Standard Operating Procedures for Tidal Monitoring

Integration of Citizen-based and Nontraditional Monitoring into the Chesapeake Bay Program Partnership

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Alliance for the Chesapeake Bay

In cooperation with
Maryland Department of Environmental Science, Alliance for Aquatic Resource Monitoring, and the Izaak Walton League of America

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Acknowledged Works

Much of the information in this manual has been adapted from the following methods manuals:

Alliance for the Chesapeake Bay. 2012. Citizen Monitoring Program Manual

EcoCheck. (2011). Sampling and data analysis protocols for Mid-Atlantic tidal tributary indicators. Wicks EC, Andreychek ML, Kelsey RH, Powell SL (eds). IAN Press, Cambridge, Maryland, USA.

Virginia Citizen Water Quality Monitoring Program. 2007. Virginia Citizen Water Quality Monitoring Program Methods Manual

Center for Marine Conservation & U. S. EPA. Volunteer Estuary Monitoring: A Methods Manual, Second Edition.

U.S. EPA. 1997. Volunteer Stream Monitoring: A Methods Manual. EPA 841-B-97-003.

U.S. EPA. 1996. Recommended Guidelines for Sampling and Analyses in the Chesapeake Bay Monitoring Program. EPA 903-R-96-006.

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1 Before You Begin

1.1 Safety, Equipment List, and Volunteer Responsibilities

1.1.1 Safety – General Precautions

a) Always perform water-monitoring activities under the guidance of an adult.

b) Read all instructions to familiarize yourself with the test procedure before you begin. Note any precautions in the instructions.

c) Keep all equipment and chemicals out of the reach of young children and pets.

d) Avoid contact between chemicals and skin, eyes, nose and mouth.

e) Read the label on each reagent container prior to use. Some containers include precautionary notices or antidote information on the back of the container.

f) In the event of an accident or suspected poisoning, immediately call the Poison Control Center phone number in the front of your local telephone directory or call your physician. Be prepared to give the name of the reagent in question and its code number. Most kit reagents are registered with POISINDEX, a computerized poison control information system available to all local poison control centers.

1.1.2 Protect Yourself & Your Equipment: Use Proper Technique

a) Wear safety goggles or glasses when handling reagent chemicals.

b) Use the test tube caps or stoppers, not your fingers, to cover test tubes during shaking or mixing.

c) When dispensing a reagent from a plastic squeeze bottle, hold the bottle vertically upside-down (not at an angle) and gently squeeze it (if a gentle squeeze does not suffice, the dispensing cap or plug may be clogged).

d) Wipe up any reagent spills, liquid or powder, as soon as they occur. Rinse area with a wet sponge, and then dry.

e) Thoroughly rinse test tubes before and after each test. Dry your hands and the outside of the tubes.

f) Tightly close all reagent containers immediately after use. Do not interchange caps from different containers.

g) Avoid prolonged exposure of equipment and reagents to direct sunlight. Protect them from extremely high temperatures. Protect them from freezing.

1.2 Monitor Responsibilities

Choose a regular sampling day: Choose a convenient day of the week for sampling. Samples should be taken at regular weekly or monthly intervals. If it is not possible to sample on the same day each week, try to sample within 2 days (either side) of your regular day spacing the sampling dates, 5 to 9 days apart. Sample at the same time of day each week; if you are sampling multiple locations, be sure to always sample your sites in the same order each monitoring run to achieve similar sample timing.

Record your test results: Record data on a data collection form provided. Always record the test results as you go along. Keep a copy of the data collected for your records and to provide a backup copy should the original be lost.

Provide comments as necessary: The "Comments" section can be used to record general observations about the site especially changes due to erosion, recent notable weather, and any problems you had with the sampling procedures.

Submit data to database: If you have access to the internet, submit your data to the project's online database.

Send datasheets once every three months. Mail the data sheets to the Alliance or your Watershed Coordinator every three months so that we can maintain a current database.

Stay certified: Attend a recertification session every other year to maintain your skills and learn new information and techniques. You can also attend any training session to refresh yourself of the concepts and procedures between re-certifications.

2 QA/QC Procedures

2.1 Certification and Re-certification

2.1.1 Certification

All monitors that wish to submit Tier II data must gain monitor certification. Monitors can become certified at their initial training session by demonstrating a mastery of the sampling procedures and complete understanding of the quality assurance protocols used during data collection to be assessed by a Project Team member or Certified Trainer. Monitors must also pass a test that assesses the monitor's understanding of QA/QC procedures outlined in this SOP and the project QAPP with a 90% score.

Monitors that attend an initial training and are unable to pass the requirements to become certified at the end of the training will be encouraged to continue practicing their monitoring procedures. Un-certified monitors are encouraged assist a certified monitors in the field until they have become comfortable with the procedures and QA/QC protocols. Un-certified monitors are allowed to retake the certification test, and demonstrate proper sampling and analysis technique up to three times in order to become a certified monitor.

When a monitor achieves certification, they may be assigned a site and begin to collect Tier II data and submit it to the project database.

2.1.2 Re-certification

The Project Team and Certified Monitors will host recertification sessions biennially for monitors that have passed the initial training and wish to maintain their certification. Recertification sessions are conducted in a fashion that is similar to a lab practical. Monitors are checked to assure that: they remain proficient in methodology and understanding of basic water quality parameters; their equipment is operational and properly calibrated / verified; and they have an adequate supply of viable chemicals, procedures, equipment verification/check, and updated information about monitoring.

The recertification session is set up with a "station" for each water quality parameter. Monitors perform the test and compare their results to a known or controlled result. Project staff observe the monitors' methods and ensure that monitors correctly perform the tests and accurately record the data. After completing and "passing" one parameter, the monitor moves through each of the other stations while completing a datasheet that serves as documentation of re-certification. Replacement equipment, datasheets, information, and chemicals are given if needed. Alliance for the Chesapeake Bay retains documentation of recertification sessions.

2.1.3 Field Audits

Project Team members, the QC manager, or Certified Trainers may accompany monitors in the field and observe field collection procedures as part of the recertification process for monitors. Monitors will demonstrate proper sample collection, analysis, labelling, and preservation in accordance with this SOP.

2.2 Pre-monitoring checks

2.2.1 Equipment Check

Prior to going out into the field, monitors should check their equipment for cleanliness, breakage, probe function and battery life, and chemical expiration dates. If a monitor finds that their equipment is damaged and will affect the quality of the data they collect they will not collect data that day and mark the reason on their data sheet. The monitor should contact their Project Team member to get the equipment repaired or replaced prior to the next scheduled sample.

Monitors measuring dissolved oxygen using the Winkler titration will check the viability of their sodium thiosulfate solution prior to each monitoring event and record the results on their field datasheet. Sodium thiosulfate is used for monitoring dissolved oxygen. By using a standard solution of iodate-iodide, with 10 mg/L dissolved oxygen value, the monitor must record a value of 9.4 – 10 mg/L with their sodium thiosulfate measurement.

If results of the first check are above or below these intended values, a second check is performed. If the second check yields unacceptable values or if the two checks are greater than 0.4 mg/L apart from each other, the monitor is instructed to abandon the dissolved oxygen test because the sodium thiosulfate is no longer viable. The monitor must replace all expired chemicals prior to sampling again.

2.2.2 Calibration

Monitors will calibrate any equipment that requires calibration prior to being used (within 24 hours of use), using standard solutions and following the manufacturer's instructions. Monitors will note on their data sheet that they calibrated their equipment.

After sampling, it is recommended that monitors check their probes against the standard solutions used for calibration to identify instrument drift. If pH is outside of +/- 0.20 units, DO is +/- 0.3 mg/L, or specific conductance is +/- 5% of verification standards, the data must be flagged and the probe must be assessed and fixed or replaced if needed.

Monitors record these calibration and verification values on their datasheet and values are entered into the online database.

Thermometers that are verified should be re-verified every year. Thermometers must be verified against the Alliance master precision thermometer that is annually verified against an NIST-traceable thermometer to 0.2° C.

2.3 Field QC

2.3.1 Duplicates

If monitors are using the Winkler titration method for measuring DO they will perform the dissolved oxygen test on the actual water sample in duplicate. Monitors are instructed to do a third titration if their two initial titrations differ by more than 0.6mg/L. The two closest values are recorded on the datasheet.

Monitors collecting samples for Tier II laboratory analysis will perform duplicate samples at least 10% of the time. Duplicates consist of either collecting a larger sample for mixing and splitting it between two containers or immersing sample containers side by side in the water at the same time.

2.3.2 Replicates

Monitors will perform replicate samples of all other parameters (DO using Winkler titration method must be done in duplicate each sample) 10% of the time. The quality control samples are prepared and analyzed for all parameters of interest. The field replicate data are used to determine the overall precision of the field and laboratory procedures.

2.3.3 Field Blanks

Monitors will perform blank samples 10% of the time for samples to be sent to a lab for analysis. Monitors will perform all field procedures including preserving the samples as required and taking to the lab for analysis using deionized water provided by the laboratory. Results from field blanks will be recorded and appropriately marked during database entry.

3 Field Monitoring Procedures

3.1 Field Sampling Procedures

3.1.1 Best Practices

a) Use of protective gloves. Gloves serve a dual purpose: 1) protecting the sample collector from potential exposure to sample constituents and 2) minimizing accidental contamination of samples by the collector. Wearing protective gloves at all times while sampling is recommended. Latex or nitrile gloves may be used for common sampling conditions.

b) Safety always comes first. All sampling should be conducted with the proper equipment and least amount of danger to field personnel.

c) Permission must be obtained from landowners before entering private property.

d) Care should be taken not to disturb the bottom when sampling. When entering a stream, always walk in an upstream direction.

e) Surface water should always be collected facing upstream and in the center of main area of flow. Therefore, unless safety is an issue, samples should be obtained from a bridge or instream.

f) Samples should be collected in the main flow representative of the stream you are monitoring (for small streams, this is usually mid-channel) just below the water surface, about 0.3 meters (1 foot) deep.

g) Whenever possible, collect field measurements directly from the sample site, not from bucket. If the field parameters need to be measured in the bucket, collect water quality samples (nutrients, etc.) first before placing the multi probe instrument in the bucket.

h) When there are obvious standing pools of water during low or no flow conditions, do not collect samples or field measurements. Make a note of this on the data sheet.

i) When collecting bacterial samples:

   i. DO NOT rinse the bacteria sample bottle before collecting the sample.
   ii. If sample bottles contain a dechlorinating tablet (usually small white tablet) and you are collecting an unchlorinated sample, dump out the tablet before collecting the sample.
   iii. Be careful not to insert fingers into the mouth of the container or on the interior of the cap.

3.1.2 Sampling from a Boat

Using a probe
If you are measuring with a multi probe and have a long enough cord, you are encouraged to do depth profiles. If the depth is ≤3 m deep take a surface and a bottom measurement. If the depth is >3 m measure 0.3 m above the bottom, then 1 meter intervals to 0.3 m below the surface. At each iteration allow the probe to stabilize before recording your reading at the corresponding depth.

If you are taking surface measurements, place your probe beneath the surface of the water, wait for the probe to stabilize, and then record your reading.

If the meter is not equipped with a pressure gauge for depth estimation and the current is strong enough to pull the meter so that the cable is at an angle noticeably different than vertical, estimation of depth will have to be corrected. Weighted probe guards may help prevent displacement by current.

Using sample bottles
For chlorophyll samples, rinse the sample bottles and syringe three times with sample water. Drain the bottle until it is empty, put the cap on, lower it one arm's length under water (about 0.5 meters) then remove the cap. Wait for the bottle to fill, then cap it and return it to the surface. For nutrient samples, water should be taken one meter from the surface facing upstream of the boat rinsing the bottle three times before final sample collection.

3.1.3 Streambank and Instream Sampling

If possible, wade into the stream to collect the sample. If wading to the sample site, always proceed upstream to allow the flow of the water to push any disturbed sediment downstream of where you will be collecting the sample.

When sampling from the streambank, care should be taken to sample from an area that will most closely represent the entire stream. Typically, this will be the area of the greatest flow in the stream and away from stagnant pools or eddies.

Step	Bacteria Samples	Nutrient and Chlorophyll Samples
1.	Walk upstream to the sample location. Be sure any sediment or debris disturbed from your movement in the streambed is not present where you will collect the sample.	Walk upstream to the sample location. Be sure any sediment or debris disturbed from your movement in the streambed is not present where you will collect the sample.
2.	Submerge the container; neck first into the water. The mouth of the bottle should be completely below the water surface approximately 3-6 inches.	Lower the sample bottle so that one edge of the opening is just below the water.
3.	Invert the bottle so the neck is upright and pointing into the water flow.	Allow the bottle to fill to the neck of the bottle.
4.	Move the bottle forward away from the body for at least six inches.	Lift the filled container. Do not pour out any excess water.
5.	Return the filled container quickly to the surface. Pour any excess water and cap.	

3.1.4 Dock or Bridge Sampling

1. Sample in the center of main flow from or as close as you can get on the dock. If sampling from a bridge sample from the safest side of the bridge and where contamination is least likely to occur. Typically, sampling on the upstream side of the bridge or dock is less likely to be contaminated.

2. During rainy periods, avoid sampling where storm water runoff from the bridge can affect sample.

3. Obtain field parameters (DO, pH, temperature) first before lowering a sample bucket.

4. When lowering the sample bucket, allow it to fill ¼ the way full and retrieve. Swirl the contents and dump the rinse away from the sample location to avoid kicking up sediment.

5. Repeat step 4 two more times and on the final time fill ½ to ¾ the way full.

6. Retrieve the bucket and collect the samples in the following order.

1. Bacteria
    - Open the bottle without touching the inner wall of the bottle or lid.
    - Invert the bottle by holding to the main body of the bottle and lower into the bucket 3-6 inches.
    - Fill the bottle in a 'U' from the side of the bucket closest to you to the opposite end.
    - At the end, bottle opening should be facing up and remove from the bucket.
    - Pour off any excess water and cap with the lid.

2. Nutrients and Chlorophyll
    - Open the bottle and tilt so that one side of the bottle will be below the waterline of the bucket.
    - Allow the bottle to fill to the neck of the bottle.
    - Remove the bottle and cap. Do not pour off any excess sample.

7. In situations where field parameters must be obtained from the bucket, all water samples must be collected prior to inserting the probe in the bucket.

3.2 Air Temperature Measurement

Equipment: armored, digital thermistor, or probe

Temperature is reported in degrees Celsius (ºC). Always measure air temperature before water temperature.

Method:

1. Locate a place near your site and hang the thermometer out of the direct sun.

2. Wait 3-5 minutes to allow the thermometer to equilibrate.

3. Record air temperature to the nearest 0.5 ºC for the armored thermometer or to the nearest tenth of a degree for the digital thermistor or probe on Page 2 of the datasheet.

3.3 Recording General Observations

Record weather and general observations on the datasheet.

3.4 Water Clarity & Turbidity Measurement

3.4.1 Secchi Disk

Equipment: 8" Secchi disk with attached line

Method:

1. Remove sunglasses if you are wearing them and stand with the sun to your back. Try to lower the disk into a shaded area.

2. Lower the disk into the water until the disk barely disappears from sight. Note the depth reading, in meters, based on the length of line submerged. Each mark is one-tenth (or 0.1) meter.

3. Slowly raise the disk and record the depth at which it reappears (i.e. is barely perceptible).

4. Average the two depth readings obtained above. The average of the two readings is considered to be the limit of visibility, or index of transparency. Record this average to the nearest tenth of a meter on your data form.

3.4.2 Transparency Tube

Transparency tubes are a type of equipment used for measuring transparency of water in streams and rivers. They are helpful for measuring transparency in situations where the stream is too shallow for the Secchi disk to be practical and for running waters where flow is too fast that the Secchi disk cannot remain vertical. Sample water collected either directly from the stream or from the sampling bucket is analyzed.

Equipment: Transparency tube

Method:

1. Close the drain tube by squeezing the crimp.

2. Fill the transparency tube with your sample water. Water may be collected directly from the stream in the vicinity of the sampling location if the stream is too small to fill the bucket, or sample water collected in the sampling bucket may be used (See 5.4, "Collecting the Water Sample"). To collect water directly from the stream, point the top of the tube in the upstream direction and collect surface water, being careful not to disturb the stream bed. To analyze water collected in the bucket, pour sample water from the bucket water directly into the transparency tube.

3. While looking down through the opening of the tube, partially open drain crimp, slowly draw off sample (Control flow by squeezing the crimp).

4. When the black and white pattern begins to appear, immediately tighten the crimp.

5. Record the level of water remaining via the centimeter ruler on the side of tube.

3.4.3 Turbidity Kit

This test is performed by comparing the turbidity of a measured amount of the sample with an identical amount of turbidity-free water containing a measured amount of standardized turbidity reagent. The readings are made by looking down through the column of liquid at a black dot. If turbidity is present, it will interfere with the passage of light through the column of liquid. Small amounts of turbidity will cause a "blurring" of the black dot in the bottom of the tube. Large amounts of turbidity may provide sufficient "cloudiness" so that it is not possible to see the black dot when looking down through the column. Any color that may be present in the sample should be disregarded. This determination is concerned only with the haziness or cloudy nature of the sample.

Equipment: Turbidity kit – LaMotte 7519-01

Method:

1. Fill one Turbidity Column to the 50 mL line with the sample water. If the black dot on the bottom of the tube is not visible when looking down through the column of liquid, pour out a sufficient amount of the test sample so that the tube is filled to the 25 mL line.

2. Fill the second Turbidity Column with an amount of turbidity-free water that is equal to the amount of sample being measured. Distilled water is preferred; however, clear tap water may be used. This is the "clear water" tube.

3. Place the two tubes side by side and note the difference in clarity. If the black dot is equally clear in both tubes, the turbidity is zero. If the black dot in the sample tube is less clear, proceed to Step 4.

4. Shake the Standard Turbidity Reagent vigorously. Add 0.5 mL to the "clear water" tube. Use the stirring rod to stir contents of both tubes to equally distribute turbid particles. Check for amount of turbidity by looking down through the solution at the black dot. If the turbidity of the sample water is greater than that of the "clear water", continue to add Standard Turbidity Reagent in 0.5 mL increments to the "clear water" tube, mixing after each addition until the turbidity equals that of the sample. Record total amount of Standard Turbidity Reagent added.

5. Each 0.5 mL addition to the 50 mL size sample is equal to 5 Jackson Turbidity Units (JTUs). If a 25 mL sample size is used, each 0.5 mL addition of the Standard Turbidity Reagent is equal to 10 Jackson Turbidity Units (JTUs). See Table 3.4-1 below. Rinse both tubes carefully after each determination.

Table 3.4-1-1. Turbidity Test Results – from LaMotte 7519-01 instructions

TURBITITY TEST RESULTS

Number of Measured Additions	Amount in mL	50 mL Graduation	25 mL Graduation
1	0.5	5 JTU	10 JTU
2	1.0	10 JTU	20 JTU
3	1.5	15 JTU	30 JTU
4	2.0	20 JTU	40 JTU
5	2.5	25 JTU	50 JTU
6	3.0	30 JTU	60 JTU
7	3.5	35 JTU	70 JTU
8	4.0	40 JTU	80 JTU
9	4.5	45 JTU	90 JTU
10	5.0	50 JTU	100 JTU
15	7.5	75 JTU	150 JTU
20	10.0	100 JTU	200 JTU

3.5 Water Temperature Measurement

Equipment: armored, digital thermistor, or probe

Method:

Depth Profile Sampling (>3m):
If you are measuring with a multi probe and have a long enough cord, you are encouraged to do depth profiles.

1. Measure 0.3 m above the bottom, then 1 meter intervals to 0.3 m below the surface. (Example: At a 3.4 m deep site, measure at 3.1, 3.0, 2.0, 1.0, and 0.3 m)

2. At each iteration allow the probe to stabilize before recording your temperature reading at the corresponding depth

3. Measure salinity and DO at each depth as well

4. Record depth, DO, temperature, and salinity on your data sheet for each depth

Depth Profile Sampling (≤3 m):

1. Measure 0.3 m above the bottom, allow the probe to stabilize and record your result

2. Measure 0.3 m below the surface, allow the probe to stabilize and record your result

Surface Sampling:

1. Place your probe or thermometer 0.3 m beneath the surface of the water

2. Wait for the probe or thermometer to stabilize

3. Record your reading

Sample with bucket:

1. Hang thermometer in the bucket

2. Wait for the probe or thermometer to stabilize

3. Record your reading

3.6 Water Depth Measurement

Equipment: Secchi disk (for <3 m deep), measuring tape with weighted end, or DO probe with marked lengths (if doing depth profile sampling)

Method:

1. At your sampling site, lower the measuring device into the water until it is resting on the bottom and the line is slack.

2. Record the depth reading, to the nearest tenth, based on the length of line submerged.

3.7 Dissolved Oxygen

3.7.1 **Winkler Titration Method**

Equipment: LaMotte Dissolved Oxygen Test Kit

Sodium Thiosulfate Check:
Prior to each sampling event (either the night before or the day of), you must run a test to make sure your Sodium Thiosulfate is still fresh and functional. Sodium Thiosulfate is fairly unstable and can degrade very suddenly, making it necessary to check it before each DO sampling. Perform this check at home before you go out. Here is how you do the check…

1. Rinse the titrating tube (small glass vial with plastic lid with hole in it) with a small amount of Iodate-Iodide Standard Solution (in large amber bottle).

2. Pour into waste container.

3. Repeat step 1 and 2 two more times

4. Pour 20 ml of the Iodate-Iodide Standard Solution into the rinsed titrating tube.

5. Add 8 drops of Sulfuric Acid (hold the bottle vertical to ensure equal drop size) to the 20 ml of solution and mix by swirling. Then place plastic cap (with hole in it) onto titrating tube.

6. Fill titrating syringe to the "0" mark with Sodium Thiosulfate.

7. Titrate using the Sodium Thiosulfate.

8. When solution turns a pale yellow color, but not clear:
    a) Remove cap, leaving syringe in cap.
    b) Add 8 drops Starch Solution (white bottle). Swirl titration sample gently to mix to a uniform blue color. Recap glass tube and continue titration process.

9. Continue adding Sodium Thiosulfate until solution turns from blue to clear.

10. Read results on syringe - Record your results under the Dissolved Oxygen portion on your field datasheet.

11. If results are less than 9.4 mg/l or greater than 10.0 mg/L, perform a 2nd test and record in the space on datasheet marked "2nd check".

12. Dispose of solution in titrating tube and syringe by pouring down sink and flushing with additional tap water.

13. Keep the amber bottle solution at home- you don't need to take into the field.

DO Sampling Method:

NOTE: Duplicate tests are run simultaneously on each sample to guard against error. If the amount of DO in the second test is more than 0.6 ppm different than the first test, you should do a third test. Record the average of the two closest results.

Since you will be doing two tests at the same time, thoroughly rinse both water sampling bottles with the sample water, filling and dumping the waste water downstream three times before collecting your sample.

1. Using the first sample bottle, submerge about 1/2 of the bottle opening allowing the water to gently flow into the bottle. Try to fill the bottle without causing a lot of bubbles. Submerge the filled bottle.

2. Turn the submerged bottle upright and tap the sides of the bottle to dislodge any air bubbles clinging to the inside of the bottle. Cap the bottle while it is still submerged.

3. Retrieve the bottle and turn it upside down to make sure that no air bubbles are trapped inside. If any air bubbles are present, empty the sample bottle downstream and refill. Fill the second sample bottle. Once two satisfactory samples have been collected, proceed immediately with Steps 4 & 5.

4. Place both sample bottles on a flat surface and uncap. While holding the bottle vertical, add 8 drops of Manganese Sulfate Solution followed by 8 drops of Alkaline Potassium Iodide Solution to each sample bottle. Always add the Manganese Sulfate first. Cap each sample bottle and mix by inverting gently several times. A precipitate will form. Allow the precipitate to settle to the shoulder of the bottle. Mix both bottles again and allow the precipitate to settle to the shoulder again.

5. Add 8 drops of the Sulfuric Acid both sample bottles. Cap the bottles and gently shake to mix, until both the reagent and the precipitate have dissolved. A clear-yellow to brown-orange color will develop. If brown flecks are present, keep mixing the samples until the flecks will not dissolve any further.

NOTE: Following the completion of Step 5, the samples have been "fixed," which means that dissolved oxygen cannot be added to the sample bottles. The titration procedure described in Steps 6-13 may be performed at a later time (but must be performed within 8 hours of sample collection). This means that several samples can be collected and "fixed" in the field and then carried back to a testing station for the remaining steps.

6. Pour 20 ml of the solution from one of the sample bottles into one of the glass tubes with a hole in its cap. Fill to white line so that the bottom of the meniscus (the curved surface of the liquid in the tube) rests on the top of the white line. The amount is critical so be sure to use the glass dropper to add or remove the sample solution from the tube. Place

cap on the tube.

7. Fill syringe (titrator) to the 0 mark with Sodium Thiosulfate solution. Be sure that there are no air bubbles in the syringe. Refer to kit manual for instructions on how to properly fill syringe.

8. To titrate the solution in the tube, insert the syringe into the cap of tube. Add 1 drop of Sodium Thiosulfate to test tube and gently swirl the glass tube to mix. Add another drop of the Sodium Thiosulfate and swirl the tube. Continue this process one drop at a time until the yellow-brown solution in the glass tube turns a pale yellow (lighter than the original yellow-brown solution but not clear). Once you reach this point, take the cap off while leaving the syringe in the cap.

9. Add 8 drops of Starch Solution to the glass tube. Swirl the tube gently to mix. The solution should turn from light yellow to dark blue.

10. Recap the glass tube and continue the titration process with the Sodium Thiosulfate remaining in the syringe (adding one drop at a time and swirling as described in Step 9), until the test tube solution turns from blue to clear. This is the endpoint. If the solution turns blue again, ignore it. Do not add any more Sodium Thiosulfate than is necessary to produce this first color change. Be sure to gently swirl the test tube after each drop.

NOTE: When the dissolved oxygen level is above 10 ppm, the solution in the tube will still be blue when the plunger tip of the titrator reaches 10 units. If it reaches this 10 unit line, do not go beyond that line. Usually, this will only happen when the water temperature is cold. In this case, refill the syringe to the 0 line from the Sodium Thiosulfate bottle and continue adding a drop at a time and swirling until reaching the endpoint.

11. Using the scale on the side of the syringe, read the total number of units of Sodium Thiosulfate used. Each line is 0.2 units. This number equals the number of parts per million (ppm) or milligrams per liter (mg/l) of dissolved oxygen in the water sample.

12. Carry out Steps 7-12 on second sample bottle and second glass tube.

13. Record the results of the two tests on the data sheet. If the difference between Test 1 and Test 2 is more than 0.6 ppm, you should do a third test and record the two results which are within 0.6 ppm.

NOTE: If using transparency tube to measure turbidity, perform this measurement now.

3.7.2 Electronic Probe Method

Equipment: Various models of dissolved oxygen probes and meters

Calibrating Dissolved Oxygen Probes and Meters

With practice and proper care for the DO probe, users can complete the entire DO probe calibration process within 5-10 minutes.

NOTE: Some probes may differ in displaying values. For DO probes, parts per million (ppm), and milligrams per liter (mg/L) are the same value. In addition, barometric pressure may be displayed in millibars (mBar) or in millimeters of mercury (mmHg).

Method:

1. Record the date of calibration. Calibration must be done each day you collect DO samples

2. Record the temperature of the probe just before you calibrate the probe

3. Set the barometric pressure (BP) mmHg or mBar- Most probes allow the user to adjust the barometric pressure readout of the probe for calibrating DO. The standard unit for barometric pressure is millimeters of mercury (mmHg) or millibars (mBar). You can get local barometric pressure readings from www.weatherunderground.com or www.noaa.gov. If using weather station data, it is important to adjust the reading by the altitude of the weather station. Appendix B explains how to calculate the correct reading.

4. Calculate the Theoretical DO Value mg/L- Prior to calibrating your probe, you should determine the theoretical DO value to confirm your probes readout. To determine the theoretical value, please follow the instructions found in Appendix B.

5. Record the mg/L reading of the calibrated DO level. If everything is working properly, the probe should display the correct DO level based on the altitude and temperature that you are calibrating at. The theoretical DO value and the probes calibrated readout should be within 0.2 mg/L. If not, try to recalibrate the probe or perform maintenance on the probe based on manufacturer instructions.

6. Turn off the probe if the manufacturer says so. If not, keep the probe on at all times while you are taking it out to the field and performing your field samples.

Measure DO

Depth Profile Sampling (>3m)
If you are measuring with a multi probe and have a long enough cord, you are encouraged to do depth profiles.

1. Measure 0.3 m above the bottom, then 1 meter intervals to 0.3 m below the surface. (Example: At 3.4 m deep site, measure at 3.1, 3.0, 2.0, 1.0, and 0.3 m)

2. At each iteration allow the probe to stabilize before recording your DO reading at the corresponding depth

3. Measure salinity and temperature at each depth as well

4. Record depth, DO, temperature, and salinity on your data sheet for each depth

Depth Profile Sampling (≤3 m)

1. Measure 0.3 m above the bottom, allow the probe to stabilize and record your result

2. Measure 0.3 m below the surface, allow the probe to stabilize and record your result

Surface Sampling

1. Place your probe 0.3 m beneath the surface of the water

2. Wait for the probe to stabilize, and then record your reading

Post Sampling Calibration Check

After the sample run is complete, return the probe to the calibration station to perform a quick post check. The post check consists of placing the probe in the DO calibration chamber and letting it equalize. This may take between 2 to 10 minutes depending on the condition of the probe.

1. Measure and record the temperature. If you did the morning calibration indoors, the probe temperature should be roughly close to the same as the morning calibration. If you are calibrating the probe outside, the temperature may be different from the earlier reading. This should not affect the post check.

2. Record the barometric pressure reading of the probe. This may have changed from the morning reading due to weather changes. You can get current local barometric pressure readings from the Internet. Remember to adjust any weather station data based on the instructions found in Appendix B.

3. As in the morning calibration, use Appendix B to determine your theoretical DO level.

4. Record the DO reading of the probe (ppm or mg/L). DO NOT recalibrate the probe. The purpose of this check is to see if the probe has drifted out of acceptable limits during the day.

5. Calculate the difference between the probe reported value and the theoretical DO value. If the probe is functioning properly there should be a difference of less than 0.50 mg/L from the afternoon theoretical DO level and the probe readout. If the calibration difference is greater than 0.50 mg/L the probe needs service and you must flag the data because the probe did not hold onto the calibration. If the calibration difference is 0.16 to 0.50 mg/L. The calibration of the probe is approaching the limits of accuracy and preventative maintenance may be required. It may be wise to clean the probe or replace the probe membrane when this occurs.

3.8 pH

3.8.1 Electronic probe method

Equipment: Various models of pH probes and meters

Calibration

The pH probe calibration procedure a similar protocol used in calibrating the DO probe. Most meters allow calibrating the pH probe using two different buffers. In most cases the use the 7.00 and 4.00 pH buffer solutions is suitable. If you are experiencing pH values above 7.00, calibrate using 7.00 and 10.00 buffer.

Use fresh buffer solution when you calibrate the probe and check the readings at the end of the day. If the probe is capable in doing so, please record the probe readings to the nearest hundredth unit place (Ex. 7.01) when performing the calibration.

1. Record the date of calibration. Calibration must be done each day you perform samples.

2. Record the temperature of the probe during calibration.

3. Record the probe reading as you place the probe in the 7.00 buffer solution. Gently swirl the buffer or the probe to obtain an accurate reading.

4. Calibrate the probe, the probe should now read a value close to 7.00 pH units. Most manufacturers of buffers provide a table showing the pH result that probes should display based on temperature. Check against this value displayed on the probe is close to this value.

5. Clean the probe with distilled or deionized water and blot dry

6. Immerse the probe in the 4.00 (or 10.00) buffer solution, record the stabilized value.

7. Calibrate the probe and it should now read a value close to 4 (or 10) pH units. Again, consult the buffer solution table to ensure accuracy.

After calibration, you may turn off the probe if the manufacturer says so. If not, the probe should be kept on at all times while going out into the field and prior to the post check. Follow manufacturer instructions regarding transporting of the probe into the field to prevent damage and drying out of the pH probe.

Field Sampling

IMPORTANT NOTE- When traveling to a sample station, keep the probe tip stored in the protective cap. This will keep the glass sensor hydrated.

1. Turn the probe on.

2. Dip the electrode about 2 to 3 cm either directly into the water or in your sampling bucket. Let the reading stabilize. This may take about 2 to 3 minutes.

3. Once the reading has stabilized record the reading on your datasheet.

4. Turn off the probe and replace the protective cap.

End of Day Calibration Check

To ensure the probe has maintained proper calibration, it is important to verify no significant probe drift has occurred. The procedures listed below will verify the probe did not drift outside QA/QC specifications. DO NOT CALIBRATE the probe during this check. Doing so will invalidate the data collected during the sample run.

1. Rinse off the probe and probe tip with distilled water and wipe dry using a soft cloth. Washing the probe will remove any material that may reduce probe life.

2. Place the probe into a container of pH 7.00 buffer. You may use the same buffer used during the morning calibration as long as the buffer was covered and appears clean.

3. Allow the probe to stabilize and record the temperature and pH reading in the "End of Day Temp C" and the "End of Day pH 7 Check" columns on the "pH Probe Calibration Form."

4. Rinse the probe and repeat the end of day check process using the 4.00 or 10.00 buffer.

If both buffer checks are within 0.20 units from the calibration values, the probe is within specifications. If the readings are greater than 0.20 units, flag all pH data collected during the sample run by typing "pH probe flag" in the "Additional comments" section when entering data into the online database. Also note "pH probe flag" at the top of the hard copy datasheet. This is because sometime during the sample run, the probe exceeded QA/QC specifications.

3.8.2 Colorimetric Kit

Equipment: LaMotte or Hach pH kits

Method:
Look on the front of black box to determine whether you have a wide range pH kit or a narrow range pH kit (i.e. cresol red, phenol red, bromthymol blue, thymol blue).

1. Rinse one sample test tube and cap twice with water from the stream or bucket

2. Fill the sample test tube to the black line with water from the stream or bucket. The bottom of the meniscus should be even with the line. Use plastic dropper to add or remove water from test tube.

3. For wide range pH kit, add ten drops of the wide range indicator while holding the reagent bottle completely upside down. For narrow range kits, add 8 drops of the indicator while holding the reagent bottle completely upside down.

4. Cap the test tube and mix the sample thoroughly.

5. Slide the tube in the comparator slot, hold it up to the sunlight, and record the pH value from the color in the comparator that most closely matches the sample tube color. When the color observed is between 2 colors on the comparator, the value is reported to the nearest 0.5 unit (for wide range kit) or 0.1 unit for other pH kits.

3.9 Salinity, Conductivity, and Total Dissolved Solids

3.9.1 Salinity Measurement with a Refractometer

Equipment: Salinity refractometer

The refractometer must be calibrated before taking salinity measurement.

Calibration:

1. Check the refractometer with distilled water. If it does not read 0 o/oo, you must calibrate the instrument. DO NOT PERFORM CALIBRATION IN THE FIELD. Calibration must take place in controlled environment at approximately 20 oC (room temperature) using distilled water of the same temperature.

2. Lift the cleat plate and add 1-2 drops of distilled water to the oval blue prism. Hold the prism at an angle close to parallel so the water drops will not run off.

3. Close the plate gently. The water drops should spread and cover the entire prism. Repeat the process if there are any gaps or if the sample is only on one portion of the prism.

