L}$

$D$  = the dilution factor

$C$  = sample concentration of nitrite-nitrogen,  $\text{NO}_2^- \text{-N, mg/L}$

- b. Using the calibration curve: When using the manual mode with a concentration-absorbance calibration curve, the absorbance reading is taken from the display and the nitrate-nitrogen concentration corrected for pre-existing nitrite-nitrogen is calculated as follows:

$$\text{NO}_3^- \text{-N, mg/L} = \left( \frac{A - b}{m \times D} \right) - C$$

where  $A$  = absorbance reading

$b$  = intercept from regression analysis of calibration standards

$m$  = slope from regression analysis of calibration standards

$D$  = the dilution factor

$C$  = sample concentration of nitrite-nitrogen,  $\text{NO}_2^- \text{-N, mg/L}$

- c. Report the concentration of nitrate-nitrogen as “mg/L  $\text{NO}_3^- \text{-N}$ ” according to the following table.

| Range of<br>Concentration,<br>mg/L | Report to the<br>Nearest mg/L |
|------------------------------------|-------------------------------|
| 0.01 to <1.00                      | 0.01                          |
| 1.0 to <10.0                       | 0.1                           |
| 10 to <30                          | 1                             |
| ≥30                                | 5                             |

## 8.0 Minimum Required Quality Control

- Continuing Calibration Verification (CCV) sample. Before analyzing any samples, analyze a CCV sample. Thereafter, analyze a CCV sample after each batch of 20 or fewer samples. Select a concentration that is in the lower-range of the calibration curve (typically either the 01.0 or 5.0 mg/L standard). Prepare a new calibration curve if the calibration check sample differs from the most recent calibration by more than 10% and the difference cannot be attributed to improper preparation of the standard.
- Reagent water blank. Analyze one reagent water blank, as any other sample, for each batch of 20 or fewer samples. The result of the reagent water blank must be less than the most recently determined method detection limit (MDL). If not, the source of the error must be determined and corrected until an acceptable reagent water blank is analyzed, and all samples since the previous acceptable reagent water blank must be reanalyzed.
- Duplicate sample or Laboratory Fortified Matrix (LFM) and Laboratory Fortified Matrix Duplicate (LFMD). Analyze at least one sample in duplicate for each batch of 20 or fewer samples. If a single sample is being analyzed, this sample must be analyzed in duplicate. When reactive nitrate is not measured in a matrix type, prepare and analyze an LFM and LFMD. Acceptance criteria (precision) for duplicate analysis using this method has not yet been established.

## 9.0 Bibliography

- Rodger B. Baird, Andrew D. Eaton, and Eugene W. Rice (2017) *Standard Methods for the Examination of Water and Wastewater*. APHA, Washington, DC, 23rd Edition.
- Hach Company, (2014) Nitrate. Cadmium reduction method, Method 8039 (DOC316.53.01066), Water Analysis Handbook, Hach Company, Loveland, CO, Edition 9.