4. Look through the eyepiece. If the scale is not in focus, adjust it by turning the eyepiece either clockwise or counterclockwise.

5. The reading is taken at the point where the boundary line of the blue and white fields crosses the scale.

6. If the reading is not at “0” turn the calibration screw with the included screwdriver while looking through the eyepiece until the boundary line falls on “0.”

7. When the measurement is complete, the sample must be cleaned using tissue paper and distilled water.

NOTE: The refractometer needs to be at the same approximate temperature as the sample water. If the refractometer has been sitting in an air-conditioned environment prior to sampling, allow it to warm to the outside air temperature.

Method:

1. Rinse the refractometer with water sample.

2. Then apply drops from water sample on refractometer and hold up to light to read salinity (right side of circle).

3. Record as parts per thousand (o/oo) using the scale located on the right hand side of

refractometer view scope.

3.9.2 Salinity, Conductivity, and TDS Probe

Equipment: Various models of conductivity probes and meters

Calibration
Most probes that test for conductivity and TDS use a pre-made calibration solution with a specific conductivity value. The probe is immersed in the solution and calibrated to the value of the solution. It is good to use a calibration solution concentration similar to what you may find in the field to ensure accuracy.

1. Record the date of calibration. Calibration must be done each day you perform samples.

2. Record the temperature of the probe while you are calibrating the probe.

3. Write down the conductivity listed on the probe when you immerse the probe into the conductivity solution and record the value prior to calibration.

4. Record the conductivity solution that you will use to calibrate the probe. The standard unit for these solutions is in microsiemens per centimeter (mS/cm) but probes may use different units.

5. Write down the conductivity reading after you have calibrated the probe in the solution. The probe should be very close to the calibrated buffer solution but may be off by a couple of units.

Measure salinity, conductivity & TDS

Depth Profile Sampling (>3m)
If you are measuring with a multi probe and have a long enough cord, you are encouraged to do depth profiles.

1. Measure 0.3 m above the bottom, then 1 meter intervals to 0.3 m below the surface. (Example: At 3.4 m deep site, measure at 3.1, 3.0, 2.0, 1.0, and 0.3 m)

2. At each iteration allow the probe to stabilize before recording your salinity reading at the corresponding depth

3. Measure DO and temperature at each depth as well

4. Record depth, DO, temperature, and salinity on your data sheet for each depth

Depth Profile Sampling (≤3 m)

1. Measure 0.3 m above the bottom, allow the probe to stabilize and record your result

2. Measure 0.3 m below the surface, allow the probe to stabilize and record your result

Surface Sampling

1. Prior to sampling, rinse the probe with deionized or distilled water.

2. Select the appropriate mode and range on the meter, beginning with the highest range and working down. Some probes will auto select the correct range.

3. Place the probe into the sample water, and read the salinity, conductivity or TDS of the water sample on the meter's scale.

NOTE: If your probe does not automatically select the appropriate measurement range, and the reading is in the lower 10 percent of the range that you selected, switch to the next lower range. If the reading is above 10 percent on the scale, then record this number on your data sheet.

4. Rinse the probe with distilled or deionized water between each sample and before post sampling calibration check. Replace the cap for storage and transport.

Post sampling calibration check

1. Record the temperature of the probe at the end of the day when you are performing the calibration check.

2. Record the temperature of the probe at the end of the day when you are performing the calibration check.

3. Write down the conductivity listed on the probe when you immerse the probe into the conductivity solution and record the value.

4. Calculate the difference between the pre and post sampling calibration values.

5. Standard rule of thumb is if the probe difference is less than 10.00%, you should be confident of the probe values. To calculate the relative percent difference use the formula:

$$RPD\% = \frac{AbsoluteValue(Sample1 - Sample2)}{Average(Sample1 + Sample2)} \times 100\%$$

6. Initial the person calibrating and using the probe for your records. This is good to know in case something happens to the probe that you may not be aware of due to someone else is using it.

3.10 Nitrate – Nitrogen and Orthophosphate Kits

Equipment:

- Nitrate – Nitrogen kit w/ all chemicals and clean glassware (Hach NI-14 14161000, LaMotte, 3110, LaMotte 3354)
- Orthophosphate kit w/ all chemicals and clean glassware (Hach PO-19 224800, Hanna HI 38061, Hanna HI 713)
- Clean polypropylene sample bottle or scintillation vial (60 ml)

Method:

1. Rinse the sample bottle with sample water and dispose of downstream

2. Repeat step 1 three times.

3. Fill the bottle with sample water and cap. Process the sample as soon as possible.

4. Make sure the sample is well mixed prior to analysis by shaking the sample bottle.

5. Follow the protocol for each nutrient type as outlined in the instructions accompanying the kit. Reagents should be maintained at about 20° C to yield best results.

6. Record your results on the data sheet.

3.11 Phosphate

Equipment:

- Hanna HI 713 Phosphate Low Range Checker
- Clean polypropylene sample bottle or scintillation vial (60 ml)

Method:

1. Rinse the sample bottle with sample water and dispose of downstream three times.

2. Fill the bottle with sample water and cap. Process the sample as soon as possible.

3. Make sure the sample is well mixed prior to analysis by shaking the sample bottle.

4. Turn the meter on by pressing the button. All segments will be displayed. When the display shows “Add”, “C.1” with “Press” blinking, the meter is ready.

5. Fill the cuvette with 10 mL of unreacted sample and replace the cap. Place the cuvette into the meter and close the meter’s cap.

6. Press the button. When the display shows “Add”, “C.2” with “Press” blinking the meter is zeroed.

7. Remove the cuvette from the meter and unscrew the cap. Add the content of one packet of HI 713-25 reagent. Replace the cap and shake gently for 2 minutes until the powder is completely dissolved. Place the cuvette back into the meter.

8. Press and hold the button until the timer is displayed on the LCD (the display will show the countdown prior to the measurement) or, alternatively, wait for 3 minutes and press the button.

9. The instrument directly displays the concentration of phosphate in ppm. The meter automatically turns off after 2 minutes.

10. Record your results on your datasheet.

4 Lab sample collection preparation and handling

4.1 Bacteria

Sample collection:

Note the amount of rainfall within 48 hours prior to sampling and record in the bacteria section of the datasheet.

Collecting by wading:

4. Wade into the main flow of the stream

5. Take a few steps upstream with minimal disturbance;

6. Un-cap the sterile and pre-labeled bottle without touching the inside of the lid

7. Using a U motion dip the bottle into the water down and away from yourself allowing the bottle to fill ¾ full.

8. Cap the bottle and place sample on ice in cooler immediately (cooler temperature should be 1°C to 4°C. NOTE: Do not freeze your sample.

Collecting using a bucket:

1. Make sure not to touch inside of bucket with your hands.

2. If sampling from a dock or pier, go as far as possible to the end of the pier to collect your sample.

3. Throw the bucket out as far as possible in the main channel, and try not to disturb the bottom.

4. Rinse the bucket three times with stream water collected downstream of your sampling location.

5. Fill the bucket with the sample water to 1/3 full.

6. Un-cap the sterile and pre-labeled bottle without touching the inside of the lid

7. Using a U motion dip the bottle into the water down and away from yourself allowing the bottle to fill ¾ full.

8. Cap the bottle and place sample on ice in cooler immediately (cooler temperature should be 1°C to 4°C. NOTE: Do not freeze your sample.

Collecting using a sampling pole (from boat or dock):

If sampling from a boat make sure that the boat motor has not stirred up the water. If the water is shallow, sampling should be done through wading.

1. Un-cap your sterile and pre-labeled bottle and secure it to the end of the pole.

2. Extend the pole outward and dip at approximately 0.3 m below the surface.

3. Cap the bottle and place sample on ice in cooler immediately (cooler temperature should be 1°C to 4°C. NOTE: Do not freeze your sample.

After sampling bacteria wash your skin that came in contact with the water with disinfectant or soap to reduce your chances of becoming sick.

4.2 Chlorophyll A

Example field supplies:

- 500-mL polypropylene (PP) sample bottles
- 50-mL syringes
- filter bodies with fourteen (14) filter caps
- 25-mm 0.7-µm porosity GF/F filter membranes
- Handheld vacuum pump
- Opaque towels
- Aluminum foil
- Filter forceps

Before going out to collect samples, prepare equipment and supplies according to the recommended sampling procedure of the laboratory where the samples will be analyzed. This can include syringe filtering or a handheld vacuum pump and filters.

Method:

1. Using the sampling pole, rinse the 500-mL labeled site specific bottle and syringe three times.

2. Drain the bottle until it is empty, put the cap on, lower it one arm's length under water (about 0.5 meters) then remove the cap. Wait for the bottle to fill, then cap it and return it to the surface.

3. Follow the recommended filtering procedure by the analytical laboratory where the samples will be analyzed. Color on the filter generally indicates a sufficient sample for analysis

4. Record the volume of water pushed through the filter on the data collection sheet.

5. Store samples in cooler. Samples must be kept cool and out of sunlight for the duration of field sampling.

6. Cap 500-mL bottle retaining sampled water and store in dark location to bring back to lab. This sample will serve as a back-up sample should there be a filter problem.

Laboratory preparation:
Following the procedures laid out by the analytical lab that will process the samples is important. Here are a few general steps:

1. Prepare pieces of aluminum foil.

2. Fold in half again, then unfold, creating a crease.

3. Create labels using labeling tape noting site number, date, and volume pressed through

filter.

4. Place filter in aluminum foil with the center of the filter centered on the crease, with side containing the intercept chlorophyll up (should have slight color to it). Folding foil and gently assisting with forceps if necessary by pressing on filter fold the filter in half.

5. Double over edges of fold, displacing air and create a little pocket in which the folded filter is located.

6. Repeat for all samples.

7. Label foil packets.

8. Place foil packets in locking plastic bag and then double bag with another locking plastic bag.

9. Place in freezer to await shipment to the analytical laboratory.

10. Rinse all filter holders and 500-mL bottles with tap water and allow to air dry.

NOTE: It is critical that the chlorophyll water samples and foil packets remain dry. The samples in foil should be double bagged and packed with ice in portable Styrofoam transport coolers with surrounding cardboard box. Samples should be mailed overnight to arrive at the analytical laboratory as soon as possible. If properly packaged and frozen (sampled filters should be stored frozen, at least -20˚C, in the dark), chlorophyll a samples can be stored for up to three and a half weeks. The package should also be marked to indicate “chlorophyll samples” as contents.

4.3 Nutrient and Grab Samples

Collecting from a boat:

1. Water samples should be taken one meter from the surface and one meter from the bottom of the water column at sites with depths greater than four meters. Because it is below the layer of mixing caused by wind, boating, and other activities, sampling one meter below the surface gives a better representation of the surface water column.

2. At sites with depths less than four meters, water samples should be taken one meter from the surface.

3. Facing upstream, extend the pole and bottle, rinse the bottle out three times, and take the sample the fourth time.

4. After samples are taken, immediately place the sample on ice up to the shoulders of the bottle. The lid should not be immersed under the ice, in case ice water leaks into the sample bottle, diluting the concentration of the sample.

5. On the field data sheet, record the time, date, and any other information about the water sampling event.

Collecting by wading:

1. Wade into the main flow of the stream

2. Take a few steps upstream with care not to disturb the sediment;

3. Un-cap the pre-labeled bottle

4. Using a U motion dip the bottle into the water down and away from yourself allowing the bottle to fill to the shoulder

5. After samples are taken, immediately place the sample on ice up to the shoulders of the bottle. The lid should not be immersed under the ice, in case ice water leaks into the sample bottle, diluting the concentration of the sample.

Collecting with a sampling pole:

1. Attach the sample bottle to the sampling pole, making sure that the clamp is tight.

2. The sampling point in the stream or river should have a low to medium flow and not be in eddies or stagnant water.

3. Facing upstream, extend the pole and bottle, rinse the bottle out three times, and take the

sample the fourth time.

4. Fill the bottle up to the shoulders and immediately cap and place on ice. The lid should not be immersed under the ice, in case ice water leaks into the sample bottle, diluting the concentration of the sample.

4.4 Chemical preservatives and reagents

The nutrient sample bottles contain a small amount of sulfuric acid as a preservative. When sampling it is important to fill the bottle to the needed level and not pour out the preservative or excess sample from the bottle.

The bacteria sample bottle contains a dechlorinating tablet. When collecting non-chlorinated water, discard the tablet. Samplers should discard the tablet just prior to collecting a bacteria sample at the site. Discard the tablet by dumping out of the bottle without touching the lip or inner wall of the sample bottle. The tablets are harmless to the environment and may be left at the site.

4.5 Sample container handling and preservation

Proper sample containers and sample preservation are essential to sample integrity. Samples not preserved properly may be rejected by the laboratory.

a) Sample containers should be inspected and any torn, punctured or cracked sample containers discarded.

b) After collecting the sample, make sure the lids are secured tightly to prevent contamination from water seepage in or out of the container.

c) Sample containers and coolers should be stored with the tops securely fastened. Containers with loose fasteners should be replaced or taped to prevent loss of sample containers during transport.

d) In the field, unless specified otherwise, all samples should be placed in an ice filled cooler immediately after collection. To ensure samples do not exceed the 4°C holding temperature, sample containers shall be placed upright and if possible, covered with ice in such a manner that the container openings are above the level of ice. Bacteria sample bottles should be stored in bags, placed in coolers and surrounded with wet ice.

e) Glass sample containers should be packed in bubble wrap or other waterproof protective materials to minimize accidental breakage.

f) The laboratory will provide temperature bottles that they use to determine sample temperature upon arrival at the lab. Make sure that every cooler used to ship samples to the lab contains one of these bottles.

4.6 Sample Bottle Identification

Each sample container must include a label with the following information.

a) Station ID or description

b) Date and time of sample collection

c) Collector's initials

d) Sample depth in meters (surface samples are reported as 0.3)

e) Parameter name and/or group code,

f) Container number

g) Preservative used and volume filtered, if applicable.

Samples will not be analyzed if this information is missing. If more than one container is needed for a parameter (such as a duplicate sample), each container collected for that parameter must have a label with identical information in addition to an indication of 1 of 3, 2 of 3, 3 of 3, etc., as required. Split samples should be designated as S1 and S2.

Please remember to fill out the labels on the bottle with a waterproof pen before taking the samples.

It is essential that the actual sampling site match the labeling information. Always check the labeling information against the actual site. Samples not labeled properly may be rejected by the laboratory.

4.7 Transport of Samples

After collecting the samples at the site:

1. Place the bottles in the cooler filled with ice. Coolers should have enough ice to come up to the necks of the sample bottles.

2. Place any chain of custody forms in the Ziploc bag taped to the inner lid of the cooler.

3. Transport the cooler with samples to the designated drop off point or laboratory by the specified time.

5 Lab Procedures

Lab work will be performed by a NELAP, federal, or state approved lab. The following are the approved methods and their corresponding SOPs for reference for laboratories. It is expected that laboratories will be in compliance with these methods and will already be in possession of the procedural documentation for these methods.

Parameter	Method	Appendix
Bacteria - Enterococcus	US EPA method 1600	Appendix C
Bacteria - Enterococcus	ASTM Method #D6503-99	See manufacturer's manual
Chlorophyll & Pheophytin	CBP IV-12.0	Appendix D
Chlorophyll & Pheophytin	US EPA method 447.0	Appendix E
Chlorophyll & Pheophytin	US EPA method 445.0	Appendix F
Silicate	US EPA method 366.0	Appendix G
Nitrate - Nitrogen	USEPA Method 352.1	Appendix H
Nitrite - Nitrate	USEPA Method 353.4	Appendix I
Ammonia - Nitrogen	USEPA Method 349.0	Appendix J
Orthophosphate	USEPA Method 365.5	Appendix K
Total Nitrogen	USEPA Method 351.2	Appendix L
Total Phosphorus	USEPA Method 365.4	Appendix M

Laboratories will perform QA/QC measures including: method blanks, matrix spikes, replicates, check standard.

6 Cleanup and Storage of Water Monitoring Equipment

a) Rinse the thermometer in tap water and store upright.

b) Pour contents of DO sampling bottles and chemical kits into the sink. Rinse all the bottles and containers thoroughly with tap water. Put all equipment away until next sampling time.

c) Store all chemical reagents in a dark, cool place and out of the reach of children and pets!

d) Save expired chemicals and give them to your monitoring coordinator or trainer at the next recertification event for proper disposal.

NOTE: If you conduct the sampling procedures away from home or on a boat, you need a special container for safe disposal of the test samples. A plastic milk jug or jar works well and is easy to obtain. Fill this container about ½ to ¾ full with kitty litter to absorb the moisture. When the litter is saturated, place the closed jar in double plastic garbage bags and dispose of in the trash.

6.1 Maintenance for pH meter

Follow maintenance and care guidelines as specified by the manufacturer manual. Below are some general day to day care tips.

1. Ensure the probe is cleaned and well maintained. After each sample run, rinse off the probe with distilled water. Use a soft cloth and gently dry the probe and glass sensor.

2. Store the probe tip in the cap provided by the manufacturer. Inside this cap, place a small cotton ball or piece of paper towel soaked with pH 4.00 buffer (or probe storage solution). This will keep the probe in working condition until the next field sampling event.

3. If you see any biological growth (mold, algae, etc.), use mild soap or warm (~30o C) pH 4.00 buffer to clean. Rinse with distilled water and dry.

4. If the calibration or end of day check indicates there is a problem with the probe, and standard cleaning does not produce acceptable results, replacement of the sensor cap may be necessary. Contact a Project Team Member to get a replacement sensor cap.

Appendix A

Field Data Sheet

Chesapeake Monitoring Cooperative

Tidal Field Data Sheet

Site Name & # _____ Stream Name _____

Date _____/_____/_____ Time (military time) _____ Rainfall (mm last 48 hrs) _____

Monitors: _____

Parameter	Method Used (Circle Applicable)	Calibration Pre / Post Sampling		Measurement 1st / 2nd / 3rd Replicate or Circle observation		
Weather Conditions (cloud cover)				Clear / Partly Cloudy Cloudy / Fog or Haze		
Tide Condition				High / Outgoing (Ebb) Low / Incoming (Flood)		
Water Color				Clear / Milky / Muddy Oil slick / Other		
Air Temperature (°C)	Armored Classic / Digital / Probe	Verified? Y / N				
pH	Kit / Probe / ColorpHast Strips					
Conductivity (µS/cm)	Probe					
TDS (mg/L)	Probe					
Turbidity (JTU)	LaMotte 7519					
Water Clarity (cm)	Secchi Disk / Turbidity Tube					
Phosphate (mg/L)	Hanna Digital Checker	Pre only:				
Orthophosphate (mg/L)	Hach PO-19 224800 Hanna HI 38061					
Nitrate (mg/L)	Hach NI-14 1416100 / LaMotte 3110 LaMotte 3354					

Use this chart to determine if your two replicates are within range of each other. If not, perform a third test.

Parameter	Acceptable Range
Temperature	Armored (+/- 1° C) / Digital (+/- 0.5° C)
Dissolved Oxygen Sodium Thiosulfate Check	Only perform 1 test. If <9.4 or >10 mg/L, do a second test. If both tests are not within 0.4 mg/L of each other, do not measure DO.
Dissolved Oxygen	+/- 0.6 mg/L
pH	+/- 1 pH unit
Salinity / TDS / Conductivity	± 2% FS
Nitrate	Low range (0–1 mg/L) = +/- 0.1 mg/L Mid range (1–10 mg/L) = +/- 1 mg/L
Phosphate	+/- 0.04 mg/L
Turbidity	+/- 5 JTU

Water Column Profile Data	Temperature (°C)	Salinity (ppt)		Dissolved Oxygen (mg/L)	
Type of device (circle one)	Armored / Digital / Probe	Refractometer / Probe		Winkler Titration / Probe	
Calibration Check Pre/Post	Verified? Y / N				
Depth (m)					
0.3 - Surface					

Notes:_____
_____
_____

Total Time Spent Monitoring:(Includes travel to and from monitoring site; equipment preparation; sample collection; water's edge time; and time spent filling out data sheets):

Name: _____ Hours: _____ (Round to nearest 15 min.)

Name: _____ Hours: _____ (Round to nearest 15 min.)

Name: _____ Hours: _____ (Round to nearest 15 min.)

Lead Monitor Signature: _____ **Date:** _____

Once datasheets have been entered in the database, send original forms to your coordinator or:

Alliance for the Chesapeake Bay
Attn: Chesapeake Monitoring Coop
612 Hull St. Suite 101C
Richmond, VA 23225

Appendix B

Theoretical DO Calculation

How to Calculate Theoretical Dissolved Oxygen Values

From: Virginia Citizen Water Quality Monitoring Program Methods Manual - October 2007

Proper calibration of Dissolved Oxygen (DO) probes is important to collect accurate data. An easy way to see if a probe is calibrated correctly is to compare the probe's results against a theoretical DO value. This value is what the DO level should be based on temperature and barometric pressure.

DO Level based on temperature

The top table on the attached chart allows users to find the DO level based on temperature. The top and side axis of the table corresponds to the temperature that the probe is reporting. The intersection of the two axes displays the DO reading. Write this number down to start calculating the theoretical DO level.

Correction factor for barometric pressure

Barometric pressure is a way to tell how much atmosphere is pressing down on a surface. Weather systems and elevation above (or below) sea level can change this value. The bottom table of the attached chart will help compensate for these changes in pressure. Dissolved oxygen probes normally show pressure in millimeters of mercury (**mmHg**) or millibars (**mBar**).

Having a barometer on hand is a good way to get pressure data. A weather station can also provide pressure data. Websites such as www.weatherunderground.com are useful to find local weather stations. Please note that most barometers and weather stations report pressure in inches of mercury (**inHg**).

Note about using weather station pressure readings

Weather stations compensate pressure readings to make it appear as if the station is at sea level. To account for this, subtract the barometric pressure by 1.01 inHg per 1,000 feet in elevation of the weather station. This final value is known as **absolute barometric pressure**.

Example: Find the absolute barometric pressure of a station located 222 feet above sea level that reported 30.12 inHg.

30.12 inHg – $\frac{1.01 \text{ inHg}}{1000/ 222 \text{ feet}}$ → 30.12 – $\frac{1.01}{4.50}$ → 30.12 – 0.22 == 29.90 inHg absolute barometric pressure

Once finding the absolute pressure, use the bottom table found on the attached chart to find the proper correction factor to use. The formulas at the bottom of the chart will help in converting inHg barometric pressure readings into **millibars** (mBar) or **millimeters of mercury** (mmHg) that are commonly used to calibrate a dissolved oxygen probe. Use this value to find the correction factor to use in the final calculation.

Example: A barometric pressure of 970 millibars you would use a correction factor of 0.96 (second column, bottom row).

Theoretical DO Calculation

To find the theoretical DO value, use the following formula.

Theoretical DO = (DO level based on temperature) x (barometric pressure correction factor)

Example: If a probe had a temperature of 18.4 C and the barometric pressure was 970 mBar, the theoretical DO value would be 9.00 mg/L (9.37mg/L x 0.96 correction factor).

Dissolved Oxygen Saturation

Directions- To determine theoretical DO saturation, multiply the O2 concentration value (found in the top chart) by the barometric pressure correction factor (bottom chart).

Example: Find the DO saturation for at a temperature of **18.4 C** at **730 mmHg** pressure: 9.37 x 0.96= **9.00 mg/L**

Temp in ^{O}C	**O_2 concentrations in mg/l**									
	0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
5	12.75	12.71	12.68	12.65	12.61	12.58	12.55	12.52	12.48	12.45
6	12.42	12.39	12.36	12.32	12.29	12.26	12.23	12.2	12.17	12.14
7	12.11	12.08	12.05	12.02	11.99	11.96	11.93	11.9	11.87	11.84
8	11.81	11.78	11.758	11.72	11.69	11.67	11.64	11.61	11.58	11.55
9	11.53	11.5	11.47	11.44	11.42	11.39	11.36	11.33	11.31	11.28
10	11.25	11.23	11.2	11.18	11.15	11.12	11.1	11.07	11.05	11.02
11	10.99	10.97	10.94	10.92	10.89	10.87	10.84	10.82	10.79	10.77
12	10.75	10.72	10.7	10.67	10.65	10.63	10.6	10.58	10.55	10.53
13	10.51	10.48	10.46	10.44	10.41	10.39	10.37	10.35	10.32	10.3
14	10.28	10.26	10.23	10.21	10.19	10.17	10.15	10.12	10.1	10.08
15	10.06	10.04	10.02	9.99	9.97	9.95	9.93	9.91	9.89	9.87
16	9.85	9.83	9.81	9.79	9.76	9.74	9.72	9.7	9.68	9.66
17	9.64	9.62	9.6	9.58	9.56	9.54	9.53	9.51	9.49	9.47
18	9.45	9.43	9.41	9.39	**9.37**	9.35	9.33	9.31	9.3	9.28
19	9.26	9.24	9.22	9.2	9.19	9.17	9.15	9.13	9.11	9.09
20	9.08	9.06	9.04	9.02	9.01	8.99	8.97	8.95	8.94	8.92
21	8.9	8.88	8.87	8.85	8.83	8.82	8.8	8.78	8.76	8.75
22	8.73	8.71	8.7	8.68	8.66	8.65	8.63	8.62	8.6	8.58
23	8.57	8.55	8.53	8.52	8.5	8.49	8.47	8.46	8.44	8.42
24	8.41	8.39	8.38	8.36	8.35	8.33	8.32	8.3	8.28	8.27
25	8.25	8.24	8.22	8.21	8.19	8.18	8.16	8.15	8.14	8.12
26	8.11	8.09	8.08	8.06	8.05	8.03	8.02	8	7.99	7.98
27	7.96	7.95	7.93	7.92	7.9	7.89	7.88	7.86	7.85	7.83
28	7.82	7.81	7.79	7.78	7.77	7.75	7.74	7.73	7.71	7.7
29	7.69	7.67	7.66	7.65	7.63	7.62	7.61	7.59	7.58	7.57
30	7.55	7.54	7.53	7.51	7.5	7.49	7.48	7.46	7.45	7.44

Barometric Pressure Correction factor:

mmHg (mBar)	**Corr. Factor**	**mmHg (mBar)**	**Corr. Factor**	**mmHg (mBar)**	**Corr. Factor**	**mmHg (mBar)**	**Corr. Factor**
775-771 (1033-1028)	**1.02**	**750-746 (1000-995)**	**0.987**	**725-721 (967-961)**	**0.953**	**700-696 (934-928)**	**0.92**
770-766 (1027-1021)	**1.014**	**745-741 (994-988)**	**0.98**	**720-716 (960-955)**	**0.947**	**695-691 (927-921)**	**0.914**
765-761 (1020-1014)	**1.007**	**740-736 (987-981)**	**0.973**	**715-711 (954-948)**	**0.94**	**690-686 (920-915)**	**0.907**
760-756 (1013-1008)	**1**	**735-731 (980-975)**	**0.967**	**710-706 (947-941)**	**0.934**	**685-681 (914-908)**	**0.9**
755-751 (1007-1001)	**0.993**	**730-726 (974-968)**	**0.96**	**705-701 (940-935)**	**0.927**	**680-676 (907-901)**	**0.893**

Appendix C
Laboratory Method – Bacteria – Enterococcus

Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)

July 2006

U.S. Environmental Protection Agency
Office of Water (4303T)
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Washington, DC 20460

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Disclaimer

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Appendices A and B are taken from Microbiological Methods for Monitoring the Environment, Water and Wastes (Reference 18.7).

Appendix A: Part II (General Operations), Section A (Sample Collection, Preservation, and Storage).

Appendix B: Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results).

Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)

July 2006

1.0 Scope and Application

1.1 Method 1600 describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. This is a single-step method that is a modification of EPA Method 1106.1 (mE-EIA). Unlike the mE-EIA method, it does not require the transfer of the membrane filter to another medium. The modified medium has a reduced amount of triphenyltetrazolium chloride (TTC) and includes indoxyl β-D-glucoside, a chromogenic cellobiose analog used in place of esculin. In this procedure, β-glucosidase-positive enterococci produce an insoluble indigo blue complex which diffuses into the surrounding media, forming a blue halo around the colony.

1.2 Enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

1.3 Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on established relationships between health effects and water quality. The significance of finding enterococci in recreational fresh or marine water samples is the direct relationship between the density of enterococci and the risk of gastrointestinal illness associated with swimming in the water (References 18.1 and 18.2).

1.4 For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

2.0 Summary of Method

2.1 Method 1600 provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (Reference 18.4). A water sample is filtered through the membrane which retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective medium, mEI agar, and incubated for 24 hours at 41°C ± 0.5°C. All colonies greater than or equal to (≥) 0.5 mm in diameter (regardless of color) with a blue halo are recorded as enterococci colonies. A fluorescent lamp with a magnifying lens is used for counting to give maximum visibility of colonies.

3.0 Definitions

3.1 In Method 1600, enterococci are those bacteria which produce colonies greater than or equal to 0.5 mm in diameter with a blue halo after incubation on mEI agar. The blue halo should not be included in the colony diameter measurement. Enterococci include *Enterococcus faecalis (E. faecalis), E. faecium, E. avium, E. gallinarium,* and their variants. The genus *Enterococcus* includes the enterococci formerly assigned to the Group D fecal streptococci.

4.0 Interferences

4.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with enumeration and identification of target colonies.

5.0 Safety

5.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

5.2 The selective medium (mEI) and azide-dextrose broth used in this method contain sodium azide as well as other potentially toxic components. Caution must be exercised during the preparation, use, and disposal of these media to prevent inhalation or contact with the medium or reagents.

5.3 This method does not address all of the safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in Method 1600 analyses.

5.4 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 Glass lens with magnification of 2-5X or stereoscopic microscope

6.2 Lamp, with a cool, white fluorescent tube

6.3 Hand tally or electronic counting device

6.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets

6.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume

6.6 Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper

6.7 Sterile membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper

6.8 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)

6.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source (In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used)

6.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing

6.11 A filter manifold to hold a number of filter bases (optional)

6.12 Flask for safety trap placed between the filter flask and the vacuum source

6.13 Forceps, straight or curved, with smooth tips to handle filters without damage

6.14 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps

6.15 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles

6.16 Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

6.17 Petri dishes, sterile, plastic, 9 x 50 mm, with tight-fitting lids; or 15 x 60 mm with loose fitting lids; or 15 x 100 mm with loose fitting lids

6.18 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, 125 mL volume

6.19 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume

6.20 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size

6.21 Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops

6.22 Incubator maintained at 41°C ± 0.5°C

6.23 Waterbath maintained at 50°C for tempering agar

6.24 Test tubes, 20 x 150 mm, borosilicate glass or plastic

6.25 Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes

6.26 Test tubes, screw-cap, borosilicate glass, 16 x 125 mm or other appropriate size

6.27 Autoclave or steam sterilizer capable of achieving 121°C [15 lb pressure per square inch (PSI)] for 15 minutes

7.0 Reagents and Standards

7.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 18.5). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 Purity of reagent water: Reagent-grade water conforming to specifications in: *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 136 or 141, as applicable), Section 9020 (Reference 18.6).

7.4 Phosphate buffered saline (PBS)

7.4.1 Composition:

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Sodium chloride (NaCl)	8.5 g
Reagent-grade water	1.0 L

7.4.2 Dissolve the reagents in 1 L of reagent-grade water and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 PSI) for 15 min. Final pH should be 7.4 ± 0.2.

7.5 mEI Agar

7.5.1 Composition:

Peptone	10.0 g
Sodium chloride (NaCl)	15.0 g
Yeast extract	30.0 g
Esculin	1.0 g
Actidione (Cycloheximide)	0.05 g
Sodium azide	0.15 g
Indoxyl β-D-glucoside	0.75 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.5.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool in a 50°C water bath.

7.5.3 After sterilization add 0.24 g nalidixic acid (sodium salt) and 0.02 g triphenyltetrazolium chloride (TTC) to the mEI medium and mix thoroughly.

Note: The amount of TTC used in this medium (mEI) is less than the amount used for mE agar in Method 1106.1.

7.5.4 Dispense mEI agar into 9 × 50 mm or 15 × 60 mm petri dishes to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. Final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

7.6 Tryptic soy agar (TSA)

7.6.1 Composition:

Pancreatic digest of casein	15.0 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.6.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool in a 50°C waterbath. Pour the medium into each 15 × 60 mm culture dish to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. Final pH should be 7.3 ± 0.2.

7.7 Brain heart infusion broth (BHIB)

7.7.1 Composition:

Calf brains, infusion from 200.0 g	7.7 g
Beef heart, infusion from 250.0 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2.5 g
Dextrose	2.0 g
Reagent-grade water	1.0 L

7.7.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense in 10-mL volumes in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2.

7.8 Brain heart infusion broth (BHIB) with 6.5% NaC1

7.8.1 Composition:

BHIB with 6.5% NaC1 is the same as BHIB above (Section 7.7), but with additional NaC1.

7.8.2 Add NaCl to formula provided in Section 7.7 above, such that the final concentration is 6.5% (65 g NaCl/L). Typically, for commercial BHIB media, an additional 60.0 g NaCl per liter of medium will need to be added to the medium. Prepare as in Section 7.7.2.

7.9 Brain heart infusion agar (BHIA)

7.9.1 Composition:

BHIA contains the same components as BHIB (Section 7.7) ,with the addition of 15.0 g agar per liter of BHIB.

7.9.2 Add agar to formula for BHIB provided in Section 7.7 above. Prepare as in Section 7.7.2. After sterilization, slant until solid. Final pH should be 7.4 ± 0.2.

7.10 Bile esculin agar (BEA)

7.10.1 Composition:

Beef Extract	3.0 g
Pancreatic Digest of Gelatin	5.0 g
Oxgall	20.0 g
Esculin	1.0 g
Ferric Citrate	0.5 g
Bacto Agar	14.0 g
Reagent-grade water	1.0 L

7.10.2 Add reagents to 1 L reagent-grade water, heat with frequent mixing, and boil 1 minute to dissolve completely. Dispense 10-mL volumes in tubes for slants or larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 PSI) for 15 minutes. Overheating may cause darkening of the medium. Cool in a 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.8 ± 0.2. Store in a refrigerator.

7.11 Azide dextrose broth (ADB)

7.11.1 Composition:

Beef extract	4.5 g
Pancreatic digest of casein	7.5 g
Proteose peptone No. 3	7.5 g
Dextrose	7.5 g
Sodium chloride (NaCl)	7.5 g
Sodium azide	0.2 g
Reagent-grade water	1.0 L

7.11.2 Add reagents to 1 L of reagent-grade water and dispense in screw cap bottles. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.2 ± 0.2.

7.12 Control cultures

7.12.1 Positive control and/or spiking organism (either of the following are acceptable)

- Stock cultures of *Enterococcus faecalis* (*E. faecalis*) ATCC #19433
- *E. faecalis* ATCC #19433 BioBalls (BTF Pty, Sydney, Australia)

7.12.2 Negative control organism (either of the following are acceptable)

- Stock cultures of *Escherichia coli* (*E. coli*) ATCC #11775
- *E. coli* ATCC #11775 BioBalls (BTF Pty, Sydney, Australia)

8.0 Sample Collection, Handling, and Storage

8.1 Sampling procedures are briefly described below. Detailed sampling methods can be found in Reference 18.7 (see Appendix A). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

8.1.1 Sampling techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge, or bank adjacent to a surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples. The sampling depth for surface water samples should be 6-12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to allow for proper mixing before analyses.

8.1.2 Storage temperature and handling conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

8.1.3 Holding time limitations

Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is 6 hours, and samples should be processed within 2 hours of receipt at the laboratory.

9.0 Quality Control

9.1 Each laboratory that uses Method 1600 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Additional recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 18.7.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1600 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.4) and matrix spike (MS) analysis (Section 9.5, disinfected wastewater only), and the routine analysis of positive and negative controls (Section 9.6), filter sterility checks (Section 9.8), method blanks (Section 9.9), and media sterility checks (Section 9.11). For the IPR, OPR and MS analyses, it is necessary to spike samples with either laboratory-prepared spiking suspensions or BioBalls as described in Section 14.

Note: Performance criteria for Method 1600 are based on the results of the interlaboratory validation of Method 1600 in PBS and disinfected wastewater matrices. The IPR (Section 9.3) and OPR (Section 9.4) recovery criteria (**Table 1**) are valid method performance criteria that should be met, regardless of the matrix being evaluated, the matrix spike recovery criteria (Section 9.5, **Table 2**) pertain only to disinfected wastewaters.

9.3 Initial precision and recovery (IPR)—The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for monitoring field samples. EPA recommends but does not require that an IPR be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). The IPR analyses are performed as follows:

9.3.1 Prepare four, 100-mL samples of PBS and spike each sample with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each IPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.3.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.3.3 Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.

9.3.4 Compare the mean recovery and RSD with the corresponding IPR criteria in Table 1, below. If the mean and RSD for recovery of enterococci meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the IPR analyses.

Table 1. Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	Lab-prepared spike acceptance criteria	BioBall™ acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	31% - 127%	85% - 106%
• Precision (as maximum relative standard deviation)	28%	14%
Ongoing precision and recovery (OPR) as percent recovery	27% - 131%	78% - 113%

9.4 Ongoing precision and recovery (OPR)—To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. OPR samples must be accompanied by an acceptable method blank (Section 9.9) and appropriate media sterility checks (Section 9.11). The OPR analysis is performed as follows:

9.4.1 Spike a 100-mL PBS sample with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each OPR sample according to the procedures in Section 11 and calculate the number of enterococci per 100 mL according to Section 13.

9.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.4.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the OPR analysis.

9.4.4 As part of the laboratory QA program, results for OPR and IPR samples should be charted and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1600 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from $R - 2s_r$ to $R + 2s_r$.

9.5 **Matrix spikes (MS)**—MS analysis are performed to determine the effect of a particular matrix on enterococci recoveries. The laboratory should analyze one MS sample when disinfected wastewater samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given disinfected wastewater source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site, an acceptable method blank (Section 9.9), and appropriate media sterility checks (Section 9.11). When possible, MS analyses should also be accompanied by an OPR sample (Section 9.4), using the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). The MS analysis is performed as follows:

9.5.1 Prepare two, 100-mL field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of enterococci for calculating MS recoveries (Section 9.5.3). The other sample will serve as the MS sample and will be spiked with *E. faecalis* ATCC #19433 according to the spiking procedure in Section 14.

9.5.2 Select sample volumes based on previous analytical results or anticipated levels of in the field sample in order to achieve the recommended target range of enterococci (20-60 CFU, including spike) per filter. If the laboratory is not familiar with the matrix being analyzed, it is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate is obtained for the MS and associated unspiked sample. If possible, 100-mL of sample should be analyzed.

9.5.3 Spike the MS sample volume(s) with a laboratory-prepared suspension as described in Section 14.2 or with BioBalls as described in Section 14.3. Immediately filter and process the unspiked and spiked field samples according to the procedures in Section 11.

Note: When analyzing smaller sample volumes (e.g, <20 mL), 20-30 mL of PBS should be added to the funnel or an aliquot of sample should be dispensed into a 20-30 mL dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.

9.5.4 For the MS sample, calculate the number of enterococci (CFU / 100 mL) according to Section 13 and adjust the colony counts based on any background enterococci observed in the unspiked matrix sample.

9.5.5 Calculate the percent recovery (R) for the MS sample (adjusted based on ambient enterococci in the unspiked sample) using the appropriate equation in Section 14.2.2 or 14.3.4 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.5.6 Compare the MS result (percent recovery) with the appropriate method performance criteria in Table 2, below. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this disinfected wastewater source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.

9.5.7 Acceptance criteria for MS recovery (Table 2) are based on data from spiked disinfected wastewater matrices and are not appropriate for use with other matrices (e.g., ambient waters).

Table 2. Matrix Spike Precision and Recovery Acceptance Criteria

Performance test	Lab-prepared acceptance criteria	BioBall™ acceptance criteria
Percent recovery for MS	29% - 122%	63% - 110%

9.5.8 Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1600. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.

9.6 Culture Controls

9.6.1 **Negative controls**—The laboratory should analyze negative controls to ensure that the mEI agar is performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed.

9.6.1.1 Negative controls are conducted by filtering a dilute suspension of viable *E. coli* (e.g., ATCC #11775) and analyzing as described in Section 11. Viability of the negative controls should be demonstrated using a non-selective media (e.g., nutrient agar or tryptic soy agar).

9.6.1.2 If the negative control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the negative control, and reanalyze the appropriate negative control.

9.6.2 **Positive controls**—The laboratory should analyze positive controls to ensure that the mEI agar is performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a positive control every day that samples are analyzed. An OPR sample (Section 9.4) may take the place of a positive control.

9.6.2.1 Positive controls are conducted by filtering a dilute suspension of viable *E. faecalis* (e.g., ATCC #19433) and analyzing as described in Section 11.

9.6.2.2 If the positive control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the positive control, and reanalyze the appropriate positive control.

9.6.3 Controls for verification media—All verification media should be tested with appropriate positive and negative controls whenever a new batch of media and/or reagents are used. On an ongoing basis, the laboratory should perform positive and negative controls on the verification media with each batch of samples submitted to verification. Examples of appropriate controls for verification media are provided in **Table 3**.

Table 3. Verification Controls

Medium	Positive Control	Negative Control
Bile esculin agar (BEA)	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) with 6.5% NaCl	*E. faecalis*	*E. coli*
Brain heart infusion broth (BHIB) incubated at 45°C	*E. faecalis*	*E. coli*

9.7 **Colony verification**—The laboratory should verify 10 typical colonies (positive) and 10 atypical colonies (negative) per month or 1 typical colony and 1 atypical colony from 10% of all positive samples, whichever is greater. Verification procedures are provided in Section 12.0.

9.8 **Filter sterility check**—Place at least one membrane filter per lot of filters on a TSA plate, and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the filter. On an ongoing basis, the laboratory should perform a filter sterility check every day that samples are analyzed.

9.9 **Method blank**—Filter a 50-mL volume of sterile PBS and place the filter on a mEI agar plate and process according to Section 11.0. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed.

9.10 **Filtration blank**—Filter a 50-mL volume of sterile PBS before beginning sample filtrations. Place the filter on a TSA plate, and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the PBS buffer and filtration assembly.

9.11 **Media sterility check**—The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium (TSA, mEI agar, and verification media) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check every day that samples are analyzed.

9.12 **Analyst colony counting variability**—Laboratories with two or more analysts should compare each analyst's colony counts from one positive field sample per month. Colony counts should be within 10% between analysts. Laboratories with a single analyst should have that analyst perform duplicate colony counts of a single membrane filter each month. Duplicate colony counts should be within 5% for a single analyst. If no positive field samples are available, a OPR sample may be substituted for these determinations.

10.0 Calibration and Standardization

10.1 Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.

10.2 Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

10.3 Refrigerators used to store media and reagents should be monitored daily to ensure proper temperature control.

11.0 Procedure

11.1 Prepare the mEI agar as directed in Section 7.5.

11.2 Mark the petri dishes and report forms with sample identification and sample volumes.

11.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base so that the membrane filter is now held between the funnel and the base.

11.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

11.5 Select sample volumes based on previous knowledge of the enterococci level, to produce 20-60 enterococci colonies on membranes. It is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate (20-60 enterococci colonies) is obtained.

11.6 Smaller sample size or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions may be filtered.

Note: When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS or phosphate-buffered dilution water should be added to the funnel or an aliquot of sample should be dispensed into a dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.

11.7 Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

11.8 Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mEI Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter outside the area of filtration, close to the edge of the dish, to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41°C ± 0.5°C for 24 ± 2 hours.

Note: If the medium is prepared in 15 × 60 mm loose lid petri dishes, they should be incubated in a tight fitting container (e.g., plastic vegetable crisper) containing a moistened paper towel to prevent dehydration of the membrane filter and medium.

11.9 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 colonies ≥0.5 mm in diameter with a blue halo regardless of colony color as an enterococci (see **Figure 1**). *Note:* When measuring colony size do not include the halo. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

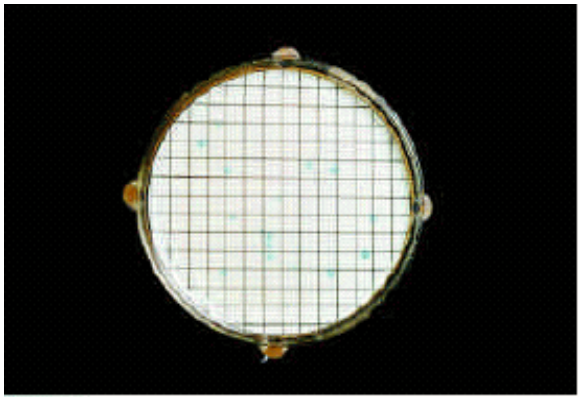

Figure 1. Enterococci colonies on mEI produce blue halos.

12.0 Verification Procedure

12.1 Colonies ≥0.5 mm in diameter of any color having a blue halo after incubation on mEI agar are considered to be “typical” enterococci colonies. Verification of colonies may be required in evidence gathering and it is also recommended as a means of quality control. The verification procedure follows.

Note: When evaluating wastewater using Method 1600, it is recommended that the false negative rate for each matrix be evaluated through biochemical confirmation and results adjusted accordingly, especially if large numbers of atypical colonies are observed in a particular matrix.

12.2 Using a sterile inoculating loop or needle, transfer growth from the centers of at least 10 well-isolated typical and at least 10 well-isolated atypical colonies into a BHIB tube and onto a BHIA slant. Incubate broth for 24 ± 2 hours and agar slants for 48 ± 3 hours at 35°C ± 0.5°C.

12.3 After a 24 hour incubation, transfer a loopful of growth from each BHIB tube to BEA, BHIB, and BHIB with 6.5% NaCl.

12.3.1 Incubate BEA and BHIB with 6.5% NaCl at 35°C ± 0.5°C for 48 ± 3 hours.

12.3.2 Incubate BHIB at 45°C ± 0.5°C for 48 ± 3 hours.

12.4 Observe all verification media for growth.

12.5 After 48 hour incubation, perform a Gram stain using growth from each BHIA slant.

12.6 Gram-positive cocci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate), and grow in BHIB with 6.5% NaCl at 35°C ± 0.5°C and BHIB at 45°C ± 0.5°C are verified as enterococci.

12.7 Alternately, commercially available multi-test identification systems (e.g., Vitek®) may be used to verify colonies. Such multi-test identification systems should include esculin hydrolysis and growth in 6.5% NaCl.

13.0 Data Analysis and Calculations

Use the following general rules to calculate the enterococci count per 100 mL of sample:

13.1 If possible, select a membrane filter with 20-60 colonies ≥0.5 mm in diameter (regardless of colony color) with a blue halo. Calculate the number of enterococci per 100 mL according to the following general formula:

$$\text{Enterococci / 100 mL} = \frac{\text{Number of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

13.2 See general counting rules in Reference 18.7 (see Appendix B).

13.3 Report results as enterococci per 100 mL of sample.

14.0 Sample Spiking Procedure

14.1 Method 1600 QC requirements (Section 9.0) include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3), OPR (Section 9.4), and MS (Section 9.5) analyses it is necessary to spike samples with either laboratory-prepared spiking suspensions (Section 14.2) or BioBalls (Section 14.3) as described below.

14.2 Laboratory-Prepared Spiking Suspensions

14.2.1 Preparation

14.2.1.1 Stock Culture. Prepare a stock culture by inoculating a TSA slant (or other non-selective media) with *E. faecalis* ATCC #19433 and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

14.2.1.2 Undiluted Spiking Suspension. Prepare a 1% solution of azide dextrose broth (ADB) by combining 99 mL of sterile phosphate buffered saline and 1 mL of sterile single strength azide dextrose broth in a sterile screw cap bottle or re-sealable dilution water container. From the stock culture of *E. faecalis* ATCC #19433 in Section 14.2.1.1, transfer a small loopful of growth to the 1 % azide dextrose broth solution and vigorously shake a minimum of 25 times. Disperse the inoculum by vigorously shaking the broth culture and incubate at 35°C ± 3°C for 20 ± 4 hours. This culture is referred to as the undiluted spiking suspension and should contain approximately 1.0×10^6 - 1.0×10^7 *E. faecalis* colony forming units (CFU) per mL of culture.

14.2.1.3 Mix the undiluted spiking suspension (Section 14.2.1.2) thoroughly by shaking the bottle a minimum of 25 times and prepare a series of dilutions (4 total) in the following manner:

14.2.1.3.1 Dilution "A"—Aseptically transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "A" and 1 mL contains 10^{-2} mL of the original undiluted spiking suspension.

14.2.1.3.2 Dilution "B"—Aseptically transfer 1.0 mL of dilution "A" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B" and 1 mL contains 10^{-4} mL of the original undiluted spiking suspension.

14.2.1.3.3 Dilution "C"—Aseptically transfer 11.0 mL of dilution "B" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C" and 1 mL contains 10^{-5} mL of the original undiluted spiking suspension.

14.2.1.3.4 Dilution "D"—Aseptically transfer 11.0 mL of dilution "C" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "D" and 1 mL contains 10^{-6} mL of the original undiluted spiking suspension.

14.2.2 Sample spiking

14.2.2.1 Add 3.0 mL of the spiking suspension dilution "D" (Section 14.2.1.3.4) to 100 mL or PBS or appropriate volume of sample and mix thoroughly by shaking the bottle a minimum of 25 times. The volume of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-6} mL, which is referred to as $V_{\text{spiked per 100 mL sample}}$ in Section 14.2.4.1 below. Filter the spiked sample and analyze the filter according to the procedures in Section 11.

14.2.3 Enumeration of spiking suspension

14.2.3.1 Prepare TSA spread plates, in triplicate, for spiking suspension dilutions "B", "C", and "D".

Note: Agar plates must be dry prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

14.2.3.2 Mix dilution "B" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "B" onto the surface of each TSA plate in triplicate.

14.2.3.3 Mix dilution "C" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "C" onto the surface of each TSA plate in triplicate.

14.2.3.4 Mix dilution "D" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "D" onto the surface of each TSA plate in triplicate.

14.2.3.5 Use a sterile bent glass rod or spreader to distribute the inoculum over the surface of plates by rotating the dish by hand or on a turntable.

Note: Ensure that the inoculum is evenly distributed over the entire surface of the plate.

14.2.3.6 Allow the inoculum to absorb into the medium of each plate completely. Invert plates and incubate at 35°C ± 0.5°C for 20 ± 4 hours.

14.2.3.7 Count and record number of colonies per plate. The number of enterococci (CFU / mL) in the undiluted spiking suspension will be calculated using all TSA plates yielding counts within the countable range of 30 to 300 CFU per plate.

14.2.4 Recovery calculations for spiked samples

14.2.4.1 Calculate the concentration of enterococci (CFU / mL) in the undiluted spiking suspension (Section 14.2.1.2) according to the following equation. Example calculations are provided in **Table 4**, below.

$$\textbf{Enterococci}_{\text{undiluted spike}} = (CFU_1 + CFU_2 + ... + CFU_n) / (V_1 + V_2 + ... + V_n)$$

$\text{Enterococci}_{\text{undiluted spike}}$ = Enterococci (CFU / mL) in undiluted spiking suspension

Where,

CFU = Number of colony forming units from TSA plates yielding counts within the countable range of 30 to 300 CFU per plate

V = Volume of undiluted sample on each TSA plate yielding counts within the countable range of 30 to 300 CFU per plate

n = Number of plates with counts within the countable range of 30 to 300 CFU per plate

Note: The example calculated numbers provided in the tables below have been rounded at the end of each step for simplification purposes. Generally, rounding should only occur after the final calculation.

Table 4. Example Calculations of Laboratory-prepared Enterococci Spiking Concentration

Examples	CFU / plate (triplicate analyses) from TSA plates			Enterococci CFU / mL in undiluted spiking suspension ($\text{Enterococci}_{\text{undiluted spike}}$) [a]
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	94, 106, 89	9, 11, 28	1, 0, 4	$(94+106+89) / (10^{-5}+10^{-5}+10^{-5})$ = $289 / (3.0 \times 10^{-5})$ = 9,633,333 = **9.6×10^6 CFU / mL**
Example 2	32, 55, 72	8, 5, 3	0, 0, 0	$(32+55+72) / (10^{-5}+10^{-5}+10^{-5})$ = $159 / (3.0 \times 10^{-5})$ = 5,300,000 = **5.3×10^6 CFU / mL**

[a] Enterococci undiluted spike is calculated using all plates yielding counts within the countable range of 30 to 300 CFU per plate

14.2.4.1 Calculate true concentration of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 5,** below.

$$\mathbf{T}_{\text{spiked Enterococci}} = (\mathbf{Enterococci}_{\text{undiluted spike}}) \times (\mathbf{V}_{\text{spiked per 100 mL sample}})$$

Where,

$\mathbf{T}_{\text{spiked Enterococci}}$ = Number of spiked Enterococci (CFU / 100 mL)
$\mathbf{Enterococci}_{\text{undiluted spike}}$ = Enterococci (CFU / mL) in undiluted spiking suspension
$\mathbf{V}_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 5. Example Calculations for Determination "True" Spiked Enterococci Concentration

$\mathbf{Enterococci}_{\text{undiluted spike}}$	$\mathbf{V}_{\text{spiked per 100 mL sample}}$	$\mathbf{T}_{\text{spiked Enterococci}}$
9.6×10^6 CFU / mL	3.0×10^{-6} mL per 100 mL of sample	(9.6×10^6 CFU / mL) x (3.0×10^{-6} mL / 100 mL) = **28.8 CFU / 100 mL**
5.3×10^6 CFU / mL	3.0×10^{-6} mL per 100 mL of sample	(2.8×10^6 CFU / mL) x (3.0×10^{-6} mL / 100 mL) = **8.4 CFU / 100 mL**

14.2.4.2 Calculate percent recovery (R) of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 6**, below.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R = Percent recovery
N_s = Enterococci (CFU / 100 mL) in the spiked sample (Section 13)
N_u = Enterococci (CFU / 100 mL) in the unspiked sample (Section 13)
T = True spiked enterococci (CFU / 100 mL) in spiked sample (Section 14.2.4.1)

Table 6. Example Percent Recovery Calculations for Lab-prepared Spiked Samples

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	$T_{\text{spiked Enterococci}}$ (CFU / 100 mL)	Percent recovery (R)
42	<1	28.8	100 x (42 - 1) / 28.8 = **142%**
34	10	28.8	100 x (34 - 10) / 28.8 = **83%**
10	<1	8.4	100 x (10 - 1) / 8.4 = **107%**

14.3 BioBall™ Spiking Procedure

14.3.1 Aseptically add 1 BioBall™ to 100 mL of PBS or appropriate volume of sample and mix by vigorously shaking the sample bottle a minimum of 25 times. Analyze the spiked sample according to the procedures in Section 11.

14.3.2 Recovery calculations for samples spiked with BioBalls—Calculate percent recovery (R) of spiked enterococci (CFU / 100 mL) according to the following equation. Example calculations are provided in **Table 7,** below.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R	=	Percent recovery
N_s	=	Enterococci (CFU / 100 mL) in the spiked sample (Section 13)
N_u	=	Enterococci (CFU / 100 mL) in the unspiked sample (Section 13)
T	=	True spiked enterococci (CFU / 100 mL) in spiked sample based on the lot mean value provided by manufacturer

Table 7. Example BioBall™ Percent Recovery Calculations

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	T (CFU / 100 mL)	Percent recovery (R)
24	<1	11.2	100 × (24 - 1) / 32 = **72%**
36	10	32	100 × (36 - 10) / 32 = **81%**

15.0 Method Performance

15.1 Performance Characteristics (Reference 18.4)

15.1.1 Precision - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. The precision among laboratories for marine water and surface water was 2.2% and 18.9%, respectively.

15.1.2 Bias - The persistent positive or negative deviation of the results from the assumed or accepted true value. The persistent positive or negative deviation of the results from the assumed or accepted true value was not significant.

15.1.3 Specificity - The ability of a method to select and/or distinguish the target bacteria from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The specificity for this medium as reported for various environmental water samples was 6.0% false positive and 6.5% false negative.

15.1.4 Multilaboratory variability - A collaborative study was conducted among fourteen collaborators at twelve laboratories to examine the interlaboratory reproducibility of the method. Reproducibility among laboratories (RSD_R) for freshwater, marine water, chlorinated secondary effluent, and non-chlorinated primary effluent ranged from 2.2% for marine water to 18.9% for freshwater with a low enterococcal density.

15.2 Interlaboratory Validation of Method 1600 in Disinfected Wastewater (Reference 18.3)

15.2.1 Twelve volunteer participant laboratories, two enterococci verification laboratories, and two research laboratories participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1600. The purposes of the study were to characterize method performance across multiple laboratories and disinfected wastewater matrices and to develop quantitative quality control (QC) acceptance criteria. A detailed description of the of the study and results are provided in the validation study report (Reference 18.3). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the September 2002 version of EPA Method 1600.

15.2.2 Recovery - Method 1600 was characterized by mean laboratory-specific recoveries of enterococci from disinfected wastewater samples spiked with BioBalls™ ranging from 77.1% to 114.9%, with an overall mean recovery of 90.8%. Mean laboratory-specific recoveries of enterococci from PBS samples spiked with BioBalls ranged from 88.0% to 105.1%, with an overall mean recovery of 95.4%.

15.2.3 Precision - Method 1600 was characterized by laboratory-specific relative standard deviations (RSDs) from disinfected wastewater samples spiked with BioBalls™ ranging from 0% to 69.5%, with an overall pooled, within-laboratory RSD of 22.6%. For PBS samples spiked with BioBalls, laboratory-specific RSDs ranged from 3.1% to 13.7%, with an overall pooled, within-laboratory RSD of 8.1%.

15.2.4 False positive confirmation rates - Method 1600 laboratory-specific false positive confirmation rates for unspiked disinfected/secondary results combined, ranging from 0.0% to 10.0%. For secondary wastewater (excluding disinfected results), only 2 of 123 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 1.6%. For disinfected wastewater (excluding secondary results), none of the 66 typical colonies submitted to verification were non-enterococci, resulting in a false positive confirmation rate of 0.0%. Since all 2184 typical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false positive result was estimated (see Table 6, Reference 18.3). It is estimated that 0.0% and 1.2% of the total colonies would have resulted in a false positive for disinfected wastewater and secondary wastewater, respectively.

15.2.5 False negative rates - Method 1600 laboratory-specific false negative rates laboratory-specific false negative confirmation rates for unspiked disinfected/secondary results combined, ranged from 28.6% to 100.0%. For secondary wastewater (excluding disinfected results), 62 of 79 atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 78.5% for secondary wastewater. For disinfected wastewater (excluding secondary results), eight of eight atypical colonies submitted to verification were identified as enterococci, resulting in a false negative confirmation rate of 100.0% for disinfected wastewater. Since all 839 atypical colonies observed during the study could not be submitted to confirmation, the percent of total colonies that would have resulted in a false negative result was estimated. It is estimated that 21.2% and 22.8% of the total colonies would have resulted in a false negative for disinfected wastewater and secondary wastewater, respectively. The false positive and negative assessments are provided in **Table 8**.

Table 8. False Positive and False Negative Assessment for Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected	391	105	66	0	0.0	0.0	8	8	100.0	21.2
Secondary	1793	734	123	2	1.6	1.2	79	62	78.5	22.8
Disinfected + Secondary	2184	839	189	2	1.1	0.8	87	70	80.5	22.3

[a] False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

[b] Percent of total colonies estimated to be false positives = [(total typical colonies FP confirmation rate) / (total number of typical and atypical colonies observed); e.g., [(1793 ×(2/123))/(1793+734)] × 100 = 1.2%

[c] False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

[d] Percent of total colonies estimated to be false negatives = [(total atypical colonies* FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(734 ×(62/79))/(1793+734)] × 100 = 22.8%

15.2.6 During evaluation of the study results, it was noted that many of the false negatives (atypical colonies submitted to verification which were identified as enterococci) were pink to red in color but simply lacked a blue halo. The predecessor to EPA Method 1600 for enterococci is EPA Method 1106.1 which uses mE and EIA media. For EPA Method 1106.1, pink to red colonies on mE, which produce a brown precipitate after transfer to EIA are considered positive for enterococci. Tetrazolium chloride (TTC), the reagent responsible for producing pink to red enterococci colonies on mE, is also included as a reagent in mEI. A follow-on study was conducted, for which pink to red colonies without halos from unspiked secondary wastewaters were submitted to verification. For pink to red colonies without halos that were ≥0.5 mm colony size, 54 of 90 colonies submitted were identified as enterococci, resulting in a 60.0% verification rate.

Results of the verification analyses from the initial study were assessed with pink to red colonies without halos being counted as enterococci. When pink to red colonies without halos are counted as enterococci, the estimated percent of total colonies that would have resulted in false positives increases slightly from 0.8% to 2.7%, for combined disinfected and secondary results. More importantly, the estimated percent of total colonies that would have resulted in false negatives decreased from 22.3% to 7.0% for combined disinfected and secondary results and from 21.2% to 2.9% for disinfected wastewater. The re-assessment of false positive and false negative initial study results with pink to red colonies without halos counted as enterococci are provided in **Table 9**.

Table 9. Re-Assessment of False Positive and False Negative Initial Study Results with Pink to Red Colonies without Halos Counted as Enterococci

Matrix (sample no.)	Total colonies		False positive (FP) assessment				False negative (FN) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [c]	Estimated % of total colonies that would have been a FN [d]
Disinfected (Samples 1-4)	477	19	69	0	0.0	0.0	4	3	75.0	2.9
Secondary (Samples 5, 6)	2291	236	166	7	4.2	3.8	32	27	84.4	7.9
Disinfected & Secondary (Samples 1-6)	2768	255	235	7	3.0	2.7	36	30	83.3	7.0

a False positive confirmation rate = number of false positive colonies / number of typical colonies submitted

b Percent of total colonies estimated to be false positives = [(total typical colonies × FP confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(2291×(7/166))/(2291+236)] × 100 = 3.8%

c False negative confirmation rate = number of false negative colonies / number of atypical colonies submitted

d Percent of total colonies estimated to be false negatives = [(total atypical colonies × FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(236 ×(27/32))/(2291+236)] × 100 = 7.9%

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

17.2 Samples, reference materials, and equipment known or suspected to have viable enterococci attached or contained must be sterilized prior to disposal.

17.3 Samples preserved with HCl to pH <2 are hazardous and must be neutralized before being disposed, or must be handled as hazardous waste.

17.4 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

18.0 References

18.1 Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, 1979. *Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches.* Amer. Jour. Public Health. 69:690-696.

18.2 Dufour, A.P. 1984. *Health Effects Criteria for Fresh Recreational Waters,* EPA-600/1-84-004. Office of Research and Development, USEPA.

18.3 USEPA. 2004. *Results of the Interlaboratory Validation of EPA Method 1600 (mEI) for Enterococci in Wastewater Effluent.* December 2004. EPA 821-R-04-008.

18.4 Messer, J.W. and A.P. Dufour. 1998. *A Rapid, Specific Membrane Filtration Procedure for Enumeration of Enterococci in Recreational Water.* Appl. Environ. Microbiol. 64:678-680.

18.5 ACS. 2000. *Reagent Chemicals, American Chemical Society Specifications.* American Chemical Society, New York. For suggestions of the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals*, BDH, Poole, Dorset, UK and the United States Pharmacopeia.

18.6 APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association, Washington D.C.

18.7 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). *Microbiological Methods for Monitoring the Environment: Water and Wastes*, EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978.

Appendix A:
Part II (General Operations), Section A (Sample Collection, Preservation, and Storage)

Sample Collection[1]

1.0 Sample Containers

1.1 **Sample Bottles:** bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw-cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see examples A and C in Figure 1). Screw-caps must not produce bacteriostatic or nutritive compounds upon sterilization.

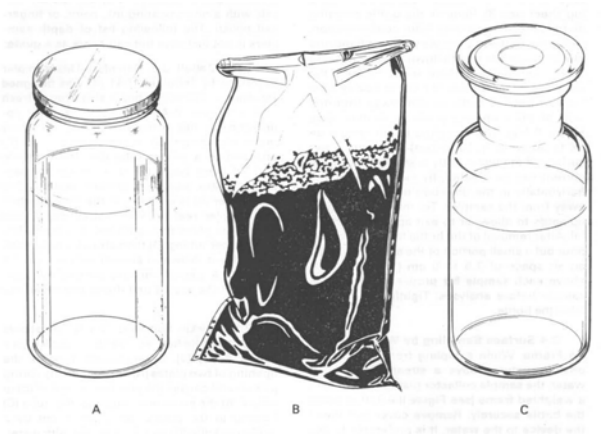

Figure 1. Suggested sample containers.

1.2 **Selection and Cleaning of Bottles:** Samples bottles should be at least 125 mL volume for adequate sampling and for good mixing. Bottles of 250 mL, 500 mL, and 1000 mL volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water.

1.3 **Dechlorinating Agent:** The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 mL of a 10% solution for each 125 mL sample volume. This concentration will neutralize approximately 15 mg/L of residue chlorine.

1.4 **Chelating Agent:** A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/L concentrations of heavy metals such as copper, nickel or zinc, etc. Add 0.3 mL of a 15% solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 mL sample volume prior to sterilization.

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[1]The text is taken from Part II, Section A, of the EPA publication "Microbiological Methods for Monitoring the Environment" EPA-600/8-78-017, December 1978.

1.5 **Wrapping Bottles:** Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 **Sterilization of Bottles:** Autoclave glass or heat-resistant plastic bottles at 121°C for 15 minutes. Alternatively, dry glassware may be sterilized in a hot oven at 170°C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate.

1.7 **Plastic Bags:** The commercially available bags (Whirl-pak) (see example B in Figure 1) are a practical substitute for plastic or glass samples bottles in sampling soil, sediment, or biosolids. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2.0 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a bridge or bank adjacent to a surface water.

2.1 **Chlorinated Samples:** When samples such as treated waters, chlorinated wastewaters or recreational waters are collected, the sample bottle must contain a dechlorinating agent (see section 1.3 above).

2.2 **Composite Sampling:** In no case should a composite sample be collected for bacteriologic examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and composition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 **Surface Sampling by Hand:** A grab sample is obtained using a sample bottle prepared as described in (1) above. Identify the sampling site on the bottle label and on a field log sheet. Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum (Figure 2). Position the mouth of the bottle into the current away from the hand of the collector and, if applicable, away from the side of the sampling platform. The sampling depth should be 15-30 cm (6-12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5-5 cm (1-2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper the bottle and place on ice (do not freeze) for transport to the laboratory.

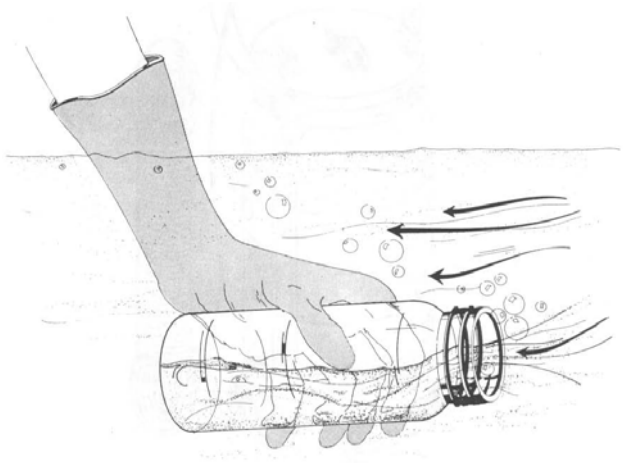

Figure 2. Grab sampling technique for surface waters.

3.0 Selection of Sampling Sites and Frequency

These will be described for streams, rivers, estuarine, marine, and recreational waters as well as domestic and industrial wastewaters.

3.1 **Stream Sampling:** The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

3.1.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined.

3.1.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (e.g., backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

3.1.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points. Occasionally, depth samples are necessary to determine vertical mixing patterns.

3.2 Estuarine and Marine Sampling: Sampling estuarine and marine waters requires the consideration of other factors in addition to those usually recognized in fresh water sampling. They include tidal cycles, current patterns, bottom currents and counter-currents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings.

The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollution loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by the Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

3.2.1 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points.

In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition.

In location sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability.

Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at the point below the fresh water river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

3.2.2 Marine Sampling: In ocean studies, the environmental conditions are most diverse along the coast where shore, atmosphere and the surf are strong influences. The shallow coastal waters are particularly susceptible to daily fluctuations in temperature and seasonal changes.

Sampling during the entire tidal cycle or during a half cycle may be required. Many ocean studies such as sampling over the continental shelf involve huge areas and no two areas of water are the same.

Selection of sampling sites and depths are most critical in marine waters. In winter, cooling of coastal waters can result in water layers which approach 0°C. In summer, the shallow waters warm much faster than the deeper waters. Despite the higher temperature, oxygen concentrations are higher in shallow than in deeper waters due to greater water movement, surf action and photosynthetic activity from macrophytes and the plankton.

Moving from the shallow waters to the intermediate depths, one observes a moderation of these shallow water characteristics. In the deeper waters, there is a marked stabilization of conditions. Water temperatures are lower and more stable. There is limited turbulence, little penetration of light, sparse vegetation and the ocean floor is covered with a layer of silts and sediments.

3.3 Recreational Waters (Bathing Beaches): Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas. Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling, preferably in the afternoon, is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.

3.4 Domestic and Industrial Waste Discharges: It is often necessary to sample secondary and tertiary wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are collected around the clock at selected intervals for a three to five day period. If it is known that the process displays little variation, fewer samples are needed. In no case should a composite sample be collected for bacteriological examination. The National Pollution Discharge Elimination System (NPDES) has established wastewater treatment plant effluent limits for all dischargers. These are often based on maximum and mean values. A sufficient number of samples must be collected to satisfy the permit and/or to provide statistically sound data and give a fair representation of the bacteriological quality of the discharge.

Appendix B:
Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results)

Counting Colonies[1]

1.0 Counting Colonies

Colonies should be counted using a fluorescent lamp with a magnifying lens. The flourescent lamp should be nearly perpendicular to the membrane filter. Count colonies individually, even if they are in contact with each other. The technician must learn to recognize the difference between two or more colonies which have grown into contact with each other and single, irregularly shaped colonies which sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and the colonies conform to the shape and size of the fiber or particulates. Colonies which have grown together almost invariably show a very fine line of contact.

2.0 Calculation of Results

2.1 Select the membrane filter with the number of colonies in the acceptable range and calculate count per 100 mL according to the general formula:

Count per 100 mL = (No. of colonies counted/Volume of sample filtered, in mL) x 100

2.2 Counts Within the Acceptable Limits

The acceptable range of colonies that are countable on a membrane is a function of the method. Different methods may have varying acceptable count ranges. All examples in this appemdix assume that the acceptable range of counts is between 20-80 colonies per membrane.

For example, assume that filtration of volumes of 50, 15, 5, 1.5, and 0.5 mL produced colony counts of 200, 110, 40, 10, and 5, respectively.

An analyst would not actually count the colonies on all filters. By inspection the analyst would select the membrane filter with the acceptable range of target colonies, as defined by the method, and then limit the actual counting to such membranes.

After selecting the best membrane filter for counting, the analyst counts colonies and applies the general formula as in section 2.1 above to calculate the count/100 mL.

2.3 More Than One Acceptable Count

2.3.1 If there are acceptable counts on replicate plates, carry counts independently to final reporting units, then calculate the arithmetic mean of these counts to obtain the final reporting value.

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[1]The text is largely taken from Part II, Section C, of the EPA publication "Microbiological Methods for Monitoring the Environment" EPA-600/8-78-017, December 1978. Some examples were kindly provided by Kristen Brenner, US EPA.

Example, if the counts are 24 and 36 for replicate plates of 100 mL each, then the arithmetic mean is calculated as follows:

$$\frac{(24\ \text{CFU}/100\ \text{mL} + 36\ \text{CFU}/100\ \text{mL})}{2} = 30\ \text{CFU}/\ 100\ \text{mL}$$

2.3.2 If there is more than one dilution having an acceptable range of counts, independently carry counts to final reporting units, then average for final reported value.

For example, if volumes of 100, 10, 1 and 0.1 mL produced colony counts of Too Numerous To Count (TNTC), 75, 30, and 1, respectively, then two volumes, 10 mL and 1 mL, produced colonies in the acceptable counting range.

Independently carry each MF count to a count per 100 mL:

$$\frac{75}{10} \times 100 = 750\ \text{CFU}/100\ \text{mL}$$

and

$$\frac{30}{1} \times 100 = 3000\ \text{CFU}/100\ \text{mL}$$

Calculate the arithmetic mean as in section 2.3.1 above:

$$\frac{(750\ \text{CFU}/100\ \text{mL} + 3000\ \text{CFU}/100\ \text{mL})}{2} = 1875\ \text{CFU}/100\ \text{mL}$$

Report this as 1875 CFU/100 mL.

2.4 If all MF counts are below the lower acceptable count limit, select the most nearly acceptable count.

2.4.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 17, 1 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 17, and report as 17 CFU/100 mL.

Note that in this case, because no calculations were done (i.e. this is the count for 100 mL), the count is reported as 17 CFU/100 mL rather than an "estimated count of 17 CFU/100 mL"

2.4.2 As a second example, assume a count in which sample volumes of 10 and 1 mL produced colony counts of 18 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in section 2.3.2 above.

$$\frac{18}{10} \times 100 = 180 \text{ CFU /100 mL}$$

Report this as an estimated count of 180 CFU/100 mL.

2.5 If counts from all membranes are zero, calculate using count from largest filtration volume.

For example, sample volumes of 25, 10, and 2 mL produced colony counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume. In this example, the largest volume filtered was 25 mL and thus the calculation would be:

$$\frac{1}{25} \times 100 = 4 \text{ CFU /100 mL}$$

Report this as < (less than) 4 CFU/100 mL.

2.6 If all membrane counts are above the upper acceptable limit, calculate count using the smallest volume filtered.

For example, assume that the volumes 1, 0.3, and 0.01 mL produced colony counts of TNTC, 150, and 110 colonies, respectively. Since all colony counts are above the acceptable limit, use the colony count from the smallest sample volume filtered and estimate the count as:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000 \text{ CFU /100 mL}$$

Report this as estimated count 1.1×10^6 CFU/100 mL

2.7 If typical colonies are too numerous to count (TNTC), use upper limit count with smallest filtration volume.

For example, assume that the volumes 1, 0.3, and 0.01 mL all produced too many typical colonies, and that the laboratory bench record indicated TNTC.

Use the upper acceptable count for the method (80 colonies in this example) as the basis of calculation with the smallest filtration volume and estimate the count as:

$$\frac{80}{0.01} \times 100 = 800{,}000 \text{ CFU /100 mL}$$

Report this as > (greater than) 8×10^5 CFU/100 mL

2.8 If colonies are both above and below the upper and lower acceptable limits (i.e., no counts are within the acceptable limits), select the most nearly acceptable count.

2.8.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 84, 8 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 84, and report as 84 CFU/100 mL.

Note that in this case, because no calculations were done (i.e. this is the count for 100 mL), the count is reported as 84 CFU/100 mL rather than an "estimated count of 84 CFU/100 mL"

2.8.2 As a second example, assume a count in which sample volumes of 100, 10 and 1 mL produced colony counts of 98, 18, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in section 2.3.2 above.

$$\frac{18}{10} \times 100 = 180 \text{ CFU /100 mL}$$

Report this as estimated count 180 CFU/100 mL.

2.9 If there is no result because of a confluent growth, > 200 atypical colonies (TNTC), lab accident, etc., report as No Data and specify the reason.

Appendix D
Laboratory Method – Chlorophyll & Pheophytin CBP IV-12.0

12. Chlorophyll and Phaeophytin

12.1 Scope and Application

12.1.1 This spectrophotometric method is used in the determination of chlorophyll *a*, *b*, and *c*, phaeophytin-*a*, and carotenoids. This method can be used to estimate phytoplankton biomass.

12.2 Summary of Method

12.2.1 The reference for this method is ASTM Method D3731-79. In brief, algal cells are ground in aqueous acetone to extract the pigments. The extract is analyzed using a spectrophotometer to measure the absorbances at the correct wavelengths.

12.3 Apparatus and Materials

12.3.1 Laboratory coat: worn at all times and with an apron when handling acids.

12.3.2 Protective eyewear: worn at all times.

12.3.3 PVC gloves: worn at all times.

12.3.4 Glass fiber filter: Whatman GF/F or equivalent.

12.3.5 Centrifuge.

12.3.6 Dual beam spectrophotometer: or scanning spectrophotometer with matched cuvettes.

12.3.7 Tissue Grinder.

12.3.8 Filtration Apparatus

12.4 Reagents

12.4.1 Solvents:

12.4.1.1 Hydrochloric acid (1N), Hcl.

12.4.1.2 Sodium bicarbonate solution (1N), ($NaH(CO_3)_2$), dissolve 8.4 g sodium bicarbonate in 100 mL water.

12.4.1.3 Magnesium carbonate suspension, ($MgCO_3$), add 1 g finely powdered magnesium carbonate to 100 Ml water.

12.4.1.4 Aqueous acetone (90%), add 1 part water to 9 parts of reagent grade acetone, add 5 drops of $NaH(CO_3)_2$ solution to every volume, made within 24-48 hours of time of use.

12.4.2 Reagent Grade Water: See section 1.9.

12.4.2.1 Reference standards: use EPA SRM reference standards if provided.

12.5 Sample Handling

12.5.1 After the addition of magnesium carbonate, samples can be held for 24 hours at 0-4°C. The extract can be store at -20 ± 2°C for a maximum 30 days.

12.6 Filtration Procedure

12.6.1 Using a 47 mm GF/F, Pour a measured amount of well mixed sample (typically 500ml) through filter. Apply vacuum to complete filtration process.

12.6.2 Add approximately 1 ml of Magnesium Carbonate Suspension and apply vacuum to dryness.

12.6.3 Release vacuum and remove filter. Store as required. Record sample volume filtered.

12.7 Grinding Procedure

12.7.1 Place filter in grinding vessel and add 2-3 ml of 90% acetone.

12.7.2 Insert pestle into grinding vessel and turn on grinder.

12.7.3 Grind filter for approximately 2 minutes being sure there are no discernible pieces remaining.

12.7.4 Pull pestle from vessel and rinse with 90% acetone.

12.7.5 Transfer sample to centrifuge tube using a glass funnel and 90% acetone for rinsing.

12.7.6 Record filtration volume.

12.8 Procedure

12.8.1 Adjust the temperature of the centrifuge so that unit cools to 4 ± 2°C. Centrifuge samples for 30 minutes at 4500 rpm. Keep centrifuged samples level, cool, and in a dark box.

12.8.2 Zero the dual beam spectrophotometer at 750 nm using the 90% acetone reagent. After zeroing the spectrophotometer, leave the cuvette in the reference cell.

12.8.3 Using a matched pair of cuvettes, one cuvette is used to zero the spectrophotometer at each wavelength; and the other cuvette contains the sample, so that the chlorophyll sample is not transferred back to the chlorophyll tube after the initial turbidity absorbance is obtained at the 750 nm wavelength. To account for any differences between the two cuvettes, the cuvette which will contain the samples is first filled with 90% acetone and the absorbance is read on the spectrophotometer. If this absorbance is not zero, the value obtained is used to adjust all of the chlorophyll readings. The acetone in the reference and zeroing cuvettes must be rehomogenized periodically throughout the analysis.

12.8.4 Record sample volume filtered and extract volume in screw-cap test tube onto the data sheet. Note

that the volume of the filter residue in the bottom of the centrifuge tube must be subtracted from the total volume to obtain extract volume. Then carefully dispense the sample into the sample cuvette using a disposable glass pipette, and measure absorbances at the following wavelengths: 750 nm, 664 nm, 647 nm, 630 nm, 480 nm, and 510 nm.

12.8.5 Re-zero spectrophotometer with 90% acetone before reading at each wavelength. After the 510 nm reading is taken, add 2 drops of 1N HCL. After 1 minute, but not longer than 2 minutes after acidification, measure sample absorbances at 750 nm and 665 nm. Zero with the blank at each absorbance before reading.

12.9 Quality Control

12.9.1 The initial absorbance reading at 750 nm must be less than or equal to .007, or sample(s) must be re-centrifuged. If after re-centrifugation the absorbance reading at 750nm is greater than .007, continue to measure the absorbances at the rest of the wavelengths and write in the comment section that the sample had been recentrifuged.

12.9.2 It is especially important to maintain the spectrophotometers in peak operating condition. This should be confirmed by the following guidelines:

12.9.2.1 Analyzing an EPA SRM reference standard for chlorophyll analysis if provided.

12.9.2.2 Periodic evaluation of the slopes of calibration curves from spectrophotometer analyses for other parameters for which there are reliable standards (e.g. orthophosphate, nitrite, etc.). If significant slope deviation or consistent unidirectional slope change over time is noted, an alternate spectrophotometer should be used until the problem is corrected by an authorized repair person.

12.9.2.3 The holmium oxide absorption spectrum should be analyzed at least annually or when problems are suspected. Details are not provided here since this and subsequent evaluation should be performed only by or under direct supervision of experienced personnel.

12.9.3 Method detection limits (MDL): Method detection limits should be established using the guidelines in Chapter II, Section D.

12.9.4 Method blank: see Chapter II, Section C.

12.9.5 Laboratory duplicate: see Chapter II, Section C.

12.9.6 Reference materials: The laboratory must analyze a standard reference material once a year, as available.

12.10 References

American Public Health Association. Standard Methods for the Examination of Water and Wastewater, 17th Edition.

Parsons, T., Y. Maita and C . Lalli. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, pp. 101-112.

Appendix E
Laboratory Method – Chlorophyll & Pheophytin – US EPA 447.0

Method 447.0

Determination of Chlorophylls *a* and *b* and Identification of Other Pigments of Interest in Marine and Freshwater Algae Using High Performance Liquid Chromatography with Visible Wavelength Detection

Elizabeth J. Arar

Version 1.0
September 1997

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

METHOD 447.0

DETERMINATION OF CHLOROPHYLLS *a* AND *b* AND IDENTIFICATION OF OTHER PIGMENTS OF INTEREST IN MARINE AND FRESHWATER ALGAE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH VISIBLE WAVELENGTH DETECTION

1.0 Scope and Application

1.1 This method provides a procedure for determination of chlorophylls *a* (chl *a*) and *b* (chl *b*) found in marine and freshwater phytoplankton. Reversed-phase high performance liquid chromatography (HPLC) with detection at 440 nm is used to separate the pigments from a complex pigment mixture and measure them in the sub-microgram range. For additional reference, other taxonomically important yet commercially unavailable pigments of interest are identified by retention time.

1.2 This method differs from previous descriptions of HPLC methods in several respects. Quality assurance/quality control measures are described in Sect. 9.0, sample collection and extraction procedures are described in Sect. 8.0 and reference chromatograms of pure pigments and reference algae are provided.

This method has also been evaluated in a multilaboratory study along with EPA Methods 445.0 and 446.0. Estimated detection limits, precision and bias are reported in Section 13.

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8
Chlorophyll *b*	519-62-0

1.3 Instrumental detection limits (IDLs) of 0.7 ng chl *a*, and 0.4 ng chl *b* in pure solutions of 90% acetone were determined by this laboratory. Method detection limit (MDL) determinations were made by analyzing seven replicate unialgal samples containing the chl *a* and *b*. Single-laboratory MDLs were chl *a* - 7 ng and chl *b* - 4 ng. A multilaboratory estimated detection limit (EDL) (in mg/L of extract is reported in Section 13.

1.4 Most taxonomically important pigments are not commercially available, therefore, a laboratory must be willing to extract and purify pigments from pure algal cultures to quantify and qualitatively identify these very important pigments. This method contains chromatographic information of select pure pigments found either in marine or freshwater algae. The information is included to aid the analyst in qualitatively identifying individual pigments and possibly algal species in natural samples.

1.5 This method uses 90% acetone as the extraction solvent because of its efficiency for extracting chl *a* from most types of algae. (**NOTE:** There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[1-3] or dimethyl sulfoxide.)[4] Using high performance liquid chromatography (HPLC), Mantoura and Llewellyn[5] found that methanol led to the formation of chl *a* derivative products, whereas 90% acetone did not. Bowles, et al.[3] found that for chl *a* 90% acetone was an effective solvent when the steeping period was optimized for the predominant species present.)

1.6 One of the limitations of visible wavelength detection is low sensitivity. It may be preferable to use fluorometry[6-8] or HPLC[913] with fluorometric detection if high volumes of water (>4 L) must be filtered to obtain detectable quantities of chl *a* or *b.*

1.7 This method is for use by analysts experienced in handling photosynthetic pigments and in the operation of HPLC or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 The HPLC is calibrated with a chl *a* and *b* solution that has been spectrophotometrically quantified

according to EPA Method 446. Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtration at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton into 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not exceeding 24 h, to ensure thorough extraction of the pigments. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is filtered through a 0.45 μm syringe filter and 50 to 200 μL is injected onto a reversed-phase column. Following separation using a ternary gradient, concentrations are reported in μg/L (ppb) or mg/L (ppm) in the whole water sample. This method is based on the HPLC work of Wright, et. al.[9]

3.0 Definitions

3.1 Calibration Standard (CAL) -- A solution prepared from dilution of a stock standard solution. The CAL solution is used to calibrate the instrument response with respect to analyte concentration or mass.

3.2 Calibration Check Standard (CALCHK) -- A mid-point calibration solution that is analyzed periodically in a sample set to verify that the instrument response to the analyte has not changed during the course of analysis.

3.3 Field Replicates -- Separate samples collected at the same time and placed under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field replicates give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.4 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent that gives an analyte signal equal to three times the standard deviation of a background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.5 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus. For this method the LRB is a blank filter that has been extracted as a sample.

3.6 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.7 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.

3.8 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. Ideally, 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.

4.0 Interferences

4.1 Any compound extracted from the filter or acquired from laboratory contamination that absorbs light at 440 nm may interfere in the accurate measurement of the method analytes.

4.2 Proper storage and good sample handling technique are critical in preventing degradation of the pigments.

4.3 Precision and recovery for any of the pigments is related to efficient extraction, i.e. efficient maceration of the filtered sample and to the steeping period of the macerated filter in the extraction solvent. Precision improves with increasing steeping periods, however, a drawback to prolonged steeping periods is the possibility of pigment degradation. The extracted sample must be kept cold and in the dark to minimize degradation.

4.4 Sample extracts must be clarified by filtration through a 0.45 μm filter prior to analysis by HPLC to prevent column fouling.

4.5 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials, and filtered samples must be stored in the dark at -20°C or -70°C to prevent rapid degradation.

5.0 Safety

5.1 Each chemical used in this method should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[15-18] A file of MSDS also should be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Centrifuge, capable of 675 g.

6.2 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity round-bottomed, glass grinding tube.

6.3 Filters, glass fiber, 47-mm or 25-mm nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.4 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.5 Aluminum foil.

6.6 Laboratory tissues.

6.7 Tweezers or flat-tipped forceps.

6.8 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg (20 KPa).

6.9 Labware -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.9.1 Assorted Class A calibrated pipets.

6.9.2 Graduated cylinders, 500-mL and 1-L.

6.9.3 Volumetric flasks, Class A calibrated, 10-mL, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.9.4 Glass rods or spatulas.

6.9.5 Pasteur Type pipets or medicine droppers.

6.9.6 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.9.7 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.9.8 Polyethylene squirt bottles.

6.9.9 Amber 2-mL HPLC autosampler vials with screw or clamp caps.

6.9.10 Glass syringe, 1 or 2-mL capacity.

6.9.11 HPLC compatible, low-volume, acetone resistant glass fiber or PTFE syringe filters.

6.10 Liquid Chromatograph

6.10.1 This method uses a ternary gradient thus requiring a programmable gradient pump with at least three pump inlets for the three different mobile phases required. A Dionex Model 4500 chromatograph equipped with a gradient pump, UV/VIS detector (cell path length, 6 mm, volume 9 µL) and PC data analysis (Dionex AI450 software, Version 3.32) system was used to generate data for this method. Tubing was made of polyether ether ketone (PEEK). A Dionex degas module was used to sparge all eluents with helium.

6.10.2 Helium or other inert gas for degassing the mobile phases OR other means of degassing such as sonication under vacuum.

6.10.3 Sample loops of various sizes (50-200 µL).

6.10.4 Guard Column -- A short column containing the same packing material as the analytical column placed before the analytical column to protect it from fouling by small particles. The guard column can be replaced periodically if it is noticed that system back pressure has increased over time.

6.10.5 Analytical Column -- A C_{18} reversed-phase column with end capping. A J.T. Baker 4.6 mm X 250 mm, 5 µm pore size column was used to generate the data in this method.

6.10.6 A visible wavelength detector with a low volume flow-through cell. Detection is at 440 nm.

6.10.7 A recorder, integrator or computer for recording detector response as a function of time.

6.10.8 Although not required, an autosampler (preferably refrigerated) is highly recommended.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Methanol, HPLC grade, (CASRN 67-56-1). Prepare ELUENT A, 80% (v/v) methanol/20% 0.5 M ammonium acetate, by adding 800 mL of methanol and 200 mL of the 0.5 M ammonium acetate (Sect. 7.5) to an eluent container.

7.3 Acetonitrile, HPLC grade, (CASRN 75-05-8). Prepare ELUENT B, 90% (v/v) acetonitrile/10% water, by adding 900 mL of acetonitrile and 100 mL of water (Sect. 7.7) to an eluent container.

7.4 Ethyl acetate, HPLC grade, (CASRN 141-78-6). ELUENT C, 100% ethyl acetate.

7.5 Ammonium acetate, ACS grade (CASRN 631-61-8). Prepare a 0.5 M solution by dissolving 38.54 g in approximately 600 mL of water in a 1-L volumetric flask. After the ammonium acetate has dissolved, dilute to volume with water.

7.6 Chl *a* free of chl *b* and chl *b* substantially free of chl *a* may be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO).

7.7 Water -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.8 Aqueous Acetone Solution -- 90% acetone/10% ASTM Type I water. Carefully measure 100 mL of the water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.9 Chlorophyll Stock Standard Solution (SSS) -- Chl *a* (MW = 893.5) and chl *b* (MW = 907.5) from a commercial supplier is shipped in amber glass ampules that have been flame sealed. The dry standards must be stored at -20 or -70°C in the dark. Tap the ampule until all the dried pigment is in the bottom of the ampule. In subdued light, carefully break the tip off the ampule. Transfer the entire contents of the ampule into a 25-mL volumetric flask. Dilute to volume with 90% acetone: (1 mg in 25 mL = 40 mg chl *a*/L) and (1 mg in 25 ml = 40 mg chl *b*/L), label the flasks and wrap with aluminum foil to protect from light. When stored in a light- and air-tight container at -20 or -70ºC, the SSS is stable for at least six months. Dilutions of the SSS should always be confirmed spectrophotometrically using EPA Method 446.

7.10 Laboratory Reagent Blank (LRB) -- A blank filter that is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.11 Quality Control Sample (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 Water Sample Collection -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth and frequency[21] at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (region in which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal populations, thus pigment concentrations, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher filtration pressures may damage cells and result in loss of chlorophyll. Care must be taken not to overload the filters. Do not increase the vacuum during filtration.

Prior to drawing a subsample from the water sample container, gently stir or invert the container several times to suspend the particles. Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). Typically, a sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20°C or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20°C or -70°C as soon as possible.

8.2 Preservation -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 Holding Time -- Filters can be stored frozen at -20°C for as long as 3½ weeks without significant loss of chl *a*.[20]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field replicates, QCSs, and CALCHKs as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (IDLs) and laboratory performance (MDLs, extraction proficiency, and analyses of QCSs) prior to sample analyses.

9.2.2 Instrumental Detection Limit (IDL) -- After a low level calibration (Sect. 10), prepare a standard solution that upon injection into the chromatograph yields an absorbance of 0.002-0.010. If using an autosampler, variable volumes may be injected and the micrograms (µg) injected calculated by multiplying the known concentration (µg/µL) of the standard by the volume injected (µL). A practical starting point may be to inject 0.05 µg (that would be a 50 µL injection of a 1.0 mg/L standard solution) and reduce or increase the mass injected according to the resulting signal. Avoid injecting really small volumes (< 10 µL). After the quantity of pigment has been selected, make three injections and calculate the IDL by multiplying the standard deviation of the calculated mass by 3.

9.2.3 Method Detection Limit (MDL) -- At least seven natural phytoplankton samples known to contain the pigments of interest should be collected, extracted and analyzed according to the procedures in Sects. 8 and 11, using clean glassware and apparatus. Mass of the pigment injected into the chromatograph should be 2 to 5 times the IDL. Dilution of the sample extract solution to the appropriate concentration or reducing the volume of sample injected may be necessary. Calculate the MDL (in micrograms) as follows.[19]

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

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

S = Standard deviation of the replicate analyses.

9.2.4 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify instrument performance with the analysis of a QCS (Sect. 7.11). If the determined value is not within ±10% of the spectrophotometrically determined value, then the instrument should be recalibrated with fresh stock standard and the QCS reanalyzed. If the redetermined value is still unacceptable then the source of the problem must be identified and corrected before continuing analyses.

9.2.5 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1). Fifteen to twenty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 samples or more should be extracted and analyzed according to Sect. 11. The percent relative standard deviation (%RSD) should not exceed 15% for samples that are at least 10X the IDL.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. If the LRB value constitutes 10% or more of the analyte level determined in a sample, fresh samples or field replicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 Calibration Check Standard (CALCHK) -- The laboratory must analyze one CALCHK for every ten samples to verify calibration. If the CALCHK is not $\pm$10% of the spectrophotometrically determined concentration, then the instrument must be recalibrated.

10.0 Calibration and Standardization

10.1 Allow the visible wavelength detector (440 nm) to warm up for at least 15 min before calibration. Prepare ELUENTS A - C and degas by sparging with an inert gas for 10 minutes or sonicating under vacuum for 5 minutes. Prime the pump for each eluent taking care to remove all air that may be in the liquid lines. Equilibrate the column for ten minutes with 100% of ELUENT A.

10.2 Remove the SSS from the freezer and allow it to come to room temperature. Add 1 mL of the SSS to a 10-mL volumetric flask and dilute to 10 mL with 90% acetone. Prepare the chl *a* and *b* separately and determine the concentrations according to EPA Method 446 using the monochromatic equations for chl *a* determination. After the concentration of the SSS is determined, add 1 mL of the chl *a* SSS plus 1 mL of the chl *b* SSS to a separate 10-mL flask and dilute to volume. Store the calibration standard in a light tight glass bottle.

10.3 Program the pump with the following gradient:

Time	Flow	%1	%2	%3	Condition
0.0	1.0	100	0	0	Injection
2.0	1.0	0	100	0	Linear Gradient
2.6	1.0	0	90	10	Linear Gradient
13.6	1.0	0	65	35	Linear Gradient
20.0	1.0	0	31	69	Linear Gradient
22.0	1.0	0	100	0	Linear Gradient
25.0	1.0	100	0	0	Linear Gradient
30.0	1.0	100	0	0	Equilibration

Flow is in mL/min.

10.4 The first analysis is a blank 90% acetone solution followed by calibration. Calibrate with at least three concentrations, covering no more than one order of magnitude, and bracketing the concentrations of samples. If an autosampler is used, variable volumes ranging from 10 - 100% of the sample injection loop volume are injected to give a calibration of detector response versus mass of pigment. If doing manual injections, variable solution concentrations are made and a fixed sample loop volume is injected for standards and samples. Calibration can be either detector response versus mass or detector response versus concentration (mg/L or µg/L). Linearity across sensitivity settings of the detector must be confirmed if samples are analyzed at a different sensitivity settings from that of the calibration.

10.5 Construct a calibration curve of analyte response (area) versus concentration (mg/L in solution) or mass (µg) of pigment and perform a linear regression to determine the slope and y-intercept. A typical coefficient of determination is > 0.99.

10.6 Calibration must be performed at least weekly although it is not necessary to calibrate daily. Daily mid-point CALCHKs must yield calculated concentrations $\pm$10% of the spectrophotometrically determined concentration.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 For convenience, a 10-mL final extraction volume is described in the following procedure. A smaller extraction volume may be used to improve detection limits.

11.1.2 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand laboratory tissues and wash bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. You may also tear the filter into smaller pieces and push them to the bottom of the tube with a glass rod. With a volumetric pipet, add 3 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has become a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 7-mL volumetric pipet, rinse the pestle and the grinding tube with the aqueous acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. To reduce the volume of reagent grade solvents used for rinsing between extractions, thoroughly rinse the grinding tube and glass rod with tap water prior to a final rinse with ASTM Type I water and acetone. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The last filter extracted should be a blank. The entire extraction with transferring and rinsing takes approximately 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.3 Again, shake each tube vigorously before placing them to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. Tubes should be shaken at least once, preferably two to three times, during the steeping period to allow the extraction solution to have maximum contact with the filter slurry.

11.1.4 After steeping is complete, centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Draw approximately 1 mL into a glass syringe, attach a 0.45 µm syringe filter, filter the extract into an amber autosampler vial, cap and label the vial. Protect the filtered samples from light and heat. If using a refrigerated autosampler, chill to 10°C.

11.2 Sample Analysis

11.2.1 Draw into a clean syringe 2-3 times the injection loop volume and inject into the chromatograph. If using an autosampler, load the sample tray, prepare a schedule and begin analysis. A typical analyses order might be: (1) blank 90% acetone, (2) CALCHK, (3) 10 samples, (4) CALCHK, (5) QCS.

11.2.2 If the calculated CALCHK is not ±10 of the spectrophotometrically determined concentration then recalibrate with fresh calibration solutions.

12.0 Data Analysis and Calculations

12.1 From the chl *a* or *b* area response of the sample, calculate the mass injected or concentration (C_E) of the solution that was analyzed using the calibration data. Mass injected must be converted to concentration in extract by dividing mass by volume injected (µL) and multiplying by 1000 to give concentration in mg/L (mg/L = µg/mL). Concentration of the natural water sample may be reported in mg/L by the following formula:

$$\frac{C_E \text{ X extract volume (L) X DF}}{\text{sample volume (L)}}$$

where:

C_E = concentration (mg/L) of pigment in extract.
DF = any dilution factors.
L = liters.

12.2 LRB and QCS data should be reported with each data set.

13.0 Method Performance

13.1 Single Laboratory Performance

13.1.1 An IDL was determined by preparing a mixed chl *a* (0.703 ppm) and chl *b* (0.437 ppm) standard. The injected mass yielded 0.004 AU for chl *a* (0.035 µg) and 0.003 AU for chl *b* (0.022 µg). Seven replicate 50 µL injections were made and the standard deviation of the calculated concentration was multiplied by three to determine an IDL. The IDL determined for chl *a* was 0.76 ng and 0.44 ng for chl *b*. The %RSDs for chl *a* and chl *b* was 0.45 and 0.67, respectively.

13.1.2 MDLs for chl *a* and chl *b* were determined by spiking seven replicate filtered samples of Pycnacoccus, extracting and processing according to this method. An

injection volume of 100 µL yielded an MDL for chl *a* of 7.0 ng and 4.0 ng for chl *b*. The RSDs were 5.1% for chl *b* and 4.7 % for chl *a*.

13.1.3 Recoveries of chl *a* and chl *b* from filtered samples of phaeodactylum were determined by spiking three filters with known amounts of the pigments, extracting, processing and analyzing the extraction solution according to the method, along with three unspiked filtered samples (to determine the native levels in the algae). The spiked levels were 1.1 ppm chl *a* and 0.53 ppm chl *b* in the 10 mL extraction volume. Chl *a* was 87% recovered and chl *b* was 94% recovered.

13.1.4 Figures 1-7 are chromatograms of seven reference unialga cultures processed according to this method.

13.1.5 Table 1 is a list of pure pigments with retention times obtained using this method. Purified pigments were prepared under contract to EPA by Moss Landing Marine Laboratory, Moss Landing, CA.

13.1.6 Table 2 contains single lab precision data for seven reference algal suspensions.

13.2 Multilaboratory Testing - A Multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 8 volunteer participants in the HPLC methods component that returned data. The primary goals of the study were to determine estimated detection limits and to assess precision and bias (as percent recovery) for select unialgal species, and natural seawater.

13.2.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et. al.[21] method that does not assume constant error variances across concentration and controls for Type II error. The approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 3. It is referred to as Pooled-EDL (p-EDL).

The p-EDL was determined in the following manner. Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 3. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs.

13.2.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different "concentrations" of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The "true" concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination . Pooled precision data are presented in Tables 4-6 and accuracy data (as percent recovery) are presented in Table 7. No significant differences in precision (%RSD) were observed across concentrations for any of the methods or species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 7) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-

trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with incresing dunaliella filtration volume. The fluorometric and athe spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 8. Only one filtration volume (100 mL) was provided in duplicate to Participant labs.

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 (e.g., Sect. 11.1.2). 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's 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 the Sect. 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Pure Pigments and Retention Times

PIGMENT	RETENTION TIME
19' butanoyloxyfucoxanthin	8.13
2,4-divinylpheoporphrin a_5	8.60
Peridinin	8.69
Fucoxanthin	8.75
19' hexanoyloxyfucoxanthin	8.90
Neoxanthin	10.07
Chlorophyll C_3	10.27
Chlorophyll C_2	10.40
Prasinoxanthin	11.20
Violaxanthin	12.00
Diadinoxanthin	15.20
Chlorophyll *b*	15.60
Myxoxanthophyll	17.00
Aphanaxanthin	17.20
Chlorophyll *a*	17.80
Monadoxanthin	17.93
Lutein	18.00
Alloxanthin	18.07
Nostaxanthin	18.70
Diatoxanthin	19.07
Zeaxanthin	19.40

Table 2. Single Lab Precision for Seven Pure Unialgal Cultures

Algae		Chlorophyll *a*	Chlorophyll *b*
Pycnacoccus provasolii	N(1)	3	3
	Mean (mg/L)(2)	2.15	1.47
	STD DEV	0.114	0.065
	% RSD	5.31	4.45
Rhodomonas salina	N(1)	3	3
	Mean (mg/L)	4.0	ND(3)
	STD DEV	0.014	ND
	% RSD	0.28	ND
Selenastrum capricornitum	N(1)	3	3
	Mean (mg/L)	4.25	0.483
	STD DEV	0.199	0.058
	% RSD	4.68	12.01
Amphidinium carterae	N(1)	3	3
	Mean (mg/L)	2.38	ND
	STD DEV	0.176	ND
	% RSD	7.40	ND
Dunaliella tertiolecti	N(1)	3	3
	Mean (mg/L)	6.68	1.42
	STD DEV	0.635	0.0412
	% RSD	9.51	2.90
Emiliania huxleyi	N(1)	3	ND
	Mean (mg/L)	1.03	ND
	STD DEV	0.008	ND
	% RSD	0.79	ND
Phaeodactylum tricornutum	N(1)	3	ND
	Mean (mg/L)	1.09	ND
	STD DEV	0.072	ND
	% RSD	7.07	ND

(1) N = Number of filtered samples.
(2) Mean concentration in extract solution.
(3) ND = none detected.

TABLE 3. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS(1)

Method(2)	N(3)	p-EDL(4) (mg/L)
FL -Mod(5)	8	0.096
FL - Std	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.2.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 4. Measured Chlorophyll *a* (mg/L) in Dunaliella Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.172	0.064	36.8
	10	5	0.276	0.074	26.8
	50	5	0.757	0.344	45.4
	100	5	1.420	0.672	47.3

(1) Not all participants labs followed the EPA method exactly.

(2) N = Number of volunteer labs whose data was used.

TABLE 5. Measured Chlorophyll *a* (mg/L) in Amphidinium Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.104	0.043	56.8
	10	5	0.172	0.083	37.5
	50	5	0.743	0.213	17.4
	100	5	1.394	0.631	14.5

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 6. Measured Chlorophyll *a* in Phaeodactylum Samples

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
HPLC	5	5	0.193	0.074	38.4
	10	5	0.317	0.114	36.1
	50	5	1.024	0.340	33.2
	100	5	1.525	0.487	29.9

(1) Not all participants labs followed the EPA method exactly.
(2) N = number of volunteer labs whose data was used.

TABLE 7. Minimum, Median, and Maximum Percent Recoveries by Genera, Method, and Concentration Level

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80
		SP-M	240	247	247	243

Table 7. Cont'd.

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318

Table 8. Chlorophyll *a* Concentrations in mg/L Determined in Filtered Seawater Samples

Method	Con.[1]	No. Obs	No. Labs	Mean	Std. Dev	RSD (%)	Minimum	Median	Maximum
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

Appendix F
Laboratory Method – Chlorophyll & Pheophytin – US EPA 445.0

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

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Revision 1.2
September 1997

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Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

1.0 Scope and Application

1.1 This method provides a procedure for low level determination of chlorophyll *a* (chl *a*) and its magnesium-free derivative, pheophytin *a* (pheo *a*), in marine and freshwater phytoplankton using fluorescence detection.[1,2] Phaeophorbides present in the sample are determined collectively as pheophytin *a*. For users primarily interested in chl *a* there is currently available a set of very narrow bandpass excitation and emission filters (Turner Designs, Sunnyvale, CA) that nearly eliminates the spectral interference caused by the presence of pheo *a* and chlorophyll *b*. The difference between the modified method and the conventional fluorometric method is that the equations used for the determination of chlor *a* without pheo *a* correction (uncorrected chlor *a*), are used instead of the equations for "corrected chlor *a*". This EPA laboratory has evaluated the modified filters and found the technique to be an acceptable alternative to the conventional fluorometric method using pheo *a* correction.[3]

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8

1.2 Instrumental detection limits (IDL) of 0.05 µg chl *a*/L and 0.06 µg pheo *a*/L in a solution of 90% acetone were determined by this laboratory. Method detection limits (MDL) using mixed assemblages of algae provide little information because the fluorescence of other pigments interferes in the fluorescence of chlorophyll *a* and pheophytin *a*.[4] A single lab estimated detection limit for chlorophyll *a* was determined to be 0.11 µg/L in 10 mL of final extraction solution. The upper limit of the linear dynamic range for the instrumentation used in this method evaluation was 250 µg chl *a*/L.

1.3 This method was multilaboratory validated in 1996.[5] Results from that study may be found in Section 13. Additional QC procedures also have been added as a result of that study.

1.4 This method uses 90% acetone as the extraction solvent because of its efficiency for most types of algae. There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[5-8] or dimethyl sulfoxide.[9] Bowles, et al.[8] found that for chlorophyll *a*, however, 90% acetone was an effective extractant when the extraction period was optimized for the dominant species present in the sample.

1.5 Depending on the type of algae under investigation, this method can have uncorrectable interferences (Sect. 4.0). In cases where taxomonic classification is unavailable, a spectrophotometric or high performance liquid chromatographic (HPLC) method may provide more accurate data for chlorophyll *a* and pheophytin *a*.

1.6 This method is for use by analysts experienced in the handling of photosynthetic pigments and in the operation of fluorescence detectors or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water are concentrated by filtering at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder and allowed to steep for a minimum of 2 h, but not to exceed 24 h, to ensure thorough extraction of the chlorophyll *a*. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is transferred to a glass cuvette and fluorescence is measured before and after acidification to 0.003 N HCl with 0.1 N HCl. Sensitivity calibration factors, which have been previously determined on solutions of

pure chlorophyll *a* of known concentration, are used to calculate the concentration of chlorophyll *a* and pheophytin *a* in the sample extract. The concentration in the natural water sample is reported in µg/L.

3.0 Definitions

3.1 ***Estimated Detection Limit*** (EDL) -- The minimum concentration of an analyte that yields a fluorescence 3X the fluorescence of blank filters which have been extracted according to this method.

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

3.3 ***Instrument Detection Limit*** (IDL) -- The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc. For this method the background is a solution of 90% acetone.

3.4 ***Stock Standard Solution*** (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

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

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

3.7 ***Response Factor*** (RF) -- The ratio of the response of the instrument to a known amount of analyte.

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

3.9 ***Field Duplicates*** (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.10 ***Quality Control Sample*** (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. Ideally, 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.

3.11 ***Material Safety Data Sheet*** (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

4.0 Interferences

4.1 Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere in the accurate measurement of both chlorophyll *a* and pheophytin *a*.

4.2 The relative amounts of chlorophyll *a*, *b* and *c* vary with the taxonomic composition of the phytoplankton. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. Due to the spectral overlap of chlorophyll *b* with pheophytin *a* and chlorophyll *a*, underestimation of chlorophyll *a* occurs accompanied by overestimation of pheophytin *a* when chlorophyll *b* is present in the sample. The degree of interference depends upon the ratio of *a*:*b*. This laboratory found that at a ratio of 5:1, using the acidification procedure to correct for pheophytin *a*, chlorophyll *a* was underestimated by approximately 5%. Loftis and Carpenter[10] reported an underestimation of 16% when the *a*:*b* ratio was 2.5:1. A ratio of 1:1 is the highest ratio likely to occur in nature. They also reported overestimation of chlorophyll *a* in the presence of chlorophyll *c* of as much as 10% when the *a*:*c* ratio was 1:1 (the theoretical maximum likely to occur in nature). The presence of chlorophyll *c* also causes the under-

estimation of pheophytin *a*. The effect of chlorophyll *c* is not as severe as the effect of chlorophyll *b* on the measurement of chlorophyll *a* and pheophytin *a*. Knowledge of the taxonomy of the algae under consideration will aid in determining if the spectrophotometric method using trichromatic equations to determine chlorophyll *a*, *b*, and *c* or an HPLC method would be more appropriate.[11-16] In the presence of chlorophyll *b* or pheopigments, the modified fluorometric method described here is also appropriate.[5]

4.3 Quenching effects are observed in highly concentrated solutions or in the presence of high concentrations of other chlorophylls or carotenoids. Minimum sensitivity settings on the fluorometer should be avoided; samples should be diluted instead.

4.4 Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, LRBs and QCSs must be at the same temperature to prevent errors and/or low precision. Analyses of samples at ambient temperature is recommended in this method. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.

4.5 Samples must be clarified by centrifugation prior to analysis.

4.6 All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials and filter samples must be stored in the dark at -20°C or -70°C to prevent degradation.

5.0 Safety

5.1 The toxicity or carcinogenicity of the chemicals used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[17-20] A file of MSDS should also be made available to all personnel involved in the chemical analysis.

5.2 The grinding of filters during the extraction step of this method should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

6.0 Apparatus and Equipment

6.1 Fluorometer -- Equipped with a high intensity F4T.5 blue lamp, red-sensitive photomultiplier, and filters for excitation (CS-5-60) and emission (CS-2-64). A Turner Designs Model 10 Series fluorometer was used in the evaluation of this method. The modified method requires excitation filter (436FS10) and emission filter (680FS10).

6.2 Centrifuge, capable of 675 g.

6.3 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity glass grinding tube.

6.4 Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.5 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.6 Aluminum foil.

6.7 Laboratory tissues.

6.8 Tweezers or flat-tipped forceps.

6.9 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg.

6.10 Room thermometer.

6.11 Labware -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.11.1 Assorted Class A calibrated pipets.

6.11.2 Graduated cylinders, 500-mL and 1-L.

6.11.3 Volumetric flasks, Class A calibrated, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.11.4 Glass rods.

6.11.5 Pasteur type pipets or medicine droppers.

6.11.6 Disposable glass cuvettes for the fluorometer.

6.11.7 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.11.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.11.9 Polyethylene squirt bottles.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Hydrochloric acid (HCl), concentrated (sp. gr. 1.19), (CASRN 7647-01-0).

7.3 Chlorophyll *a* free of chlorophyll *b*. May be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO). Turner Designs (Sunnyvale, CA) supplies ready-made standards.

7.4 **Water** -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.5 0.1 N HCl Solution -- Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 L.

7.6 Aqueous Acetone Solution -- 90% acetone /10% water. Carefully measure 100 mL of water into the 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store.

7.7 Chlorophyll Stock Standard Solution (SSS) -- Chlorophyll *a* from a commercial supplier will be shipped in an amber glass ampoule which has been flame sealed. This dry standard should be stored at -20 or -70°C in the dark and the SSS prepared just prior to use. Tap the ampoule until all the dried chlorophyll is in the bottom of the ampoule. In subdued light, carefully break the tip off the ampoule. Transfer the entire contents of the ampoule into a 50-mL volumetric flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. The concentration of the solution must be determined spectrophotometrically using a multiwavelength spectrophotometer.[10] When stored in a light and airtight container at freezer temperatures, the SSS is stable for at least six months. The concentration of all dilutions of the SSS must be determined spectrophotometrically each time they are made.

7.8 Laboratory Reagent Blank (LRB) -- A blank filter which is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.9 Chlorophyll a Primary Dilution Standard Solution (PDS) -- Add 1 mL of the SSS (Sect. 7.8) to a clean 100-mL flask and dilute to volume with the aqueous acetone solution (Sect. 7.7). If exactly 1 mg of pure chlorophyll *a* was used to prepare the SSS, the concentration of the PDS is 200 µg/L. Prepare fresh just prior to use.

7.10 Quality Control Sample (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 Water Sample Collection -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (depth at which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal poulations, thus chlorophyll *a* concentration, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher

filtration pressures and excessively long filtration times (> 10 min) may damage cells and result in loss of chorophyll.

Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates (stir or invert several times). Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). A sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, then wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20 or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20 or -70°C as soon as possible.

8.2 ***Preservation*** -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 ***Holding Time*** -- Filters can be stored frozen at -20 or -70°C for as long as 3½ weeks without significant loss of chlorophyll *a.*[21]

9.0 Quality Control

9.1 Each Laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field duplicates and quality control samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data thus generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (instrumental detection limits, linear dynamic range and MDLs) and laboratory performance (analyses of QCSs) prior to sample analyses.

9.2.2 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of 5 calibration standards ranging in concentration from 0.2 µg/L to 200 µg chl *a*/L across all sensitivity settings of the fluorometer. If using an analog fluorometer or a digital fluorometer requiring manual changes in sensitivity settings, normalize responses by dividing the response by the sensitivity setting multiplier. 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 fluorescence response, *R*, of a standard no longer yields a calculated concentration, C_c, that is ± 10% of the known concentration, *C*, where $C_c = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument. Should samples be encountered that have a concentration which is 90% of the upper limit of the LDR, these samples must be diluted and reanalyzed.

9.2.3 Instrumental Detection Limit (IDL) -- Zero the fluorometer with a solution of 90% acetone on the maximum sensitivity setting. Pure chlorophyll *a* in 90% acetone should be serially diluted until it is no longer detected by the fluorometer on a maximum sensitivity setting.

9.2.4 Estimated Detection Limit (EDL) -- Several blank filters should be extracted according to the procedure in Sect. 11, using clean glassware and apparatus, and the fluorescence measured. A solution of pure chlorophyll *a* in 90% acetone should be serially diluted until it yields a response which is 3X the average response of the blank filters.

9.2.5 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify the calibration standards and acceptable instrument performance with the analysis of a QCS (Sect. 7.10). If the determined value is not within the confidence limits established by project data quality objectives, then the determinative step of this method is unacceptable. The source of the problem must be identified and corrected before continuing analyses.

9.2.6 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1).

Twenty to thirty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 or more samples should be extracted and analyzed according to Sect. 11.2. The percent relative standard deviation (%RSD) of uncorrected values of chlorophyll *a* should not exceed 15% for samples that are approximately 10X the IDL. RSD for pheophytin *a* might typically range from 10 to 50%.

9.2.7 Corrected Chl *a* -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining corrected chl *a*. The problem manifests itself by highly erratic pheo-*a* results, high %RSDs for corrected chl *a* and poor agreement between corrected and uncorrected chl *a*. To determine if a new analyst is performing the acidification step properly, perform the following QC procedure:

Prepare 100 mL of a 50 ppb chl *a* solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using separate cuvettes, according to instructions in Section 11.2. Process the results according to Section 12 and calculate separate means and %RSDs for corrected and uncorrected chl *a*. If the means differ by more than 10%, then the stock chl *a* has probably degraded and fresh stock should be prepared. The %RSD for corrected chl *a* should not exceed 5%. If the %RSD exceeds 5%, repeat the procedure until the %RSD 5%.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one blank filter with each sample batch. The LRB should be the last filter extracted. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the IDL indicate contamination from the laboratory environment. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

10.0 Calibration and Standardization

10.1 Calibration -- Calibration should be performed bimonthly or when there has been an adjustment made to the instrument, such as replacement of lamp, filters or photomultiplier. Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (Sect. 7.11). Allow the instrument to warm up for at least 15 min. Measure the fluorescence of each standard at sensitivity settings that provide midscale readings. Obtain response factors for chlorophyll *a* for each sensitivity setting as follows:

$$F_s = C_a/R_s$$

where:

F_s = response factor for sensitivity setting, S.

R_s = fluorometer reading for sensitivity setting, S.

C_a = concentration of chlorophyll *a*.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify. Use the "uncorrected" chl *a* calculation described in Section 12.1.

If pheophytin *a* determinations will be made, it will be necessary to obtain before-to-after acidification response ratios of the chlorophyll *a* calibration standards as follows: (1) measure the fluorescence of the standard, (2) remove the cuvette from the fluorometer, (3) acidify the solution to .003 N HCl[6] with the 0.1 N HCl solution, (4) use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample, (5) wait 90 sec and measure the fluorescence of the standard solution again. Addition of the acid may be made using a medicine dropper. It will be necessary to know how many drops are equal to 1 mL of acid. For a cuvette that holds 5 mL of extraction solution, it will be necessary to add 0.15 mL of 0.1 N HCl to reach a final acid concentration of 0.003N in the 5 mL. Calculate the ratio, r, as follows:

$$r = R_b/R_a$$

where:

R_b = fluorescence of pure chlorophyll *a* standard solution before acidification.

R_a = fluorescence of pure chlorophyll *a* standard solution after acidification.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 If sampled filters have been frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand tissues and squirt bottles containing water and acetone. Workspace lighting should be the minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push it to the bottom of the tube with a glass rod. With a volumetric pipet, add 4 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has been converted to a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipet, rinse the pestle and the grinding tube with 90% acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The entire extraction with transferring and rinsing steps takes 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.2 Shake each tube vigorously before placing them to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. The tubes should be shaken at least once during the steeping period.

11.1.3 After steeping is complete, shake the tubes vigorously and centrifuge samples for 15 min at 675 g or for 5 min at 1000 g. Samples should be allowed to come to ambient temperature before analysis. This can be done by placing the tubes in a constant temperature water bath or by letting them stand at room temperature for 30 min. Recalibrate the fluorometer if the room temperature fluctuated ± 3°C from the last calibration date.

11.2 SAMPLE ANALYSIS

11.2.1 After the fluorometer has warmed up for at least 15 min, use the 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.

11.2.2 Pour or pipet the supernatant of the extracted sample into a sample cuvette. The volume of sample required in your instrument's cuvette should be known so that the correct amount of acid can be added in the pheophytin *a* determinative step. For a cuvette that holds 5 mL of extraction solution, 0.15 mL of the 0.1 N HCl solution should be used. Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting. If the concentration of chlorophyll *a* in the sample is ≥ 90% of the upper limit of the LDR, then dilute the sample with the 90% acetone solution and reanalyze. Record the fluorescence measurement and sensitivity setting used for the sample. Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 N HCl using the 0.1 N HCl solution. Use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample. Wait 90 sec before measuring fluorescence again. NOTE: Proper mixing is critical for precise and accurate results. Twenty-five to thirty-five samples can be extracted and analyzed in one 8 hr day.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify samples. Use the "uncorrected" chl *a* calculations described in Section 12.1.

12.0 Data Analysis and Calculations

12.1 For "uncorrected chlorophyll *a*," calculate the chlorophyll *a* concentration in the extract as:

$$C_{E,u} = R_b \times F_s$$

where $C_{E,u}$ = uncorrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

R_b = fluoresence response of sample extract before acidification, and

F_s = fluoresence respnse factor for sensitivity setting S.

Calculate the "uncorrected" concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{S,u} = \frac{C_{E,u} \; x \; extract\ volume\ (L) \; X \; DF}{sample\ volume\ (L)}$$

where $C_{S,u}$ = uncorrected chlorophyll *a* concentration (µg/L) in the whole water sample,

extract volume = volume (L) of extraction prepared before any dilutions,

DF = dilution factor,

sample volume = volume (L) of whole water sample.

12.2 For "corrected chlorophyll *a*", calculate the chlorophyll *a* concentration in the extract as :

$C_{E,c} = F_s\ (r/r\text{-}1)\ (R_b - R_a)$

where:

$C_{E,c}$ = corrected chlorophyll *a* concentration (µg/L) in the extract solution analyzed,

F_s = response factor for the sensitivity setting S,

r = the before-to-after acidification ratio of a pure chlorophyll *a* solution (Sect. 10.1),

R_b = fluorescence of sample extract before acidification, and

R_a = fluorescence of sample extract after acidification.

Calculate the "corrected" concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{s,c} = \frac{C_{E,u} \; x \; extract\ volume\ (L) \; X \; DF}{sample\ volume\ (L)}$$

where $C_{S,c}$ = corrected chlorophyll *a* concetration (µg/L) in the whole water sample,

extract volume = volume (L) of extract prepared before dilution,

12.3 Calculate the pheophytin *a* concentration as follows:

$$P_E = F_s\ (r/r\text{-}1)\ (rR_a - R_b)$$

$$P_s = \frac{P_E \; X \; extract\ volume\ (L) \; X \; DF}{sample\ \ volume\ (L)}$$

where P_E = pheophytin *a* concentration (µg/L) in the sample extract; and

P_s = pheophytin *a* concentration (µg/L) in the whole water sample.

12.4 LRB and QCS data should be reported with each sample data set.

13.0 Method Performance

13.1 The single lab EDL for the instrument used in the evaluation of this method was 0.05 µg/L for chlorophyll *a* and 0.06 µg/L pheophytin *a*.

13.2 The precision (%RSD) for chlorophyll *a* in mostly blue-green and green phytoplankton natural samples which were steeped for 2 h vs 24 h is reported in Table 1. Although the means were the same, precision was better for samples which were allowed to steep for 24 h prior to analysis. Since pheophytin *a* was found in the samples, the chlorophyll *a* values are "corrected" (Sect. 12.2). Table 2 contains precision data for pheophytin *a*. A statistical analysis of the pheophytin *a* data indicated a significant difference in the mean values at the 0.05 significance level. The cause of the lower pheophytin *a* values in samples extracted for 24 h is not known.

13.3 Three QCS ampoules obtained from the USEPA were analyzed and compared to the reported confidence limits in Table 3. **NOTE:** The USEPA no longer provides these QCSs.

13.4 **Multilaboratory Testing** - A multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 21 volunteer participants in the fluorometric methods

component that returned data; 10 that used the modified fluorometric method and 11 that used the conventional method. The primary goals of the study were to determine estimated detection limits and to assess precision (%RSD) and bias (as percent recovery) for select unialgal species, and natural seawater.

13.4.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). An EPA MDL determination is not possible nor practical for a natural water or pure species sample due to known spectral interferences and to the fact that it is impossible to prepare solutions of known concentrations that incorporate all sources of error (sample collection, filtration, processing). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et.al.[22] method that does not assume constant error variances across concentration and controls for Type II error. The statistical approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 4. It is referred to as pooled-EDL (p-EDL).

Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 4. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs. Due to the large dilutions required to analyze these solutions, the fluorometric p-EDLs are unrealistically high compared to what is achievable by a single lab. Typical single lab EDLs can easily be 1000 fold lower than the p-EDL reported in Table 4.

13.4.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different "concentrations" of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The "true" concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination. Pooled precision (as determined by %RSD) data are presented in Tables 5-7 and accuracy data (as percent recovery) are presented in Table 8. No significant differences in precision were observed across concentrations for any of the species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 8) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The fluorometric and the spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 9. Only one filtration volume (100 mL) was provided in duplicate to participant labs.

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 EPA 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 (e.g., Sect. 11.1.1). 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's 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 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 the Sect. 14.2.

16.0 References

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17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. COMPARISON OF PRECISION OF TWO EXTRACTION PERIODS

CORRECTED CHLOROPHYLL *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	49.6	52.9	78.6	78.8
Standard Deviation (µg/L)	4.89	2.64	6.21	2.77
Relative Standard Deviation (%)	9.9	5.0	7.9	3.5

1 Values reported are the mean measured concentrations (n=6) of chlorophyll *a* in the natural water based on a 100-mL filtration volume.

2 Values reported are the mean measured concentrations (n=9) of the extraction solution. Sample filtration volume was 300 mL.

3 The length of time that the filters steeped after they were macerated.

TABLE 2. COMPARISON OF PRECISION OF TWO EXTRACTIONS PERIODS FOR Pheophytin *a*

Pheophytin *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	9.22	8.19	13.1	10.61
Standard Deviation (µg/L)	2.36	3.55	3.86	2.29
Relative Standard Deviation (%)	25.6	43.2	29.5	21.6

1 Values reported are the mean measured concentrations (n=6) of pheophytin *a* in the natural water based on a 100-mL filtration volume.

2 Values reported are the mean measured concentrations (n=9) of pheophytin *a* the extraction solution. Sample filtration volume was 300 mL.

3 The length of time that the filters steeped after they were macerated.

TABLE 3. ANALYSES OF USEPA QC SAMPLES

ANALYTE	REFERENCE VALUE	CONFIDENCE LIMITS
Chlorophyll *a*	2.1 µg/L	0.5 to 3.7 µg/L
Pheophytin *a*	0.3 µg/L	-0.2 to 0.8 µg/L

ANALYTE	MEAN MEASURED VALUE	% Relative Standard Deviation[1]
Chlorophyll *a*	2.8 µg/L	1.5
Pheophytin *a*	0.3 µg/L	33

[1] N = 3

TABLE 4. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std[5]	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.4.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 5. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.163	0.037	22.8
	10	7	0.298	0.080	26.7
	50	7	1.684	0.385	22.9
	100	7	3.311	0.656	19.8
Fl-Std	5	8	0.185	0.056	30.4
	10	8	0.341	0.083	24.4
	50	8	1.560	0.311	19.9
	100	8	3.171	0.662	20.9

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 6. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.066	0.010	14.6
	10	7	0.142	0.045	31.5
	50	7	0.757	0.208	27.5
	100	7	1.381	0.347	25.1
Fl-Std	5	8	0.076	0.018	23.2
	10	8	0.165	0.040	24.3
	50	8	0.796	0.140	17.5
	100	8	1.508	0.324	21.5

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 7. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.221	0.040	18.0
	10	7	0.462	0.094	20.3
	50	7	2.108	0.491	23.3
	100	7	3.568	1.186	33.2
Fl-Std	5	8	0.214	0.053	24.8
	10	8	0.493	0.091	18.4
	50	8	2.251	0.635	28.2
	100	8	4.173	0.929	22.3

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

NOTE: The phaeodactylum extract contained significant amounts of chlorophyll *c* and chlorophyllide *a* which interferes in chlorophyll *a* measurement in the fluorometric method, therefore, the concentration of chlorophyll *a* is overestimated compared to the HPLC method which separates the three pigments. The FL-Mod interference filters minimize this interference more so than the conventional filters.

TABLE 8. MINIMUM, MEDIAN, AND MAXIMUM PERCENT RECOVERIES BY GENERA, METHOD, AND CONCENTRATION LEVEL

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80

Table 8 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-M	240	247	247	243
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318
		FL-STD	371	415	415	334
		HPLC	394	289	182	139
		SP-M	446	344	330	328
		SP-T	357	316	318	299

TABLE 9. CHLOROPHYLL *A* CONCENTRATIONS IN MG/L DETERMINED IN FILTERED SEAWATER SAMPLES

Method	Con.(1)	No. Obs.	No. Labs	Mean	Std. Dev.	RSD(%)	Minimum	Median	Maxium
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

Appendix G
Laboratory Method – Silicate – US EPA 366.0

Method 366.0

Determination of Dissolved Silicate in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

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

Method 366.0

Determination of Dissolved Silicate in Estuarine and Coastal Waters by Gas Segmented Continuous Flow Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of dissolved silicate concentration in estuarine and coastal waters. The dissolved silicate is mainly in the form of silicic acid, H_4SiO_4, in estuarine and coastal waters. All soluble silicate, including colloidal silicic acid, can be determined by this method. Long chain polymers containing three or more silicic acid units do not react at any appreciable rate[1], but no significant amount of these large polymers exists in estuarine and coastal waters.[2,3] This method is based upon the method of Koroleff,[4] adapted to automated gas segmented continuous flow analysis.[5-7]

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Silicate	12627-13-3

1.2 A statistically determined method detection limit (MDL) of 0.0012 mg Si/L has been determined by one laboratory in seawaters of three different salinities.[8] The method is linear to 6.0 mg Si/L using a Flow Solution System (Perstorp Analytical Inc., Silver Spring, MD).

1.3 Approximately 60 samples per hour can be analyzed.

1.4 This method should be used by analysts experienced in the use of automated gas segmented continuous flow colorimetric analyses, and familiar with matrix interferences and procedures for their correction. A minimum of 6-months experience under supervision is recommended.

2.0 Summary of Method

2.1 An automated gas segmented continuous flow colorimetric method for the analysis of dissolved silicate concentration is described. In the method, β-molybdosilicic acid is formed by reaction of the silicate contained in the sample with molybdate in acidic solution. The β-molybdosilicic acid is then reduced by ascorbic acid to form molybdenum blue. The absorbance of the molybdenum blue, measured at 660 nm, is linearly proportional to the concentration of silicate in the sample. A small positive error caused by differences in the refractive index of seawater and reagent water, and negative error caused by the effect of salt on the color formation, are corrected prior to data reporting.

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 Dissolved Analyte (DA) -- The concentration of analyte in an aqueous sample that will pass through a 0.45 μm membrane filter assembly prior to sample acidification or other processing.

3.3 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 basically a standard prepared in reagent water that is analyzed as a sample.

3.4 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.

3.5 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.

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

3.7 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]

3.8 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.

3.9 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 refractive index correction in this method.

3.10 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.

3.11 Quality Control Sample (QCS) - A solution of method analyte 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 most 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 Interferences caused by hydrogen sulfide, such as occur in samples taken from deep anoxic basins can be eliminated by oxidation with bromine or stripping with nitrogen gas after acidification. Interferences of phosphate at concentrations larger than 0.15 mg P/L is eliminated by the use of oxalic acid in the color development step of this method. Interferences of fluoride at concentrations greater than 50 mg F/L can be reduced by complexing the fluoride with boric acid.[4]

4.2 Glassware made of borosilicate glass should be avoided for use in silicate analysis. Plastic labware such as polyethylene volumetric flasks and plastic sample vials, should be used.

4.3 Sample turbidity and particles are removed by filtration through a 0.45 µm non-glass membrane filters after sample collection.

4.4 This method corrects for refractive index and salt error interferences which occur if sampler wash solution and calibration standards are not matched with samples in salinity.

4.5 Frozen samples should be filled about 3/4 full in the sample bottles. The expansion of water on freezing will squeeze some of the brine out of the bottle if the bottle was overfilled. The overfill of the sample bottle during freezing will drastically alter the nutrient concentrations in the sample that remains.

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. A lab coat, safety goggles, and gloves should be worn when preparing the sulfuric acid reagent.

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 silicate analysis.

6.1.3 Proportioning pump.

6.1.4 Monochromator or spectrophotometer equipped with a tungsten lamp (380-800 nm) and a low refractive index flowcell.

6.1.5 Strip chart recorder or computer based data acquisition system.

6.2 Glassware and Supplies

6.2.1 All labware used in the analysis must be low in residual silicate 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 was found to be effective.

6.2.2 Glassware made of borosilicate glass should be avoided for storage of solutions for silicate analysis. Plastic containers are preferable for silicate analysis.

6.2.3 Non-glass membrane filters with 0.45 µm nominal pore size. Plastic syringes with syringe filters, pipets, 60 mL polyethylene bottles, and polyethylene volumetric flasks, plastic sample vials.

6.2.4 Drying oven, desiccator and analytical balance.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Sulfuric Acid Solution (0.05 M) - Cautiously add 2.8 mL of concentrated Analytical Reagent Grade sulfuric acid (H_2SO_4) to approximately 800 mL of reagent water, mix then bring up to 1 L with reagent water.

7.1.2 Ammonium Molybdate Solution (10 g/L) - Dissolve 10 g of ammonium molybdate (VI) tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) in approximately 800 mL of 0.05 M sulfuric acid solution and dilute to 1000 mL with 0.05 M sulfuric acid solution. Store in an amber plastic bottle. This solution is stable for one month. Inspect the solution before use. If a white precipitation forms on the wall of container, discard the solution and make a fresh one.

7.1.3 Stock Silicate Solution (100 mg Si/L) - Quantitatively transfer 0.6696 g of pre-dried (105°C for 2 hours) sodium hexafluorosilicate (Na_2SiF_6) to a 1000 mL polypropylene flask containing approximate 800 mL of reagent water, cover with plastic film and dissolve on a stir plate using a Teflon-coated stirring bar. Complete dissolution usually takes 2-24 hours. Dilute the solution to 1000 mL in polyethylene volumetric flask with reagent water. Store the stock solution in a plastic bottle. This solution is stable for one year if care is taken to prevent contamination and evaporation.

7.1.4 Low Nutrient Sea Water (LNSW) - Obtain natural low nutrient seawater from surface seawater in the Gulf Stream or Sargasso Sea (salinity 36 ‰, < 0.03 mg Si/L) and filter through 0.45 µm pore size non-glass membrane filters. In addition, commercially available low nutrient sea water (< 0.03 mg Si/L) with salinity of 35 ‰ (Ocean Scientific International, Wormley, U.K.) can be used.

7.2 Working Reagents

7.2.1 Dowfax Start-up Solution - Add 2 mL of Dowfax 2A1 surfactant (Dow Chemical Company) to 1000 mL reagent water and mix gently.

Note: Dowfax 2A1 contains (w/w) 47% benzene, 1,1-oxybis, tetrapropylene derivatives, sulfonate, sodium salt, 1% sodium sulfate, 3% sodium chloride and 49% water.

7.2.2 Working Molybdate Reagent - Add 0.5 mL Dowfax 2A1 to 250 mL of ammonium molybdate solution, mix gently. Prepare this solution daily. This volume of solution is sufficient for an 8-hour run.

7.2.3 Ascorbic Acid Solution - Dissolve 4.4 g of ascorbic acid ($C_6H_8O_6$) in 200 mL of reagent water and 12.5 mL of acetone(C_3H_6O), dilute to 250 mL with reagent water. Store in a plastic container. This solution is stable for one week if stored at 4°C. Discard the solution if it turns brown.

7.2.4 Oxalic Acid Solution - Dissolve 50 g of oxalic acid ($C_2H_2O_4$) in approximately 800 mL of reagent water and dilute to 1000 mL with reagent water. Store in a plastic container. This solution is stable for approximately 3-months.

7.2.5 Refractive Index Matrix Solution - Add 0.5 mL Dowfax 2A1 to 250 mL of 0.05 M sulfuric acid solution and mix gently.

7.2.6 Colored SYNC Peak Solution - Add 50 µL of blue food coloring solution to 1000 mL reagent water and mix thoroughly. The solution should give a peak of between 25 to 100 percent full scale, otherwise the volume of food coloring added needs to be adjusted.

7.2.7 Calibration Standards - Prepare a series of calibration standards (CAL) by diluting suitable volumes of Stock Silicate Solution (Section 7.1.3) 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.

When working with samples of a narrow range of salinities (± 2 ‰), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and the Sampler Wash Solution also be Low Nutrient Seawater (Section 7.1.4) diluted to that salinity. If this procedure is performed, it is not necessary to perform the salt error and refractive index corrections outlined in Sections 12.2 and 12.3.

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 salt error and refractive index be made to the sample concentrations (Section 12.2 and 12.3).

7.2.8 Saline Silicate Standards - If CAL solutions will not be prepared to match sample salinity, then saline silicate standards must be prepared in a series of salinities in order to quantify the salt error, the change in the colorimetric response of silicate due to the change in the ionic strength of the solution. The following dilutions prepared in 100 mL volumetric flasks, diluted to volume with reagent water, are suggested.

Salinity (‰)	Volume of LNSW(mL)	Volume(mL) Si stock std	Conc. mg Si/L
0	0	1.5	1.5
9	25	1.5	1.5
18	50	1.5	1.5
27	75	1.5	1.5
35	98	1.5	1.5

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 convenient samplers. Wash the sampler three times with sample water before collecting samples.

8.1.4 Samples must be filtered through a 0.45 µm non-glass membrane filters 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. The expansion of water on freezing will squeeze some of the brine out of the bottle if the bottle was overfilled.

8.2 Sample Preservation - After collection and filtration, samples should be analyzed as soon as possible. If samples will be analyzed within 24 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 - If samples are to be frozen for long-term storage ensure that none of the sample bottles are filled more than 3/4 full and the cap is firmly screwed on. Place the bottles upright on a rack and store in the freezer (-20°C).

Before analysis, frozen samples must be taken out of the freezer and allowed to thaw in a refrigerator at 4°C in the dark. Thawing times depend upon sample salinities. It was found that the frozen low salinity estuarine water took 4 days to thaw. After completely thawing, take samples out of the refrigerator and mix thoroughly. Keep samples in the dark at room temperature overnight before analysis.

Effects of thawing conditions on the recoveries of frozen samples are more pronounced in low salinity estuarine

waters than high salinity coastal waters as shown in following results:

Day	Recovery (%)			Remark
	S=35.85	S=18.07	S=2.86	
0	100.00	100.00	100.00	
7	102.44	102.65	89.37	a
14	98.59	101.06	86.49	a
21	99.51	99.30	83.49	a
27	98.86			a
		98.86	91.43	b
35	98.70			b
		98.66	92.98	b
42	100.87			b
49		102.44	79.12	c
		103.92	79.10	d
		99.92	89.68	e
56	103.47			c
	104.12			d
	99.35			e
84		100.80	91.71	f
		99.90	93.81	g
91	100.65			f
	100.22			g

S = Salinity
a, overnight thawing at room temperature
b, 20 hours thawing at room temperature
c, 24 hours thawing at room temperature
d, 8 hours thawing at room temperature then heating at 80°C for 16 hours
e, 24 hours thawing at room temperature in the dark
f, 4 days thawing at room temperature in the dark
g, 4 days thawing at 4°C in a refrigerator in the dark

To ensure a slow process of depolymerization of polysilicate to occur, thawing the frozen samples in the dark at 4°C for 4 days is critical condition for obtaining high recoveries of silicate in frozen samples. A maximum holding time for frozen estuarine and coastal waters is two months.[9-11]

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 consists 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 Method Detection Limits (MDLs) should be established 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 6-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 0.03 to 5.00 mg Si/L across all sensitivity settings (Absorbance Units Full Scale) 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 the 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 Si/L)

C = sample background concentration (mg Si/L)

S = concentration in mg Si/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 matrix related and the sample data should be flagged.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared daily for system calibration.

10.2 A calibration curve should be constructed for each run by analyzing a set of calibration standard solutions. A run should contain no more than 60 samples.

It is suggested that a large set of samples be analyzed in several sets with individual calibration curves.

10.3 Place the calibration standards before samples for each run. All the calibration solutions should be analyzed in duplicate.

10.4 The calibration curve containing five data points or more should have a correlation coefficient $\geq$ 0.995.

10.5 Place a high standard solution cup and follow 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 standard cup. The carry-over coefficient, k, is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}} X\ 100$$

where, P_{high} = the peak height of the high silicate 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 in order to obtain a statistically significant number. The k should be remeasured when a change in the plumbing of the manifold or replacement of pump tube occur.

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

$$CO = kxP_{i-1}$$

To correct a given peak height reading, P_i, one subtracts the carry over correction.[12,13]

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

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 should be less than 2%.

10.6 Place a high standard solution at the end of a run to check sensitivity drift. The sensitivity drift should be ± 5% during the run.

11.0 Procedure

11.1 If samples are frozen, thaw the sample at 4°C in the dark as outlined in Section 8.3. Mix samples thoroughly prior to analyses.

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

11.3 Set up the cartridge and pump tubes as shown in Figure 1.

Note: Fluctuation of ambient temperature can cause erratic results due to the effect of temperature on kinetics of color development. The laboratory temperature should be maintained as close to a constant temperature as possible. The cartridge should be away from the direct path of air flow from a heater or air conditioner. In cases such as on a ship where the fluctuation of temperature can be extreme, the temperature effect can be minimized by increasing the length of mixing coil 1 (Figure 1) to bring the formation of silicomolybdic acid reaction to completion.

11.4 Set the wavelength at 660 nm on the spectrometer/monochrometer.

Note: The absorption spectra of silicomolybdeum blue complex has two maxima at 820 nm and 660 nm with 820 nm higher than 660 nm. This method measures absorbance at 660 nm because the detector works in the range of 380 to 800 nm. The sensitivity of the method is satisfactory at 660 nm. The sensitivity, however, can be improved by using 820 nm if this wavelength is available on the detector.

11.5 On the monochromator, set the Absorbance Unit Full Scale at an appropriate setting according to the highest concentration of silicate in the samples. The highest setting used in this method was 0.2 for 6 mg Si/L.

11.6 Prepare all reagents and standards.

11.7 Begin pumping the Dowfax 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 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, 1N 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 a syringe for a few minutes or, if not, dislodged by pumping the syringe pistion. Alternatively, flushing the flowcell with alcohol was found to be effective in removing air bubbles from the flowcell.

For analysis of samples with a narrow range of salinities (± 2 ‰), it is recommended that the wash water in the sampler be prepared in Low Nutrient Seawater diluted to the salinity of samples in place of reagent water. For samples with varying salinities, it is suggested that reagent waters and procedures in Sections 12.2 and 12.3 be employed.

11.8 A good sampling rate is approximately 60 samples per hour with 40 seconds of sample time and 20 seconds of wash time.

11.9 Use 10% HCl followed by reagent water to rinse sample cups. 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 every ten samples and between samples of high and low concentrations.

11.10 Commence analysis.

11.11 If the reagent water is used as wash solution instead of Low Nutrient Seawater and an operator wants to quantify the refractive index correction due to the difference in salinities between sample and wash solution, the following procedures are used. Replace ammonium molybdate solution (Section 7.1.2) with refractive index matrix solution (Section 7.2.5). All other reagents remain the same. Replace the synchronization cup with the colored SYNC peak solution (Section 7.2.6). Commence analysis and obtain a second set of peak heights for all standards and samples. The peak heights obtained from these measurements must be subtracted from the peak heights of samples analyzed with color developing reagent pumping through the system. If a low refractive index flowcell is used, the correction for refractive index is negligible. This procedure is optional.

12.0 Data Analysis and Calculations

12.1 Concentrations of silicate 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 (optional)

12.2.1 Obtain a second set of peak heights for all standards and samples with refractive index matrix solution being pumped through the system in place of color reagent (ammonium molybdate solution). All other reagents remain the same. The peak heights for the refractive index correction must be obtained at the same Absorbance Unit Full Scale range setting and on the same monochromator as the corresponding samples and standards.

12.2.2 Subtract the refractive index peak heights from the peak heights obtained from the silicate determination.

12.2.3 An alternative approach is to measure the relationship between the sample salinity and refractive index on a particular detector.

First analyze a set of silicate standards in reagent water with color reagent and obtain a linear regression from the standard curve.

Prepare a set of different salinity samples with LNSW. Analyze these samples with refractive index matrix solution being pumped through the system in place of color reagent (ammonium molybdate solution). All other reagents remain the same. The peak heights for the refractive index correction must be obtained at the same Absorbance Unit Full Scale setting and on the same monochromator as the corresponding standards.

For each sample, the apparent silicate concentration due to refractive index is then calculated from its peak height obtained with refractive index reagent and the regression of silicate standards obtained with color reagent pumping

through the system. Salinity is entered as the independent variable and the apparent silicate concentration due to refractive index in that detector is entered as the dependent variable. The resulting regression allows the operator to calculate apparent silicate concentration due to refractive index when the salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.4 Refractive index correction can be minimized by using a low refractive index flowcell. An example of typical results using a low refractive index flowcell follows:

Salinity (‰)	Apparent silicate conc. due to refractive index (mg Si/L)
4.5	0.0003
9.0	0.0005
18.0	0.0016
27.0	0.0027
36.0	0.0042

12.2.5 An example of a typical equation is:

$$\text{Apparent silicate (mg Si/L)} = 0.00001953 * S^{1.5}$$

where S is sample salinity. The form of fitted equation might vary as the design of flowcell, so the operators are advised to obtain the appropriate equation which has the best fit of their own data with the least fitting coefficients.

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 wash solution prepared in reagent water, it is usual to first correct for refractive index errors, then correct for the change in color development due to the differences in ionic strength between samples and standards (salt error). The refractive index correction is negligible, so is optional, but correction for salt error is necessary.

12.3.2 Plot the salinity of the saline standards (Section 7.2.8) as the independent variable, and the apparent concentration of silicate (mg Si/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for all 1.50 mg Si/L standards. The resulting regression equation allows the operator to correct the concentrations of samples of known salinity for the color suppression due to salinity effect, e.g., salt error. An example of typical results follows:

Salinty (‰)	Peak height of 1.50 mg Si/L	Uncorrected Si conc.calculated from standards in reagent water
0	2503	1.50
9	2376	1.32
18	2282	1.27
27	2250	1.25
36	2202	1.23

12.3.3 An example of a typical equation to correct for salt error is:

$$\text{Corrected mg Si/L} = \frac{\text{Uncorrected mg Si/L}}{1 - 0.02186 \times \sqrt{S}}$$

where S is salinity.

12.3.4 Results of sample analyses should be reported in mg Si/L or in µg Si/L.

mg Si/L = ppm (parts per million)
µg Si/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 0.0012 mg Si/L has been determined by one laboratory in seawaters of three different salinities.

Salinity (‰)	SD (µg/L)	Recovery (%)	MDL (µg/L)
36	0.3924	105	1.233
36	0.4980	107	1.565
27	0.2649	104	0.832
27	0.3362	104	1.056
27	0.4671	100	1.468
18	0.3441	101	1.081
18	0.2809	105	0.883
18	0.2432	104	0.764
3	0.3441	101	1.081
3	0.2331	102	0.733
3	0.1963	98	0.617
3	0.2809	99	0.883

13.1.2 Single Analyst Precision - A single laboratory analyzed three samples collected from the Miami River and Biscayne Bay areas of Florida. Seven replicates of

each sample were processed and analyzed with salinities ranging from 2.86 to 35.85. The results were as follows:

Sample	Salinity (‰)	Concentration (mg Si/L)	RSD (%)
1	35.85	0.097	1.2
2	18.07	1.725	1.4
3	2.86	3.322	0.9

13.1.3 Laboratory Fortified Sample Matrix - Laboratory fortified sample matrixes were processed in three different salinities ranging from 2.86 to 35.85 and ambient concentrations from 0.095 to 3.322 mg Si/L with three fortified levels at each salinity. Seven replicates of each sample were analyzed and the results were as follows:

Salinity (‰)	Concentration (mg Si/L)		RSD (%)	Recovery (%)
	Ambient	Fortified		
35.85	0.095	0.1647	0.82	99.37
35.85	0.095	0.2196	1.34	100.61
35.85	0.095	0.2747	1.74	99.62
18.07	1.725	0.5517	1.11	107.18
18.07	1.725	1.1008	0.77	104.69
18.07	1.725	1.6508	0.98	103.62
2.86	3.322	0.5421	0.99	101.03
2.86	3.322	1.0801	1.26	103.22
2.86	3.322	1.6188	0.98	100.59

13.2 Multi-Laboratory Validation

Multi-laboratory validation has not been conducted for this method and, therefore, multi-laboratory data is currently unavailable.

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.

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

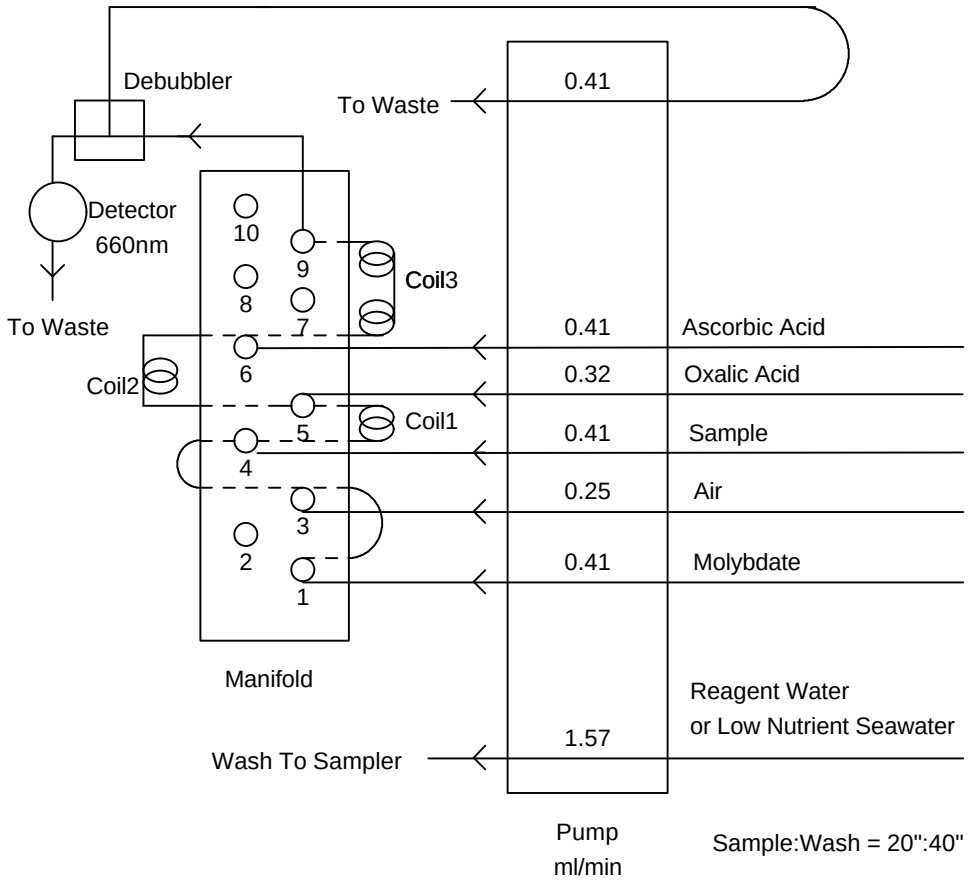

Figure 1. Manifold Configuration for Silicate Analysis.

Appendix H
Laboratory Method – Nitrate - Nitrogen – US EPA 352.1

METHOD #: 352.1 Approved for NPDES and SDWA (Issued 1971)

TITLE: Nitrogen, Nitrate (Colorimetric, Brucine)

ANALYTE: CAS # N Nitrogen 7727-37-9
NO3 Nitrate

INSTRUMENTATION: Spectrophotometer

STORET No. Total 00620

1.0 Scope and Application

1.1 This method is applicable to the analysis of drinking, surface and saline waters, domestic and industrial wastes. Modification can be made to remove or correct for turbidity, color, salinity, or dissolved organic compounds in the sample.

1.2 The applicable range of concentrations is 0.1 to 2 mg NO_3-N/liter.

2.0 Summary of Method

2.1 This method is based upon the reaction of the nitrate ion with brucine sulfate in a 13 N H_2SO_4 solution at a temperature of 100°C. The color of the resulting complex is measured at 410 nm. Temperature control of the color reaction is extremely critical.

3.0 Sample Handling and Preservation

3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with sulfuric acid (2 mL conc. H_2SO_4 per liter) and refrigeration.

4.0 Interferences

4.1 Dissolved organic matter will cause an off color in 13 N H_2SO_4 and must be compensated for by additions of all reagents except the brucine-sulfanilic acid reagent. This also applies to natural color present not due to dissolved organics.

4.2 The effect of salinity is eliminated by addition of sodium chloride to the blanks, standards and samples.

4.3 All strong oxidizing or reducing agents interfere. The presence of oxidizing agents may be determined with a total residual chlorine test kit.

4.4 Residual chlorine interference is eliminated by the addition of sodium arsenite.

4.5 Ferrous and ferric iron and quadrivalent manganese give slight positive interferences, but in concentrations less than 1 mg/L these are negligible.

4.6 Uneven heating of the samples and standards during the reaction time will result in erratic values. The necessity for absolute control of temperature

during the critical color development period cannot be too strongly emphasized.

5.0 Apparatus

5.1 Spectrophotometer or filter photometer suitable for measuring absorbance at 410 nm.
5.2 Sufficient number of 40-50 mL glass sample tubes for reagent blanks, standards and samples.
5.3 Neoprene coated wire racks to hold sample tubes.
5.4 Water bath suitable for use at 100°C. This bath should contain a stirring mechanism so that all tubes are at the same temperature and should be of sufficient capacity to accept the required number of tubes without significant drop in temperature when the tubes are immersed.
5.5 Water bath suitable for use at 10-15°C.

6.0 Reagents

6.1 Distilled water free of nitrite and nitrate is to be used in preparation of all reagents and standards.
6.2 Sodium chloride solution (30%): Dissolve 300 g NaCl in distilled water and dilute to 1 liter.
6.3 Sulfuric acid solution: Carefully add 500 mL conc. H_2SO_4 to 125 ml distilled water. Cool and keep tightly stoppered to prevent absorption of atmospheric moisture.
6.4 Brucine-sulfanilic acid reagent: Dissolve 1 g brucine sulfate $[(C_{23}H_{26}N_2O_4)_2 \bullet H_2SO_4 \bullet 7H_2O]$ and 0.1 g sulfanilic acid ($NH_2C_6H_4SO_3H \bullet H_2O$) in 70 mL hot distilled water. Add 3 mL conc. HCl, cool, mix and dilute to 100 mL with distilled water. Store in a dark bottle at 5 °C. This solution is stable for several months; the pink color that develops slowly does not effect its usefulness. Mark bottle with warning: CAUTION: Brucine Sulfate is toxic; take care to avoid ingestion.
6.5 Potassium nitrate stock solution: 1.0 mL = 0.1 mg NO_3-N. Dissolve 0.7218 g anhydrous potassium nitrate (KNO_3) in distilled water and dilute to 1 liter in a volumetric flask. Preserve with 2 mL chloroform per liter. This solution is stable for at least 6 months.
6.6 Potassium nitrate standard solution: 1.0 mL = 0.001 mg NO_3-N. Dilute 10.0 mL of the stock solution (6.5) to 1 liter in a volumetric flask. This standard solution should be prepared fresh weekly.
6.7 Acetic acid (1 + 3): Dilute 1 volume glacial acetic acid (CH_3COOH) with 3 volumes of distilled water.
6.8 Sodium hydroxide (lN): Dissolve 40 g of NaOH in distilled water. Cool and dilute to 1 liter.

7.0 Procedure

7.1 Adjust the pH of the samples to approximately 7 with acetic acid (6.7) or sodium hydroxide (6.8). If necessary, filter to remove turbidity.
7.2 Set up the required number of sample tubes in the rack to handle reagent blank, standards and samples. Space tubes evenly throughout the rack to allow

for even flow of bath water between the tubes. This should assist in achieving uniform heating of all tubes.

7.3 If it is necessary to correct for color or dissolved organic matter which will cause color on heating, a set of duplicate samples must be run to which all reagents except the brucine-sulfanilic acid have been added.

7.4 Pipette 10.0 mL of standards and samples or an aliquot of the samples diluted to 10.0 mL - into the sample tubes.

7.5 If the samples are saline, add 2 mL of the 30% sodium chloride solution (6.2) to the reagent blank, standards and samples. For fresh water samples, sodium chloride solution may be omitted. Mix contents of tubes by swirling and place rack in cold water bath (0 - 10°C).

7.6 Pipette 10.0 mL of sulfuric acid solution (6.3) into each tube and mix by swirling. Allow tubes to come to thermal equilibrium in the cold bath. Be sure that temperatures have equilibrated in all tubes before continuing.

7.7 Add 0.5 mL brucine-sulfanilic acid reagent (6.4) to each tube (except the interference control tubes, 7.3) and carefully mix by swirling, then place the rack of tubes in the 100°C water bath for exactly 25 minutes.
CAUTION: Immersion of the tube rack into the bath should not decrease the temperature of the bath more than 1 to 2°C. In order to keep this temperature decrease to an absolute minimum, flow of bath water between the tubes should not be restricted by crowding too many tubes into the rack. If color development in the standards reveals discrepancies in the procedure, the operator should repeat the procedure after reviewing the temperature control steps.

7.8 Remove rack of tubes from the hot water bath and immerse in the cold water bath and allow to reach thermal equilibrium (20-25°C).

7.9 Read absorbance against the reagent blank at 410 nm using a 1 cm or longer cell.

8.0 Calculation

8.1 Obtain a standard curve by plotting the absorbance of standards run by the above procedure against mg NO_3-N/L. (The color reaction does not always follow Beer's law).

8.2 Subtract the absorbance of the sample without the brucine-sulfanilic reagent from the absorbance of the sample containing brucine-sulfanilic acid and determine mg NO_3-N/L. Multiply by an appropriate dilution factor if less than 10 mL of sample is taken.

9.0 Precision and Accuracy

9.1 Twenty-seven analysts in fifteen laboratories analyzed natural water samples containing exact increments of inorganic nitrate, with the following results:

Increment as Nitrogen, Nitrate mg N/liter	Precision as Standard Deviation mg N/liter	Accuracy as Bias, %	Bias, mg N/liter
0.16	0.92	-6.79	-0.01
0.19	0.083	+8.30	+0.02
1.08	0.245	+4.12	+0.04
1.24	0.214	+2.82	+0.04

(FWPCA Method Study 2, Nutrient Analyses).

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Appendix I
Laboratory Method – Nitrite - Nitrate – US EPA 353.4

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

Method 353.4

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

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 (‰)	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

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

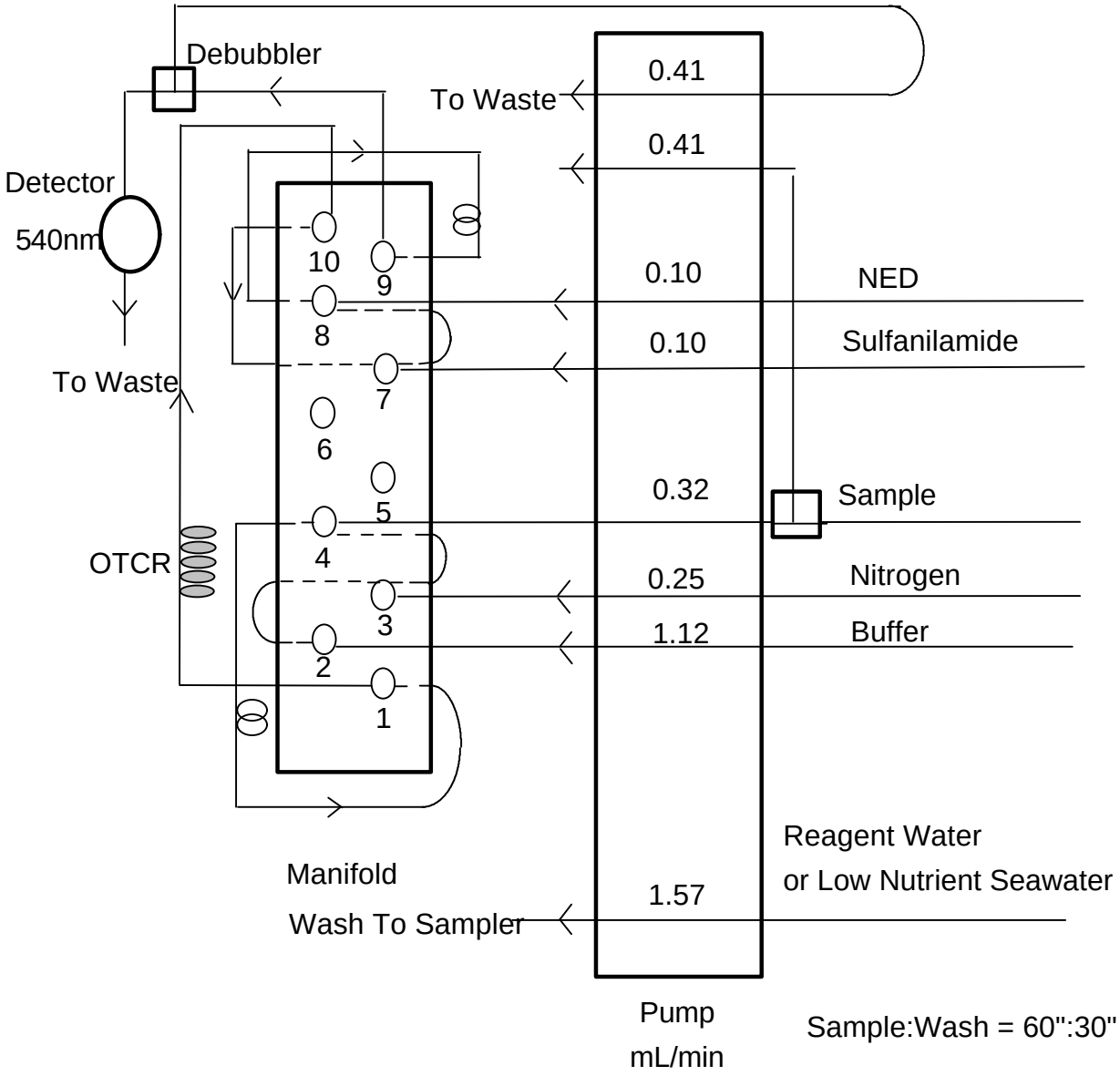

Figure 1. Manifold configuration for nitrate + nitrite analysis using an Open Tubular Cadmium Reactor.

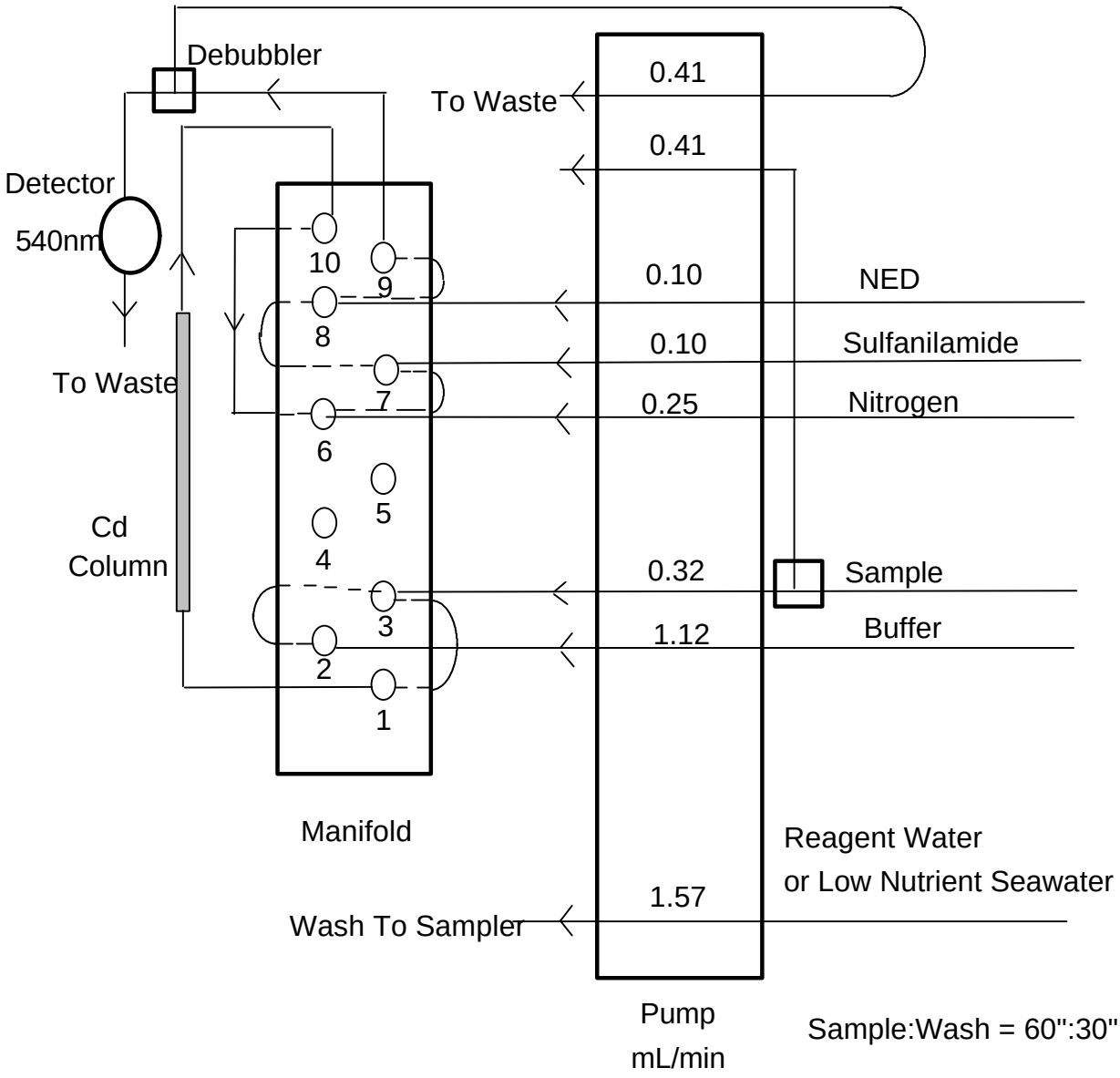

Figure 2. Manifold configuration for nitrate + nitrite analysis using a homemade packed copper-coated cadmium reduction column.

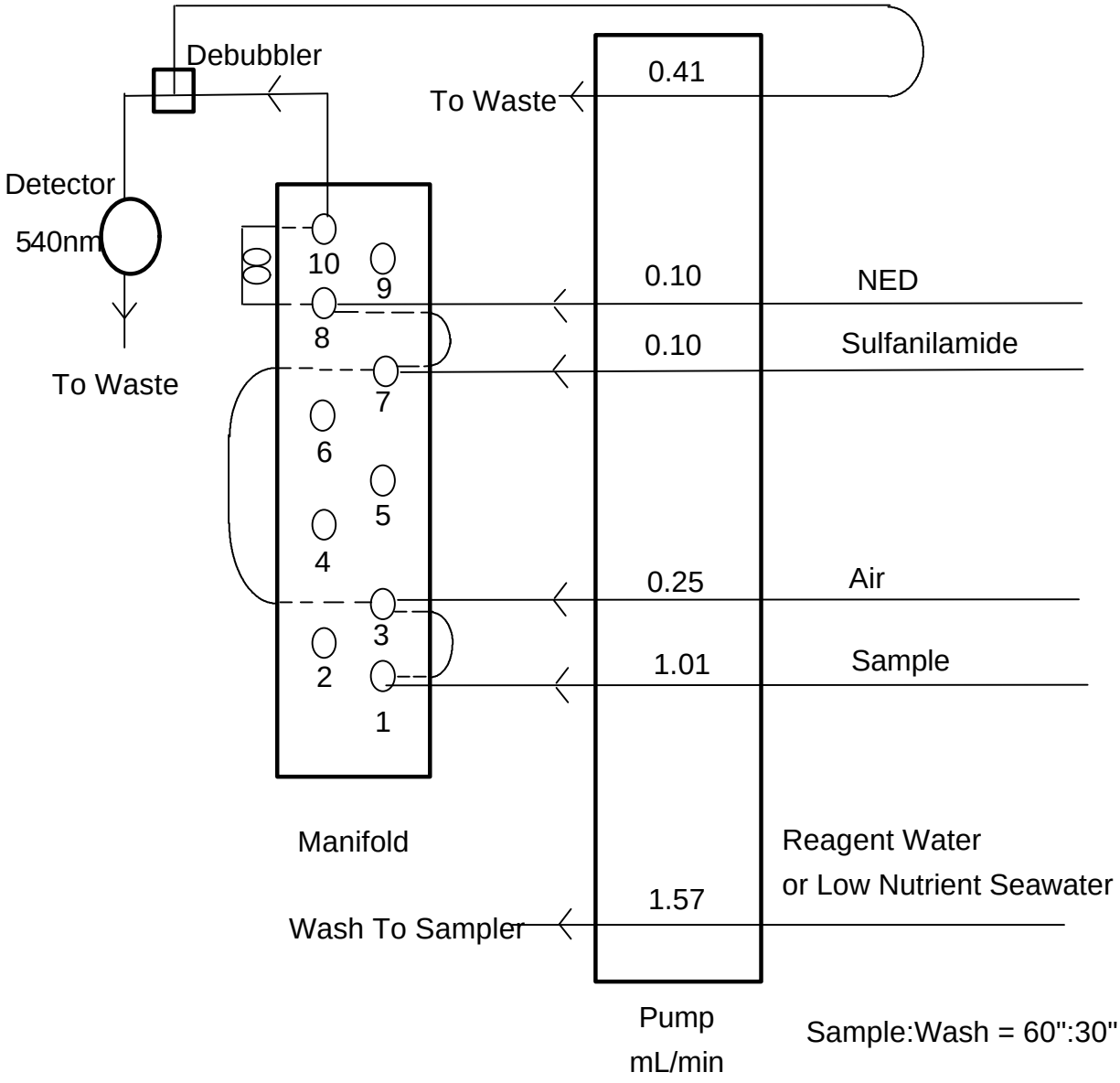

Figure 3. Manifold configuration for nitrite analysis.

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.

Appendix J
Laboratory Method – Ammonia - Nitrogen – US EPA 349.0

Method 349.0

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

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Version 1.0
September 1997

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

Method 349.0

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

1.0 Scope and Application

1.1 This method provides a procedure for the determination of ammonia in estuarine and coastal waters. The method is based upon the indophenol reaction,[1-5] here adapted to automated gas-segmented continuous flow analysis.

The term ammonia as used in this method denotes total concentration of ammonia, including both chemical forms, NH_3 and NH_4^+. Because ionization of NH_4^+ has a pK value of about 9.3, NH_4^+ is the dominant chemical form in natural waters. At pH of 8.2 and 25°C only 8.1% is present as NH_3, the form that can be toxic to fish and other aquatic organisms.

The concentration of ammonia in estuarine and coastal water shows considerable temporal and spatial variability. It rarely exceeds 0.005 mg N/L in oxygenated, unpolluted estuarine and coastal water, but in anoxic water, the amount of ammonia can be as high as 0.28 mg N/L.[6]

Although other forms of nitrogen contribute to primary productivity and nutrient cycling in marine and estuarine waters, ammonia is particularly important. Because ammonia represents the most reduced form of inorganic nitrogen available, it is preferentially assimilated by phytoplankton. Whereas nitrate is the source of nitrogen, it must first be reduced to ammonia before it can be assimilated and incorporated into amino acids and other compounds. Ammonia is released during the decomposition of organic nitrogen compounds by proteolytic bacteria, but also excreted directly by invertebrates along with urea and peptides.[7] In regions of coastal upwelling, ammonia released by zooplankton can play a significant role in supplying the nitrogen that supports phytoplankton production.[8]

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Ammonia	7664-41-7

1.2 A statistically determined method detection limit (MDL)[9] of 0.3 µg N/L has been determined by one laboratory from seawaters of four different salinities. The method is linear to 4.0 mg N/L using a Flow Solution System (Alpkem, Wilsonville, Oregon).

1.3 Approximately 60 samples per hour can be analyzed.

1.4 This method should be used by analysts both experienced in the use of automated gas segmented continuous flow colorimetric analyses, and also familiar with matrix interferences and the procedures used in their correction. A minimum of 6-months experience under the close supervision of a qualified analyst is recommended.

2.0 Summary of Method

2.1 The automated gas segmented continuous flow colorimetric method is used for the analysis of ammonia concentration. Ammonia in solution reacts with alkaline phenol and NaDTT (Sect. 7.2.5) at 60°C to form indophenol blue in the presence of sodium nitroferricyanide as a catalyst. The absorbance of indophenol blue at 640 nm is linearly proportional to the concentration of ammonia in the sample. A small systematic negative error caused by differences in the refractive index of seawater and reagent water, and a positive error caused by the matrix effect on the color formation, may be 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.[9]

3.7 Reagent Water (RW) -- Type 1 reagent grade water equal to or exceeding the standards established by the 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 of ammonia from the air, the reagent water should be stored in a sealed or a collapsible container and used the day of preparation.

3.8 Refractive Index (RI) -- The ratio of the 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 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 analyte 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 Synchronization Peak Solution -- A synchronization peak is required by most 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 concentration standard, but can be any sample that generates a peak at least 25% of full scale.

3.13 Color SYNC Peak Solution -- A colored solution used to produce a synchronization peak in the refractive index measurement in which no color reagent is pumped through system.

3.14 Sensitivity Drift -- The change in absorbance for a given concentration of analyte due to instrumental or chemical drift during the course of measurement.

3.15 Matrix Effect -- The change of absorbance in different matrices due to the effect of ionic strength and composition on the kinetics of color forming reactions.

4.0 Interferences

4.1 Hydrogen sulfide at concentrations greater than 2 mg S/L can negatively interfere with ammonia analysis. Hydrogen sulfide in samples should be removed by acidification with sulfuric acid to a pH of about 3, then stripping with gaseous nitrogen.

4.2 The addition of sodium citrate and EDTA complexing reagent eliminates the precipitation of calcium and magnesium hydroxides when calcium and

magnesium in seawater samples mix with high pH (about 13) reagent solution.[4]

4.3 Sample turbidity is eliminated by filtration or centrifugation after sample collection.

4.4 As noted in Section 2.1 refractive index and salt error interferences occur when sampler wash solution and calibration standards are not matched with samples in salinity, but are correctable. For low concentration samples (< 20 µg N/L), low nutrient seawater (LNSW) with salinity matched to samples, sampler wash solutions and calibration standards is recommended to eliminate matrix interferences.

5.0 Safety

5.1 Water samples collected from the estuarine and coastal environment are rarely 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 al chemicals used in this method. A lab coat, safety goggles, and gloves should be worn when handling the concentrated acid.

5.3 Chloroform is used as a preservative in this method. Use in a properly ventilated area, such as a fume hood.

6.0 Equipment and Supplies

6.1 Gas Segmented Continuous Flow Autoanalyzer Consisting of:

6.1.1 Automatic sampler.

6.1.2 Analytical cartridge with reaction coils and heater.

6.1.3 Proportioning pump.

6.1.4 Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 640 nm interference filter (maximum 2 nm bandwidth).

6.1.5 Strip chart recorder or computer based data acquisition system.

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

6.2 Glassware and Supplies

6.2.1 Gaseous ammonia concentration in the laboratory air should be minimal to avoid sample or reagent contamination. Remove any NH_4OH solution stored in the laboratory. Smoking should be strictly forbidden. An air filtration unit might also be used to obtain ammonia-free lab air.

6.2.2 All labware used in the analysis must be free of residual ammonia to avoid sample or reagent contamination. Soaking with laboratory grade detergent, rinsing with tap water, followed by rinsing with 10% HCl (v/v) and then thoroughly rinsing with reagent water was found to be sufficient when working at moderate and high concentration of ammonia. Ammonia is known for its high surface reactivity.[10] When working at low levels of ammonia (< 20 µg N/L), further cleaning of labware is mandatory. Plastic bottles and glass volumetric flasks should be cleaned in an ultrasonic bath with reagent water for 60 minutes. Bottles and sample tubes made of glass can be easily cleaned by boiling in reagent water. Repeat the cleaning process with fresh reagent water prior to use if necessary.

6.2.3 Automatic pipetters with disposable pipet tips capable of delivering volumes ranging from 100 µL to 1000 µL and 1 mL to 10 mL.

6.2.4 Analytical balance, with accuracy to 0.1 mg, for preparing standards.

6.2.5 60-mL glass or high density polyethylene sample bottles, glass volumetric flasks and glass sample tubes.

6.2.6 Drying oven.

6.2.7 Desiccator.

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

6.2.9 Centrifuge.

6.2.10 Ultrasonic water bath cleaner.

7.0 Reagents and Standards

Note: All reagents must be of analytical reagent grade.

7.1 Stock Reagent Solutions

7.1.1 Complexing Reagent - Dissolve 140 g of sodium citrate dihydrate($Na_3C_6H_5O_7.2H_2O$, FW 294.11), 5 g of sodium hydroxide (NaOH, FW 40) and 10 g of disodium EDTA ($Na_2C_{10}H_{14}O_8N_2.2H_2O$, FW 372.24), in approximately 800 mL of reagent water, mix and dilute to 1 L with reagent water. The pH of this solution is approximately 13. This solution is stable for 2 months.

7.1.2 Stock Ammonium Sulfate Solution (100 mg N/L) - Quantitatively transfer 0.4721 g of pre-dried (105°C for 2 hours) ammonium sulfate ($(NH_4)_2SO_4$, FW 132.15) to a 1000 mL glass volumetric flask containing approximately 800 mL of reagent water and dissolve the salt. Add a few drops of chloroform as a preservative. Dilute the solution to the mark with reagent water. Store in a glass bottle in the refrigerator at 4°C. It is stable for 2 months.[11]

7.1.3 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. **NOTE:** Don't use artificial seawater in this method.

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 Complexing Reagent - Add 1 mL Brij-35 to 200 mL of stock complexing reagent, mix gently. Prepare this solution daily. This volume of solution is sufficient for an 8-hour run.

7.2.3 Sodium Nitroferricyanide Solution - Dissolve 0.25 g of sodium nitroferricyanide ($Na_2Fe(CN)_5NO.2H_2O$, FW 297.97) in 400 mL of reagent water, dilute to 500 mL with reagent water. Store in an amber bottle at room temperature.

7.2.4 Phenol Solution - Dissolve 1.8 g of solid phenol (C_6H_5OH, FW 94.11) and 1.5 g of sodium hydroxide (NaOH, FW 40) in 100 mL of reagent water. Prepare this solution fresh daily.

7.2.5 NaDTT Solution - Dissolve 0.5 g of sodium hydroxide (NaOH, FW 40) and 0.2 g dichloroisocyanuric acid sodium salt (NaDTT, $NaC_3Cl_2N_3O_3$, FW 219.95) in 100 mL of reagent water. Prepare this solution fresh daily.

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

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

Note: This solution should be prepared to give an intermediate concentration appropriate for further dilution in preparing the calibration solutions. Therefore, the concentration of a primary dilution standard solution must be adjusted according to the desired 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 span more than two orders of magnitude. At least five calibration standards with equal increments in concentration should be used to construct the calibration curve.

When working with samples of a narrow range of salinities (± 2 ‰) or samples containing low ammonia concentration (< 20 µg N/L), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and the Sampler Wash Solution also be Low Nutrient Seawater (Section 7.1.4) diluted to the same salinity. **NOTE:** *If this procedure is employed, it is not necessary to perform the matrix effect and refractive index corrections outlined in Sections 12.2 and 12.3.*

When analyzing samples of moderate and high ammonia concentration (> 20 µg N/L) with varying salinities, calibration standard solutions and sampler wash solutions can be prepared in reagent water. The corrections for matrix effect and refractive index should be subsequently applied (Sections 12.2 and 12.3).

7.2.9 Saline Ammonia Standards - If CAL solutions are not prepared to match sample salinity, then saline ammonia standards must be prepared in a series of salinities in order to quantify the matrix effect (the change in the colorimetric response of ammonia due to the change in the composition of the solution). The following dilution of Primary Dilution Standard Solution (Section 7.2.7) and LNSW with reagent water to 100 mL in volumetric flasks, are suggested.

Salinity (‰)	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

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) 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 convenient samplers. Wash the sampler three times with sample water before collecting samples.

8.1.4 Turbid samples must be filtered through a 0.45 µm membrane filter as soon as possible after collection. Wash the filter with reagent water before use. Pass at least 100 mL of sample through the filter and discard before taking the final sample. Care must be taken to avoid the contamination of ammonia especially handling low concentrations of ammonia (< 20 µg N/L) samples.[10] An alternative technique to remove particulate is centrifugation.

8.1.5 60-mL glass or 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 or centrifugation, samples should be analyzed as soon as possible. If samples will be analyzed within 3 hours then keep refrigerated in tightly sealed, glass or high density polyethylene bottles in the dark at 4°C until the analysis can be performed.

8.3 Sample Storage - At low concentrations of ammonia (< 20 µg N/L), no preservation technique is satisfactory. *Samples must be analyzed within 3 hours of collection*. At moderate and high concentrations of ammonia (> 20 µg N/L) samples can be preserved by the addition of 2 mL of chloroform per liter of sample and refrigerated in the dark at 4°C. Samples can be stored in either glass or high density polyethylene bottles. A maximum holding time for preserved estuarine and coastal water samples with moderate to high concentrations of ammonia is two weeks.[12]

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 consists 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 analyte, 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 6 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 to avoid the necessity to correct for salt error, or refractive index. 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 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 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:

$$\text{Upper Control Limit} = x + 3S$$
$$\text{Lower Control Limit} = x - 3S$$

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 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 LFM 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 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 matrix related and the sample data should be flagged accordingly.

10.0 Calibration and Standardization

10.1 At least five calibration standards should be prepared fresh daily for system calibration.

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 the actual samples.

10.4 The calibration curve containing five data points or more that bracket the conentrations of samples should have a correlation coefficient, r, of 0.995 or better and the range should not be greater than two orders of magnitude.

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

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

where, P_{high} = the peak height of the high ammonia standard

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

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

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

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

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

To correct a given peak height reading, P_i, subtract the carryover correction.[13,14]

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

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

10.6 Place a high standard solution at the end of each sample run 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 stored in a refrigerator, remove samples and equilibrate to room temperature 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 cartridge and pump tubes as shown in Figure 1.

11.4 Set spectrophotometer wavelength to 640 nm, and turn on lamp.

11.5 Set the Absorbance Unit Full Scale (AUFS) range on the spectrophotometer at an appropriate setting according to the highest concentration of ammonia in the samples. The highest setting appropriate for this method is 0.2 AUFS for 6 mg N/L.

11.6 Prepare all reagents and standards.

11.7 Choose an appropriate wash solution for sampler wash. For analysis of samples with a narrow range of salinities (± 2 ‰) or for samples containing low ammonia concentrations (< 20 µg N/L), it is recommended that the CAL solutions be prepared in Low Nutrient Seawater (Section 7.1.4) diluted to the salinity of samples, and that the Sampler Wash Solution also be Low Nutrient Seawater diluted to the same salinity. For samples with varying salinities and higher ammonia concentrations (> 20 µg N/L), it is suggested that the reagent water used for the sampler wash solution and for preparing calibration standards and procedures in Section 12.2 and 12.3 be employed.

11.8 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 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 few minutes each at tahe 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 a syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohhol was found to be effective in removing air bubbles from the flowcell.

11.9 The sampling rate is approximately 60 samples per hour with 30 seconds of sample time and 30 seconds of wash time.

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 ammonia 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, the operator has to quantify the refractive index correction due to the difference in salinity between sample and wash solution. The following procedures are used to measure the relationship between the sample salinity and refractive index on a particular detector.

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

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

Note: In ammonia 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 the wash solution to bring the baseline down.

12.2.4 Third, replace the phenol solution (Section 7.2.4) and NaDTT solution (Section 7.2.5) 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 series of different salinity samples by diluting the 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 LNSW 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 ammonia concentration due to refractive index from its peak height corrected to reagent water baseline (Section 12.2.5) and the regression equation of ammonia standards obtained with color reagent being pumped through the system (Section 12.2.2). Salinity is entered as the independent variable and the apparent ammonia concentration due to refractive index is entered as the dependent variable. The resulting regression allows the operator to calculate apparent ammonia concentration due to refractive index when the sample salinity is known. Thus, the operator would not be required to obtain refractive index peak heights for all samples.

12.2.8 The magnitude of refractive index correction can be minimized by using a low refractive index flowcell. An example of a typical result using a low refractive index flowcell follows:

Salinity (‰)	Apparent ammonia conc. due to refractive index (µg N/L)
0.0	0.00
4.5	0.18
9.1	0.45
13.9	0.66
17.9	0.86
27.6	1.30
36.2	1.63

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

12.2.9 An example of a typical equation is:

Apparent ammonia (µg N/L) = 0.0134 + 0.0457S

where S is sample salinity in parts per thousand. The apparent ammonia concentration due to refractive index so obtained should then be added to samples of

corresponding salinity when reagent water was used as the wash solution for samples analysis.

If a low refractive index flowcell is used and ammonia concentration is greater than 200 µg N/L, the correction for refractive index becomes negligible.

12.3 Correction for Matrix Effect in Estuarine and Coastal Samples

12.3.1 When calculating concentrations of samples of varying salinities from standards and wash solution prepared in reagent water, it is necessary to first correct for refractive index errors, then correct for the change in color development due to the differences in composition between samples and standards (matrix effect). Even where the refractive index correction may be small, the correction for matrix effect can be appreciable.

12.3.2 Plot the salinity of the saline standards (Section 7.2.9) as the independent variable, and the apparent concentration of ammonia (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. An example of a typical result follows:

Salinity (‰)	Peak height of 0.140 mg N/L	Uncorrected NH_3 conc. calculated from standards in reagent water (mg N/L)
0	2420	0.1400
4.5	2856	0.1649
9.1	2852	0.1649
13.9	2823	0.1635
17.9	2887	0.1673
27.6	2861	0.1663
36.2	2801	0.1633

12.3.3 Using the reagent described in Section 7.0, as shown above, matrix effect becomes a single factor independent of sample salinity. An example of a typical equation to correct for matrix effect is:

Corrected concentration (mg N/L)
= Uncorrected concentration /1.17(mg N/L)

12.3.4 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 0.3 µg N/L has been determined by one laboratory from spiked LNSW of three different salinities as follows:

Salinity (‰)	$[NH_3]$ (µg N/L)	SD (µg N/L)	Recovery (%)	MDL (µg N/L)
36.2	0.7	0.0252	95.4	0.0792
36.2	0.7	0.0784	100.8	0.2463
36.2	1.4	0.0826	104.7	0.2595
36.2	1.4	0.0966	105.6	0.3035
17.9	0.7	0.0322	106.5	0.1012
17.9	0.7	0.0182	92.2	0.0572
17.9	1.4	0.0938	109.1	0.2947
17.9	1.4	0.0882	100	0.2771
4.5	0.7	0.0672	95.1	0.2111
4.5	1.4	0.1008	94.1	0.3167
4.5	1.4	0.126	106.7	0.3959
0.0	0.7	0.077	98.2	0.2419
0.0	0.7	0.0784	100.8	0.2463
0.0	1.4	0.0854	101.9	0.2683

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 4.8 to 35.0. The results were as follows:

Sample	Salinity (‰)	Concentration (µg N/L)	RSD (%)
1	35.5	6.3	7.19
2	20.0	72.1	1.57
3	4.8	517.6	0.64

13.1.3 Laboratory Fortified Sample Matrix - Laboratory fortified sample matrices were processed in three different salinities ranging from 4.8 to 35.0 and ambient ammonia concentrations from 0.0 to 72.1 µg N/L. Seven replicates of each sample were analyzed and the results were as follows:

Salinity	Concentration		RSD	Recovery
	ambient	fortified		
(‰)	(µg N/L)		(%)	(%)
35.5	6.3	70	5.01	98.3
20.0	72.1	140	1.71	98.3
4.8	0.0	280	1.81	98.1

13.1.4 Linear Dynamic Range - A linear dynamic range (LDR) of 4.0 mg N/L has been determined by one laboratory from spiked LNSW using a Flow Solution System (Alpkem, Wilsonville, Oregon).

13.1.5 Sample Preservation Study - Natural samples have been preserved by freezing, acidification and addition of chloroform and phenol as preservatives to the samples stored in glass and high density polyethylene bottles. Table 1 summarized the results of preservation study.

There is no significant difference in recovery of ammonia from samples stored in glass and high density polyethylene bottles, suggesting either glass or high density polyethylene bottles can be used for storage of ammonia samples.

For low concentration of ammonia samples (< 20 µg N/L, sample 1 in table 1), no preservation technique is satisfactory. Samples must be analyzed within 3 hours of collection.

Freezing cannot preserve ammonia in samples for more than one week. Acidified samples must be neutralized with NaOH solution prior to analysis. Addition of NaOH to acidified samples induces the precipitation of $Mg(OH)_2$ and $Ca(OH)_2$. Centrifuging the samples cannot completely eliminate the interference, therefore, acidification is not suitable preservation technique. Addition of phenol increases the absorbance of samples. Phenol is not recommended as a suitable preservative although samples preserved with phenol were stable as those preserved by chloroform.[12]

For moderate and high concentrations of ammonia (> 20 µg N/L) samples, it is suggested samples be preserved by the addition of 2 mL of chloroform per liter of sample and refrigerated in the dark at 4°C. A maximum holding time for preserved estuarine and coastal water samples with moderate to high concentrations of ammonia is two weeks.[10]

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

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

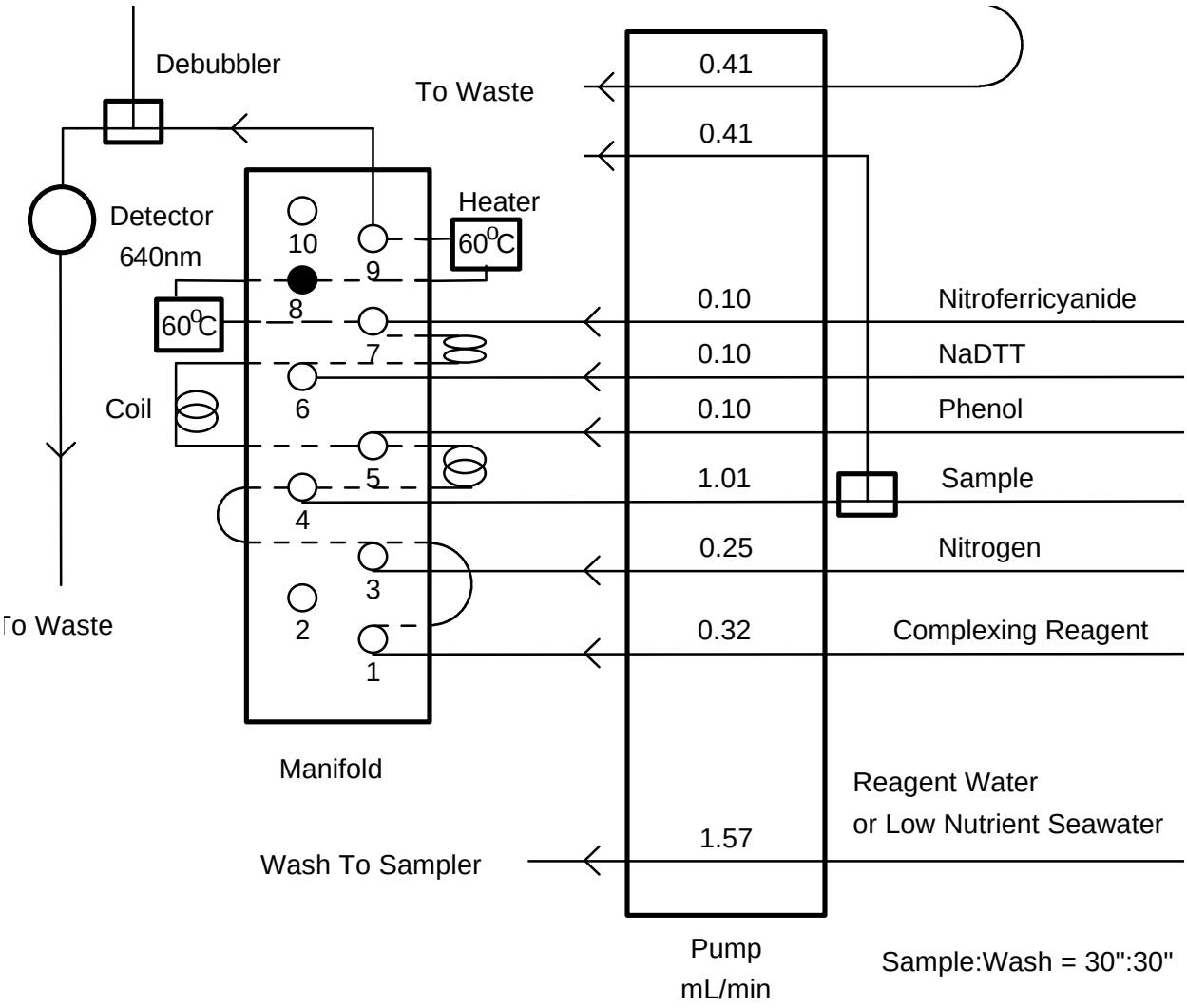

Figure 1. Manifold Configuration for Ammonia Analysis.

Table 1 . Percentage Recovery[A] of Ammonia From Natural Water Samples Preserved by Freezing, Acidification, Addition of Chloroform and Phenol.

sample[B]	method[C]	bottle[D]	time(day)				
			0	7	14	21	28
1	none	HDPE	100	349	345	18	91
	freezing	glass	100	100	0	0	0
		HDPE	100	102	0	0	0
	H_2SO_4 [E]	glass	200	564	285	73	55
		HDPE	200	113	64	45	36
	$CHCl_3$	glass	193	135	29	47	36
		HDPE	193	193	18	44	36
	phenol [F]	glass	153	36	44	0	0
		HDPE	153	36	0	0	0
1+	freezing	glass	100	101	82	77	102
		HDPE	100	97	76	61	81
	H_2SO_4 [E]	glass	95	105	69	54	37
		HDPE	95	91	91	88	116
	$CHCl_3$	glass	96	105	85	78	89
		HDPE	96	102	85	78	92
	phenol [F]	glass	130	133	110	148	123
		HDPE	130	128	102	103	118
2	none	HDPE	100	32	0	0	0
	freezing	glass	100	109	93	77	88
		HDPE	100	107	82	67	91
	H_2SO_4 [E]	glass	252	162	66	62	50
		HDPE	252	193	45	41	27
	$CHCl_3$	glass	99	114	83	75	96
		HDPE	99	98	80	70	83
	phenol [F]	glass	108	107	88	74	93
		HDPE	108	101	83	74	86
2+	freezing	glass	99	108	109	111	106
		HDPE	99	106	95	78	91
	H_2SO_4 [E]	glass	100	107	51	86	88
		HDPE	100	102	39	98	107
	$CHCl_3$	glass	99	106	116	94	105
		HDPE	99	107	98	95	103
	phenol [F]	glass	117	121	106	105	116
		HDPE	117	124	107	106	117
3	none	HDPE	100	104	14	1	0
	freezing	glass	100	-	116	64	106
		HDPE	100	108	105	65	75
	H_2SO_4 [E]	glass	101	106	44	74	61
		HDPE	101	108	111	106	109
	$CHCl_3$	glass	100	96	98	96	94
		HDPE	100	93	97	95	95
	phenol [F]	glass	112	106	107	112	125
		HDPE	112	112	108	110	112

[A] Recovery is calculated based on the ammonia concentration in non-preserved sample at day 0. Samples with recoveries higher than 100% are subject to interference either from precipitation or phenol.

[B] For salinity and concentration of ammonia in samples 1, 2, 3 see Section 13.1.2.

Sample 1+ and 2+ are the fortified samples 1 and 2 at ammonia concentrations 76.3 and 202.1 µg N/L, respectively.

C Methods of preservation:
None: stored the samples in high density polyethylene carboys at room temperature without any preservative added.
Freezing: Frozen and stored at -20°C.
H_2SO_4 : Acidified to pH 1.8 with H_2SO_4, and stored at 4°C. Neutralized the acid with NaOH solution before analysis.
$CHCl_3$: Added 2 mL chloroform per 1000 mL sample, and stored at 4°C.
Phenol: Added 8 g phenol per 1000 mL sample, and stored at 4°C.

C Glass and high density polyethylene bottles were compared to determine the effect of sample bottle type on the preservation.

E Adding NaOH to neutralize acidified samples induced the precipitation of $Mg(OH)_2$ and $Ca(OH)_2$. Centrifuging the samples can not completely eliminate the interference, therefore, acidification is not suitable preservation technique.

F Although samples preserved with phenol were stable as those preserved by chloroform, an absorbance increase was observed, therefore, this is not recommended as a suitable preservation technique.

Appendix K
Laboratory Method – Orthophosphate – US EPA 365.5

Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

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Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of low-level orthophosphate concentrations normally found in estuarine and/or coastal waters. It is based upon the method of Murphy and Riley[1] adapted for automated segmented flow analysis[2] in which the two reagent solutions are added separately for greater reagent stability and facility of sample separation.

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Phosphate	14265-44-2

1.2 A statistically determined method detection limit (MDL) of 0.0007 mg P/L has been determined by one laboratory in 3 parts per thousand (ppt) saline water.[3] The method is linear to 0.39 mg P/L using a Technicon AutoAnalyzer II system (Bran & Luebbe, Buffalo Grove, IL).

1.3 Approximately 40 samples per hour can be analyzed.

1.4 This method should be used by analysts experienced in the use of automated colorimetric analyses, and familiar with matrix interferences and procedures for their correction. A minimum of 6-months experience under experienced supervision is recommended.

2.0 Summary of Method

2.1 An automated colorimetric method for the analysis of low-level orthophosphate concentrations is described. Ammonium molybdate and antimony potassium tartrate react in an acidic medium with dilute solutions of phosphate to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color produced is proportional to the phosphate concentration present in the sample. Positive bias caused by differences in the refractive index of seawater and reagent water is corrected for prior to data reporting.

3.0 Definitions

3.1 Calibration Standard (CAL) -- A solution prepared from the stock standard solution that is used to calibrate the instrument response with respect to analyte concentration. One of the standards in the standard curve.

3.2 Dissolved Analyte (DA) -- The concentration of analyte in an aqueous sample that will pass through a 0.45-μm membrane filter assembly prior to sample acidification or other processing.

3.3 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. This is basically a standard prepared in reagent water that is analyzed as a sample.

3.4 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.

3.5 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, 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.

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

3.7 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.

3.8 Reagent Water (RW) -- Type 1 reagent grade water equal to or exceeding standards established by American Society of Testing Materials (ASTM). Reverse osmosis systems or distilling units that produce 18 megohm water are two examples of acceptable water sources.

3.9 Refractive Index (RI) -- The ratio of the 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 sea or estuarine water versus reagent water. The correction for this difference is referred to as the refractive index correction in this method.

3.10 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.

4.0 Interferences

4.1 Interferences caused by copper, arsenate and silicate are minimal relative to the orthophosphate determination because of the extremely low concentrations normally found in estuarine or coastal waters. High iron concentrations can cause precipitation of and subsequent loss of phosphate from the dissolved phase. Hydrogen sulfide effects, such as occur in samples collected from deep anoxic basins, can be treated by simple dilution of the sample since high sulfide concentrations are most often associated with high phosphate values.[4]

4.2 Sample turbidity is removed by filtration prior to analysis.

4.3 Refractive Index interferences are corrected for estuarine/coastal samples (Section 12.2).

5.0 Safety

5.1 Water samples collected from the estuarine and/or ocean 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. A lab coat, safety goggles, and gloves should be worn when preparing the sulfuric acid reagent.

6.0 Equipment and Supplies

6.1 Continuous Flow Automated Analytical System Consisting of:

6.1.1 Sampler.

6.1.2 Manifold or Analytical Cartridge equipped with 37°C heating bath.

6.1.3 Proportioning pump.

6.1.4 Colorimeter equipped with 1.5 X 50 mm tubular flow cell and a 880 nm filter.

6.1.5 Phototube that can be used for 600-900 nm range.

6.1.6 Strip chart recorder or computer based data system.

6.2 Phosphate-Free Glassware and Polyethylene Bottles

6.2.1 All labware used in the determination must be low in residual phosphate to avoid sample or reagent contamination. Washing with 10% HCI (v/v) and thoroughly rinsing with distilled, deionized water was found to be effective.

6.2.2 Membrane or glass fiber filters, 0.45 μm nominal pore size.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Ammonium Molybdate Solution (40 g/L) -- Dissolve 20.0 g of ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\bullet 4H_2O$, CASRN 12027-67-7) in approximately 400 mL of reagent water and dilute to 500 mL. Store in a plastic bottle out of direct sunlight. This reagent is stable for approximately three months.

7.1.2 Antimony Potassium Tartrate Solution (3.0 g/L) -- Dissolve 0.3 g of antimony potassium tartrate [$(K(SbO)C_4H_4O_6\bullet 1/2H_2O$, CASRN 11071-15-1] in approximately 90 mL of reagent water and dilute to 100 mL. This reagent is stable for approximately three months.

7.1.3 Ascorbic Acid Solution (18.0 g/L) -- Dissolve 18.0 g of ascorbic acid ($C_6H_6O_6$, CASRN 50-81-7) in approximately 800 mL of reagent water and dilute to 1 L. Dispense approximately 75 mL into clean polyethylene bottles and freeze. The stability of the frozen ascorbic acid is approximately three months. Thaw overnight in the refrigerator before use. The stability of the thawed, refrigerated reagent is less than 10 days.

7.1.4 Sodium Lauryl Sulfate Solution (30.0 g/L) -- Sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$, CASRN 151-21-3). Dissolve 3.0 g of sodium lauryl sulfate (SLS) in approximately 80 mL of reagent water and dilute to 100 mL. This solution is the wetting agent and its stability is approximately three weeks.

7.1.5 Sulfuric Acid Solution (4.9 N) -- Slowly add 136 mL of concentrated sulfuric acid (H_2SO_4, CASRN 7664-93-9) to approximately 800 mL of reagent water. After the solution is cooled, dilute to 1 L with reagent water.

7.1.6 Stock Phosphorus Solution -- Dissolve 0.439 g of pre-dried (105°C for 1 hr) monobasic potassium phosphate (KH_2PO_4, CASRN 7778-77-0) in reagent water and

dilute to 1000 mL. (1.0 mL = 0.100 mg P.) The stability of this stock standard is approximately three months when kept refrigerated.

7.1.7 Low Nutrient Seawater -- Obtain natural low nutrient seawater (36 ppt salinity; <0.0003 mg P/L) or dissolve 31 g analytical reagent grade sodium chloride, (NaCl, CASRN 7647-14-5); 10 g analytical grade magnesium sulfate, ($MgSO_4$) CASRN 10034-99-8); and 0.05 g analytical reagent grade sodium bicarbonate, ($NaHCO_3$, CASRN 144-55-8), in 1 L of reagent water.

7.2 Working Reagents

7.2.1 Reagent A -- Mix the following reagents in the following proportions for 142 mL of Reagent A: 100 mL of 4.9 N H_2SO_4 (Section 7.1.5), 30 mL of ammonium molybdate solution (Section 7.1.1), 10 mL of antimony potassium tartrate solution (Section 7.1.2), and 2.0 mL of SLS solution (Section 7.1.4). Prepare fresh daily.

7.2.2 Reagent B -- Add approximately 0.5 mL of the SLS solution (Section 7.1.4) to the 75 mL of ascorbic acid solution (Section 7.1.3). Stability is approximately 10 days when kept refrigerated.

7.2.3 Refractive Reagent A -- Add 50 mL of 4.9 N H_2SO_4 (Section 7.1.5) to 20 mL of reagent water. Add 1 mL of SLS (Section 7.1.4) to this solution. Prepare fresh every few days.

7.2.4 Secondary Phosphorus Solution -- Take 1.0 mL of Stock Phosphorus Solution (Section 7.1.6) and dilute to 100 mL with reagent water. (1.0 mL = 0.0010 mg P.) Refrigerate and prepare fresh every 10 days.

7.2.5 Prepare a series of standards by diluting suitable volumes of standard solutions (Section 7.2.4) to 100 mL with reagent water. Prepare these standards daily. When working with samples of known salinity, it is recommended that the standard curve concentrations be prepared in low-level natural seawater (Section 7.1.7) diluted to match the salinity of the samples. Doing so obviates the need to perform the refractive index correction outlined in Section 12.2. When analyzing samples of varying salinities, it is recommended that the standard curve be prepared in reagent water and refractive index corrections be made to the sample concentrations (Section 12.2). The following dilutions are suggested.

mL of Secondary Phosphorus Solution (7.2.4)	Conc. mg P/L
0.1	0.0010
0.2	0.0020
0.5	0.0050
1.0	0.0100
2.0	0.0200
4.0	0.0400
5.0	0.0500

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. Filtration of the sample through a 0.45-μm membrane or glass fiber filter immediately after collection is required.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Nansen, 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 When a submersible pump system is used, 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 processing.

8.1.3 Another method used to collect surface samples involves the use of a plastic bucket or large plastic bottle. While not the most ideal method, it is commonly used in citizen monitoring programs.

8.2 Sample Preservation -- After collection and filtration, samples should be analyzed as quickly as possible. If the samples are to be analyzed within 24 hr of collection, then refrigeration at 4°C is acceptable.

8.3 Sample Storage -- Long-term storage of frozen samples should be in clearly labeled polyethylene bottles or polystyrene cups compatible with the analytical system's automatic sampler (Section 6.1.1). If samples cannot be analyzed within 24 hr, then freezing at -20°C for a maximum period of two months is acceptable.[5-8]

9.0 Quality Control

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, the continued analysis of LRBs, laboratory duplicates, and LFBs 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 (MDLs and linear dynamic range) and laboratory performance (analysis of QC samples) prior to analyses of samples using this method.

9.2.2 MDLs should be established using a low-level estuarine water sample fortified to approximately five

times the estimated detection limit.[3] To determine MDL values, analyze seven replicate aliquots of water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

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

where, S = the standard deviation of the replicate analyses.

t = the 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 when a new matrix is encountered.

9.2.3 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of five calibration standards ranging in concentration from 0.001 mg P/L to 0.20, mg P/L across all sensitivity settings of the autoanalyzer. Normalize responses by dividing the response by the sensitivity setting multiplier. 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 ± 10% of the known concentration, C, where $C_C = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument. Should samples be encountered that have a concentration that is ≥90% of the upper limit of the 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 (Section 3.5) 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 LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates of the samples 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 (Section 3.3) with each batch of samples. Calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the analyte is judged out of control and the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB data to assess laboratory performance against the required control limits of 90-110% (Section 9.3.2). 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 the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

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

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

9.4.2 Calculate the percent recovery of the analyte, corrected for background concentrations measured in the unfortified sample, and compare these values with the values obtained from the LFBs.

Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_s - C)}{S} x\ 100$$

where, R = percent recovery
C_S = measured fortified sample concentration (background + concentrated addition in mg P/L)
C = sample background concentration (mg P/L)
S = concentration in mg P/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the designated range of 90-110% recovery, but the laboratory performance for that analyte is in control, 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 matrix related, not system related.

10.0 Calibration and Standardization

10.1 Calibration (Refer to Sections 11.5 and 12.0).

10.2 Standardization (Refer to Section 12.2).

11.0 Procedure

11.1 If samples are frozen, thaw the samples to room temperature.

11.2 Set up manifold as shown in Figure 1. The tubing, flow rates, sample:wash ratio, sample rate, etc., are based on a Technicon AutoAnalyzer II system. Specifications for similar segmented flow analyzers vary, so slight adjustments may be necessary.

11.3 Allow both colorimeter and recorder to warm up for 30 min. Obtain a steady baseline with reagent water pumping through the system, add reagents to the sample stream and after the reagent water baseline has equilibrated, note that rise (reagent water baseline), and adjust baseline.

For analysis of samples with a narrow salinity range, it is advisable to use low nutrient seawater matched to sample salinity as wash water in the sampler in place of reagent water. For samples with a large salinity range, it is suggested that reagent wash water and procedure (Section 12.2) be employed.

11.4 A good sampling rate is approximately 40 samples/hr with a 9:1, sample:wash ratio.

11.5 Place standards (Section 7.2.5) in sampler in order of decreasing concentration. Complete filling the sampler tray with samples, LRBs, LFBs, and LFMs.

11.6 Commence analysis.

11.7 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). This "apparent" concentration due to coloration of the water should be subtracted from concentrations obtained with Reagent A pumping through the system.

12.0 Data Analysis and Calculations

12.1 Concentrations of orthophosphate 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 the corresponding peak height is the dependent variable.

12.2 Refractive Index Correction for Estuarine/ Coastal Systems

12.2.1 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). Reagent B (Section 7.2.2) remains the same and is also pumped through the system. Peak heights for the refractive index correction must be obtained at the same Standard Calibration Setting and on the same colorimeter as the corresponding samples and standards.[9]

12.2.2 Subtract the refractive index peak heights from the heights obtained for the orthophosphate determination. Calculate the regression equation using the corrected standard peak heights. Calculate the concentration of samples from the regression equation using the corrected sample peak heights.

12.2.3 When a large data set has been amassed in which each sample's salinity is known, a regression for the refractive index correction on a particular colorimeter can be calculated. For each sample, the apparent orthophosphate concentration due to refractive index is calculated from its peak height obtained with Refractive Reagent A (Section 7.2.3) and Reagent B (Section 7.2.2) and the regression of orthophosphate standards obtained with orthophosphate Reagent A (Section 7.2.1) and Reagent B (Section 7.2.2) for each sample. Its salinity is entered as the independent variable and its apparent orthophosphate concentration due to its refractive index in that colorimeter is entered as the dependent variable. The resulting regression equation allows the operator to subtract an apparent orthophosphate concentration when the salinity is known, as long as other matrix effects are not present. Thus, the operator would not be required to obtain the refractive index peak heights for all samples after a large data set has been found to yield consistent apparent orthophosphate concentrations due to salinity. An example follows:

Salinity (ppt)	Apparent orthophosphate conc. due to refractive index (mg P/L)
1	0.0002
5	0.0006
10	0.0009
20	0.0017

12.2.4 An example of a typical equation is:

mg P/L apparent PO_4^{3-} = 0.000087 X Salinity (ppt) where, 0.000087 is the slope of the line.

where, 0.000087 is the slope of the line.

12.3 Results should be reported in mg PO_4^{3-}- P/L or µg PO_4^{3-}- P/L.

mg PO_4^{3-} - P/L = ppm (parts per million)

µg PO_4^{3-} - P/L = ppb (parts per billion)

13.0 Method Performance

13.1 Single Analyst Precision -- A single laboratory analyzed three samples collected from Chesapeake Bay, Maryland, and East Bay, Florida. Seven replicates of each sample were processed and analyzed randomly throughout a group of 75 samples with salinities ranging from 3 to 36 ppt. The results were as follows:

Sample	Salinity (ppt)	Concentration (mg P/L)	Percent Relative Standard Deviation
1	36	0.0040	6.5
2	18	0.0024	10
3	3	0.0007	24

13.2 Multilaboratory Testing

13.2.1 This method was tested by nine laboratories using reagent water, high salinity seawater from the Sargasso Sea (36 ppt) and three different salinity waters from Chesapeake Bay, Maryland (8.3 ppt, 12.6 ppt, and 19.5 ppt). The reagent water and the Sargasso Seawater were fortified at four Youden pair concentrations ranging from 0.0012 mg P/L to 0.1000 mg P/L.[10] The Chesapeake Bay waters were fortified at three Youden pair concentrations ranging from 0.0050 mg P/L to 0.0959 mg P/L with the highest salinity waters containing the lowest Youden pair and the lowest salinity waters containing the highest Youden pair. Analysis of variance (ANOVA) at the 95% confidence level found no statistical differences between water types indicating that the refractive index correction for different salinity waters is an acceptable procedure. Table 1 contains the linear equations that describe the single-analyst standard deviation, overall standard deviation, and mean recovery of orthophosphate from each water type.

13.2.2 Pooled Method Detection Limit (p-MDL) -- The p-MDL is derived from the pooled precision obtained by single laboratories for the lowest analyte concentration level used in the multilaboratory study. The p-MDLs using reagent water and Sargasso Sea water were 0.00128 and 0.00093 mg P/L, respectively.

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 EPA 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's 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 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

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Single-Analyst Precision, Overall Precision and Recovery from Multilaboratory Study

Reagent Water	
(0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.972C - 0.000018
Overall Standard Deviation	S_R = 0.033X + 0.000505
Single-Analyst Standard Deviation	S_r = 0.002X + 0.000448
Sargasso Sea Water	
(0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.971C - 0.000002
Overall Standard Deviation	S_R = 0.021X + 0.000550
Single-Analyst Standard Deviation	S_r = 0.010X + 0.000249
Chesapeake Bay Water	
(0.005 - 0.100 mg P/L)	
Mean Recovery	
X = 1.019C - 0.000871	
Overall Standard Deviation	S_R = 0.066X + 0.000068
Single-Analyst Standard Deviation	S_r = 0.030X + 0.000165

C True value of spike concentration, mg P/L
X Mean concentration found, mg P/L, exclusive of outliers.
S_R Overall standard deviation, mg P/L, exclusive of outliers.
S_r Single-analyst standard deviation, mg P/L, exclusive of outliers.

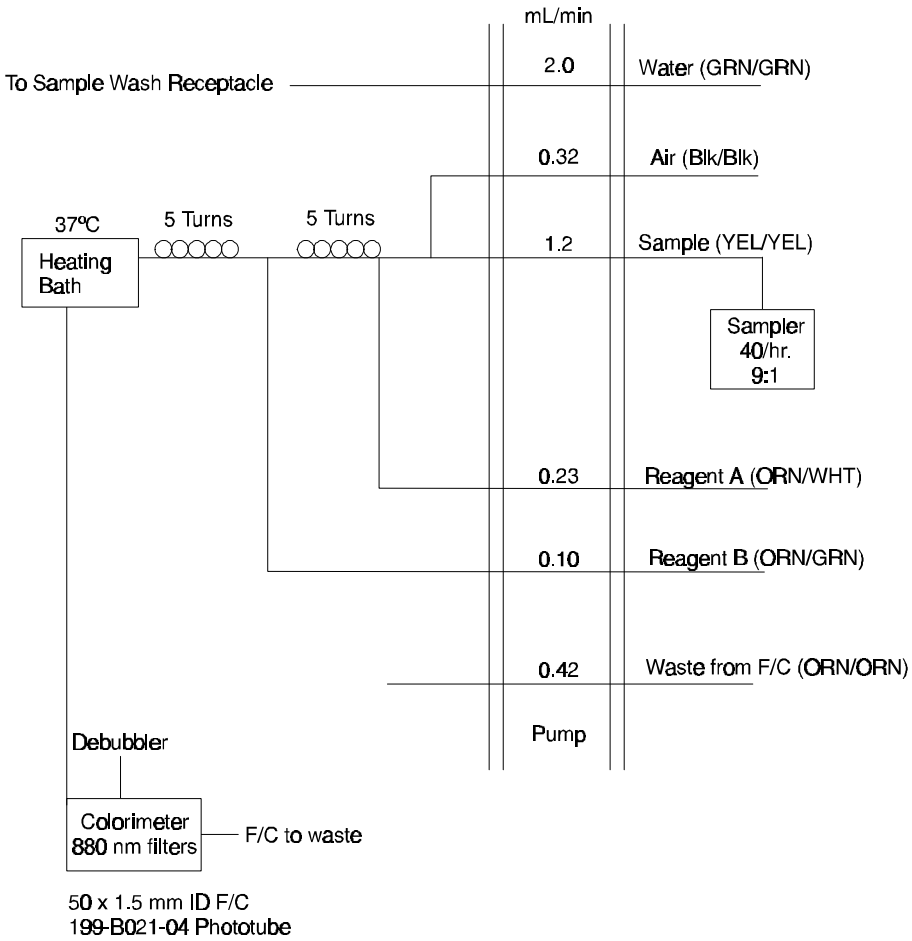

Figure 1. Manifold Configuration for Orthophosphate.

Appendix L
Laboratory Method – Total Nitrogen – US EPA 351.2

Detailed Methodology for Data Collection and Reporting Procedures for Tidal Monitoring
Alliance for the Chesapeake Bay
CB96334901 for Citizen-Based/Non-Traditional Monitoring Grant

United States Environmental Protection Agency

www.epa.gov August 1993

Method 351.2, Revision 2.0: Determination of Total Kjeldahl Nitrogen by Semi-Automated Colorimetry

METHOD 351.2

DETERMINATION OF TOTAL KJELDAHL NITROGEN BY SEMI-AUTOMATEDCOLORIMETRY

Edited by James W. O'Dell
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Revision 2.0
August 1993

ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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METHOD 351.2

DETERMINATION OF TOTAL KJELDAHL NITROGEN BY SEMI-AUTOMATED COLORIMETRY

1.0 SCOPE AND APPLICATION

1.1 This method covers the determination of total Kjeldahl nitrogen in drinking, ground, and surface waters, domestic and industrial wastes. The procedure converts nitrogen components of biological origin such as amino acids, proteins and peptides to ammonia, but may not convert the nitrogenous compounds of some industrial wastes such as amines, nitro compounds, hydrazones, oximes, semicarbazones and some refractory tertiary amines.

1.2 The applicable range is 0.1-20 mg/L TKN. The range may be extended with sample dilution.

2.0 SUMMARY OF METHOD

2.1 The sample is heated in the presence of sulfuric acid, H_2SO_4 for two and one half hours. The residue is cooled, diluted to 25 mL and analyzed for ammonia. This digested sample may also be used for phosphorus determination.

2.2 Total Kjeldahl nitrogen is the sum of free-ammonia and organic nitrogen compounds which are converted to ammonium sulfate $(NH_4)_2SO_4$, under the conditions of digestion described.

2.3 Organic Kjeldahl nitrogen is the difference obtained by subtracting the free-ammonia value from the total Kjeldahl nitrogen value.

2.4 Reduced volume versions of this method that use the same reagents and molar ratios are acceptable provided they meet the quality control and performance requirements stated in the method.

2.5 Limited performance-based method modifications may be acceptable provided they are fully documented and meet or exceed requirements expressed in Section 9.0, Quality Control.

3.0 DEFINITIONS

3.1 **Calibration Blank (CB)** -- A volume of reagent water fortified with the same matrix as the calibration standards, but without the analytes, internal standards, or surrogate analytes.

3.2 **Calibration Standard (CAL)** -- A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and

surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 **Instrument Performance Check Solution (IPC)** -- A solution of one or more method analytes, surrogates, internal standards, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.4 **Laboratory Fortified Blank (LFB)** -- An aliquot of reagent water or other blank matrices 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 the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.5 **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.

3.6 **Laboratory Reagent Blank (LRB)** -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates 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 the apparatus.

3.7 **Linear Calibration Range (LCR)** -- The concentration range over which the instrument response is linear.

3.8 **Material Safety Data Sheet (MSDS)** -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.9 **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.

3.10 **Quality Control Sample (QCS)** -- A solution of method analytes of known concentrations that 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.

3.11 **Stock Standard Solution (SSS)** -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

4.0 INTERFERENCES

4.1 High nitrate concentrations (10X or more than the TKN level) result in low TKN values. If interference is suspected, samples should be diluted and reanalyzed.

4.2 Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that bias analyte response.

5.0 SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.

5.2 Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data Sheets (MSDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

5.3 The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.

5.3.1 Mercury (Sections 7.2 and 7.3)

5.3.2 Sulfuric acid (Sections 7.2, 7.3, and 7.4)

5.3.3 Sodium nitroprusside (Section 7.9)

6.0 EQUIPMENT AND SUPPLIES

6.1 Balance - Analytical, capable of accurately weighing to the nearest 0.000l g.

6.2 Glassware - Class A volumetric flasks and pipets as required.

6.3 Block digestor with tubes.

6.4 Automated continuous flow analysis equipment designed to deliver and react sample and reagents in the required order and ratios.

6.4.1 Sampling device (sampler)

6.4.2 Multichannel pump

6.4.3 Reaction unit or manifold

6.4.4 Colorimetric detector

6.4.5 Data recording device

7.0 REAGENTS AND STANDARDS

7.1 Reagent water: Ammonia free distilled or deionized water, free of the analyte of interest. ASTM Type II or equivalent.

7.2 Mercuric sulfate: Dissolve 8 g red mercuric oxide (HgO) (CASRN 21908-53-2) in 50 mL of 1:4 sulfuric acid (10 mL conc. H_2SO_4: [CASRN 7664-93-9] 40 mL reagent water) and dilute to 100 mL with reagent water.

7.3 Digestion solution: (Sulfuric acid-mercuric sulfate-potassium sulfate solution): Dissolve 133 g of K_2SO_4 (CASRN 7778-80-5) in 700 mL of reagent water and 200 mL of conc. H_2SO_4. Add 25 mL of mercuric sulfate solution (Section 7.1) and dilute to 1 L.

Note 1: An alternate mercury-free digestion solution can be prepared by dissolving 134 g K_2SO_4 and 7.3 g $CuSO_4$ in 800 mL reagent water and then adding 134 mL conc. H_2SO_4 and diluting to 1 L. Use 10 mL solution per 25 mL of sample.

7.4 Sulfuric Acid solution (4%): Add 40 mL of conc. sulfuric acid to 800 mL of reagent water, cool and dilute to 1 L.

Note 2: If alternate mercury-free digestion solution is used, adjust the above solution to equal the acid concentration of the digested sample (Section 11.6).

7.5 Stock Sodium Hydroxide (20%): Dissolve 200 g of sodium hydroxide (CASRN 1310-73-2) in 900 mL of reagent water and dilute to 1 L.

7.6 Stock Sodium Potassium Tartrate solution (20%): Dissolve 200 g sodium potassium tartrate (CASRN 6381-59-5) in about 800 mL of reagent water and dilute to 1 L.

7.7 Stock Buffer solution: Dissolve 134.0 g of sodium phosphate, dibasic (Na_2HPO_4) (CASRN 7558-79-4) in about 800 mL of reagent water. Add 20 g of sodium hydroxide and dilute to 1 L.

7.8 Working Buffer solution: Combine the reagents in the stated order, add 250 mL of stock sodium potassium tartrate solution (Section 7.6) to 200 mL of stock buffer solution (Section 7.7) and mix. Add xx mL sodium hydroxide solution

(Section 7.5) and dilute to 1 L. See concentration ranges, Table 2, for composition of working buffer.

7.9 Sodium Salicylate/Sodium Nitroprusside solution: Dissolve 150 g of sodium salicylate (CASRN 54-21-7) and 0.3 g of sodium nitroprusside (CASRN 13755-38-9 or 14402-89-2) in about 600 mL of reagent water and dilute to 1 L.

7.10 Sodium Hypochlorite solution: Dilute 6.0 mL sodium hypochlorite solution (CASRN 7681-52-9) (Clorox) to 100 mL with reagent water.

7.11 Ammonium chloride, stock solution: Dissolve 3.819 g NH_4Cl (CASRN 12125-02-9) in reagent water and bring to volume in a 1 L volumetric flask. 1 mL = 1.0 mg NH_3-N.

7.12 Teflon boiling chips.

8.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 Samples should be collected in plastic or glass bottles. All bottles must be thoroughly cleaned and rinsed with reagent water. Volume collected should be sufficient to insure a representative sample, allow for replicate analysis (if required), and minimize waste disposal.

8.2 Samples must be preserved with H_2SO_4 to a pH <2 and cooled to 4°C at the time of collection.

8.3 Samples should be analyzed as soon as possible after collection. If storage is required, preserved samples are maintained at 4°C and may be held for up to 28 days.

9.0 QUALITY CONTROL

9.1 Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated.

9.2 INITIAL DEMONSTRATION OF PERFORMANCE

9.2.1 The initial demonstration of performance is used to characterize instrument performance (determination of linear calibration ranges and analysis of QCS) and laboratory performance (determination of MDLs) prior to performing analyses by this method.

9.2.2 Linear Calibration Range (LCR) -- The LCR must be determined initially and verified every 6 months or whenever a significant change

in instrument response is observed or expected. The initial demonstration of linearity must use sufficient standards to insure that the resulting curve is linear. The verification of linearity must use a minimum of a blank and three standards. If any verification data exceeds the initial values by ±10%, linearity must be reestablished. If any portion of the range is shown to be nonlinear, sufficient standards must be used to clearly define the nonlinear portion.

9.2.3 Quality Control Sample (QCS) -- When beginning the use of this method, on a quarterly basis, or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS. If the determined concentrations are not within ±10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with on-going analyses.

9.2.4 Method Detection Limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit.[6] To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

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

where, t = Student's t value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates]
S = standard deviation of the replicate analyses

MDLs should be determined every six months, when a new operator begins work, or whenever there is a significant change in the background or instrument response.

9.3 ASSESSING LABORATORY PERFORMANCE

9.3.1 Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one LRB with each batch of samples. Data produced are used to assess contamination from the laboratory environment. Values that exceed the MDL indicate laboratory or reagent contamination should be suspected and corrective actions must be taken before continuing the analysis.

9.3.2 Laboratory Fortified Blank (LFB) -- The laboratory must analyze at least one LFB with each batch of samples. Calculate accuracy as percent recovery (Section 9.4.2). If the recovery of any analyte falls outside the required control limits of 90-110%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing 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 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the 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 five to ten new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an on-going precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.3.4 Instrument Performance Check Solution (IPC) -- For all determinations the laboratory must analyze the IPC (a mid-range check standard) and a calibration blank immediately following daily calibration, after every 10th sample (or more frequently, if required), and at the end of the sample run. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subsequent analyses of the IPC solution must verify the calibration is still within ±10%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

9.4 ASSESSING ANALYTE RECOVERY AND DATA QUALITY

9.4.1 Laboratory Fortified Sample Matrix (LFM) -- The laboratory must add a known amount of analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis. The analyte concentration must be high enough to

be detected above the original sample and should not be less than four times the MDL. The added analyte concentration should be the same as that used in the laboratory fortified blank.

9.4.2 Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range 90-110%. Percent recovery may be calculated using the following equation:

$$R = \frac{C_s - C}{s} \times 100$$

where, R = percent recovery
C_s = fortified sample concentration
C = sample background concentration
s = concentration equivalent of analyte added to sample

9.4.3 If the recovery of any analyte falls outside the designated LFM recovery range and the laboratory performance for that analyte is shown to be in control (Section 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.

9.4.4 Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

10.0 CALIBRATION AND STANDARDIZATION

10.1 Prepare a series of at least three standards, covering the desired range, and a blank by diluting suitable volumes of standard solution (Section 7.11) with reagent water.

10.2 Process standards and blanks as described in Section 11.0, Procedure.

10.3 Set up manifold as shown in Figure 1 and Table 2.

10.4 Prepare flow system as described in Section 11.0, Procedure.

10.5 Place appropriate standards in the sampler in order of decreasing concentration and perform analysis.

10.6 Prepare standard curve by plotting instrument response against concentration values. A calibration curve may be fitted to the calibration solutions concentration/response data using computer or calculator based regression curve fitting techniques. Acceptance or control limits should be established

using the difference between the measured value of the calibration solution and the "true value" concentration.

10.7 After the calibration has been established, it must be verified by the analysis of a suitable quality control sample (QCS). If measurements exceed ±10% of the established QCS value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis. Periodic reanalysis of the QCS is recommended as a continuing calibration check.

11.0 PROCEDURE

11.1 Pipet 25.0 mL of sample, standard or blank in the digestor tube.

11.2 Add 5 mL of digestion solution (Section 7.3) and mix with a vortex mixer (See Note 1).

11.3 Add four to eight Teflon boiling chips (Section 7.12). **CAUTION:** An excess of Teflon chips may cause the sample to boil over.

11.4 Place tubes in block digestor preheated to 160°C and maintain temperature for one hour.

11.5 Reset temperature to 380°C and continue to heat for one and one half hour.

(380°C MUST BE MAINTAINED FOR 30 MINUTES)

11.6 Remove digestion tubes, cool and dilute to 25 mL with reagent water.

11.7 Excluding the salicylate line, place all reagent lines in their respective containers, connect the sample probe to the sampler and start the pump.

11.8 Flush the sampler wash receptacle with about 25 mL of 4% sulfuric acid (Section 7.4) (See Note 2).

11.9 When reagents have been pumping for at least five minutes, place the salicylate line in its respective container and allow the system to equilibrate. If a precipitate forms after the addition of salicylate, the pH is too low. Immediately stop the proportioning pump and flush the coils with water using a syringe. Before restarting the system, check the concentration of the sulfuric acid solutions and/or the working buffer solution.

11.10 To prevent precipitation of sodium salicylate in the waste tray, which can clog the tray outlet, keep the nitrogen flowcell pump tube and the nitrogen Colorimeter "To Waste" tube separate from all other lines or keep tap water flowing in the waste tray.

11.11 After a stable baseline has been obtained, start the sampler and perform analysis.

12.0 DATA ANALYSIS AND CALCULATIONS

12.1 Prepare a calibration curve by plotting instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve. Multiply answer by appropriate dilution factor.

12.2 Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.

l2.3 Report results in mg N/L.

13.0 METHOD PERFORMANCE

13.1 In a single laboratory (EMSL-Cincinnati) using sewage samples at concentrations of 1.2, 2.6, and 1.7 mg N/L, the precision was ±0.07, ±0.03, and ±0.15, respectively.

13.2 In a single laboratory (EMSL-Cincinnati) using sewage samples at concentrations 4.7 and 8.74 mg N/L, the recoveries were 99% and 99%, respectively.

13.3 The interlaboratory precision and accuracy data in Table 1 were developed using a reagent water matrix. Values are in mg N/L.

l4.0 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 EPA 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 The quantity of chemicals purchased should be based on expected usage during its shelf life and disposal cost of unused material. Actual reagent preparation volumes should reflect anticipated usage and reagent stability.

14.3 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's Department of Government Regulations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036, (202) 872-4477.

15.0 WASTE MANAGEMENT

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. Excess Reagents and samples and method process wastes should be characterized and disposed of in an acceptable manner. 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 waste discharge permit 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 14.3.

16.0 REFERENCES

1. McDaniel, W.H., Hemphill, R.N. and Donaldson, W.T., "Automatic Determination of total Kjeldahl Nitrogen in Estuarine Water", Technicon Symposia, pp. 362-367, Vol. 1, 1967.

2. Gales, M.E. and Booth, R.L., "Evaluation of Organic Nitrogen Methods", EPA Office of Research and Monitoring, June, 1972.

3. Gales, M.E. and Booth, R.L., "Simultaneous and Automated Determination of Total Phosphorus and Total Kjeldahl Nitrogen", Methods Development and Quality Assurance Research Laboratory, May 1974.

4. Technicon "Total Kjeldahl Nitrogen and Total Phosphorus BD-40 Digestion Procedure for Water", August 1974.

5. Gales, M.E., and Booth, R.L., "Evaluation of the Technicon Block Digestor System for the Measurement of Total Kjeldahl Nitrogen and Total Phosphorus", EPA-600/4-78-015, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, 1978.

6. Code of Federal Regulations 40, Ch. 1, Pt. 136, Appendix B.

17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. INTERLABORATORY PRECISION AND ACCURACY DATA

Number of Values Reported	True Value (T)	Mean (X)	Residual for X	Standard Deviation (S)	Residual for S
115	0.380	0.3891	-0.0091	0.0750	-0.0135
134	0.451	0.4807	0.0125	0.1181	0.0238
127	1.00	1.0095	-0.0000	0.1170	-0.0227
164	3.10	3.0992	0.0191	0.2821	-0.0310
138	3.50	3.4765	0.0020	0.3973	0.0512
115	5.71	5.6083	-0.0452	0.4869	-0.0417
175	7.00	6.9246	-0.0008	0.6623	0.0272
121	8.00	7.9991	0.0877	0.6283	-0.0894
120	15.0	15.0213	0.2080	1.2495	-0.0462
127	21.0	20.4355	-0.2937	1.7267	-0.0644
164	25.0	24.7157	0.0426	2.0147	-0.1067
175	26.9	26.1464	-0.4000	2.9743	0.6960

REGRESSIONS: $X = 0.986T + 0.024$, $S = 0.083T + 0.057$

TABLE 2. CONCENTRATION RANGES

Range mg/LN	Pump mL/min.		mL NaOH Buffer (Section 7.7)
	Sample	Resample	
0-1.5	0.80	0.32	250
0-5.0	0.16	0.32	120
0-10.0	0.16	0.16	80

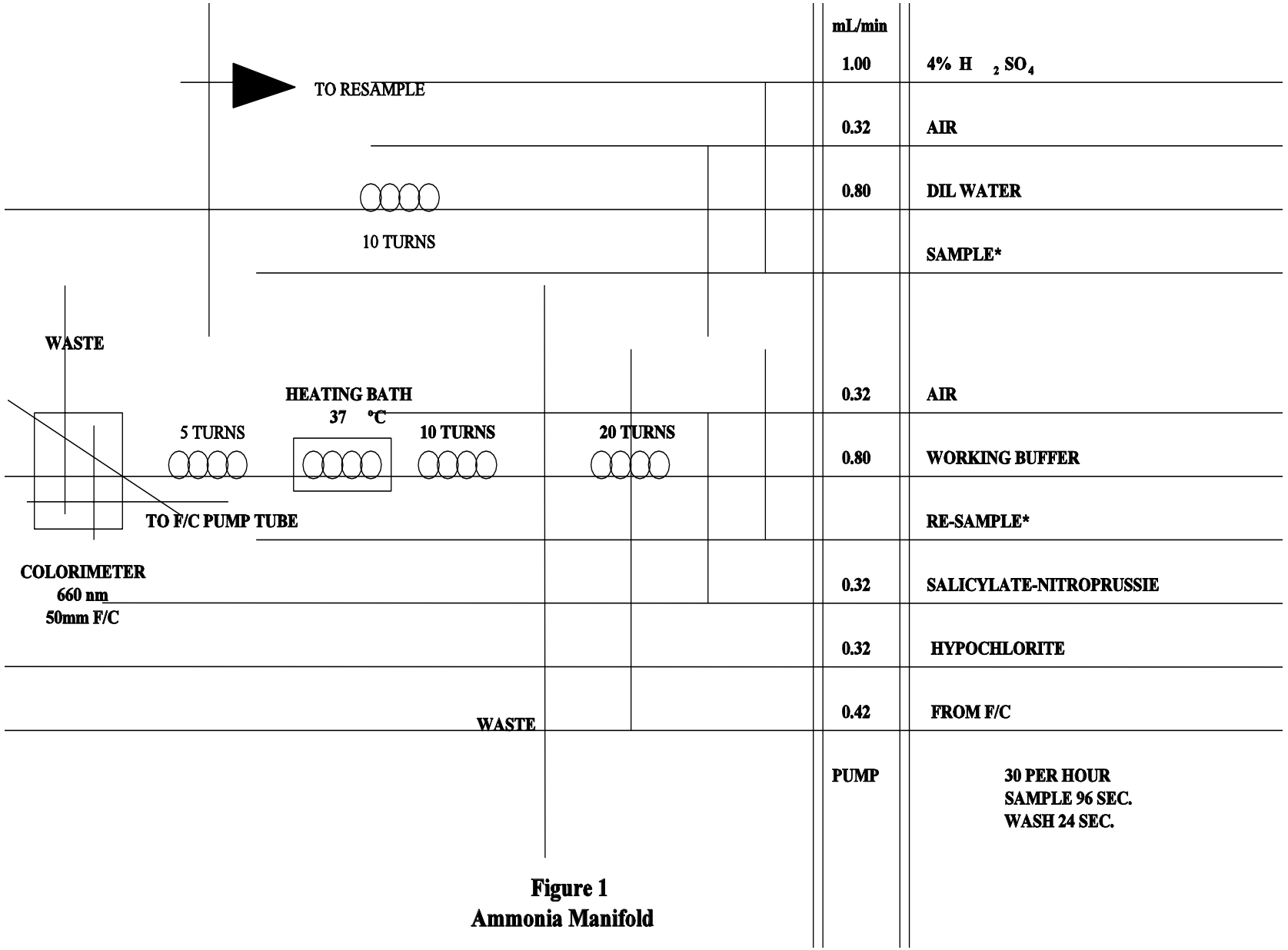

Figure 1
Ammonia Manifold

Appendix M
Laboratory Method – Total Phosphorus– US EPA 365.4

METHOD #: 365.4 Pending Approval for NPDES, CWA (Issued 1974)

TITLE: Phosphorous, Total (Colorimetric, Automated, Block Digester AA II)

ANALYTE: CAS # P Phosphorus 7723-14-0

INSTRUMENTATION: Autoanalyzer

STORET No. 00665

1.0 Scope and Application

1.1 This method covers the determination of total phosphorus in drinking water, surface water and domestic and industrial wastes. The applicable range of this method is 0.01 to 20 mg P/L.

2.0 Summary of Method

2.1 The sample is heated in the presence of sulfuric acid, K_2SO_4 and $HgSO_4$ for two and one half hours. The residue is cooled, diluted to 25 mL and placed on the AutoAnalyzer for phosphorus determination.

3.0 Sample Handling and Preservation

3.1 Sample containers may be of plastic material, such as a cubitainer, or of Pyrex glass.
3.2 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 mL of conc. H_2SO_4 per liter and refrigeration at 4°C.

4.0 Apparatus

4.1 Block Digester BD-40
4.2 Technicon Method No. 327-74W for Phosphorus

5.0 Reagents

5.1 Mercuric sulfate: Dissolve 8 g red mercuric oxide (HgO) in 50 mL of 1:4 sulfuric acid (10 conc. H_2SO_4: 40 mL distilled water) and dilute to 100 mL with distilled water.
5.2 Digestion solution: (Sulfuric acid-mercuric sulfate-potassium sulfate solution): Dissolve 133 g of K_2SO_4in 600 mL of distilled water and 200 mL of conc. H_2SO_4. Add 25 mL of mercuric sulfate solution (5.1) and dilute to 1 liter.
5.3 Sulfuric acid solution (0.72 N): Add 20 mL of conc. sulfuric acid to 800 of distilled water, mix and dilute to 1 liter.
5.4 Molybdate/antimony solution: Dissolve 8 g of ammonium molybdate and 0.2g of antimony potassium tartrate in about 800 mL of distilled water and dilute to

1 liter.

5.5 Ascorbic acid solution: Dissolve 60 g of ascorbic acid in about 600 mL of distilled water. Add 2 mL of acetone and dilute to 1 liter.

5.6 Diluent water: Dissolve 40 g of NaCl in about 600 mL of distilled water and dilute to 1 liter.

5.7 Sulfuric acid solution, 4%: Add 40 mL of conc. sulfuric acid to 800 mL of ammonia-free distilled water, cool and dilute to 1 liter.

6.0 Procedure

Digestion

6.1 To 20 or 25 mL of sample, add 5 mL of digestion solution and mix. (Use a vortex mixer).

6.2 Add 4-8 Teflon boiling chips. Too many boiling chips will cause the sample to boil over.

6.3 With Block Digester in manual mode set low and high temperature at 160°C and preheat unit to 160°C. Place tubes in digester and switch to automatic mode. Set low temperature timer for 1 hour. Reset high temperature to 380°C and set timer for 2 1/2 hours.

6.4 Cool sample and dilute to 25 mL with distilled water. If TKN is determined the sample should be diluted with ammonia-free water.

Colorimetric Analysis

6.4.1 Check the level of all reagent containers to ensure an adequate supply.

6.4.2 Excluding the molybdate/antimony line, place all reagent lines in their respective containers, connect the sample probe to the Sampler IV and start the proportioning pump.

6.4.3 Flush the Sampler IV wash receptacle with about 25 mL of 4% sulfuric acid (5.7).

6.4.4 When reagents have been pumping for at least five minutes, place the molybdate/antimony line in its container and allow the system to equilibrate.

6.4.5 After a stable baseline has been obtained, start the sampler.

7.0 Calculations

7.1 Prepare a standard curve by plotting peak heights of processed standards against concentration values. Compute concentrations by comparing sample peak heights with the standard curve.

8.0 Precision and Accuracy

8.1 In a single laboratory (EMSL) using sewage sample containing total P at levels of 0.23, 1.33, and 2.0, the precision was ± 0.01, ±0.04, and ± 0.06, respectively.

8.2 In a single laboratory (EMSL) using sewage samples of concentration 1.84 and 1.89, the recoveries were 95 and 98%, respectively.

Bibliography

1. McDaniel, W.H., Hemphill, R.N. and Donaldson, W.T., "Automatic Determination of Total Kjeldahl Nitrogen in Estuarine Water", Technicon Symposia, pp. 362-367, Vol. 1,

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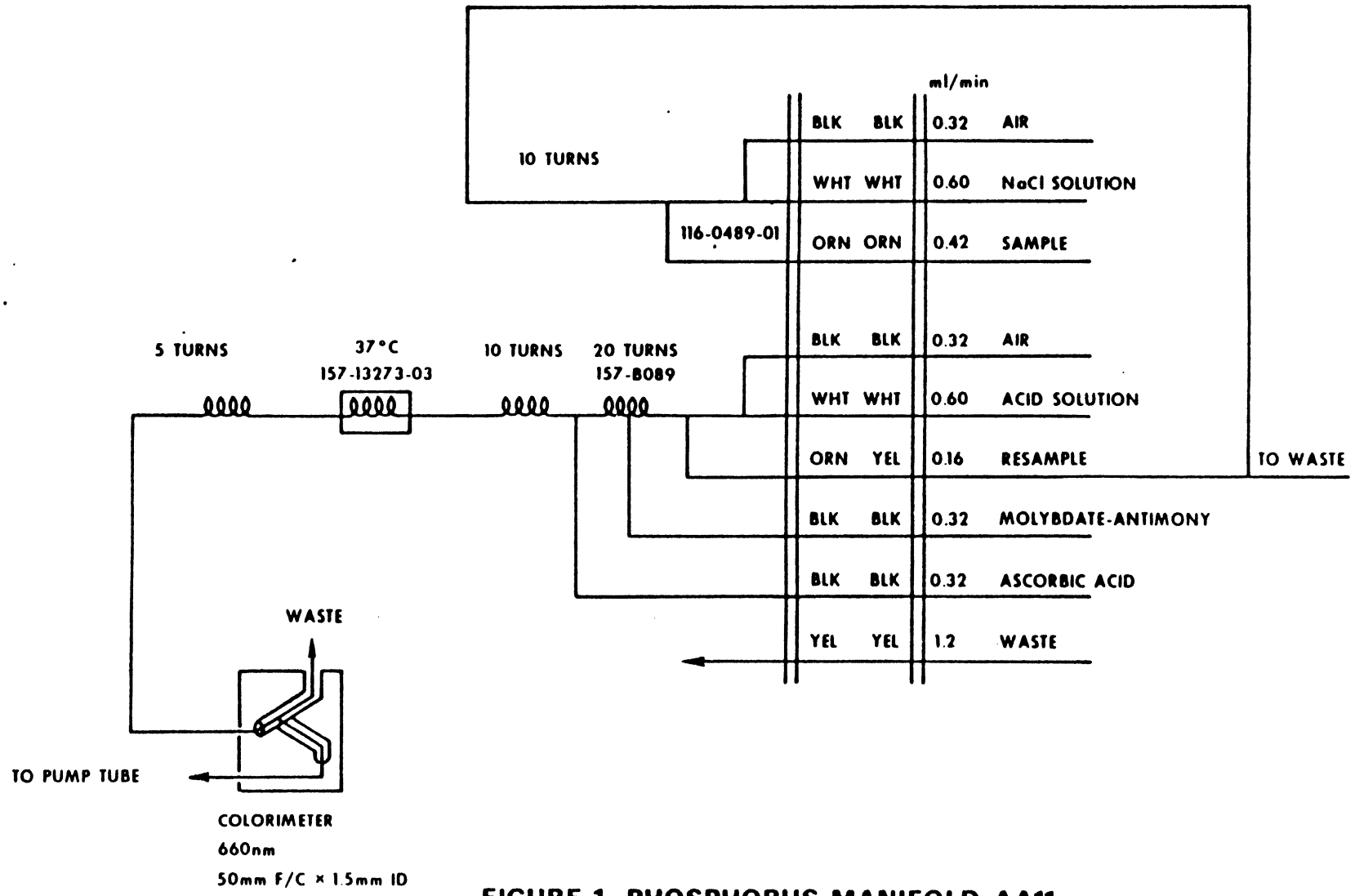

FIGURE 1. PHOSPHORUS MANIFOLD AA11

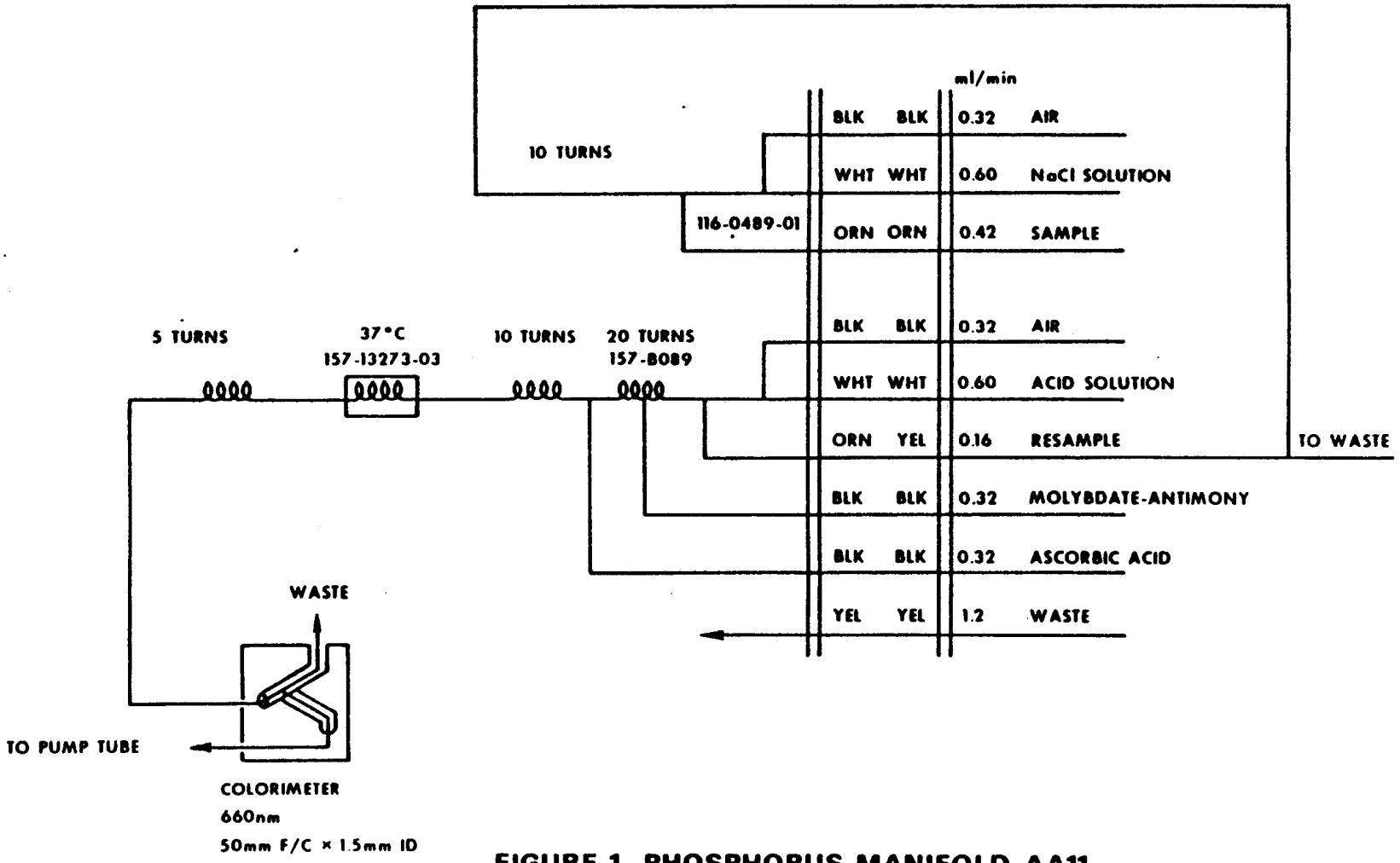

FIGURE 1. PHOSPHORUS MANIFOLD AA11